SPRINGER
REFERENCE

Sangdun Choi
Editor

Encyclopedia of Signaling Molecules

Springer

Encyclopedia of Signaling Molecules

Sangdun Choi
Editor

Encyclopedia of Signaling Molecules

With 686 Figures and 86 Tables

Springer

Editor
Sangdun Choi
Department of Molecular Science and Technology
Ajou University
Suwon, Korea

ISBN 978-1-4419-0460-7 ISBN 978-1-4419-0461-4 (eBook)
DOI 10.1007/978-1-4419-0461-4
ISBN 978-1-4419-0462-1 (print and electronic bundle)
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012942647

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Biological processes are driven by a complex system of functionally interacting signaling components within the cell. These signaling processes are initiated when a class of cell surface proteins, called receptors, receives information from the surrounding environment. This information is then routed through complex signaling pathways and decoded in the nucleus and other areas of the cell. In order to fully understand cell signaling, we must first appreciate the spatial and temporal dynamics of cell surface receptors as well as the downstream components of signaling pathways. The functional states and downstream interactions of cell signaling components are critical to the understanding of both normal and pathologic biological processes. In recent years, a steady increase in both clinical and experimental data on cell signaling has emerged. As we progress through the twenty-first century, it is clear that a systems biology approach, concomitant with the understanding of individual cell signaling components, is needed to delineate biologically relevant signaling networks. Furthermore, it is well understood that almost all diseases exhibit signaling pathway dysfunction. As a result, there has been a profound interest in identifying novel drug targets that regulate key signaling components in disease states.

Currently, there are more than 20,000 genes reported in the human genome; however, not all of the encoded proteins work equally to maintain homeostasis. Achieving a thorough understanding of the most potent signaling components and their associated signaling pathways will significantly improve our knowledge of the molecular mechanisms that regulate disease. Moreover, this insight will lead to the development of novel therapeutics. In recent years, there were multiple attempts to build molecule databases, which were still very partial and brief. Amid the excitement over the recent discoveries of new functional molecules, one of this century's greatest scientific tasks is to compile all information pertaining to signaling components into a single resource. Such an attempt may be arduous but, at the end, will fill the intellectual voids of the field and drastically streamline the understanding of critical signaling networks.

The *Encyclopedia of Signaling Molecules* is a testament to how far we have come in terms of identifying the function of and the interconnection between signaling molecules. This book represents biologically important signaling components from the level of a single gene, to that of gene families. The contents of this encyclopedia are built on the core concepts of the function of signaling components along with the early historical findings to show readers the progress the field has achieved. The encyclopedia not only focuses on individual and groups of signaling components, but also explores the interactions between these groups of signaling components within signal transduction networks. Additionally, it also provides an abundance of

information on the conversion of external signals generated by growth factors, hormones, neurotransmitters, chemokines, cytokines, etc., to the resultant cellular response. Applications of these data to disease and drug discovery efforts have also been discussed. Overall, the encyclopedia is designed to aid those who wish to investigate the function of specific signaling molecules and its role in signaling networks.

The encyclopedia is a Springer Major Reference Work, published in print and online. It consists of entries organized alphabetically. Each entry is concise, clearly written, and contains references to the literature for readers who wish to study each topic in depth. The broad coverage is expected to make the encyclopedia an indispensable reference tool in the field of biomedical research. The online version features colored illustrations and is fully searchable. In addition, cross-references are listed as hyperlinks to easily access related topics in the book.

There are many people to thank and whose help was critical for completing the *Encyclopedia of Signaling Molecules*. This encyclopedia is based on the expertise of hundreds of biomedical professionals who must receive my sincere gratitude for their dedication, efforts, and polite responsiveness to the continuous inquiries. Most importantly, the chapter authors have written outstanding pieces that provide the latest information in their respective field of research. I am grateful to the editors and staffs of Springer: Ann H. Avouris, Anil Chandy, Meetu Lall, Mansi Seth, and Rajneesh Roy for their outstanding help and assistance. Special thanks go to Mrs. Melanie Tucker who made certain that all the molecules were included for the final draft of the encyclopedia.

I hope that the information presented in the *Encyclopedia of Signaling Molecules* will not only aid in understanding the subject matter but also in using the biological information for the benefit of humankind.

Sangdun Choi

About the Editor

Prof. Dr. Sangdun Choi
Department of Molecular Science and Technology
Ajou University
Suwon, 443-749
Korea
sangdunchoi@ajou.ac.kr

Sangdun Choi is currently the Chair of the Department of Biological Sciences and a Professor at the Department of Molecular Science and Technology in Ajou University, Korea. He joined the faculty of the Department of Molecular Science and Technology, Ajou University, as an Associate Professor in 2006. His research interests include Toll-like receptor signaling, innate immunity, cellular signaling and systems biology. He earned his PhD at the Texas A&M University, USA. He was one of the pioneers in the development of Bacterial Artificial Chromosomes (BACs). He was instrumental in the construction of a complete human BAC library for the Human Genome Project (HGP). He was also involved in the consortium, Alliance for Cellular Signaling (AfCS). Furthermore, he served as the Director of the Transcription Analysis Laboratory at the California Institute of Technology (Caltech), USA. He has published more than 200 scientific articles in international journals including *Nature*, *Science*, *Cell*, *Nature Biotechnology*, *Nature Immunology*, *Immunity*, *Blood* and *Proceedings of the National Academy of Sciences*. He has edited seven books including *Encyclopedia of Signaling Molecules*, *Systems Biology for Signaling Networks* and *Introduction to Systems Biology*. He is an academic editor of *PLoS ONE*, an associate editor of *Experimental and Molecular Medicine*, and an active editor for several other international journals. He has also served as a reviewer for several international journals.

Contributors

Brian T. Abe Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA

Jun-ichi Abe Aab Cardiovascular Research Institute, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA

Stephanie Affet Department of Bioscience Technologies, Program in Biotechnology, JSHP, Thomas Jefferson University, Philadelphia, PA, USA

Jacob O. Agola Department of Pathology and Cancer Center, MSC08-4640, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Krassimira Alexieva-Botcheva Biology Department, Brookhaven National Laboratory, Upton, NY, USA

Irene Aligianis MRC Human Genetics Unit, Western General Hospital, Edinburgh, Scotland, UK

Carl W. Anderson Biology Department, Brookhaven National Laboratory, Upton, NY, USA

Douglas A. Andres Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Alberto Anel Departamento de Bioquímica, Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain

Adrienn Angyal Department of Cardiovascular Science, University of Sheffield, Sheffield, South Yorkshire, UK

Alexander Annenkov Bone and Joint Research Unit, William Harvey Research Institute, Queen Mary University of London, London, UK

Bryan A. Anthony Department of Surgery, The Ohio State University Medical Center, Columbus, OH, USA

Ferenc András Antoni Division of Preclinical Research, EGIS Pharmaceuticals PLC, Budapest, Hungary

Kathryn M. Appleton Department of Pharmaceutical and Biomedical Sciences, The Medical University of South Carolina, Charleston, SC, USA

Marie Arsenian Henriksson Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden

Najla Arshad Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka, India

Shaikh M. Atif Center for Comparative Medicine, University of California Davis, Davis, CA, USA

Manuela Baccarini Department of Microbiology and Immunobiology, Center for Molecular Biology, University of Vienna, Max F. Perutz Laboratories, Vienna, Austria

C. T. Baldari Department of Evolutionary Biology, Universty of Siena, Siena, Italy

Ramiro Barcia Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Santiago de Compostela, Lugo, Spain

Betsy J. Barnes Department of Biochemistry and Molecular Biology, New Jersey Medical School-University Hospital Cancer Center, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA

Giancarlo Barone Department of Biochemistry, University of Leicester, Leicester, UK

Blaine Bartholomew Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, IL, USA

Anna Baruzzi Department of Pathology and Diagnostics, Section of General Pathology, University of Verona, Verona, Italy

Shaherin Basith Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Nirmalya Basu Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka, India

Soumik BasuRay Department of Pathology and Cancer Center, MSC08-4640, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Timothy J. Bauler Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT, USA

Joanne Baxter Department of Biochemistry, University of Leicester, Leicester, UK

Carine Becamel Dépt. de Neurobiologie, Institut de Génomique Fonctionnelle, Unité Mixte de Recherche 5203, Centre National de la Recherche Scientifique, Institut National de Santé et de la Recherche Médicale U661, Universités Montpellier 1 and 2, Montpellier Cedex 5, France

Elisa A. Bellomo Section of Cell Biology, Division of Diabetes, Endocrinology and Metabolism, Faculty of Medicine, Imperial College London, South Kensington, London, UK

Arnauld Belmer Université Pierre et Marie Curie, Institut du Fer à Moulin, Paris, France

Armand Bensussan INSERM U976, Saint Louis Hospital, Paris cedex 10, France

Ora Bernard Cytoskeleton and Cancer Unit, St Vincent's Institute of Medical Research, Fitzroy, VIC, Australia

Giorgio Berton Department of Pathology and Diagnostics, Section of General Pathology, University of Verona, Verona, Italy

Anton Bespalov Neuroscience Research, GPRD, Abbott, Ludwigshafen, Germany

Daniel D. Billadeau Department of Immunology, College of Medicine, Mayo Clinic, Rochester, MN, USA

Division of Oncology Research, Schulze Center for Novel Therapeutics, College of Medicine, Mayo Clinic, Rochester, MN, USA

Robert J. Binder Department of Immunology, University of Pittsburgh, Pittsburgh, PA, USA

Gail A. Bishop Department of Microbiology & Internal Medicine, The University of Iowa and VA Medical Center, Iowa City, IA, USA

Arunima Biswas Department of Developmental and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA

Esther E. Biswas-Fiss Department of Bioscience Technologies, Program in Biotechnology, JSHP, Thomas Jefferson University, Philadelphia, PA, USA

Department of Molecular Biology, University of Medicine and Dentistry of New Jersey, SOM, Stratford, NJ, USA

Haruhiko Bito Department of Neurochemistry, The University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan

CREST–Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

Sandra Blanco Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Giovanni Blandino Translational Oncogenomics Unit, Regina Elena Cancer Institute, Rome, Italy

Francesco Blasi IFOM (Fondazione Istituto FIRC di Oncologia Molecolare), Milan, Italy

Joelle Blot Department of Biochemistry, University of Leicester, Leicester, UK

Richard Blouin Département de Biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada

Mitsi A. Blount Renal Division, Emory University Woodruff Memorial Research Building, Atlanta, GA, USA

Joe B. Blumer Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

Department of Neurosciences, Medical University of South Carolina, Charleston, SC, USA

Matthew D. Blunt Department of Pharmacy & Pharmacology, University of Bath, Bath, Somerset, UK

Joël Bockaert Dépt. de Neurobiologie, Institut de Génomique Fonctionnelle, Unité Mixte de Recherche 5203, Centre National de la Recherche Scientifique, Institut National de Santé et de la Recherche Médicale U661, Universités Montpellier 1 and 2, Montpellier Cedex 5, France

Jean Marie Boeynaems Department of Laboratory Medicine, Erasme Academic Hospital, Université Libre de Bruxelles, Brussels, Belgium

Subbarao Bondada Department of Microbiology, Immunology and Molecular Genetics, Markey Cancer Center, University of Kentucky, Lexington, KY, USA

Kaya Bork Institute for Physiological Chemistry, Martin-Luther-University Halle-Wittenberg, Halle(Saale), Germany

Dave Boucher Department of pharmacology, Université de Sherbrooke, Sherbrooke, QC, Canada

Robert R. Bowers Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

Charles D. Boyd John A. Burns School of Medicine, University of Hawaii, Honolulu, HI, USA

Jennifer Boylston Department of Biochemistry and Program in Molecular & Cellular Biology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Maria Elena Bravo-Adame Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Catherine Brenner INSERM UMR-S 769, Université Paris-Sud XI, Châtenay-Malabry, France

Charles Brenner Department of Biochemistry and Program in Molecular & Cellular Biology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Gary Brewer Department of Molecular Genetics, Microbiology and Immunology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ, USA

James P. Brody Department of Biomedical Engineering, University of California, Irvine, CA, USA

Gordon D. Brown Section of Immunology and Infection, Division of Applied Medicine, Institute of Medical Sciences, Aberdeen Fungal Group, Aberdeen, UK

Joshua D. Brown-Clay Department of Biochemistry and Molecular and Cellular Biology, Georgetown University, Washington, DC, USA

Laurence L. Brunton Pharmacology, University of California San Diego, San Diego, CA, USA

Brad Allen Bryan Department of Biology, Ghosh Science and Technology Center, Worcester State University, Worcester, MA, USA

Center of Excellence in Cancer Research, Department of Biomedical Sciences, Texas Tech University Health Sciences Center, El Paso, TX, USA

Thomas P. Burris Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, FL, USA

Stephanie L. Burroughs Department of Ophthalmology, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Scott A. Busby Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, FL, USA

Xosé R. Bustelo Centro de Investigación del Cáncer/Cancer Research Center, CSIC-University of Salamanca, Salamanca, Spain

Aisha Qasim Butt Department of Biology, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

Bettina Büttner Institute for Physiological Chemistry, Martin-Luther-University Halle-Wittenberg, Halle(Saale), Germany

Jennifer Anne Byrne Children's Cancer Research Unit, Kids Research Institute, The Children's Hospital at Westmead, Westmead, NSW, Australia

Tatiana V. Byzova Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Lerner Research Institute, The Cleveland Clinic, Cleveland, OH, USA

Weikang Cai Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Mario R. Calderon Department of Physiology, McGill University McIntyre Medical Sciences Building, Montréal, Québec, Canada

Chunhua Cao Department of Medicine, Division of Nephrology, University of Maryland, Baltimore, Baltimore, MD, USA

Astrid E. Cardona Department of Biology and South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, TX, USA

Graeme K. Carnegie Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, USA

Lai N. Chan Molecular Biology Institute, Los Angeles, CA, USA

Department of Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles, CA, USA

Rakesh Chandarana Bombay College of Pharmacy, Mumbai, India

Nilanjana Chatterjee Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, IL, USA

Jian Chen Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI, USA

Xingwang Chen Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, USA

Yuyan Chen Children's Cancer Research Unit, Kids Research Institute, The Children's Hospital at Westmead, Westmead, NSW, Australia

Frank S. Chen Department of Biochemistry and Molecular Biology, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA

Ching-Kang (Jason) Chen Department of Biochemistry and Molecular Biology, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA

Heung-Chin Cheng Department of Biochemistry and Molecular Biology, University of Melbourne Bio21 Molecular Science and Biotechnology Institute, Parkville, VIC, Australia

Chin Chiang Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, USA

Pallavi Chittoor Department of Biochemistry, Sri Venkateswara University College of Sciences, Tirupati, AP, India

Sangdun Choi Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Yuh-Ping Chong Edinburgh Cancer Research Centre, Western General Hospital, Edinburgh, UK

Avik Choudhuri Department of Developmental and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA

Laurie T. C. Chow Clinical Pharmacology Unit, Department of Medicine Austin Health/Northern Health, University of Melbourne, Heidelberg, VIC, Australia

Matthew L. H. Chu Department of Structural Biology, Stanford University School of Medicine, Stanford, CA, USA

Jonathan H. Clarke Department of Pharmacology, University of Cambridge, Cambridge, UK

Jaime O. Claudio Toronto General Research Institute, University Health Network, Toronto Medical Discovery Tower, Toronto, ON, Canada

John Colicelli Department of Biological Chemistry, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Lynne M. Coluccio Boston Biomedical Research Institute, Watertown, MA, USA

Didier Communi Institute of Interdisciplinary Research, School of Medicine, Université Libre de Bruxelles, Brussels

Christopher T. Cottage San Diego State Heart Institute, San Diego State University, San Diego, CA, USA

Evans C. Coutinho Bombay College of Pharmacy, Mumbai, India

Jean-Philippe Couture Département de Biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada

Dianne Cox Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, Bronx, NY, USA

Carl E. Creutz Department of Pharmacology, University of Virginia, Virginia, VA, USA

Katalin Csiszar John A. Burns School of Medicine, University of Hawaii, Honolulu, HI, USA

Fernando Queiroz Cunha Inflammation lab, Department of Pharmacology, School of Medicine of Ribeirão Preto, São Paulo University – FMRP/USP, Ribeirao Preto, SP, Brazil

Ian Cushman Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, USA

Karin Dahlman-Wright Department of Biosciences and Nutrition, Novum, Karolinska Institutet, Huddinge, Sweden

Alexander H. Dalpke Department of Infectious Diseases, Medical Microbiology and Hygiene, University of Heidelberg, Heidelberg, Germany

Ben Davies Wellcome Trust Centre for Human Genetics, University of Oxford Roosevelt Drive, Oxford, UK

Dima A. Decker Department of Microbiology & Internal Medicine, The University of Iowa and VA Medical Center, Iowa City, IA, USA

Francesca Deflorian Laboratory of Bioorganic Chemistry & Molecular Recognition Section, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

James H. DeFord Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA

Jennifer Defren Department of Molecular Genetics, Microbiology and Immunology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ, USA

Austin Della-Franca Children's Cancer Research Unit, Kids Research Institute, The Children's Hospital at Westmead, Westmead, NSW, Australia

Brian R. Dempsey Department of Biochemistry, The University of Western Ontario, London, ON, Canada

Jean-Bernard Denault Department of pharmacology, Université de Sherbrooke, Sherbrooke, QC, Canada

Carmen W. Dessauer Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, Houston, TX, USA

Sumit Deswal Max Planck Institute of Immunobiology and Faculty of Biology, Biology III, University of Freiburg, Freiburg, Germany

Spemann Graduate School of Biology and Medicine, Freiburg, Germany

Sandeepa Dey Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA

Silvina Laura Diaz Université Pierre et Marie Curie, Institut du Fer à Moulin, Paris, France

Jonathan R. Dimmock Drug Design and Discovery Research Group, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

Shabana Din San Diego State Heart Institute, San Diego State University, San Diego, CA, USA

Rachel Doidge School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK

Stéphane Doly Université Pierre et Marie Curie, Institut du Fer à Moulin, Paris, France

Johnna Dominick Department of Molecular and Cellular Biochemistry, Center for Molecular Neurobiology, Ohio State Biochemistry Program Ohio State University, Columbus, OH, USA

Molecular & Cellular Biochemistry, Ohio State University, Columbus, OH, USA

Dominique M. Donato Physics of Life Processes, Leiden Institute of Physics, Leiden University, CA, Leiden, The Netherlands

H. Henry Dong Division of Immunogenetics, Department of Pediatrics, Rangos Research Center, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Athanassios Dovas Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, Bronx, NY, USA

Gerard Drewes Discovery Research, Cellzome AG, Heidelberg, Germany

Kirk M. Druey Laboratory of Allergic Diseases, National Institute of Allergy and infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Jacinta S. D'Souza UM-DAE-Centre for Excellence in Basic Sciences, Kalina campus, Santacruz (E), Mumbai, India

Xiaoyu Du The Rosalind & Morris Goodman Cancer Research Center, McGill University, Québec, Canada

Department of Medicine, McGill University Health Center, Montréal, Québec, Canada

Vikas V. Dukhande Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Denis J. Dupré Department of Pharmacology, Dalhousie University, Nova Scotia, Canada

Norman L. Eberhardt Departments of Medicine and Biochemistry & Molecular Biology, Mayo Clinic, Rochester, MN, USA

Sean E. Egan Program in Developmental & Stem Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada

Aristides G. Eliopoulos Molecular & Cellular Biology Laboratory, Division of Basic Sciences, University of Crete Medical School, Heraklion, Crete, Greece

Institute for Molecular Biology & Biotechnology, Foundation of Research & Technology Hellas (FORTH), Heraklion, Crete, Greece

Ari Elson Department of Molecular Genetics, Arnold R. Meyer Institute of Biological Sciences, The Weizmann Institute of Science, Rehovot, Israel

Marina Rode von Essen Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, DK, Denmark

Patrick A. Eyers YCR Institute for Cancer Studies, University of Sheffield, Sheffield, UK

Zakaria Ezzoukhry Laboratoire de Biochimie, Inserm ERI12 – EA4292, Université de Picardie Jules Verne (UPJV), Amiens Cedex, France

Björn H. Falkenburger Department of Neurology, RWTH University Medical Center, Aachen, Germany

Angharad E. Fenton-May Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Isabel F. Fernández Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Alberto Fernández-Medarde Centro de Investigación del Cáncer, IBMCC (CSIC/USAL), University of Salamanca, Salamanca, Spain

M. Ferro Department of Evolutionary Biology, Universty of Siena, Siena, Italy

Katherine Figella Department of Biology, Ghosh Science and Technology Center, Worcester State University, Worcester, MA, USA

Kathryn Finton Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Giulia Fontemaggi Translational Oncogenomics Unit, Regina Elena Cancer Institute, Rome, Italy

Sonia-Vanina Forcales Institute of Predictive and Personalized Medicine of Cancer (IMPPC), Badalona, Barcelona, Spain

Albert J. Fornace Jr. Department of Biochemistry and Molecular and Cellular Biology, Georgetown University, Washington, DC, USA

Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

Anna Frenzel Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden

Jeffrey A. Frost Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, Houston, TX, USA

David A. Fruman Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Andrew Fry Department of Biochemistry, University of Leicester, Leicester, UK

Masakazu Fujiwara Department of Molecular Pathology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School, Kawasaki, Kanagawa, Japan

Masaki Fukata Division of Membrane Physiology, Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, Aichi, Japan

Department of Physiological Sciences, School of Life Science, The Graduate University for Advanced Studies (SOKENDAI), Aichi, Japan

Yuko Fukata Division of Membrane Physiology, Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, Aichi, Japan

Department of Physiological Sciences, School of Life Science, The Graduate University for Advanced Studies (SOKENDAI), Aichi, Japan

Mitsunori Fukuda Laboratory of Membrane Trafficking Mechanisms, Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi, Japan

Matthias Gaestel Hannover Medical School, Institute of Biochemistry, Hannover, Germany

Thierry Galli Membrane Traffic in Neuronal & Epithelial Morphogenesis, INSERM ERL U950, Paris, France

Program in Development & Neurobiology, Jacques Monod Institute, CNRS UMR7592, Paris, France

University Denis Diderot–Paris 7, Paris, France

Kathleen A. Gallo Department of Physiology, Michigan State University, East Lansing, MI, USA

Cell & Molecular Biology Program, Michigan State University, East Lansing, MI, USA

Lorenzo Galluzzi INSERM, U848, Institut Gustave Roussy, Université Paris-Sud XI, Villejuif, France

Antoine Galmiche Laboratoire de Biochimie, Inserm ERI12 – EA4292, Université de Picardie Jules Verne (UPJV), Amiens Cedex, France

Zhan-Guo Gao Laboratory of Bioorganic Chemistry & Molecular Recognition Section, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

Gustavo Pompermaier Garlet OSTEOimmunology lab, Department of Biological Sciences, School of Dentistry of Bauru, São Paulo University – FOB/USP, Bauru, SP, Brazil

Thiago Pompermaier Garlet Inflammation lab, Department of Pharmacology, School of Medicine of Ribeirão Preto, São Paulo University – FMRP/USP, Ribeirao Preto, SP, Brazil

Joanna E. Gawecka University of Hawai'i Cancer Center, University of Hawai'i at Manoa, Honolulu, HI, USA

Carsten Geisler Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, DK, Denmark

Matthew S. Gentry Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Mesfin Gewe Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Andreas Gewies Laboratory for Signaling in the Immune System, Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany

Mohammad Ghazizadeh Department of Molecular Pathology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School, Kawasaki, Kanagawa, Japan

N. Giommoni Department of Evolutionary Biology, Universty of Siena, Siena, Italy

Daryl L. Goad Department of Ophthalmology and Department of Basic Medical Science, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Catherine Godson UCD Diabetes Research Centre, UCD Conway Institute, School of Medicine and Medical Sciences, University College Dublin, Dublin, Ireland

Susan Goebel-Goody Child Study Center, School of Medicine, Yale University, New Haven, CT, USA

Julian Gomez-Cambronero Department of Biochemistry and Molecular Biology, Wright State University School of Medicine, Dayton, OH, USA

João Gonçalves Centro de Química e Bioquímica, Lisbon, Portugal

Instituto Gulbenkian de Ciência, Oeiras, Portugal

Jose-Luis González de Aguilar Laboratory of Molecular Signaling and Neurodegeneration, INSERM, Faculty of Life Sciences, University of Strasbourg, Strasbourg, France

Luisa Gorza Department of Biomedical Sciences, University of Padova, Padova, Italy

Benjamin J. Gosney Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales, UK

Noriko Gotoh Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan

Michael T. Greenwood Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Kingston, ON, Canada

Michael A. Grillo Department of Ophthalmology and Department of Basic Medical Science, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Julian Hendrik Gronau Division of Cancer, Department of Surgery & Cancer, Imperial College London, Hammersmith Hospital, London, UK

Haihua Gu Department of Pathology, University of Colorado Denver, Aurora, CO, USA

Marga Gual-Soler Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

Donald Gullberg Department of Biomedicine, University of Bergen, Bergen, Norway

Mads Gyrd-Hansen Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Copenhagen, Denmark

Malissa Ha Department of Bioscience Technologies, Program in Biotechnology, JSHP, Thomas Jefferson University, Philadelphia, PA, USA

Gregg A. Hadley Department of Surgery, The Ohio State University Medical Center, Columbus, OH, USA

Tsonwin Hai Department of Molecular and Cellular Biochemistry, Center for Molecular Neurobiology, Ohio State Biochemistry Program Ohio State University, Columbus, OH, USA

Molecular & Cellular Biochemistry, Ohio State University, Columbus, OH, USA

Michelle L. Halls Department of Pharmacology, University of Cambridge, Cambridge, UK

Mark Handley MRC Human Genetics Unit, Western General Hospital, Edinburgh, Scotland, UK

Dorit Hanein Bioinformatics and Systems Biology Program, Sanford-Burnham Medical Research Institute, La Jolla, CA, USA

Steven K. Hanks Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN, USA

Hiromitsu Hara Division of Molecular and Cellular Immunoscience, Department of Biomolecular Sciences, Saga University, Saga, Japan

Kenneth W. Harder Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada

Nirmala Hariharan San Diego State Heart Institute, San Diego State University, San Diego, CA, USA

Christian Harteneck Department of Pharmacology and Experimental Therapy, Institute of Experimental and Clinical Pharmacology and Toxicology, Interfaculty Center of Pharmacogenomics and Pharmaceutical Research (ICePhA), Eberhard-Karls-University, Tübingen, Germany

Amanda Harvey Brunel Institute for Cancer Genetics and Pharmacogenomics, Brunel University, West London, UK

Debbie L. Hay School of Biological Sciences, University of Auckland, Auckland, New Zealand

Terence E. Hébert Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada

Jyrki Heino Department of Biochemistry and Food Chemistry, University of Turku, Turku, Finland

Karen M. Henkels Department of Biochemistry and Molecular Biology, Wright State University School of Medicine, Dayton, OH, USA

Jonathan J. Henry Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Jeniffer B. Hernandez Institute for Immunology and Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Deike Hesse Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Randa Hilal-Dandan Pharmacology, University of California San Diego, San Diego, CA, USA

Ping-Chih Ho Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN, USA

Thomas G. Hofmann Cellular Senescence (A210), German Cancer Research Center, Heidelberg, Germany

Arie Horowitz Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic Foundation and Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH, USA

Rüdiger Horstkorte Institute for Physiological Chemistry, Martin-Luther-University Halle-Wittenberg, Halle(Saale), Germany

Richard Horuk Department of Pharmacology, UC Davis, Davis, CA, USA

Mohammed Iqbal Hossain Department of Biochemistry and Molecular Biology, University of Melbourne Bio21 Molecular Science and Biotechnology Institute, Parkville, VIC, Australia

Bruce S. Hostager Department of Pediatrics, University of Iowa, Iowa City, IA, USA

Miles D. Houslay Molecular Pharmacology Group, Institute of Neuroscience and Psychology, CMVLS, University of Glasgow, Glasgow, Scotland, UK

Ching-Chyuan Hsieh Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA

Yuan-Hao Hsu Department of Chemistry, Tunghai University, Taichung, Taiwan

Dongli Hu Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, USA

Kun Huang Department of Biomedical Informatics, OSUCCC Biomedical Informatics Shared Resources, Ohio State University, Columbus, OH, USA

Masatoshi Inoue Department of Neurochemistry, The University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan

Giorgio Iotti IFOM (Fondazione Istituto FIRC di Oncologia Molecolare), Milan, Italy

Nancy Y. Ip Division of Life Science, Molecular Neuroscience Center and State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science and Technology, Kowloon, Hong Kong

Robin F. Irvine Department of Pharmacology, University of Cambridge, Cambridge, UK

Clare M. Isacke Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, London, UK

Jeff S. Isenberg Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh School of Medicine and the Vascular Medicine Institute of the University of Pittsburgh, Pittsburgh, PA, USA

Hiroshi Itoh Department of Medical Science, Nara Institute of Science and Technology, Ikoma, Nara, Japan

Ryo Iwamoto Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

Maria C. Izquierdo IIS-Fundacion Jimenez Diaz and Universidad Autonoma de Madrid, Madrid, Spain

Kenneth A. Jacobson Laboratory of Bioorganic Chemistry & Molecular Recognition Section, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

David A. Jans Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

Christine Janson Department of Biological Chemistry, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Alexander Jaschke Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

M. P. Suresh Jayasekara Laboratory of Bioorganic Chemistry & Molecular Recognition Section, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

Patricia A. Jim Department of Pathology and Cancer Center, MSC08-4640, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Pauline Johnson Department of Microbiology and Immunology, Life Sciences Institute, 2350 Health Sciences Mall, University of British Columbia, Vancouver, BC, Canada

Faye M. Johnson Department of Thoracic/Head and Neck Medical Oncology, Unit 432, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX, USA

Simon Kaja Department of Ophthalmology, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Wataru Kakegawa Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Theodosia A. Kalfa Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, OH, USA

Mohd Aizuddin Kamaruddin Department of Biochemistry and Molecular Biology, University of Melbourne Bio21 Molecular Science and Biotechnology Institute, Parkville, VIC, Australia

Venkateswarlu Kanamarlapudi Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales, UK

Ana Kasirer-Friede Department of Medicine, University of California, La Jolla, San Diego, CA, USA

Masaru Katoh Division of Integrative Omics and Bioinformatics, National Cancer Center, Tokyo, Japan

Gurpreet Kaur Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

Thyaga Raju Kedam Department of Biochemistry, Sri Venkateswara University College of Sciences, Tirupati, AP, India

Bethany A. Kerr Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Lerner Research Institute, The Cleveland Clinic, Cleveland, OH, USA

Anupriya Khare Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, and Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Sandeep Khurana Department of Medicine, Division of Gastroenterology, University of Maryland, Baltimore, Baltimore, MD, USA

Friedemann Kiefer Department Vascular Cell Biology, Max Planck Institute for Molecular Biomedicine, Mammalian Cell Signaling Laboratory, Münster, Germany

Werner Kilb Institute of Physiology & Pathophysiology, University Medical Center Mainz, Mainz, Germany

Yong-Sung Kim Department of Molecular Science and Technology, Ajou University, Yeongtong-Gu, Suwon, South Korea

Young Woo Kim College of Pharmacy, Seoul National University, Seoul, South Korea

Dae Hyun Kim Division of Immunogenetics, Department of Pediatrics, Rangos Research Center, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Sang Geon Kim College of Pharmacy, Seoul National University, Seoul, South Korea

Adam J. Kimple Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Akihiro Kimura Laboratory of Immune Regulation, Osaka University Graduate School of Frontier Biosciences, Osaka, Japan

Philip D. King Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA

Christiane Kirchhoff Department of Andrology, University Hospital Hamburg-Eppendorf, Hamburg, Germany

Hatice Zeynep Kirli Department of Molecular Biosciences, University of Oslo, Oslo, Norway

Tadamitsu Kishimoto Laboratory of Immune Regulation, Osaka University Graduate School of Frontier Biosciences, Osaka, Japan

Endre Kiss-Toth Department of Cardiovascular Science, University of Sheffield, Sheffield, South Yorkshire, UK

Janet D. Klein Renal Division, Emory University Woodruff Memorial Research Building, Atlanta, GA, USA

Sonia Klinger Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada

Masaaki Kobayashi Department of Physiology, Toho University School of Medicine, Ohta-ku, Toyko, Japan

Karl-Wilhelm Koch Department of Biology and Environmental Sciences, University of Oldenburg, Oldenburg, Germany

Kazuhisa Kohda Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Walter Kolch Systems Biology Ireland, University College Dublin, Dublin, Ireland

UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland

Shigeru Komaba Boston Biomedical Research Institute, Watertown, MA, USA

Sebastian Königsberger Department Vascular Cell Biology, Max Planck Institute for Molecular Biomedicine, Mammalian Cell Signaling Laboratory, Münster, Germany

Diamantis Konstantinidis Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, OH, USA

Jun Kotera Advanced Medical Research Laboratories, Mitsubishi Tanabe Pharma Corporation, Yokohama, Kanagawa, Japan

Alexey Kotlyarov Institute of Biochemistry, Hannover Medical School, Hannover, Germany

Peter Koulen Department of Ophthalmology and Department of Basic Medical Science, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Eva Krieghoff-Henning Cellular Senescence (A210), German Cancer Research Center, Heidelberg, Germany

Sergei Krirschuk Institute of Physiology & Pathophysiology, University Medical Center Mainz, Mainz, Germany

Nandini Krishnamoorthy Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, and Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Jayalakshmi Krishnan Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Guido Kroemer INSERM U848, Institut Gustave Roussy, Villejuif cedex, France

University Paris 11, Orsay, France

Institut Gustave Roussy, Villejuif cedex, France

Satoshi Kubota Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Okayama, Japan

Sujeet Kumar Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Cancer Research Unit, Saskatchewan Cancer Agency, Saskatoon, SK, Canada

Ashok Kumar Center for Cancer Research, Children's Hospital Oakland Research Institute, Oakland, CA, USA

Divya Kurumala Department of Biochemistry, Sri Venkateswara University College of Sciences, Tirupati, AP, India

Pradeep Kurup Child Study Center, School of Medicine, Yale University, New Haven, CT, USA

Rozalia Laczko John A. Burns School of Medicine, University of Hawaii, Honolulu, HI, USA

Jill M. Lahti Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, USA

Kwok-On Lai Division of Life Science, Molecular Neuroscience Center and State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science and Technology, Kowloon, Hong Kong

Pui Man Rosalind Lai Neuroscience Program, Wellesley College, Wellesley, MA, USA

Harvard Medical School, Boston, MA, USA

Maréne Landström Department of Medical Biosciences, Department of Pathology, Umeå University, Umeå, Sweden

Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden

Stephen M. Lanier Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

Philip E. Lapinski Department of Microbiology and Immunology, University of Michigan, Medical School, Ann Arbor, MI, USA

Kelvin Lau Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

Pedro A. Lazo Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Antigone Lazou School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece

Nhat-Tu Le Aab Cardiovascular Research Institute, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA

Woo Hyung Lee College of Pharmacy, Seoul National University, Seoul, South Korea

Jong Ran Lee Department of Life Science, College of Natural Sciences, Ewha Womans University, Seoul, South Korea

Yong-Hun Lee Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

Philippe Lenormand Institute of Developmental Biology and Cancer, CNRS UMR6543, Université de Nice, Nice, France

Nicholas R. Leslie Division of Cell Signalling and Immunology, College of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dundee, UK

Shoshana Levy School of Medicine – Division of Oncology Center for Clinical Sciences Research, Stanford University, Stanford, CA, USA

Feng-Qian Li Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, USA

Linfang Li Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao, China

Willis X. Li Department of Medicine, University of California San Diego, La Jolla, CA, USA

Bruce T. Liang Calhoun Cardiovascular Center, University of Connecticut Health Center, Farmington, CT, USA

Su Jun Lim University of Rochester Medical Center, Rochester, NY, USA

Fan-ching Lin Laboratory of Experimental Immunology, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA

Rafael Linden Laboratorio de Neurogenese, Instituto de Biofisica da UFRJ, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

David W. Litchfield Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

Department of Oncology, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

Mingyao Liu Department of Molecular and Cellular Medicine, Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, TX, USA

Zhenhui Liu Department of Marine Biology, Key Laboratory of Marine Genetics and Breeding, Ocean University of China, Qingdao, PR, China

Paul Lombroso Child Study Center, School of Medicine, Yale University, New Haven, CT, USA

Inmaculada López-Sánchez Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

William J. Louis Clinical Pharmacology Unit, Department of Medicine Austin Health/Northern Health, University of Melbourne, Heidelberg, VIC, Australia

Simon N. S. Louis Clinical Pharmacology Unit, Department of Medicine Austin Health/Northern Health, University of Melbourne, Heidelberg, VIC, Australia

Thomas J. Lukas Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, IL, USA

Sarah C. R. Lummis Department of Biochemistry, University of Cambridge, Cambridge, UK

Alex Lyakhovich Duke-National University of Singapore Graduate Medical School, Singapore

Fernando Macian Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA

Jeffrey P. MacKeigan Center for Cancer Genomics and Quantitative Biology, Van Andel Research Institute, Grand Rapids, MI, USA

Kathrin Maedler Center for Biomolecular Interactions, University of Bremen, Bremen, Germany

Luiz Alexandre V. Magno INCT de Medicina Molecular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Umadas Maitra Department of Developmental and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA

Romit Majumdar Department of Developmental and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA

Balachandran Manavalan Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Anne Marie-Cardine INSERM U976, Saint Louis Hospital, Paris cedex 10, France

Philippe Marin Dépt. de Neurobiologie, Institut de Génomique Fonctionnelle, Unité Mixte de Recherche 5203, Centre National de la Recherche Scientifique, Institut National de Santé et de la Recherche Médicale U661, Universités Montpellier 1 and 2, Montpellier Cedex 5, France

Barbara Mariniello Endocrinology Unit, Department of Medical and Surgical Sciences, University of Padua, Padua, Italy

Thomais Markou School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece

Luc Maroteaux Université Pierre et Marie Curie, Institut du Fer à Moulin, Paris, France

Kirill A. Martemyanov Department of Neuroscience, The Scripps Research Institute, Jupiter, Florida, USA

Luis Martinez-Lostao Departamento de Bioquímica, Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain

Vilma R. Martins International Center for Research and Education Antonio Prudente Foundation, A.C. Camargo Hospital, São Paulo, SP, Brazil

Hisao Masai Genome Dynamics Project, Department of Genome Medicine, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Shinji Matsuda Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Keiko Matsuda Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Motozumi Matsui Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

Julie A. Maupin-Furlow Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, USA

Jean-Claude Maziere Laboratoire de Biochimie, Inserm ERI12 – EA4292, Université de Picardie Jules Verne (UPJV), Amiens Cedex, France

Michael T. McCarthy Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Stephen J. McSorley Center for Comparative Medicine, University of California Davis, Davis, CA, USA

Thomas E. Meigs Dept. of Biology, University of North Carolina Asheville, Asheville, NC, USA

Eisuke Mekada Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

Sylvain Meloche Department of Pharmacology, Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada

Program of Molecular Biology, Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada

Triana Merced Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Sinéad M. Miggin Department of Biology, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

Oscar Arturo Migueles-Lozano Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Toru Miki Laboratory of Cellular Signaling, Nagaoka University of Technology, Niigata, Japan

Francesca Milanesi IFOM, Milan, Italy

Laurence J. Miller Department of Molecular Pharmacology and Experimental Therapeutics and Department of Internal Medicine, Division of Gastroenterology, Mayo Clinic, Scottsdale, AZ, USA

Yu Ming Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden

Hugo V. Miranda Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, USA

Anita R. Mistry Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Maria Mittelbrunn Centro Vascular Biology and Inflamation Department, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain

Tooru Mizuno University of Manitoba, Winnipeg, MB, Canada

Achim Möller Neuroscience Research, GPRD, Abbott, Ludwigshafen, Germany

Nazanine Modjtahedi INSERM U848, Institut Gustave Roussy, Villejuif cedex, France

University Paris 11, Orsay, France

Institut Gustave Roussy, Villejuif cedex, France

Elek Molnár MRC Centre for Synaptic Plasticity, School of Physiology and Pharmacology, University of Bristol, Medical Sciences Building, University Walk, Bristol, UK

Diana M. Monsalve Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Jean Morisset Service de gastroentérologie, Département de médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada

Joel Moss Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

Makoto Murakami Lipid Metabolism Project, The Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo, Japan

Natarajan Muthusamy Department of Hematology, Ohio State Comprehensive Cancer Center, Ohio State University, Columbus, OH, USA

Maria Aparecida Nagai Disciplina de Oncologia, Departamento de Radiologia da Faculdade de Medicina da, Centro de Investigação Translacional em Oncologia, Laboratório de Genética Molecular, Instituto do Cancer de São Paulo, Universidade de São Paulo, São Paulo, Brazil

Tetsuji Naka Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan

Hiroyuki Nakanishi Department of Molecular Pharmacology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

Tomoki Nakashima Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

Japan Science and Technology Agency (JST), Explorative Research for Advanced Technology (ERATO) Program, Takayanagi Osteonetwork Project, Bunkyo-ku, Tokyo, Japan

Javier Naval Departamento de Bioquímica, Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain

Scott A. Ness Department of Molecular Genetics & Microbiology, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Ryan H. Newton Institute for Immunology and Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Hoa B. Nguyen Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, USA

Natalie M. Niemi Van Andel Institute Graduate School, Van Andel Research Institute, Grand Rapids, MI, USA

Center for Cancer Genomics and Quantitative Biology, Van Andel Research Institute, Grand Rapids, MI, USA

Volker Nimmrich Neuroscience Research, GPRD, Abbott, Ludwigshafen, Germany

Hiroshi Nishihara Graduate School of Medicine, Laboratory of Translational Pathology, Hokkaido University, Sapporo, Japan

Karen Nolan UCD Diabetes Research Centre, UCD Conway Institute, School of Medicine and Medical Sciences, University College Dublin, Dublin, Ireland

Sofia Nolasco Instituto Gulbenkian de Ciência, Oeiras, Portugal

Escola Superior de Tecnologia da Saúde de Lisboa, Lisboa, Portugal

Instituto de Investigação Científica Tropical CIISA, Lisboa, Portugal

Mio Nonaka Department of Neurochemistry, The University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan

Christopher A. O'Callaghan Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Masato Okada Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

Shinichiro Oku Division of Membrane Physiology, Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, Aichi, Japan

Department of Physiological Sciences, School of Life Science, The Graduate University for Advanced Studies (SOKENDAI), Aichi, Japan

Hiroyuki Okuno Department of Neurochemistry, The University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan

Martin Olivier Departments of Medicine and of Microbiology and Immunology, The Research Institute of The McGill University Health Centre, McGill University, Montréal, Québec, Canada

Michael F. Olson Molecular Cell Biology Laboratory, Beatson Institute for Cancer Research, Glasgow, UK

Kenji Omori Advanced Medical Research Laboratories, Mitsubishi Tanabe Pharma Corporation, Yokohama, Kanagawa, Japan

Dana Onica Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

Laura O'Regan Department of Biochemistry, University of Leicester, Leicester, UK

Alberto Ortiz Unidad de Dialisis, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain

Siân-Eleri Owens Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales, UK

Thomas L. Pallone Department of Medicine, Division of Nephrology, University of Maryland, Baltimore, Baltimore, MD, USA

Søren Paludan Sheikh Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, University of Southern Denmark, Odense, Denmark

John Papaconstantinou Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA

Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX, USA

Pooja Parameswaran Department of Pharmacology, University of Minnesota, Minneapolis, Minneapolis, MN, USA

Morag Park Goodman Cancer Research Centre, McGill University, Montréal, Canada

Department of Biochemistry, McGill University, Montréal, Quebec, Canada

Department of Medicine, McGill University, Montréal, Canada

Department of Oncology, McGill University, Montréal, Canada

Gibeom Park Department of Biomedical Sciences, Seoul National University College of Medicine, Jongnogu, Seoul, South Korea

Haein Park Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, Bronx, NY, USA

Woong-Yang Park Department of Biomedical Sciences, Seoul National University College of Medicine, Jongnogu, Seoul, South Korea

Federico Paroni Center for Biomolecular Interactions, University of Bremen, Bremen, Germany

Surojit Paul Neurology, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Sudesh Pawaria Department of Immunology, University of Pittsburgh, Pittsburgh, PA, USA

Andrew J. Payne Department of Ophthalmology, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Andrew A. Peden Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

Kimberly J. Perry Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

João Bosco Pesquero Biophysics Department, Universidade Federal de São Paulo, São Paulo, Brazil

Yuri K. Peterson Department of Pharmaceutical and Biomedical Sciences, The Medical University of South Carolina, Charleston, SC, USA

Carlo Petosa Institut de Biologie Structurale Jean-Pierre Ebel, UMR 5075 (CEA/CNRS/Université Joseph Fourier), Grenoble, France

Pavel P. Philippov Department of Cell Signalling, A.N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, Moscow, Russia

Paula A. Pino Department of Biology and South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, TX, USA

Joshua T. Piotrowski Department of Immunology, College of Medicine, Mayo Clinic, Rochester, MN, USA

Randy Y. C. Poon Division of Life Science, Hong Kong University of Science and Technology, Kowloon, Hong Kong

Jacques Pouysségur Institute of Developmental Biology and Cancer, CNRS UMR6543, Université de Nice, Nice, France

Jonay Poveda Unidad de Dialisis, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain

David R. Poyner School of Life and Health Sciences, Aston University, Birmingham, UK

Marco A. M. Prado Robarts Research Institute, University of Western Ontario, London, ON, Canada

John J. Priatel Department of Pathology and Laboratory Medicine, Child and Family Research Institute, University of British Columbia, Vancouver, BC, Canada

Claude Prigent CNRS, UMR 6061, Institut Génétique et Développement de Rennes, Rennes, France

Université Rennes 1, UEB, IFR, 140, Faculté de Médecine, Rennes, France

Shiri Procaccia Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel

Rainer Prohaska Max F. Perutz Laboratories (MFPL), Medical University of Vienna, Vienna, Austria

Véronique Proux-Gillardeaux Membrane Traffic in Neuronal & Epithelial Morphogenesis, INSERM ERL U950, Paris, France

Program in Development & Neurobiology, Jacques Monod Institute, CNRS UMR7592, Paris, France

University Denis Diderot–Paris 7, Paris, France

Membrane Traffic in Neuronal and Epithelial Morphogenesis; Program in Development, Jacques Monod Institute CNRS UMR7592, Equipe INSERM ERL U950, CNRS & Univ. Denis Diderot/Paris 7, Paris, Cedex 13, France

Lawrence A. Quilliam Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, USA

Leonard Rabinow Université Paris Sud, CNRS UMR 8195, Orsay, France

Pia Ragno Department of Chemistry and Biology, University of Salerno, Fisciano, Salerno, Italy

Joe W. Ramos The Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI, USA

Chotirat Rattanasinchai Cell & Molecular Biology Program, Michigan State University, East Lansing, MI, USA

Jens Rauch Systems Biology Ireland, University College Dublin, Dublin, Ireland

Moran Rawet-Slobodkin Department of Biological Chemistry Ullman Building, Weizmann Institute of Science, Rehovot, Israel

Prabir Ray Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, and Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Anuradha Ray Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, and Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

David Reboutier CNRS, UMR 6061, Institut Génétique et Développement de Rennes, Rennes, France

Université Rennes 1, UEB, IFR, 140, Faculté de Médecine, Rennes, France

Annegret Reinhold Institute for Molecular and Clinical Immunology, Otto von Guericke University Magdeburg, Magdeburg, Germany

Carlos Eduardo Repeke OSTEOimmunology lab, Department of Biological Sciences, School of Dentistry of Bauru, São Paulo University – FOB/USP, Bauru, SP, Brazil

Michael Reschen Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Nicolas Reymond Randall Division of Cell and Molecular Biophysics, King's College London, London, UK

Anne J. Ridley Randall Division of Cell and Molecular Biophysics, King's College London, London, UK

Steven Ringquist Division of Immunogenetics, Department of Pediatrics, Rangos Research Center, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Anne C. Rintala-Dempsey Department of Biochemistry, The University of Western Ontario, London, ON, Canada

Bernard Robaye Institute of Interdisciplinary Research, School of Medicine, Université Libre de Bruxelles, Gosselies

David D. Roberts Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Aaron M. Robitaille Growth and Development, Biozentrum, University of Basel, Basel, Switzerland

Melissa Rodriguez Center for Cancer and Stem Cell Biology, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, TX, USA

Marco A. Romano-Silva INCT de Medicina Molecular; Departamento de Saúde Mental, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Daniela V. Rosa INCT de Medicina Molecular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Yvonne Rosenstein Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Anne Roumier Université Pierre et Marie Curie, Institut du Fer à Moulin, Paris, France

Liat Rousso-Noori Department of Molecular Genetics, Arnold R. Meyer Institute of Biological Sciences, The Weizmann Institute of Science, Rehovot, Israel

Philippe P. Roux Department of Pathology and Cell Biology, Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, Montreal, QC, Canada

Jennifer L. Rudolph Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Rosamaria Ruggieri Oncology & Cell Biology, The Feinstein Institute for Medical Research, Manhasset, NY, USA

Jürgen Ruland Laboratory for Signaling in the Immune System, Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany

Third Medical Department, Institute for Molecular Immunology, Technical University of Munich, Klinikum rechts der Isar, Munich, Germany

Constance E. Runyan Department of Pediatrics, Northwestern University, Chicago, IL, USA

Guy A. Rutter Section of Cell Biology, Division of Diabetes, Endocrinology and Metabolism, Faculty of Medicine, Imperial College London, South Kensington, London, UK

Kaitlyn Ryan Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, USA

Fahri Saatcioglu Department of Molecular Biosciences, University of Oslo, Oslo, Norway

Julie D. Saba Center for Cancer Research, Children's Hospital Oakland Research Institute, Oakland, CA, USA

Sarah Sabir Department of Biochemistry, University of Leicester, Leicester, UK

Rachna Sadana Department of Natural Sciences, University of Houston-Downtown, Houston, TX, USA

Anthony John Sadler Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Clayton, VIC, Australia

Navdeep Sahota Department of Biochemistry, University of Leicester, Leicester, UK

Zuzana Saidak Laboratoire de Biochimie, Inserm ERI12 – EA4292, Université de Picardie Jules Verne (UPJV), Amiens Cedex, France

Vicência Sales Biophysics Department, Universidade Federal de São Paulo, São Paulo, Brazil

Michael W. Salter Program in Neurosciences & Mental Health, The Hospital for Sick Children, Toronto, ON, Canada

Ulrich Salzer Max F. Perutz Laboratories (MFPL), Medical University of Vienna, Vienna, Austria

Asanga Samarakoon Department of Microbiology and Immunology, Life Sciences Institute, 2350 Health Sciences Mall, University of British Columbia, Vancouver, BC, Canada

Michael S. Samuel Centre for Cancer Biology, SA Pathology, Adelaide, SA, Australia

Francisco Sánchez-Madrid Servicio de Inmunología, Hospital Universitario La Princesa Instituto Investigación Sanitaria Princesa, Madrid, Spain

Maria D. Sanchez-Niño IdI-Paz, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain

Monserrat Alba Sandoval-Hernandez Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Jeff M. Sands Renal Division, Emory University Woodruff Memorial Research Building, Atlanta, GA, USA

Eugenio Santos Centro de Investigación del Cáncer, IBMCC (CSIC/USAL), University of Salamanca, Salamanca, Spain

Leopoldo Santos-Argumedo Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), México, DF, México

Ana B. Sanz IdI-Paz, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain

Marta Sanz-García Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Takaya Satoh Department of Biological Science, Graduate School of Science, Osaka Prefecture University, Sakai, Osaka, Japan

Takashi Sasaki Advanced Medical Research Laboratories, Mitsubishi Tanabe Pharma Corporation, Yokohama, Kanagawa, Japan

Amy E. Saunders Department of Microbiology and Immunology, Life Sciences Institute, 2350 Health Sciences Mall, University of British Columbia, Vancouver, BC, Canada

Wolfgang W. A. Schamel Max Planck Institute of Immunobiology and Faculty of Biology, Biology III, University of Freiburg, Freiburg, Germany

Centre for Biological Signalling Studies (BIOSS), University of Freiburg, Freiburg, Germany

Centre of Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Freiburg, Germany

Tobias M. H. Schenk Laboratory of Signal Transduction, Inositol Signaling Section, National Institute of Environmental Health Sciences (NIEHS/NIH), NC, USA

William Schiemann Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

Gerd Schmitz Institute of Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany

H. William Schnaper Department of Pediatrics, Northwestern University, Chicago, IL, USA

Burkhart L. Schraven Institute for Molecular and Clinical Immunology, Otto von Guericke University Magdeburg, Magdeburg, Germany

Annette Schürmann Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Giorgio Scita IFOM, Milan, Italy

Dipartimento di Medicina, Chirurgia ed Odontoiatria, Universita' degli Studi di Milano, Milan, Italy

John D. Scott Department of Pharmacology, Howard Hughes Medical Institute, University of Washington School of Medicine, Seattle, WA, USA

Matthew N. Seaman Department of Clinical Biochemistry, Cambridge Institute for Medical Research, Cambridge, UK

Benedict Seddon Division of Immune Cell Biology, MRC National Institute for Medical Research, Mill Hill, London, UK

Rony Seger Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel

Ponniah Selvakumar Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Cancer Research Unit, Saskatchewan Cancer Agency, Saskatoon, SK, Canada

Payel Sen Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, IL, USA

Banibrata Sen Department of Thoracic/Head and Neck Medical Oncology, Unit 432, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Ameet S. Sengar Program in Neurosciences & Mental Health, The Hospital for Sick Children, Toronto, ON, Canada

Jean Sévigny Department of Microbiology-Infectiology and Immunology, Centre de Recherche en Rhumatologie et Immunologie, CHUQ Research Centre, Université Laval, Quebec, QC, Canada

Ana Sevilla Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Patrick M. Sexton Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, VIC, Australia

Rajendra K. Sharma Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Cancer Research Unit, Saskatchewan Cancer Agency, Saskatoon, SK, Canada

Gary S. Shaw Department of Biochemistry, The University of Western Ontario, London, ON, Canada

Stephen B. Shears Laboratory of Signal Transduction, Inositol Signaling Section, National Institute of Environmental Health Sciences (NIEHS/NIH), NC, USA

Jian-Bing Shen Calhoun Cardiovascular Center, University of Connecticut Health Center, Farmington, CT, USA

Amanda R. Sherwood Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Enda Shevlin Department of Biology, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

Hoon Shim Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA, USA

Takashi Shimizu Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

Marina Tiemi Shio Department of Microbiology and Immunology, The Research Institute of The McGill University Health Centre, McGill University, Montréal, Québec, Canada

Kazuhiro Shiozaki Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan

Department of Microbiology, University of California, Davis, Davis, CA, USA

Vladimir P. Shirinsky Institute of Experimental Cardiology, Russian Cardiology Research Center of the Ministry of Health, Moscow, Russia

David P. Siderovski Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Elcia Maria Silveira OSTEOimmunology lab, Department of Biological Sciences, School of Dentistry of Bauru, São Paulo University – FOB/USP, Bauru, SP, Brazil

Bernhard B. Singer Department of Anatomy, University Hospital Essen, University Duisburg-Essen, Essen, NRW, Germany

Simona Sivori Dipartimento di Medicina Sperimentale (DI.ME.S.) and Centro di Eccellenza per la Ricerca Biomedica, Università di Genova, Genova, Italy

Lomon So Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Helena Soares Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

Instituto Gulbenkian de Ciência, Oeiras, Portugal

Escola Superior de Tecnologia da Saúde de Lisboa, Lisboa, Portugal

Florentina Soto Department of Ophthalmology & Visual Sciences, Washington University in St. Louis, St. Louis, MO, USA

David R. Soto-Pantoja Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Erica L. Southgate Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL, USA

Bruno R. Souza Department of Cell & Systems Biology, Centre for the Analysis of Genome Evolution and Function, University of Toronto, Toronto, ON, Canada

Annina C. Spilker Goodman Cancer Research Centre, McGill University, Montréal, Canada

Department of Biochemistry, McGill University, Montréal, Quebec, Canada

Laura Spinelli Division of Cell Signalling and Immunology, College of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dundee, UK

Nicole A. St. Denis Centre for Systems Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada

A. Keith Stewart Division of Hematology-Oncology, Mayo Clinic, Scottsdale, AZ, USA

Peter Storz Department of Cancer Biology, Mayo Clinic Comprehensive Cancer Center, Jacksonville, FL, USA

Jennifer L. Stow Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

Lisa Stowers Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA

Julia Strebovsky Department of Infectious Diseases, Medical Microbiology and Hygiene, University of Heidelberg, Heidelberg, Germany

Emanuel E. Strehler Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, USA

Roland K. Strong Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Justin Sturge Division of Cancer, Department of Surgery & Cancer, Imperial College London, Hammersmith Hospital, London, UK

Hiroko Sugiura Department of Neuropharmacology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan

Sarah Sullivan Department of Immunology, Duke University Medical Center, Durham, NC, USA

Roger J. Summers Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia

Reshma Sundar Department of Medical Biosciences, Department of Pathology, Umeå University, Umeå, Sweden

Balaji Sundararaman San Diego State Heart Institute, San Diego State University, San Diego, CA, USA

Eun-Sil Sung Department of Molecular Science and Technology, Ajou University, Yeongtong-Gu, Suwon, South Korea

Mark A. Sussman SDSU Heart Institute, San Diego State University Biology Department, San Diego, CA, USA

Kornelia Molnarne Szauter John A. Burns School of Medicine, University of Hawaii, Honolulu, HI, USA

Tomohiko Taguchi Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

Yoshimi Takai Division of Molecular and Cellular Biology, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, Kobe, Japan

Ken Takamatsu Department of Physiology, Toho University School of Medicine, Ohta-ku, Toyko, Japan

Hiroshi Takayanagi Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

Japan Science and Technology Agency (JST), Explorative Research for Advanced Technology (ERATO) Program, Takayanagi Osteonetwork Project, Bunkyo-ku, Tokyo, Japan

Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Bunkyo-ku, Tokyo, Japan

Ken-Ichi Takemaru Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, USA

Masaharu Takigawa Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Okayama, Japan

Gregory G. Tall Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY, USA

Fuyuhiko Tamanoi Molecular Biology Institute, Los Angeles, CA, USA

Department of Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles, CA, USA

Hisashi Tatebe Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan

Gregory A. Taylor Departments of Medicine; Molecular Genetics and Microbiology; and Immunology, Duke University, Durham VA Medical Center, Geriatric Research, Education, and Clinical Center, Durham, NC, USA

Colin W. Taylor Department of Pharmacology, University of Cambridge, Cambridge, UK

Lars Terenius Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden

Martina Tesikova Department of Molecular Biosciences, University of Oslo, Oslo, Norway

Marc J. Tetel Neuroscience Program, Wellesley College, Wellesley, MA, USA

Masafumi Tetsuka Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

Kenneth D. Tew Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

Aiysha Thompson Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales, UK

Priyanka Tibarewal Division of Cell Signalling and Immunology, College of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dundee, UK

Pamela Y. Ting Department of Biological Chemistry, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Hiroshi Tokuo Boston Biomedical Research Institute, Watertown, MA, USA

Danyelle M. Townsend Dept of Pharmaceutical and Biomedical Sciences, Medical University of South Carolina, Charleston, SC, USA

Maria Traver Departments of Medicine; Molecular Genetics and Microbiology; and Immunology, Duke University, Durham VA Medical Center, Geriatric Research, Education, and Clinical Center, Durham, NC, USA

Ana Paula Favaro Trombone Pathology lab, Instituto Lauro de Souza Lima - ILSL, Bauru, SP, Brazil

Kevin Tsai Department of Pathology and Laboratory Medicine, Child and Family Research Institute, University of British Columbia, Vancouver, BC, Canada

Christos Tsatsanis Department of Clinical Chemistry, University of Crete Medical School, Heraklion, Crete, Greece

Grégory Tufo INSERM UMR-S 769, Université Paris-Sud XI, Châtenay-Malabry, France

Felipe Francisco Tuon Division of Infectious and Parasitic Diseases, Hospital Universitario Evangelico de Curitiba, Curitiba, PR, Brazil

Jacob P. Turowec Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

Alvaro C. Ucero IIS-Fundacion Jimenez Diaz and Universidad Autonoma de Madrid, Madrid, Spain

Daisuke Urano Department of Biology, University of North Carolina, Chapel Hill, NC, USA

Mikko Uusi-Oukari Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku, Turku, Finland

Richard Vaillancourt Goodman Cancer Research Centre, McGill University, Montréal, Canada

Department of Biochemistry, McGill University, Montréal, Quebec, Canada

Alberto Valbuena Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Rut Valdor Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA

Filip Van Petegem Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

Frans van Roy Molecular Cell Biology Unit, Department for Molecular Biomedical Research, VIB and Ghent University, Ghent, Belgium

Andrea Varga Department of Microbiology and Immunobiology, Center for Molecular Biology, University of Vienna, Max F. Perutz Laboratories, Vienna, Austria

Naghmeh Varghayee Clinical Pharmacology Unit, Department of Medicine Austin Health/Northern Health, University of Melbourne, Heidelberg, VIC, Australia

Martha Vaughan Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

Marta Vázquez-Cedeira Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Francisco M. Vega Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Andreia Espíndola Vieira OSTEOimmunology Lab, Department of Biological Sciences, School of Dentistry of Bauru, São Paulo University – FOB/USP, Bauru, SP, Brazil

Dimitra Virla Molecular & Cellular Biology Laboratory, Division of Basic Sciences, University of Crete Medical School, Heraklion, Crete, Greece

Sandhya S. Visweswariah Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka, India

Maurizio Vitadello CNR-Institute of Neuroscience, Padova section, Padova, Italy

Niels Volkmann Bioinformatics and Systems Biology Program, Sanford-Burnham Medical Research Institute, La Jolla, CA, USA

Vladana Vukojević Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden

Craig M. Walsh Institute for Immunology and Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Angela Wandinger-Ness Department of Pathology and Cancer Center, MSC08-4640, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Yongping Wang Department of Pathology, University of Colorado Denver, Aurora, CO, USA

Stephen G. Ward Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, Somerset, UK

Heather H. Ward Department of Pathology and Cancer Center, MSC08-4640, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Nimmi R. Weerasinghe Division of Cell Signalling and Immunology, College of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dundee, UK

Li-Na Wei Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN, USA

Brian M. Weist Institute for Immunology and Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Emma T. van der Westhuizen Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montréal, QC, Canada

Laura M. Westrate Van Andel Graduate School, Grand Rapids, MI, USA

Van Andel Research Institute, Grand Rapids, MI, USA

John H. White Department of Physiology, McGill University McIntyre Medical Sciences Building, Montréal, Québec, Canada

Carol Wicking Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

Janet A. Willment Institute for Medical Sciences, Aberdeen, UK

G. Sebastiaan Winkler School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK

James Woodgett Samuel Lunenfeld Research Institute, Mount Sinai Hospital & Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada

Hsin-Jung Wu Department of Immunobiology, University of Arizona, Tuscon, AZ, USA

Zhihui Xie Laboratory of Allergic Diseases, National Institute of Allergy and infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Bin Xu Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Jian Xu Child Study Center, School of Medicine, Yale University, New Haven, CT, USA

Nan Yagishita-Kyo Department of Neurochemistry, The University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan

Kanato Yamagata Department of Neuropharmacology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan

Department of Pharmacology, Shukutoku University, Chuo–ku, Chiba, Japan

Sheng-Wei Yang Department of Chemistry, Tunghai University, Taichung, Taiwan

Jay Yang Department of Anesthesiology, University of Wisconsin, Madison, WI, USA

Xiang-Jiao Yang The Rosalind & Morris Goodman Cancer Research Center, McGill University, Montréal, Québec, Canada

Departments of Biochemistry and Anatomy & Medicine, McGill University Health Center, Montréal, Québec, Canada

Departments of Medicine, McGill University Health Center, Montréal, Québec, Canada

Shin Yasuda Department of Neuropharmacology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan

Richard D. Ye Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL, USA

Byong Kwon Yoo Emory University School of Medicine, Division of Digestive Diseases, Department of Medicine, Atlanta, GA, USA

Linya You The Rosalind & Morris Goodman Cancer Research Center, McGill University, Montréal, Québec, Canada

Departments of Medicine, McGill University Health Center, Montréal, Québec, Canada

Howard A. Young Laboratory of Experimental Immunology, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA

C. Chris Yun Emory University School of Medicine, Division of Digestive Diseases, Department of Medicine, Atlanta, GA, USA

Michisuke Yuzaki Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Detina Zalli Department of Biochemistry, University of Leicester, Leicester, UK

Cédric Zeltz Department of Biomedicine, University of Bergen, Bergen, Norway

Evgeni Yu Zernii Department of Cell Signalling, A.N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, Moscow, Russia

Jiang-Ping Zhang The Rosalind & Morris Goodman Cancer Research Center, McGill University, Québec, Canada

Department of Medicine, McGill University Health Center, Montréal, Québec, Canada

Weiguo Zhang Department of Immunology, Duke University Medical Center, Durham, NC, USA

Min Zhang Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao, China

Chunyan Zhao Department of Biosciences and Nutrition, Novum, Karolinska Institutet, Huddinge, Sweden

Yixing Zhou Laboratory of Signal Transduction, Inositol Signaling Section, National Institute of Environmental Health Sciences (NIEHS/NIH), NC, USA

Renping Zhou Department of Chemical Biology, Susan Lehman Cullman Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA

Yuan Xiao Zhu Division of Hematology-Oncology, Mayo Clinic, Scottsdale, AZ, USA

Georgios Zilidis Division of Cell Signalling and Immunology, College of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dundee, UK

0–9

2-, 3pk (MAPKAPK-2, -3)

► p38 MAPK Family of Signal Transduction Proteins

5-HT

► The 5-HT_3 Receptor

5-HT 2B Receptor

► 5-Hydroxytryptamine Receptor 2B

5-HT(2B)

► 5-Hydroxytryptamine Receptor 2B

5-HT1C

► 5-Hydroxytryptamine Receptor 2C

5-HT2B

► 5-Hydroxytryptamine Receptor 2B

5-HT-2B

► 5-Hydroxytryptamine Receptor 2B

5-HT2C

► 5-Hydroxytryptamine Receptor 2C

5-Hydroxytryptamine

► The 5-HT_3 Receptor

5-Hydroxytryptamine (Serotonin) Receptor 2B

► 5-Hydroxytryptamine Receptor 2B

5-Hydroxytryptamine 2B Receptor

► 5-Hydroxytryptamine Receptor 2B

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

5-Hydroxytryptamine Receptor 2B

Stéphane Doly, Silvina Laura Diaz, Arnauld Belmer, Anne Roumier and Luc Maroteaux
Université Pierre et Marie Curie, Institut du Fer à Moulin, Paris, France

Synonyms

5-Hydroxytryptamine (serotonin) receptor 2B; 5-Hydroxytryptamine 2B receptor; 5-HT 2B receptor; 5-HT(2B); 5-HT-2B; 5-HT2B; Serotonin receptor 2B

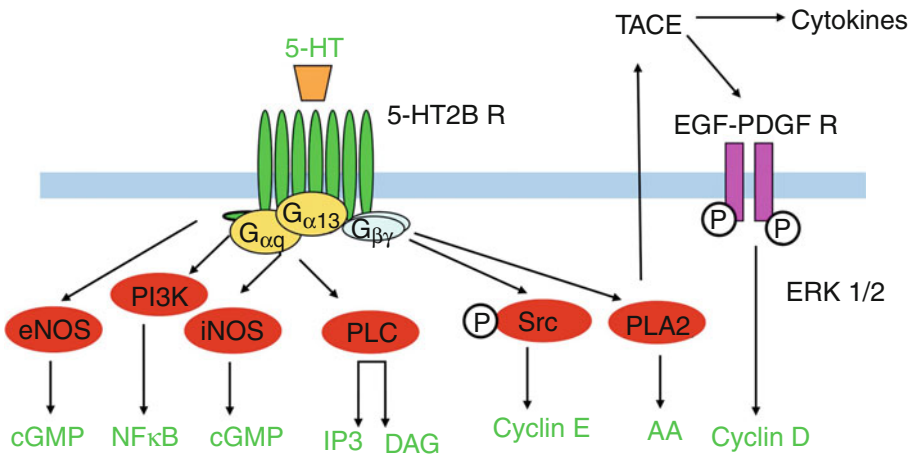
Historical Background

Because of its exquisite sensitivity to serotonin, the rat stomach fundus was used as a bioassay for serotonin before the development of more quantitative analytical assays for this biogenic amine (Vane 1957). Although the potency for the contractile effects of serotonin has been known for a long time, the receptors mediating such a response have eluded definitive characterization for long time. Pharmacological studies attempting to characterize the contractile serotonergic receptor in the rat stomach fundus documented the similarity to the 5-HT_{2C} receptor. In the absence of detectable 5-HT_{2C} receptor mRNA in the rat stomach fundus, only molecular cloning allowed the identification of a new receptor in 1992 in rat and mouse, and later (1994) human species, called 5-HT_{2B} receptor. Subsequent pharmacological characterization of this receptor subtype in different species including humans identified species difference in its pharmacology and confirmed the proximity of this pharmacology to that of 5-HT_{2C} receptors.

Pharmacological analysis has shown that the 5-HT_{2B} receptor displays high-affinity binding to its endogenous ligand serotonin (Kd ~10 nM), a value significantly different from that for the 5-HT_{2A} receptor. 5-HT_{2B} receptors are the targets of many 5-HT2 nonselective compounds, metabolites of therapeutic compounds, and drugs of abuse. Agonists include nordexfenfluramine (metabolite of dexfenflamine), methylergonovine (metabolite of methysergide), and MDA (metabolite of MDMA). Tryptamine, α-methyl-serotonin, BW 723C86: 1-methyl-2-[5-(2-thienylmethoxy)-1H-indole-3-yl]ethylamine hydrochloride, D-norfenfluramine, Ro 60-0175: 2(S)-1-(6-chloro-5-fluoro-1H-indol-1-yl)-2-propanamine fumarate, and LSD all exhibit the highest potencies for 5-HT_{2B}. BW 723C86 has been reported to have over a 10-fold higher selectivity for the 5-HT_{2B} receptor than the 5-HT_{2C} receptor and a 100-fold higher selectivity than the 5-HT_{2A} receptor. DOI is a nearly full agonist at 5-HT_{2B} receptors with similar affinity to 5-HT_{2A} and 5-HT_{2B} receptors. Nonselective 5-HT2 receptor antagonists such as ritanserin, metergoline, and LY 53857 antagonize 5-HT_{2B} receptor mediated effects. Also, the adrenergic α2 receptor antagonists yohimbine and rauwolscine are potent antagonists and have a low affinity for the 5-HT_{2C} and 5-HT_{2A} receptor sites. The first highly selective antagonists have been recently reported, LY266097: 1-(2-chloro-3,4-dimethoxybenzyl)-6-methyl-1,2,3,4-tetrahydro-9Hpyrido[3,4-b]indole hydrochloride, has a pKi of 9.7 for the human cloned 5-HT_{2B} receptor with a 100-fold greater selectivity for this receptor than the human 5-HT_{2C} and 5-HT_{2A} sites. SB 204741: N-(1-methyl-5-indolyl)-N′-(3-methyl-5-isothiazolyl)urea, has been reported as a selective 5-HT_{2B} receptor antagonist with lower potency (Ki around 100 nM). RS 127445: 2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine was found to have sub-nanomolar affinity for the 5-HT_{2B} receptor (pKi = 9.5) and a 1,000-fold selectivity for this receptor as compared to numerous other receptor and ion channel binding sites and appears to be the most selective, high affinity 5-HT_{2B} receptor antagonist currently available. The tetrahydro-β-carboline, LY272015: 6-chloro-5-methyl-N-(5-quinolinyl)-2,3-dihydro-1H-indole-1-carboxamide is also a selective and potent antagonist. The SB 206553: 5-methyl-N-(3-pyridyl)-1,2,3,5-tetrahydrobenzo[1,2-b:4,5-b′]dipyrrole-1-carboxamide and SB 215505: 6-chloro-5-methyl-N-(5-quinolinyl)-2,3-dihydro-1H-indole-1-carboxamide behave as mixed 5-HT_{2C}/5-HT_{2B} receptor antagonist.

Like 5-HT_{2A} and 5-HT_{2C} receptors, the 5-HT_{2B} receptor when expressed in transfected cells can induce GTPase activation and inositol 1,4,5-trisphosphate production upon agonist stimulation. GTPase activation is blocked by antibodies against Gαq/11, but not by pertussis or cholera toxins or by anti-Gαi or anti-Gαs antibodies, indicating that this GTPase activation is mediated by the G-protein Gαq/11, but not by Gαs or Gαi. Interestingly, the GTPase activation can also be blocked by anti-β1-4, or -γ2 subunit antibodies. Agonist stimulation of the 5-HT_{2B} receptor causes a rapid

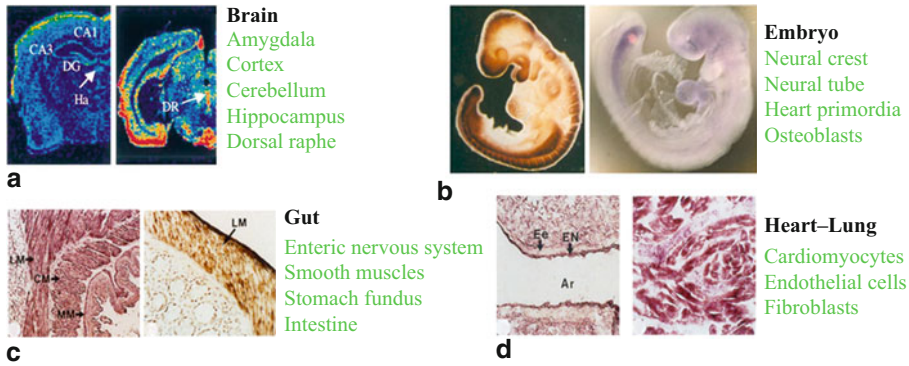
0–9



5-Hydroxytryptamine Receptor 2B, Fig. 1 Summary of some of the identified transduction pathways that can be used by 5-HT2B receptors adapted from Nebigil et al. (2000b), Manivet et al. (2000) and Nebigil et al. (2003)

and transient activation of the proto-oncogene product $p21^{ras}$ in response to serotonin, as measured by an increase in GTP bound-Ras. Furthermore, 5-HT_{2B} receptor stimulation activates the mitogen-activated protein kinases (MAPKs) p42mapk/p44mapk (ERK2/ERK1). In addition to a mitogenic action, a transforming activity of serotonin can be mediated by the 5-HT_{2B} receptor as it leads to the formation of foci and to the formation of tumors from foci in nude mice. Moreover, the 5-HT_{2B} receptors-dependent cell cycle progression occurs through retinoblastoma protein hyperphosphorylation and the activation of both cyclin D1/cdk4 and cyclin E/cdk2 kinases. The induction of cyclin D1 expression, but not that of cyclin E, is under mitogen-activated MAPK control, indicating an independent regulation of these two cyclins in 5-HT_{2B} receptor mitogenesis. Moreover, platelet derived growth factor receptor (PDGFR) kinase activity is essential for 5-HT_{2B}-triggered MAPK/cyclin D1, but not cyclin E, signaling pathways. The 5-HT_{2B} receptor activation also increases activity of the Src family kinases c-Src, Fyn, and c-Yes. Strikingly, c-Src, but not Fyn or c-Yes, is the crucial molecule between the Gq protein-coupled 5-HT_{2B} receptor and the cell cycle regulators. Inhibition of c-Src activity is sufficient to abolish the serotonin-induced: (1) PDGFR tyrosine kinase phosphorylation and MAPK activation, (2) cyclin D1 and cyclin E expression levels, and (3) thymidine incorporation. Thus, c-Src activation by the 5-HT_{2B} receptor controls cyclin E induction and, in concert with the receptor tyrosine kinase PDGFR, induces cyclin D1 expression via the MAPK/ERK pathway (Nebigil et al. 2000b). The 5-HT_{2B} receptor can also couple to the phospholipase A2 (PLA2)-mediated release of arachidonic acid. In addition, stimulation of the 5-HT_{2B} receptor triggers intracellular cGMP production through dual activation of constitutive nitric-oxide synthase (cNOS) and inducible NOS (iNOS). The group I PDZ motif at the carboxy terminus of the 5-HT_{2B} receptor is required for recruitment of the cNOS transduction pathways, and iNOS stimulation is under control of the Gα13 pathways. 5-HT_{2B} shares the C-terminal E-X-V/I-S-X-V sequence with 5-HT_{2C} receptors and also binds MUPP1-PDZ domains in vitro (Manivet et al. 2000) (Fig. 1).

Expression of 5-HT_{2B} receptors has been reported in mouse embryos in migrating neural crest cells, neural tube, heart primordia, and somites by immunohistochemistry and in situ hybridization. By Northern blot and immunohistochemistry, 5-HT_{2B} receptor expression has been detected in the stomach fundus, intestine, liver, kidney, pancreas, spleen, and lung, as well as in the myocardium of several species including rats, mice, and humans. In brain, 5-HT_{2B} expression has been reported in the cerebral cortex, cerebellar nuclei and their projection areas, lateral septum, dorsal hypothalamus, and medial amygdala. Expression of the serotonin 5-HT_{2B} receptor mRNA was also confirmed in several brain nuclei including dorsal raphe nuclei by gene expression profiling in the mammalian brain, and by in-situ hybridization. Reports using pharmacological or molecular studies indicate that the 5-HT_{2B}



5-Hydroxytryptamine Receptor 2B, Fig. 2 Major sites of expression of 5-H2B receptors are shown. This includes (**a**) brain as illustrated by in situ hybridization on rat brain sections, (**b**) embryos as illustrated by in situ hybridization and immunohistochemistry on mouse embryos, and (**c**) immmunohistochemistry on mouse gut and (**d**) cardiopulmonary systems, adapted from Choi and Maroteaux (1996), Choi et al. (1997) and Bonaventure et al. (2002)

receptor is also expressed in blood vessels, including both endothelial and smooth muscle cells as well as on the endothelial cells of meningeal blood vessels. In addition, 5-HT_{2B} receptor expression has been detected in spleen, thymus, peripheral blood lymphocytes, spinal cord tissue, superior cervical ganglion, and in the organ of Corti lateral wall and spiral ganglion subfractions. Lately, 5-HT_{2B} receptor expression was also demonstrated in osteocytes, osteoblasts, and a population of periosteal fibroblasts containing osteoblast precursor cells (Fig. 2).

The human 5-hydroxytryptamine receptor $_{2B}$ (5-HT_{2B} receptor) gene (HTR_{2B}; MIM 601122) located in chromosome 2q37.1 (Le Coniat et al. 1996) was identified as a candidate gene in obsessive-compulsive disorder: one single nucleotide polymorphism was described in intron 1, but no evidence for functional mutation was found in the sequenced regions of the 5-HT_{2B} receptor. Novel single nucleotide polymorphisms (SNPs), two of which confer a double-mutant R6G/E42G of the receptor protein that tends to associate with drug abuse, suggest that HTR_{2B} contributes to brain architecture and pathways that are involved in illegal drug reward. More recently, by investigating the 5-HT_{2B} receptor gene in patients who developed pulmonary hypertension after intake of fenfluramine, a heterozygous mutation was found in one female diagnosed with pulmonary hypertension who 5 years earlier had followed a 9-month anorexigen treatment. This heterozygous mutation R393X in the 5-HT_{2B} receptor generates a carboxy terminus-truncated receptor characterized by a switch of coupling to Gα13, reduced nitric oxide synthase (NOS) activation, and an increase in cell proliferation – modifications that are relevant to pathophysiological vasoconstriction.

Physiological Functions in Embryonic Development

The 5-HT_{2B} receptor mRNA was detected in mouse embryos since 8.5 days postcoitum (dpc), whereas there were only low levels of 5-HT_{2A} and no 5-HT_{2C} receptor mRNA expression at this stage. Expression of this receptor was confirmed by pharmacological assays. Teratogenesis by retinoic acid, or blockade of serotonergic signaling by 5-HT2 receptor antagonists, perturbs development, resulting in forebrain and olfactory placode anomalies, malformations of the face, eye, and lens, as well as posterior neural tube and cardiac defects (Bhasin et al. 2004). These pathways may act as opposing signals for common targets in the mouse embryo. The importance of 5-HT_{2B} receptors in cardiac development has been validated by genetic inactivation by homologous recombination of the 5-HT_{2B} receptor gene, which leads to embryonic and neonatal death due to the following defects in the heart: 5-HT_{2B} receptor mutant embryos exhibit a lack of trabeculae in the heart leading to mid-gestation lethality and newborn 5-HT_{2B} receptor mutant mice exhibit cardiac dilation resulting from contractility deficits and structural deficits at the intercellular junctions between cardiomyocytes (Nebigil et al. 2000a). Recent report

showed that serotonin acts downstream of lactogen signaling to stimulate beta cell proliferation. Blocking 5-HT_{2B} signaling in pregnant mice also blocked beta cell expansion and caused glucose intolerance (Kim et al. 2010).

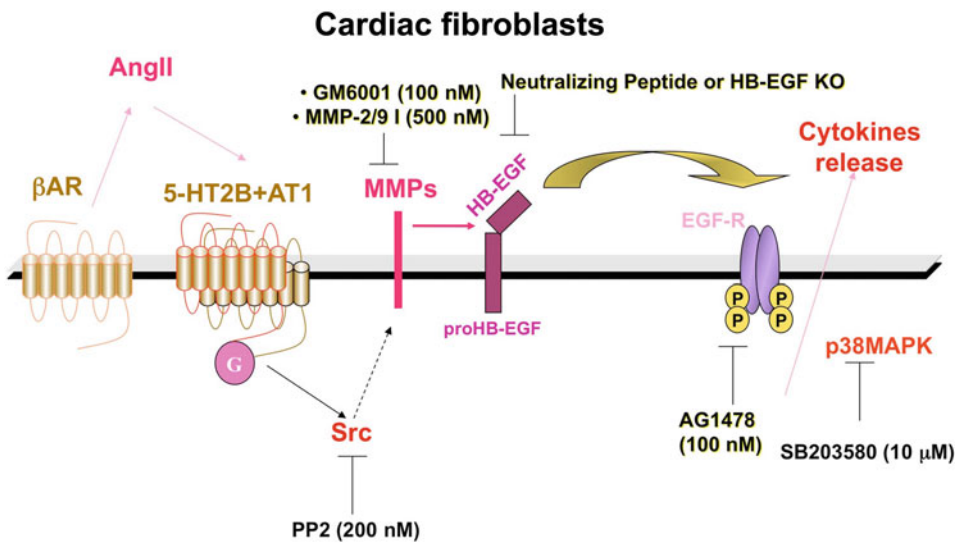
Physiological Cardiopulmonary Functions

Inactivation of the 5-HT_{2B} receptor gene by homologous recombination leads to partial embryonic lethality due to defects in the heart development. Neonates exhibit a second wave of partial lethality due to cardiac dilation resulting from contractility deficits and structural deficits at the intercellular junctions between cardiomyocytes. Echocardiography and electrocardiography studies in animals that past the first week and survive until adulthood, confirm the presence of left ventricular dilation and decreased systolic function. Serotonin, via the 5-HT_{2B} receptor, regulates differentiation and proliferation during development as well as cardiac structure and function in adults (Nebigil et al. 2000a). In adults, 5-HT_{2B} receptors are overexpressed in hearts from patients with congestive heart failure, this overexpression being positively correlated with cytokine and catecholamine plasma levels. The 5-HT_{2B} receptor has been shown functionally coupled to reactive oxygen species synthesis through NAD(P)H oxidase stimulation in neuronal cells and in angiotensin II and isoproterenol-induced cardiac hypertrophy. Recently, 5-HT_{2B} receptor blockade has been shown to prevent the cardiac hypertrophy induced by angiotensin II or isoproterenol infusion. The 5-HT_{2B} receptor has also been shown to be involved in cardiac hypertrophy by acting directly on cardiac myocytes. Serotonin plasma level and serotonin activity are increased in patients with heart failure and in animal studies with cardiac hypertrophy induced by aortic constriction. These findings may indicate that serotonin induces cardiac hypertrophy or heart failure through the 5-HT_{2B} receptor. These findings may have important clinical implications because sympathetic activity, 5-HT_{2B} receptor expression, and plasma cytokines were all increased in patients with heart failure (Jaffre et al. 2009).

In human pulmonary artery endothelial cells, 5-HT_{2B} receptor stimulation elicits a reversible endothelium-dependent relaxation of precontracted arterial ring segments and is associated with an increase in cyclic GMP. The vasoconstrictor serotonergic response induced in the autoperfused rat mesenteric vascular bed in situ seems mainly mediated by 5-HT_{2B} receptor activation. The contraction of the aorta in response to serotonin is primarily mediated by 5-HT_{2A} receptors in normotensive rats; however, it is mediated by both 5-HT_{2A} and 5-HT_{2B} receptors in hypertensive rats. The endothelium-denuded isolated superior mesenteric artery of hypertensive (DOCA-salt) rats displays a marked increase in maximum contraction to 5-HT_{2B} receptor agonists when compared with that of arteries from control rats, confirming that the 5-HT_{2B} receptor plays a greater role in serotonin-induced contraction in arteries from hypertensive rats (Banes and Watts 2003).

A role for serotonin in migraines has been supported by changes in circulating levels of serotonin and its metabolites during the phases of a migraine attack. Correlation of the receptor affinities with the potencies used in migraine prophylaxis showed significant correlations for the 5-HT_{2B} receptor. A migraine headache is thought to be transmitted by the trigeminal nerve from the meninges and their associated blood vessels. The 5-HT_{2B} receptor can activate the release of nitric oxide and induce relaxation of the cerebral arteries and the jugular vein. Various human meningeal tissues express 5-HT_{2B} mRNAs. 5-HT_{2B} receptors located in endothelial cells of meningeal blood vessels may trigger migraine headache through the formation of nitric oxide, which results in the dilation of cerebral blood vessels and the concomitant activation of sensory trigeminovascular afferents, thus initiating the manifestation of head pain. A recent report identified 5-HT_{2B} receptors as a susceptibility gene to migraine (Corominas et al. 2010).

In cardiac fibroblasts, expression of HB-EGF and Src activity are critical for either angiotensin II (AngII) or 5-HT-dependent cytokine release process. Matrix metalloproteinases (MMPs) are responsible for HB-EGF shedding and subsequent EGF-receptor transactivation that is induced by agonists such as AngII or 5-HT. TNF-α converting enzyme (TACE, ADAM-17) was found to control HB-EGF shedding in fibroblasts and 5-HT_{2B} receptors can directly regulate this enzyme activity in neuronal cells. These results also highlighted the importance of p38 but not ERK1/2 pathway for cytokine release. All these findings support that AT-1Rs and 5-HT_{2B} receptors share



5-Hydroxytryptamine Receptor 2B, Fig. 3 Signal transduction mediated by 5-HT2B receptors in cardiac fibroblasts to control the release of hypertrophic cytokines by interacting with AT1 receptors. Inhibitors used to determine this transduction pathway are shown, adapted from Jaffre et al. (2009)

common EGF receptor-dependent signaling pathways in adult cardiac fibroblasts. Blockade of one of the two receptors prevents cytokine release induced by the other receptor, supporting interactions between 5-HT_{2B} receptors and AT-1Rs. Using co-immunolocalization and a pull-down assay, the two receptors were shown to interact in a common cell compartment. Reports have suggested that these receptors exist in heterodimeric complexes that may play a key role in receptor maturation and trafficking to the plasma membrane and/or signaling. Together, these findings are consistent with the hypothesis that AT-1Rs and 5-HT_{2B} receptors exist in common signaling complexes and that they may interact together to regulate hypertrophic factors in heart (Jaffre et al. 2009) (Fig. 3).

Carcinoid heart disease occurs in over 65% of patients with the carcinoid tumors and is characterized by fibrous thickening of cardiac valves, leading ultimately to heart failure. The anorectic compound dexfenfluramine has been reported to be associated with cardiovascular disease, including pulmonary hypertension and valvular heart defects. The valvular changes (myofibroblast proliferation) are histopathologically indistinguishable from those observed in carcinoid disease or after long-term exposure to 5-HT2-preferring ergot drugs (such as ergotamine and methysergide). The mitogenic action of 5-HT2 receptor stimulation could contribute to this lesion. Fenfluramine or its N-de-ethylated metabolite norfenfluramine and other medications known to produce valvular heart disease have preferential high affinities for the 5-HT_{2B} subtype capable of stimulating mitogenesis (with or without accompanying 5-HT_{2A} receptor activation). Norfenfluramine, ergotamine, pergolide, cabergoline, and methysergide metabolite methylergonovine all are partial to full agonists at the 5-HT_{2B} receptor. MDMA and MDA, like norfenfluramine, elicit prolonged mitogenic responses in human valvular interstitial cells via activation of 5-HT_{2B} receptors and have been associated with valvulopathies. Thus, preferential stimulation of valvular 5-HT_{2B} receptors by norfenfluramine, ergot drugs, or serotonin released from carcinoid tumors has been proposed to contribute to valvular fibroplasia in humans (Roth 2007).

Primary pulmonary hypertension is a progressive and often fatal disorder in humans that results from an increase in pulmonary blood pressure associated with abnormal vascular proliferation. In the chronic-hypoxic-mouse model of pulmonary hypertension, the hypoxia-dependent increase in pulmonary blood pressure and lung remodeling are associated with an increase in vascular proliferation, elastase activity, and transforming growth factor-β levels. These parameters are all potentiated by dexfenfluramine treatment. In contrast, hypoxic mice with genetically or

pharmacologically inactive 5-HT_{2B} receptors manifest no change in any of these parameters. Pulmonary hypertension is associated with a substantial increase in 5-HT_{2B} receptor expression in pulmonary arteries in mice and humans. Activation of 5-HT_{2B} receptors is, therefore, a limiting step in the development of pulmonary hypertension (Launay et al. 2002). More recently, the restricted expression of 5-HT_{2B} receptors to bone-marrow cells was shown as necessary and sufficient for pulmonary hypertension to develop via an action at hematopoietic stem cells differentiation (Launay et al. 2012).

Physiological Functions in Central Nervous System

5-HT_{2B} receptor antagonists exhibit anxiolytic-like properties in both the rat social interaction test and two conflict models of anxiety: the rat Geller-Seifter and marmoset conflict test. The presence of neurons expressing 5-HT_{2B} receptor protein has been reported in the medial amygdaloid nucleus of the adult rat brain. Injection of 5-HT_{2B} receptor agonist into the medial amygdaloid nuclei increased the total interaction time of a pair of male rats in the social interaction test without altering locomotor activity, indicative of anxiolytic activity. Activation of 5-HT_{2B} receptors in the medial amygdala induces anxiolysis in the social interaction model but has little effect on behavior in a punished conflict model of anxiety (Kennett et al. 1996).

The "club drug" 3,4-methylenedioxymethamphetamine (MDMA also known as ecstasy) binds preferentially to and reverses the activity of the serotonin transporter, causing release of serotonin stores from nerve terminals. Subsequent activation of postsynaptic 5-HT receptors by released 5-HT has been shown to be critical for the unique psychostimulatory effects of MDMA. Acute pharmacological inhibition or genetic ablation of the 5-HT_{2B} receptor in mice completely abolishes MDMA-induced hyperlocomotion and 5-HT release in nucleus accumbens and ventral tegmental area. Furthermore, the 5-HT_{2B} receptor dependence of MDMA-stimulated release of endogenous 5-HT from superfused midbrain synaptosomes suggests that 5-HT_{2B} receptors act, unlike any other 5-HT receptor, presynaptically to affect MDMA-stimulated 5-HT release. Thus, the 5-HT_{2B} receptor is a novel regulatory component in the actions of MDMA (Doly et al. 2008). However, the role of serotonin–dopamine interactions in the behavioral effects of MDMA remains unclear. 5-HT_{2B}-/- mice do not exhibit behavioral sensitization or conditioned place preference following MDMA injections. In addition, MDMA-induced reinstatement of conditioned place preference after extinction and locomotor sensitization development are each abolished by a 5-HT2) receptor antagonist (RS127445) in wild type mice. Accordingly, MDMA-induced dopamine D1 receptor-dependent phosphorylation of extracellular regulated kinase in nucleus accumbens is abolished in mice lacking functional 5-HT_{2B} receptors. These results underpin the importance of 5-HT_{2B} receptors in the reinforcing properties of MDMA and illustrate the importance of dose-dependent effects of MDMA on serotonin–dopamine interactions (Doly et al. 2009). Moreover, another selective 5-HT_{2B} receptor antagonist LY 266097, which has no influence on basal accumbal and striatal DA outflow, reduces significantly accumbal DA outflow. A significant reduction of basal DA outflow in the nucleus accumbens (NAc) was also observed after i.p. administration of the 5-HT_{2B} receptor antagonist RS 127445. In contrast, the 5-HT_{2B} receptor agonist BW 723C86 had no influence on basal DA outflow in either brain region. The increase in striatal and accumbal DA outflow induced by the 5-HT_{2C}R inverse agonist SB 206553 was unaltered by LY 266097 pretreatment. Conversely, LY 266097 significantly diminished the increase in DA outflow induced by haloperidol or amphetamine in the NAc, but not in the striatum. Amphetamine-induced hyperlocomotion was also attenuated by LY 266097. Thus 5-HT_{2B} receptors exert a facilitatory control on mesoaccumbens DA pathway activity, and may constitute a new target for improved treatment of DA-related neuropsychiatric disorders (Auclair et al. 2010).

Rare alleles can be linked to complex behavior by applying deep sequencing in severely affected individuals. Recently a stop codon in 5-HT_{2B} receptors (Q20*) that is common but exclusive to Finns was found associated with psychiatric diseases marked by impulsivity (Bevilacqua et al. 2010). The receptor with the stop codon is non-functional and may act directly to lead to associated psychiatric diseases, severe impulsive criminal behavior, and suicide. Accordingly, 5-$HT_{2B}^{-/}$ mice display increased

impulsive behaviors validating the Human findings (Bevilacqua et al. 2010). This work is the first documented case of unique behavioral effects of a 5-HT_{2B} in human impulsivity.

The now-banned anorectic molecule, dexfenfluramine, promotes also 5-HT release through a SERT-dependent mechanism, and it has been widely prescribed for the treatment of obesity. Interestingly, the hypophagic response to dexfenfluramine observed in WT mice was eliminated in mice lacking 5-HT_{2B} receptors (5-$HT_{2B}^{-/-}$) or in WT mice treated with the selective 5-HT_{2B} receptor antagonist RS127445. Using microdialysis, we observed that in 5-$HT_{2B}^{-/-}$ awake mice, the dexfenfluramine-induced hypothalamic peak of 5-HT release was strongly reduced. Moreover, 5-HT release was only observed upon dexfenfluramine stimulation of synaptosome preparation from WT but not from 5-$HT_{2B}^{-/-}$ mice. The hypophagia induced by 5-HT_{2C} agonists is detected in 5-$HT_{2B}^{-/-}$ or $SERT^{-/-}$ mice (Banas et al. 2011). These findings strongly support an epistatic role of 5-HT_{2B} receptors on 5-HT_{2C} receptors in dexfenfluramine action with respect to feeding behavior.

The most widely used antidepressants are the so called serotonin-selective reuptake inhibitor (SSRI) since they initially block SERT uptake activity. Whereas blockade of SERT and increase of 5-HT levels are immediately attained after SSRI administration, therapeutic effects are only observed after weeks of treatment. The delay before the onset of clinical effects in depressive individuals appears to rely on the time required for stabilization of monoamine levels and other neuroadaptations, including adult hippocampal neurogenesis. Recent work showed that 5-HT_{2B} receptors are necessary not only for acute behavioral effects of SSRIs (Diaz and Maroteaux 2011), but also for long-term behavioral and neurogenic SSRI effects (Diaz et al. 2012). Conversely, direct agonist stimulation of 5-HT_{2B} receptors induces an SSRI-like response in behavioral and neurogenic assays. These work showed also that this receptor is expressed by raphe serotonergic neurons, and that the SSRI-induced increase in hippocampal extracellular 5-HT concentration is strongly reduced in the absence of functional 5-HT_{2B} receptors. The 5-HT_{2B} receptor thus positively regulates serotonergic neurons and is required for the therapeutic actions of SSRIs (Diaz et al. 2012). As novel antidepressants are being developed to both ameliorate efficacy of treatments and increase the number of responders, the positive contribution of the 5-HT_{2B} receptor to serotonergic homeostasis should be considered.

Pathophysiological Functions

In Fibrosis

The liver can regenerate its volume after major tissue loss. In a mouse model of liver regeneration, thrombocytopenia, or impaired platelet activity, results in the failure to initiate cellular proliferation in the liver. The expression of 5-HT_{2A} and 5-HT_{2B} subtype serotonin receptors in the liver increased after hepatectomy. Antagonists of 5-HT_{2A} and 5-HT_{2B} receptors inhibited liver regeneration. Liver regeneration was also blunted in mice lacking tryptophan hydroxylase 1, which is the rate-limiting enzyme for the synthesis of peripheral serotonin. This failure of regeneration was rescued by reloading serotonin-free platelets with a serotonin precursor molecule (Lesurtel et al. 2006). Bleomycin-induced fibrosis that was independently shown to be reduced in 5-$HT_{2B}^{-/-}$ mice as for dermal fibrosis (Dees et al. 2011). In the liver, fibrogenic hepatic stellate cells, which are negative regulators of hepatocyte regeneration, are known to express also 5-HT_{2A} and 5-HT_{2B} receptors that may regulate TGFβ-1 and Smads. Antagonism of 5-HT_{2B} attenuates fibrogenesis and improves liver function in disease models in which fibrosis is pre-established and progressive (Ebrahimkhani et al. 2011). These findings suggest that 5-HT_{2B} signaling links cardiovascular damage and platelet activation to tissue remodeling and identify 5-HT_{2B} as a novel therapeutic target to treat fibrotic diseases.

Serotonin is known to increase proliferation and collagen synthesis by fibroblasts. Serotonin concentrations in lung homogenates increased significantly over the time course of bleomycin-induced fibrosis, with a maximum at day 7. The expression of serotonin receptors 5-HT_{2A} and 5-HT_{2B} increased also in the lung after bleomycin treatment. Blockade of either 5-HT_{2A} receptors by ketanserin or 5-HT_{2B} receptors by SB 215505 reduced bleomycin-induced lung fibrosis, as demonstrated by reduced lung collagen content and reduced procollagen 1 and procollagen 3 mRNA expression. Serotonin antagonists promoted an antifibrotic environment by decreasing the lung mRNA levels of transforming growth factor-beta 1,

connective growth factor, and plasminogen activator inhibitor-1 mRNA, but had minimal effects on lung inflammation as assessed by bronchoalveolar lavage cytology analysis. Interestingly, the 5-HT_{2B} receptor was strongly expressed by fibroblasts in the fibroblastic foci in human idiopathic pulmonary fibrosis samples (Fabre et al. 2008).

In Gut

5-HT_{2B} receptor initially characterized as the receptor responsible for the rat fundus contraction in response to 5-HT (Vane 1957) is expressed in the small intestine as well as the stomach and it is expressed by enteric neurons as well as by muscle. By stimulating 5-HT_{2B} receptors, 5-HT affects the fate of the large subset of enteric neurons that arises after the development of endogenous sources of 5-HT (Fiorica-Howells et al. 2000). High levels of both mRNA and protein for 5-HT_{2B} receptors were found predominantly in the muscle layers and in the myenteric nerve plexus throughout the colon where they caused neuronally mediated contractile responses of longitudinal muscle (Borman et al. 2002). The 5-HT_{2B} receptor appears involved in regulating sensory pathways only under hyperalgesic conditions, suggesting the possible utility of 5-HT_{2B} receptor antagonism in reducing visceral hypersensitivity in patients with irritable bowel syndrome (IBS). 5-HT_{2B} receptors are involved in signaling from the colon in rats in which there is visceral hypersensitivity and a selective 5-HT_{2B} receptor antagonist could have therapeutic potential for the treatment of gut disorders characterized by visceral hypersensitivity (O'Mahony et al. 2010). Interstitial cells of Cajal (ICC) proliferate in adult mice and activation of 5-HT_{2B} receptors results in increased proliferation of ICC in vivo. Furthermore, lack of 5-HT_{2B} receptor signaling reduces the density of ICC networks in mature mice. Targeting 5-HT availability may have the potential to protect ICC networks from injury or can assist in repairing ICC networks after injury (Tharayil et al. 2010).

In Immune System

Positive signals were obtained for 5-HT_{2B} receptor mRNAs in spleen, thymus, and peripheral blood lymphocytes (Stefulj et al. 2000). The neurotransmitter serotonin can be stored at peripheral sites in mast cells and released from this peripheral source upon IgE cross-linking. Study of the expression of serotonergic receptors on human dendritic cells (DC) showed that immature DC preferentially expressed mRNA for the heptahelical 5-HT_{1B}, 5-HT_{1E}, and 5-HT_{2B} receptors, while mature DC mostly expressed 5-HT_4 and 5-HT_7. Moreover, 5-HT_{2B} receptor stimulation induced intracellular Ca2+ mobilization in immature, but not mature, DC. 5-HT stimulates, in a maturation-dependent manner, different signaling pathways in DC. These data point to a role for 5-HT in regulating the immune response at peripheral sites (Idzko et al. 2004).

In Cancer Cells

The 5-HT_{2B} receptor mRNA has been recently identified as a marker of adrenocortical carcinoma using genomewide gene expression profiling (Fernandez-Ranvier et al. 2008). High transcript levels of 5-HT_{2B} receptor gene were also found in uveal melanomas with monosomy 3 (metastatic) but not in tumors with disomy 3, identifying the 5-HT_{2B} receptor expression as a marker for patients with poor prognosis (Tschentscher et al. 2003). Expression of 5-HT_{2B} receptors has been detected in all prostate cancers (Dizeyi et al. 2005) and antagonists of these receptors were reported to inhibit prostatic cancers. The levels of eNOS were found to increase in carcinomas and to promote cancer progression by providing a selective growth advantage to tumor cells. Recent work showed that pharmacologic blockade of the 5-HT_{2B} receptor inhibits implanted cancer tumors in mice. A 5-HT_{2B} receptor antagonist, which suppress the phosphorylation of ERK1/2 and eNOS in HUVEC, also reduced tumor growth by suppressing angiogenesis (Asada et al. 2009).

In Bones

The 5-HT_{2B} receptor contributes in an autocrine manner to osteogenic differentiation and highlights a switch in the downstream targets of the receptor at the terminal stage of the program. The mRNA for the 5-HT_{2B} receptor, which was undetectable in anaplastic osteoblasts, became detectable in differentiated and matured osteoblasts. The differentiation and maturation of osteoblasts might be regulated by the activation of the 5-HT_{2B} receptor under the control of 5-HT inactivation (Hirai et al. 2009). Of interest, 5-HT_{2B} receptor mutant female mice displayed reduced bone density that was significant from age 4 months and had intensified by 12 and 18 months.

This histomorphometrically confirmed osteopenia seems to be due to reduced bone formation because (1) the alkaline phosphatase-positive colony-forming unit capacity of bone marrow precursors was markedly reduced in the 5-5-HT_{2B} receptor mutant mice from 4 to 12 months of age, (2) ex vivo primary osteoblasts from mutant mice exhibited reduced proliferation and delayed differentiation, and (3) calcium incorporation was markedly reduced in osteoblasts after 5-HT_{2B} receptor depletion (produced genetically or by pharmacological inactivation). The 5-HT_{2B} receptor facilitates osteoblast recruitment and proliferation and its absence leads to osteopenia that worsens with age (Collet et al. 2008). A functional link between the 5-HT_{2B} receptor and the activity of the tissue-nonspecific alkaline phosphatase (TNAP) was established using an osteoprogenitor cell line. During osteogenic differentiation, both 5-HT_{2B} receptor and TNAP mRNA translations are delayed with respect to extracellular matrix deposition. Once the receptor is expressed, it constitutively controls TNAP activity at a posttranslational level along the overall period of mineral deposition. Indeed, inhibition of the 5-HT_{2B} receptor intrinsic activity prevents TNAP activation. In contrast, agonist stimulation of the receptor further increases TNAP activity during the initial mineralization phase. Previous observations indicated that the 5-HT_{2B} receptor couples with the phospholipase A2 (PLA2) pathway and prostaglandin production at the beginning of mineral deposition. The 5-HT_{2B} receptor controls also leukotriene synthesis via PLA2 at the terminal stages of differentiation. These two 5-HT_{2B} receptor-dependent eicosanoid productions delineate distinct time-windows of TNAP regulation during the osteogenic program. Finally, prostaglandins or leukotrienes are shown to relay the posttranslational activation of TNAP via stimulation of the phosphatidylinositol-specific phospholipase C. In agreement with the above findings, primary calvarial osteoblasts from 5-HT_{2B} receptor null mice exhibit defects in TNAP activity (Baudry et al. 2010).

Summary

Although expressed quite widely but at low levels, 5-HT_{2B} receptors have been reported to play an important role at cardiac, intestinal, and central levels. Therapeutic interest concerning this receptor subtype has been delayed due to the lack of selective tools, radioligands, antibodies, etc. However, in the last decade, antagonists at 5-HT_{2B} receptors have gained much attention as new targets in therapeutics: 5-HT_{2B}R antagonists are under active investigation for pulmonary hypertension, since they produced encouraging results in animal models, and promising human trials are underway; 5-HT_{2B} receptor may represent a new therapeutic target to reduce cardiac hypertrophy and hypertrophy-induced heart failure. If the safety and efficacy of a 5-HT_{2B} receptor antagonist to treat cardiac hypertrophy or hypertrophy-induced heart failure can be confirmed, application of this kind of therapy to human study may be warranted.

These receptors have also been recognized as off-targets because their agonists play a significant role in the pathogenesis of valvulopathy.

References

Asada M, Ebihara S, Yamanda S, Niu K, Okazaki T, Sora I, Arai H. Depletion of serotonin and selective inhibition of 2B receptor suppressed tumor angiogenesis by inhibiting endothelial nitric oxide synthase and extracellular signal-regulated kinase 1/2 phosphorylation. Neoplasia. 2009;11 (4):408–17.

Auclair A, Cathala A, Sarrazin F, Depoortère R, Piazza P, Newman-Tancredi A, Spampinato U. The central serotonin2B receptor: a new pharmacological target to modulate the mesoaccumbens dopaminergic pathway activity. J Neurochemistry. 2010;114(5):1323–32.

Banas S, Doly S, Boutourlinsky K, Diaz S, Belmer A, Callebert J, Collet C, Launay J-M, Maroteaux L. Deconstructing antiobesity compound action: requirement of serotonin 5-HT2B receptors for dexfenfluramine anorectic effects. Neuropsychopharmacology. 2011;36:423–433.

Banes AK, Watts SW. Arterial expression of 5-HT2B and 5-HT1B receptors during development of DOCA-salt hypertension. BMC Pharmacol. 2003;3(1):12.

Baudry A, Bitard J, Mouillet-Richard S, Locker M, Poliard A, Launay J-M, Kellermann O. The serotonergic 5-HT2B receptor controls tissue non-specific alkaline phosphatase activity in osteoblasts via eicosanoids and phosphatidylinositol-specific phospholipase C. J Biol Chem. 2010;285 (34):26066–73.

Bevilacqua L, Doly S, Kaprio J, Yuan Q, Tikkanen R, Paunio T, Zhou Z, Wedenoja J, Maroteaux L, Diaz S, Belmer A, Hodgkinson C, Dell'Osso L, Suvisaari J, Coccaro E, Rose R, Peltonen L, Virkkunen M, Goldman D. A population-specific HTR2B stop codon predisposes to severe impulsivity. Nature. 2010;468:1061–1066

Bhasin N, LaMantia AS, Lauder JM. Opposing regulation of cell proliferation by retinoic acid and the serotonin2B receptor in the mouse frontonasal mass. Anat Embryol (Berl). 2004;208(2):135–43

Bonaventure P, Guo H, Tian B, Liu X, Bittner A, Roland B, Salunga R, Ma XJ, Kamme F, Meurers B, Bakker M, Jurzak M, Leysen JE, Erlander MG. Nuclei and subnuclei gene expression profiling in mammalian brain. Brain Res. 2002;943(1):38–47.

Borman RA, Tilford NS, Harmer DW, Day N, Ellis ES, Sheldrick RL, Carey J, Coleman RA, Baxter GS. 5-HT2B receptors play a key role in mediating the excitatory effects of 5-HT in human colon in vitro. Br J Pharmacol. 2002;135 (5):1144–51.

Choi D-S, Maroteaux L. Immunohistochemical localisation of the serotonin 5-HT2B receptor in mouse gut, cardiovascular system, and brain. FEBS Lett. 1996;391:45–51.

Choi D-S, Ward S, Messaddeq N, Launay J-M, Maroteaux L. 5-HT2B receptor-mediated serotonin morphogenetic functions in mouse cranial neural crest and myocardiac cells. Development. 1997;124:1745–55.

Collet C, Schiltz C, Geoffroy V, Maroteaux L, Launay J-M, de Vernejoul M-C. The serotonin 5-HT2B receptor controls bone mass via osteoblast recruitment and proliferation. FASEB J. 2008;22(2):418–27.

Corominas R, Sobrido MJ, Ribasés M, Cuenca-León E, Blanco-Arias P, Narberhaus B, Roig M, Leira R, López-González J, Macaya A, Cormand B. Association study of the serotoninergic system in migraine in the Spanish population. Am J Med Genet B Neuropsychiatr Genet. 2010;153B(1):177–84.

Dees C, Akhmetshina A, Reich N, Jüngel A, Beyer C, Krönke G, Zwerina J, Reiter R, Alenina N, Maroteaux L, Gay S, Schett G, Distler O, Distler JHW. Platelet derived serotonin links vascular disease and tissue fibrosis. J Exp Med. 2011;208:961–972.

Diaz S, Doly S, Narboux-Nême N, Fernandez S, Mazot P, Banas S, Boutourlinsky K, Moutkine I, Belmer A, Roumier A, Maroteaux L. 5-HT2B receptors are required for serotonin-selective antidepressant actions. Mol Psychiatry. 2012;17:154–163.

Diaz S, Maroteaux L. Implication of 5-HT2B receptors in the serotonin syndrome. Neuropharmacology. 2011; 61:495–502.

Dizeyi N, Bjartell A, Hedlund P, Tasken KA, Gadaleanu V, Abrahamsson PA. Expression of serotonin receptors 2B and 4 in human prostate cancer tissue and effects of their antagonists on prostate cancer cell lines. Eur Urol. 2005;47 (6):895–900.

Doly S, Valjent E, Setola V, Callebert J, Herve D, Launay JM, Maroteaux L. Serotonin 5-HT2B receptors are required for 3, 4-methylenedioxymethamphetamine-induced hyperlocomotion and 5-HT release in vivo and in vitro. J Neurosci. 2008;28(11):2933–40.

Doly S, Bertran-Gonzalez J, Callebert J, Bruneau A, Banas SM, Belmer A, Boutourlinsky K, Herve D, Launay JM, Maroteaux L. Role of serotonin via 5-HT2B receptors in the reinforcing effects of MDMA in mice. PLoS ONE. 2009;4(11):e7952.

Ebrahimkhani M, Oakley F, Murphy L, Mann J, Moles A, Perugorria M, Ellis E, Burt A, Douglass A, Wright M, White S, Jaffré F, Maroteaux L, Mann D. Stimulating healthy tissue regeneration by targeting the 5-HT2B receptor in chronic liver disease. Nat Med. 2011;17:1668–1673.

Fabre A, Marchal-Sommé J, Marchand-Adam S, Quesnel C, Borie R, Dehoux M, Ruffié C, Callebert J, Launay J-M, Hénin D, Soler P, Crestani B. Modulation of bleomycin-induced lung fibrosis by serotonin receptor antagonists in mice. Eur Respir J. 2008;32(2):426–36.

Fernandez-Ranvier GG, Weng J, Yeh RF, Khanafshar E, Suh I, Barker C, Duh QY, Clark OH, Kebebew E. Identification of biomarkers of adrenocortical carcinoma using genomewide gene expression profiling. Arch Surg. 2008;143(9):841–6. discussion 846.

Fiorica-Howells E, Maroteaux L, Gershon MD. Serotonin and the 5-HT2B receptor in the development of enteric neurons. J Neurosci. 2000;20:294–305.

Hirai T, Tokumo K, Tsuchiya D, Nishio H. Expression of mRNA for 5-HT2 receptors and proteins related to inactivation of 5-HT in mouse osteoblasts. J Pharmacol Sci. 2009;109(2): 319–23.

Idzko M, Panther E, Stratz C, Müller T, Bayer H, Zissel G, Dürk T, Sorichter S, Virgilio F, Geissler M, Fiebich B, Herouy Y, Elsner P, Norgauer J, Ferrari D. The serotoninergic receptors of human dendritic cells: identification and coupling to cytokine release. J Immunol. 2004;172(10): 6011–19.

Jaffre F, Bonnin P, Callebert J, Debbabi H, Setola V, Doly S, Monassier L, Mettauer B, Blaxall BC, Launay JM, Maroteaux L. Serotonin and angiotensin receptors in cardiac fibroblasts coregulate adrenergic-dependent cardiac hypertrophy. Circ Res. 2009;104(1):113–23.

Kennett GA, Bright F, Trail B, Baxter GS, Blackburn TP. Effects of the 5-HT2B receptor agonist, BW 723C86, on three rat models of anxiety. Br J Pharmacol. 1996;117(7): 1443–8.

Kim H, Toyofuku Y, Lynn FC, Chak E, Uchida T, Mizukami H, Fujitani Y, Kawamori R, Miyatsuka T, Kosaka Y, Yang K, Honig G, Hart M, Kishimoto N, Wang J, Yagihashi S, Tecott LH, Watada H, German MS. Serotonin regulates pancreatic beta cell mass during pregnancy. Nat Med. 2010;16 (7):804–8.

Launay JM, Hervé P, Peoc'h K, Tournois C, Callebert J, Nebigil C, Etienne N, Drouet L, Humbert M, Simonneau G, Maroteaux L. Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. Nat Med. 2002;8:1129–35.

Launay J-M, Hervé P, Callebert J, Mallat Z, Collet C, Doly S, Belmer A, Diaz SL, Hatia S, Côté F, Humbert M, Maroteaux L. Serotonin 5-HT2B receptors are required for bone-marrow contribution to pulmonary arterial hypertension. Blood. 2012;119:1772–1780.

Le Coniat M, Choi DS, Maroteaux L, Launay JM, Berger R. The 5-HT2B receptor gene maps to 2q36.3-2q37.1. Genomics. 1996;32(1):172–3.

Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA. Platelet-derived serotonin mediates liver regeneration. Science. 2006;312(5770): 104–7.

Manivet P, Mouillet-Richard S, Callebert J, Nebigil CG, Maroteaux L, Hosoda S, Kellermann O, Launay J-M. PDZ-dependent activation of nitric-oxide synthases by the serotonin 2B receptor. J Biol Chem. 2000;275:9324–31.

Nebigil CG, Choi D-S, Dierich A, Hickel P, Le Meur M, Messaddeq N, Launay J-M, Maroteaux L. Serotonin 2B receptor is required for heart development. Proc Natl Acad Sci USA. 2000a;97:9508–13.

Nebigil CG, Launay J-M, Hickel P, Tournois C, Maroteaux L. 5-Hydroxytryptamine 2B receptor regulates cell-cycle progression: Cross talk with tyrosine kinase pathways. Proc Natl Acad Sci USA. 2000b;97(6):2591–6.

Nebigil CG, Etienne N, Messaddeq N, Maroteaux L. Serotonin is a novel survival factor of cardiomyocytes: mitochondria as a target of 5-HT2B-receptor signaling. FASEB J. 2003;17(10):1373–5.

O'Mahony SM, Bulmer DC, Coelho A-M, Fitzgerald P, Bongiovanni C, Lee K, Winchester W, Dinan TG, Cryan JF. 5-HT2B receptors modulate visceral hypersensitivity in a stress-sensitive animal model of brain-gut axis dysfunction. Neurogastroenterol Motil. 2010;22(5):573–8.

Roth BL. Drugs and valvular heart disease. N Engl J Med. 2007;356(1):6–9.

Stefulj J, Jernej B, Cicin-Sain L, Rinner I, Schauenstein K. mRNA expression of serotonin receptors in cells of the immune tissues of the rat. Brain Behav Immun. 2000;14(3):219–24.

Tharayil VS, Wouters MM, Stanich JE, Roeder JL, Lei S, Beyder A, Gomez-Pinilla PJ, Gershon MD, Maroteaux L, Gibbons SJ, Farrugia G. Lack of serotonin 5-HT2B receptor alters proliferation and network volume of interstitial cells of Cajal in vivo. Neurogastroenterol Motil 2010;22:462–471

Tschentscher F, Husing J, Holter T, Kruse E, Dresen IG, Jockel KH, Anastassiou G, Schilling H, Bornfeld N, Horsthemke B, Lohmann DR, Zeschnigk M. Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities. Cancer Res. 2003;63(10):2578–84.

Vane JR. A sensitive method for the assay of 5-hydroxytryptamine. Br J Pharmacol Chemother. 1957;12(3):344–9.

5-Hydroxytryptamine Receptor 2C

Carine Becamel, Philippe Marin and Joël Bockaert
Dépt. de Neurobiologie, Institut de Génomique Fonctionnelle, Unité Mixte de Recherche 5203, Centre National de la Recherche Scientifique, Institut National de Santé et de la Recherche Médicale U661, Universités Montpellier 1 and 2, Montpellier Cedex 5, France

Synonyms

5-HT2C; 5-HT1C

Historical Background

The 5-hydroxytryptamine type 2C receptor (5-HT2C, previously 5-HT1C) is one of the 13 G protein-coupled receptor subtypes activated by serotonin. 5-HT2C receptors are involved in a large variety of physiological functions such as nociception, motor behavior, endocrine secretion, thermoregulation, modulation of appetite, and the control of exchanges between the central nervous system and the cerebrospinal fluid. They have also been implicated in numerous pathologies such as schizophrenia, anxiety, depression, Parkinson's disease, drug addiction, and obesity (Berg et al. 2008). In line with their implication in various pathological situations, 5-HT2C receptors are considered as a major pharmacological target for the development of new treatments.

With regard to signaling, 5-HT2C receptors are positively coupled to phospholipase Cß protein via Gαq in several brain regions (Berg et al. 2008). Native 5-HT2C receptors also activate phospholipase D through a mechanism involving Gα13 and Gβγ subunits in choroid plexus. Transactivation of the small GTPase RhoA seems to be required for 5-HT2C receptor-mediated phospholipase D activation. Activation of phospholipase A2 and ERK1,2 by 5-HT2C receptors has also been reported in heterologous cells and/or native tissues (Berg et al. 2008; Labasque et al. 2008).

Protein Function

5-HT2C receptors expressed along ascending dopaminergic pathways play a prominent role in the control of mesocorticolimbic and nigrostriatal dopaminergic systems. Constitutive and/or agonist-induced 5-HT2C receptor activation inhibits dopamine release (Berg et al. 2008; Bubar and Cunningham 2008). Atypical antipsychotics and antidepressants, in particular those exhibiting inverse agonist activity at 5-HT2C receptors, relieve this inhibition, leading to activation of the mesocorticolimbic dopaminergic system (Berg et al. 2008). Systemic administration of RO600175 (agonist) and SB242084 (antagonist) inhibits and potentiates cocaine-induced behaviors, respectively. Microdialysis of RO600175 or SB242084 in the ventral tegmental area (VTA) or nucleus accumbens also revealed that dopamine efflux induced by systemic cocaine injection is controlled by 5HT2C receptors expressed in the VTA and the Nac (Berg et al. 2008; Bubar and Cunningham 2008).

5-HT2C receptor activation likewise exerts a tonic inhibitory influence on the activity of locus

coeruleus-derived noradrenergic pathways innervating cortico-limbic structures.

5-HT2C receptors are expressed in pro-opiomelanocortin (POMC)/cocaine amphetamine-regulated transcript neurons of the arcuate nucleus of hypothalamus (Heisler et al. 2003). A series of elegant studies have shown that 5-HT2C receptor activation by administration of fenfluramine, a 5-HT reuptake inhibitor/5-HT releasing compound, or by selective agonists like BVT.X regulates melanocortin signaling and inhibits food intake (Heisler et al. 2003; Miller 2005). More recent studies showed that mice lacking 5-HT2C receptors displayed hepatic insulin resistance. Moreover, 5-HT2C receptor-deficient mice are resistant to the antidiabetic effects of the 5-HT2C agonist mCPP. Specific reexpression of 5-HT2C receptors in POMC neurons restored the wild type phenotype (Xu et al. 2010), underlying the potential of 5-HT2C agonists for the treatment of obesity.

Indeed, 5HT2C receptor-deficient mice are obese due to an abnormal control of feeding behavior (Berg et al. 2008). Developmental studies on food intake have revealed a chronic hyperphagia in young mutants (from the first 2 months of life through the first year of age) without changes in their body weights, adiposity levels, and in total energy expenditures, compared to wild type animals (Nonogaki et al. 1998, 2003). In contrast, by 9/10 months of age, mutants exhibit elevations of body weight and adiposity, accompanied by an increase in leptin and insulin levels, and a reduction in the total energy expense in older mutant animals (Nonogaki et al. 1998, 2003).

Mice lacking 5HT2C receptors exhibit lower thresholds for the expression of generalized seizures (Applegate and Tecott 1998), an effect modulated by other genetic factors and aging (Brennan et al. 1997).

Mutant mice also exhibit an anxiolytic phenotype (Heisler et al. 2007) and an increased responsiveness to novelty. They are also more sensitive to the effects of acute cocaine treatment on both locomotor activity and on nucleus accumbens dopamine levels (Bubar and Cunningham 2008). Mutant mice display an approximately 50% reduction in DOI-induced head-twitch response compared to their wild type littermates, suggesting the involvement of 5-HT2C receptors in the psychoactive response to hallucinogenic drugs (Canal et al. 2010).

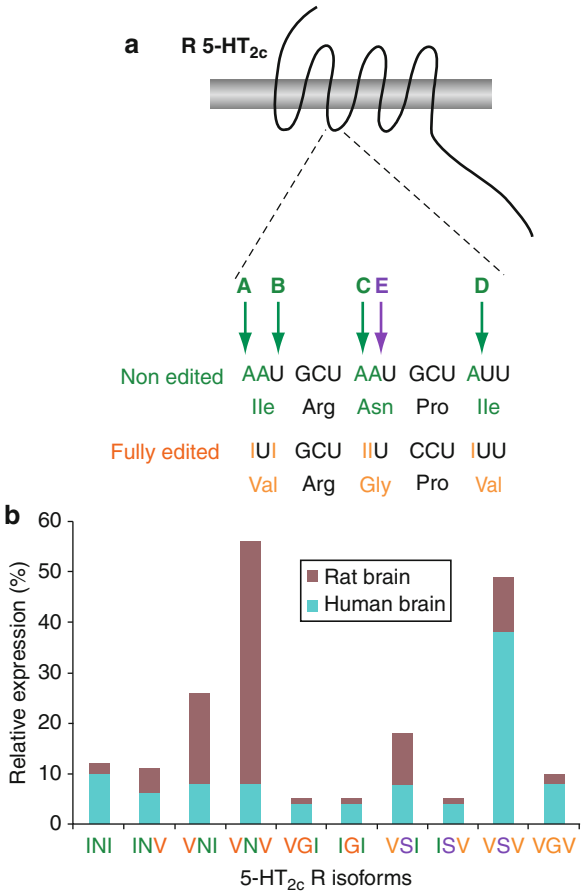
Regulation of Concentration and Activity

Exposure of 5-HT2C receptors to agonists leads to receptor desensitization (i.e., a decrease in receptor responsiveness) and down-regulation (i.e., a reduction in the total number of specific receptor binding sites without a change in apparent affinity for 5-HT) in vivo and in vitro (Bockaert et al. 2006). Several studies indicated that chronic treatment with antagonists and monoamine oxidase inhibitors induces an atypical down-regulation of 5-HT2C receptor in the choroid plexus as well as in several heterologous models (Berg et al. 2008). Chronic administration of SSRIs increases 5HT2C receptors density in the choroid plexus (Bockaert et al. 2006; Berg et al. 2008). Paradoxically, the density and functional status of 5-HT2C receptors is also elevated in experimental models of depression as well as in depressed patients (Berg et al. 2008).

Desensitization is initiated by receptor phosphorylation by G protein receptor kinase (GRK)2 (Berg et al. 2008). Receptor phosphorylation is followed by the recruitment of β-arrestins, which uncouple receptor from G protein and promote its internalization into endosomes. This phenomenon not only contributes to receptor desensitization but also allows receptor dephosphorylation and recycling to the plasma membrane in a fully resensitized state.

In addition to agonist-dependent activation, 5-HT2C receptors undergo constitutive activity. Constitutive activity toward phospholipase C effector pathway is accompanied by constitutive receptor desensitization and internalization (Berg et al. 2008). Constitutive activity at 5-HT2C receptors expressed on VTA GABAergic interneurons is responsible for tonic inhibition of mesocorticolimbic dopaminergic neurons (De Deurwaerdere et al. 2004).

RNA transcripts encoding the 5-HT2C receptor undergo adenosine to inosine editing at five sites located in the second intracellular loop in the receptor sequence, theoretically generating up to 32 different mRNAs that encode 24 receptor isoforms, ranging from the nonedited form (INI) to the fully edited one (VGV) (Fig. 1). The 5-HT2C receptor is to date the only known GPCR for which pre-mRNA editing generates multiple functional variants. RNA editing has multiple consequences on receptor function: (1) edited forms exhibit decreased constitutive activity, decreased agonist affinity, and decreased potency to



5-Hydroxytryptamine Receptor 2C, Fig. 1 RNA editing of 5-HT2C receptor transcripts. (**a**) The position of the editing sites (A, B, C, D, and the minor site E) within the exon V of human 5-HT2C receptor mRNA and the predicted amino acid sequences are shown for the nonedited INI isoform and for the fully edited VGV isoform. (**b**) 5-HT2C receptor editing efficiency at each editing site for human brain (*blue bars*) and rat brain (*brown bars*)

activate phospholipase C (Sanders-Bush et al. 2003; Werry et al. 2008); (2) the nonedited 5-HT2C receptor is capable of coupling to Gα13 protein, whereas the fully edited one fails to activate Gα13 (Sanders-Bush et al. 2003; Werry et al. 2008); (3) the nonedited form exhibits a constitutive activity at β-arrestin- and calmodulin-dependent ERK signaling (Labasque et al. 2010); and (4) editing alters 5-HT2C receptor trafficking in and out of the plasma membrane, a process reflecting their ability to associate with β-arrestins (Marion et al. 2004). Editing is modulated by 5-HT itself. Depletion of 5-HT increases the expression of receptor forms exhibiting the highest agonist affinity, whereas opposite changes of editing profile are detected following 5-HT2C receptor activation (Sanders-Bush et al. 2003; Werry et al. 2008). A recent study performed on mutant mice solely expressing the fully edited form of the receptor has suggested that editing may regulate the density of 5-HT2C receptor binding sites in brain (Olaghere da Silva et al. 2010).

Altered patterns of 5-HT2C receptor editing are observed in postmortem brains from suicide victims with a history of major depression (Iwamoto et al. 2009). Chronic treatments with antidepressants generate opposite changes of editing profiles (Sanders-Bush et al. 2003; Werry et al. 2008). Moreover, early life stress alters adult 5-HT2C receptor mRNA editing and expression of Gαq protein. Kishore and Stamm reported that Prader–Willy syndrome patients (characterized by neonatal muscular hypotonia, early childhood obesity, hypogonadism, and mental retardation) do not express the small nucleolar RNA HBII-52, a regulator of 5-HT2C receptor mRNA splicing. These patients exhibit abnormally low levels of nonedited 5-HT2C-INI receptor. Moreover, mutant mice solely expressing the fully edited form of the receptor display phenotypic characteristics of Prader–Willy syndrome, suggesting a role of serotonergic systems in Prader–Willi syndrome (Morabito et al. 2010).

Several studies have also demonstrated a critical role of 5HT2C receptors in the onset time of therapeutic response to antidepressants (Berg et al. 2008). Long-term treatment with selective 5-HT reuptake inhibitors (SSRIs) and other antidepressant classes progressively down-regulate 5HT2C receptor in rats and humans, a process paralleling their gradual onset of actions.

The 5-HT2C receptor is certainly one of the G protein-coupled receptors for which the largest number of G protein-coupled receptors-interacting proteins has been identified. Those proteins clearly control G protein-coupled receptors subcellular localization, as well as nature, kinetics, strength, and fine-tuning of G protein-coupled receptors signaling. 5-HT2C receptor interacts with a number of PDZ domain-containing proteins including Multiple PDZ domain protein (MUPP1), PostSynaptic Density protein 95 (PSD95), MAGUK p55 subfamily member 3 (MPP3), Veli3, Synapse-associated protein 97 (SAP97), Synapse-associated protein 102 (SAP102), and MAGI2

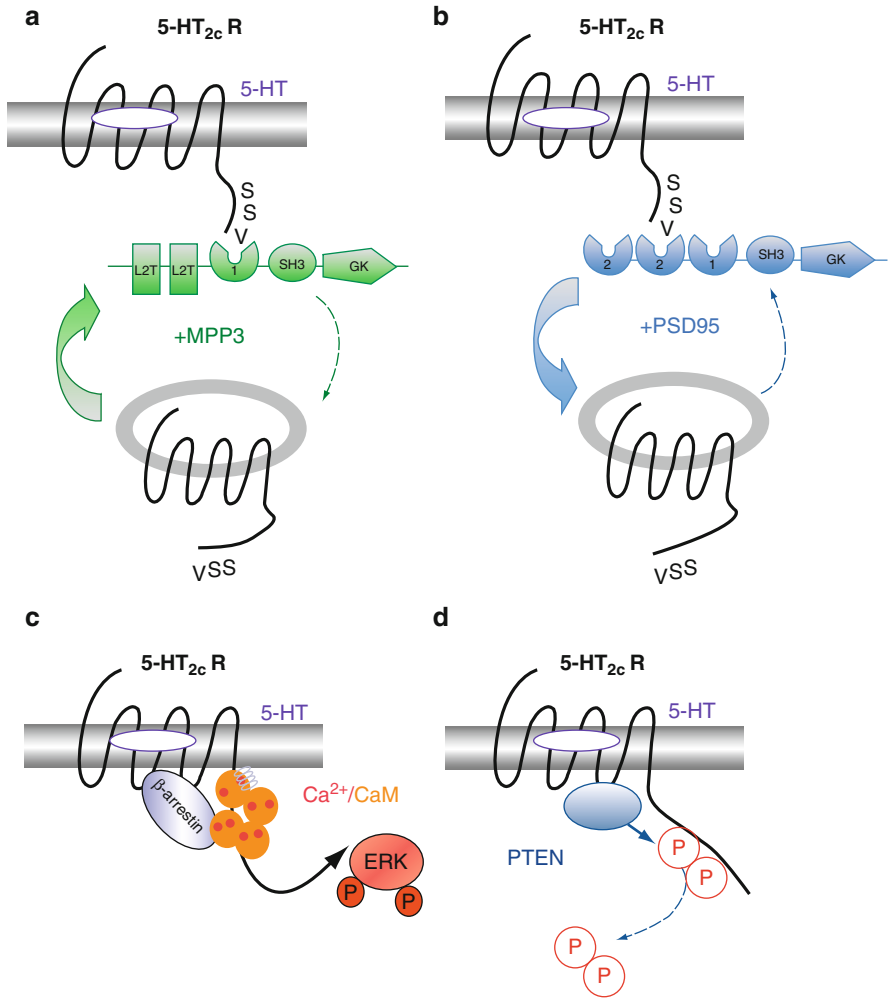
(for membrane associated guanylate kinase, WW and PDZ domain containing 2) via its C-terminal PDZ binding motif (SSV) (Becamel et al. 2002; Becamel et al. 2004). MUPP1 was the first 5-HT2C receptor-interacting protein identified using the yeast two-hybrid system (Ullmer et al. 1998). The interaction between 5-HT2C receptors and MUPP1 is dynamically regulated by agonist-dependent receptor phosphorylation of serine residues located in the PDZ binding motif (Parker et al. 2003). Receptor/MUPP1 interaction induces both conformational changes in the MUPP1 protein (Parker et al. 2003) and receptor clustering at the cell surface (Becamel et al. 2001). The 5-HT2 like *Caenorhabditis elegans* receptor SER-1 similarly interacts with a multi-PDZ domain containing protein similar to MUPP1 (designated as MPZ-1) in vulval muscle cell. This interaction facilitates SER-1 signaling (Xiao et al. 2006). PDZ partners of 5-HT2C receptor exhibit both presynaptic and post-synaptic localizations consistent with the differential distribution of the receptors at the synaptic junction. Several studies using receptors mutated on the PDZ ligand indicate that 5-HT2C receptor/PDZ protein interactions play a critical role in modulating their signal transduction properties and their desensitization (Gavarini et al. 2006). Interestingly, the effects depend on the nature of the PDZ protein associated with the receptor. For instance, association of the receptor with PSD-95 increases receptor desensitization and internalization, whereas its association with MPP3 prevents receptor internalization (Fig. 2a and b). These opposite actions highlight the importance of identifying which PDZ protein is associated with the receptor at a given time within a given neuron in native brain tissue.

PSD95 is essential for maintaining normal 5-HT2C receptor expression level and downstream signaling in vivo. $PSD95^{null}$ mice exhibit a significant reduction in 5-HT2C expression level particularly in the striatal and hippocampal regions and display a decrease in c-fos induction upon 5-HT2C receptor activation (Abbas et al. 2009). As previously reported for the association of 5-HT2C receptors with MUPP1, the interactions between the receptor and both PSD-95 and MPP3 are negatively regulated by the phosphorylation of 5-HT2C receptors on the PDZ ligand (Ser457, Ser458) (Gavarini et al. 2006).

5-HT2C receptors also interact with non-PDZ proteins. These include calmodulin, which binds to the receptor C-terminus (Becamel et al. 2002; Labasque et al. 2008). Additional calmodulin binding motifs have been identified in intracellular loops of the 5-HT2C receptor (Labasque et al. 2008). A combination of genetic and molecular approaches indicated that activation of Erk1,2 by 5-HT2C receptor, which is entirely independent of receptor' cognate G proteins, requires a physical association of CaM with the juxtamembrane region of the receptor C-terminus. CaM requirement was established in both heterologous system and authentic cellular contexts such as cortical neurons and choroid plexus epithelial cells. Differing from the classic mechanism of Erk1,2 activation by numerous GPCRs, phosphorylation of Erk1,2 induced by 5-HT2C receptor activation was entirely dependent on β-arrestins, which thereby acts in concert with CaM to activate Erk (Fig. 2c).

5-HT2C receptors interact with β-arrestins (mainly β-arrestin2) via their second intracellular loop (Berg et al. 2008). RNA editing of 5-HT2C receptors directly influences receptor interaction with β-arrestin2 (Marion et al. 2004). The unedited 5-HT2C-INI receptor is capable of interacting with β-arrestin2 in the absence of agonist, leading to constitutive receptor internalization and its accumulation within endocytic vesicles. Application of inverse agonists induces a marked redistribution of 5-HT2C-INI receptors to the plasma membrane of HEK-293 cells (Chanrion et al. 2008; Marion et al. 2004). Fully edited 5-HT2C-VGV receptors, which display the lowest level of constitutive activity, do not associate with β-arrestin2 in the absence of agonist and are mainly detected at the cell surface. Nevertheless, upon agonist treatment, the fully edited receptor associates with β-arrestin2 and undergoes rapid internalization.

5-HT2C receptors physically interact via their third intracellular loop with the tumor suppressor ▶ PTEN, an enzyme exhibiting both lipid and protein phosphatase activities (Ji et al. 2006). PTEN (for phosphatase and tensin homolog) association with 5-HT2C receptors prevents agonist-induced receptor phosphorylation at serine residues located in the receptor PDZ motif (Fig. 2d). Interaction between PTEN and 5-HT2C receptors occurs in dopaminergic neurons of the ventral tegmental area innervating the NAC, which are tonically inhibited by activated 5-HT2C receptors. This interaction plays an important role in mediating the reinforcing role of drugs (Ji et al. 2006).



5-Hydroxytryptamine Receptor 2C, Fig. 2 Interaction of 5-HT2C receptors with accessory proteins: modulation of receptor phosphorylation state and plasma membrane localization. (**a**) and (**b**) Opposite effect of PDZ proteins on 5HT2C receptors desensitization and trafficking. 5-HT2C receptors interact with MPP3 and PSD95 PDZ-containing proteins via their PDZ ligand (SSV) located at their carboxy terminal extremity. PSD95 increases desensitization of the Ca^{2+} response as well as constitutive and agonist-induced receptor internalization, whereas MPP3 stabilized the receptor at the plasma membrane and prevented desensitization of the receptor Ca^{2+} response. (**c**) Model of assembly of liganded 5-HT2C receptor with calmodulin and b-arrestin 2. In the presence of 5-HT, β-arrestin 2 binds to 5-HT2C receptor, probably via a recognition motif located in the i2 loop and common to the rhodopsin family GPCRs. β-arrestin 2 is also connected to receptor C-terminus by a Ca^{2+}-CaM dimer, that binds to the receptor upon agonist stimulation. This CaM-dependent scaffold might function to stabilize 5-HT_{2C} receptor/β-arrestin complex. (**d**) PTEN associated with the third intracellular loop of the 5-HT2C receptor induces receptor dephosphorylation and thereby reinforces effects of drugs of abuse

Major Sites of Expression and Subcellular Localization

5-HT2C receptors are exclusively expressed in the CNS. 5-HT2C receptors were first identified by radioligand binding using [^{3}H]-5-HT and [^{3}H]-mesulergine in pig choroid plexus. Further studies using autoradiography with [^{3}H]-mesulergine confirmed highest receptor density in choroid plexus in all mammalian species. 5-HT2C receptors expressed in choroid plexus control the secretion of the cerebrospinal fluid (Sanders-Bush et al. 2003). High receptor densities were detected in substantia nigra, globus pallidus, and ventromedial thalamus. A large amount of 5-HT2C receptors was also

detected in the suprachiasmic nucleus where they may be involved in the circadian rhythm. Recent studies have shown that 5HT2C receptors are expressed on GABAergic and dopaminergic neurons within the rat ventral tegmental area and on GABAergic neurons (preferentially in layers V/VI) within the prelimbic prefrontal cortex, a subregion of the medial prefrontal cortex (Berg et al. 2008; Bubar and Cunningham 2008).

[^{3}H]-mesulergine binding in monkey and human brains followed by receptor autoradiography has revealed both pre- and postsynaptic localizations of 5-HT2C receptor (Berg et al. 2008), which were further confirmed by electron microscopy studies (Becamel et al. 2002). 5-HT2C receptors exist as constitutive homodimers at the plasma membrane of living cells (Berg et al. 2008; Mancia et al. 2008). Two distinct dimerization interfaces have been characterized: the first one, located at TMI, is insensitive to receptor activation state, whereas the second one, located between TMs IV and V, depends on the nature of the ligand bound to receptors (Herrick-Davis et al. 2007).

As previously mentioned, cell surface expression of 5-HT2C receptor isoforms decreases in parallel with the degree of their constitutive activity (Marion et al. 2004). Inverse agonist treatments induce redistribution to the cell surface of constitutively active 5-HT2C receptors (Marion et al. 2004).

Ligands Interacting with the 5-HT2C Receptor

In line with its broad localization and physiological functions, the 5-HT2C receptor is considered as a therapeutic target for treating obesity, obsessive-compulsive disorder, drug abuse, sleep disorders, and anxiodepressive states (Jensen et al. 2010). During the last decade, the characterization of selective 5HT2C receptor ligands has been complicated by its close structural homology with the two other 5-HT2 receptor subtypes, namely, the 5-HT2A and 5-HT2B receptors. Accordingly, many compounds bind with high affinity to all three receptor subtypes.

1. Anorexic properties of Agonists
   5-HT2C receptors are activated by a variety of synthetic agonists such as RO60-0175, WAY163909, WAY 161503, YM348, VER2692, and BTV.X, which reduce food intake in rodents and enhance satiety (Jensen et al. 2010). More recently, Lorcaserin (ADP356) was characterized as a novel selective, high-affinity 5HT2C receptor agonist, which reduces food intake in rats (Thomsen et al. 2008).
2. Antidepressant effects of 5-HT2C ligands
   Several studies have suggested that some of the therapeutic effects of serotonin reuptake inhibitors (SSRIs) are mediated in part by the 5HT2C receptor. Chronic treatment with SSRI is associated with the down-regulation of 5HT2C receptors. This may lead to a disinhibition of the mesolimbic dopamine system that might contribute to their antidepressant action (Berg et al. 2008; Bubar and Cunningham 2008; Jensen et al. 2010). Numerous clinically effective antidepressants act as antagonists at 5-HT2C receptors. These include amitriptyline and clomipramine (tricyclics), trazodone and nefazodone (both behave as weak SSRI), citalopram and fluoxetine (SSRIs) (Berg et al. 2008), and agomelatine (a mixed 5-HT2C antagonist/melatonin agonist) (de Bodinat et al. 2010). Tetracyclic antidepressants such as mianserin and mirtazapine, which behave as antagonists of α2-adrenergic receptors and 5-HT3 receptors, were recently identified as inverse agonists at 5-HT2C receptors based on their ability to suppress basal inositol phosphate production and to increase plasma membrane localization of unedited 5-HT2C-INI receptors in HEK-293 cells as well as in cortical cultured neurons (Chanrion et al. 2008). Paradoxically, 5-HT2C receptor agonists such as WAY161503, RO 60-0175, and RO 60-0332 also exhibited antidepressant-like activity in selected models of depression, an effect that might reflect their ability to promote neurogenesis (Millan 2005).
3. Anxiolytic effects of antagonists
   Several 5-HT2C receptor antagonists (e.g., SB206553) exert a robust anxiolytic activity. These compounds also behave as inverse agonists at 5-HT2C receptors (Chanrion et al. 2008). Corroborating the anxiolytic action of antagonists, agonists like RO60-0175, WAY163909, and mCPP (*m*-chlorophenylpiperazine) display anxiogenic properties in the social interaction test (Millan 2005).
4. Antipsychotic properties of antagonists
   Numerous antipsychotics act as antagonists (or inverse agonists) at 5-HT2C receptors

(Berg et al. 2008; Jensen et al. 2010). Moreover, the 5HT2C inverse agonist SB 206553 potentiates the effect of haloperidol on dopamine release (Berg et al. 2008; Bubar and Cunningham 2008).

5. Other properties of 5HT2C ligands
   SB242084 behaves as a neutral antagonist at 5HT2C receptor signaling via Phospholipase C, whereas it acts as partial inverse agonist at Phospholipase A2, suggesting that it might be a protean ligand (Chanrion et al. 2008).
   PNU-69176E, a positive allosteric modulator, highly selective for 5-HT2C receptors has been characterized in different mammalian expression systems. PNU-69176E enhances [^{3}H]-5-HT binding to the human 5-HT2C receptor by selectively increasing 5-HT affinity for its low-affinity sites without affecting antagonist binding (Berg et al. 2008).

Summary

5-HT2C receptors still raise particular attention in view of their implication in many physiological functions and behaviors and in a large spectrum of psychiatric disorders such as anxiodepressive states, schizophrenia, obsessive-compulsive behaviors, and obesity. Accordingly, 5-HT2C receptors are considered as a major therapeutic target for the development of improved treatments of these diseases. Moreover, 5-HT2C is unique within G protein-coupled receptor superfamily to exhibit mRNA editing. Editing generates numerous 5-HT2C isoforms exhibiting different coupling properties, subcellular localization, and constitutive activity levels. Getting further insight into mechanisms controlling 5-HT2C receptor mRNA editing, signal transduction properties of receptor variants and their interaction with accessory proteins in neurons as well as their role in synaptic transmission and synaptic plasticity is, therefore, essential to discover innovative approaches in psychiatric disorders related to receptor dysfunction.

References

Abbas AI, Yadav PN, Yao WD, Arbuckle MI, Grant SG, Caron MG, Roth BL. PSD-95 is essential for hallucinogen and atypical antipsychotic drug actions at serotonin receptors. J Neurosci. 2009;29:7124–36.

Applegate CD, Tecott LH. Global increases in seizure susceptibility in mice lacking 5-HT2C receptors: a behavioral analysis. Exp Neurol. 1998;154:522–30.

Becamel C, Figge A, Poliak S, Dumuis A, Peles E, Bockaert J, Lubbert H, Ullmer C. Interaction of serotonin 5-hydroxytryptamine type 2C receptors with PDZ10 of the multi-PDZ domain protein MUPP1. J Biol Chem. 2001;276:12974–82.

Becamel C, Alonso G, Galeotti N, Demey E, Jouin P, Ullmer C, Dumuis A, Bockaert J, Marin P. Synaptic multiprotein complexes associated with 5-HT(2C) receptors: a proteomic approach. EMBO J. 2002;21:2332–42.

Becamel C, Gavarini S, Chanrion B, Alonso G, Galeotti N, Dumuis A, Bockaert J, Marin P. The serotonin 5-HT2A and 5-HT2C receptors interact with specific sets of PDZ proteins. J Biol Chem. 2004;279:20257–66.

Berg KA, Clarke WP, Cunningham KA, Spampinato U. Fine-tuning serotonin2c receptor function in the brain: molecular and functional implications. Neuropharmacology. 2008;55:969–76.

Bockaert J, Claeysen S, Becamel C, Dumuis A, Marin P. Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. Cell Tissue Res. 2006;326:553–72.

Brennan TJ, Seeley WW, Kilgard M, Schreiner CE, Tecott LH. Sound-induced seizures in serotonin 5-HT2c receptor mutant mice. Nat Genet. 1997;16:387–90.

Bubar MJ, Cunningham KA. Prospects for serotonin 5-HT2R pharmacotherapy in psychostimulant abuse. Prog Brain Res. 2008;172:319–46.

Canal CE, Olaghere da Silva UB, Gresch PJ, Watt EE, Sanders-Bush E, Airey DC. The serotonin 2C receptor potently modulates the head-twitch response in mice induced by a phenethylamine hallucinogen. Psychopharmacology (Berl). 2010;209:163–74.

Chanrion B, Mannoury la Cour C, Gavarini S, Seimandi M, Vincent L, Pujol JF, Bockaert J, Marin P, Millan MJ. Inverse agonist and neutral antagonist actions of antidepressants at recombinant and native 5-hydroxytryptamine2C receptors: differential modulation of cell surface expression and signal transduction. Mol Pharmacol. 2008;73:748–57.

de Bodinat C, Guardiola-Lemaitre B, Mocaer E, Renard P, Munoz C, Millan MJ. Agomelatine, the first melatonergic antidepressant: discovery, characterization and development. Nat Rev Drug Discov. 2010;9:628–42.

De Deurwaerdere P, Navailles S, Berg KA, Clarke WP, Spampinato U. Constitutive activity of the serotonin2C receptor inhibits in vivo dopamine release in the rat striatum and nucleus accumbens. J Neurosci. 2004;24:3235–41.

Gavarini S, Becamel C, Altier C, Lory P, Poncet J, Wijnholds J, Bockaert J, Marin P. Opposite effects of PSD-95 and MPP3 PDZ proteins on serotonin 5-hydroxytryptamine2C receptor desensitization and membrane stability. Mol Biol Cell. 2006;17:4619–31.

Heisler LK, Cowley MA, Kishi T, Tecott LH, Fan W, Low MJ, Smart JL, Rubinstein M, Tatro JB, Zigman JM, et al. Central serotonin and melanocortin pathways regulating energy homeostasis. Ann NY Acad Sci. 2003;994:169–74.

Heisler LK, Zhou L, Bajwa P, Hsu J, Tecott LH. Serotonin 5-HT (2C) receptors regulate anxiety-like behavior. Genes Brain Behav. 2007;6:491–6.

Herrick-Davis K, Grinde E, Weaver BA. Serotonin 5-HT(2C) receptor homodimerization is not regulated by agonist or inverse agonist treatment. Eur J Pharmacol. 2007; 568:45–53.
Iwamoto K, Bundo M, Kato T. Serotonin receptor 2C and mental disorders: genetic, expression and RNA editing studies. RNA Biol. 2009;6:248–53.
Jensen NH, Cremers TI, Sotty F. Therapeutic potential of 5-HT2C receptor ligands. Sci World J. 2010;10:1870–85.
Ji SP, Zhang Y, Van Cleemput J, Jiang W, Liao M, Li L, Wan Q, Backstrom JR, Zhang X. Disruption of PTEN coupling with 5-HT2C receptors suppresses behavioral responses induced by drugs of abuse. Nat Med. 2006;12:324–9.
Labasque M, Reiter E, Becamel C, Bockaert J, Marin P. Physical interaction of calmodulin with the 5-hydroxytryptamine2C receptor C-terminus is essential for G protein-independent, arrestin-dependent receptor signaling. Mol Biol Cell. 2008;19:4640–50.
Labasque M, Meffre J, Carrat G, Becamel C, Bockaert J, Marin P. Constitutive activity of Serotonin2C receptors at G protein-independent signaling: modulation by RNA editing and antidepressants. Mol Pharmacol. 2010;78: 818–26.
Mancia F, Assur Z, Herman AG, Siegel R, Hendrickson WA. Ligand sensitivity in dimeric associations of the serotonin 5HT2c receptor. EMBO Rep. 2008;9:363–9.
Marion S, Weiner DM, Caron MG. RNA editing induces variation in desensitization and trafficking of 5-hydroxytryptamine 2c receptor isoforms. J Biol Chem. 2004;279:2945–54.
Millan MJ. Serotonin 5-HT2C receptors as a target for the treatment of depressive and anxious states: focus on novel therapeutic strategies. Therapie. 2005;60:441–60.
Miller KJ. Serotonin 5-ht2c receptor agonists: potential for the treatment of obesity. Mol Interv. 2005;5:282–91.
Morabito MV, Abbas AI, Hood JL, Kesterson RA, Jacobs MM, Kump DS, Hachey DL, Roth BL, Emeson RB. Mice with altered serotonin 2C receptor RNA editing display characteristics of Prader-Willi syndrome. Neurobiol Dis. 2010;39:169–80.
Nonogaki K, Strack AM, Dallman MF, Tecott LH. Leptin-independent hyperphagia and type 2 diabetes in mice with a mutated serotonin 5-HT2C receptor gene. Nat Med. 1998;4:1152–6.
Nonogaki K, Abdallah L, Goulding EH, Bonasera SJ, Tecott LH. Hyperactivity and reduced energy cost of physical activity in serotonin 5-HT(2C) receptor mutant mice. Diabetes. 2003;52:315–20.
Olaghere da Silva UB, Morabito MV, Canal CE, Airey DC, Emeson RB, Sanders-Bush E. Impact of RNA editing on functions of the serotonin 2C receptor in vivo. Front Neurosci. 2010;4:26.
Parker LL, Backstrom JR, Sanders-Bush E, Shieh BH. Agonist-induced phosphorylation of the serotonin 5-HT2C receptor regulates its interaction with multiple PDZ protein 1. J Biol Chem. 2003;278:21576–83.
Sanders-Bush E, Fentress H, Hazelwood L. Serotonin 5-ht2 receptors: molecular and genomic diversity. Mol Interv. 2003;3:319–30.
Thomsen WJ, Grottick AJ, Menzaghi F, Reyes-Saldana H, Espitia S, Yuskin D, Whelan K, Martin M, Morgan M, Chen W, et al. Lorcaserin, a novel selective human 5-hydroxytryptamine2C agonist: in vitro and in vivo pharmacological characterization. J Pharmacol Exp Ther. 2008;325:577–87.
Ullmer C, Schmuck K, Figge A, Lubbert H. Cloning and characterization of MUPP1, a novel PDZ domain protein. FEBS Lett. 1998;424:63–8.
Werry TD, Loiacono R, Sexton PM, Christopoulos A. RNA editing of the serotonin 5HT2C receptor and its effects on cell signalling, pharmacology and brain function. Pharmacol Ther. 2008;119:7–23.
Xiao H, Hapiak VM, Smith KA, Lin L, Hobson RJ, Plenefisch J, Komuniecki R. SER-1, a Caenorhabditis elegans 5-HT2-like receptor, and a multi-PDZ domain containing protein (MPZ-1) interact in vulval muscle to facilitate serotonin-stimulated egg-laying. Dev Biol. 2006;298:379–91.
Xu Y, Berglund ED, Sohn JW, Holland WL, Chuang JC, Fukuda M, Rossi J, Williams KW, Jones JE, Zigman JM, et al. 5-HT(2C)Rs expressed by pro-opiomelanocortin neurons regulate insulin sensitivity in liver. Nat Neurosci. 2010;13:1457–9.

23 kDa Photoreceptor Cell-Specific Protein

► Recoverin

90 kDa Ribosomal Protein S6 Kinase 5

► MSK1

90 kDa Ribosomal S6 Kinase

► RSK (p90 Ribosomal S6 Kinase)

94 kDa Glucose-Regulated Protein

► Grp94 (HSP90B1)

β-Glucan Receptor

► CLEC7A

γ-Aminobutyrate$_A$ Receptor

► GABA$_A$ Receptor

γ-Aminobutyric Acid Type A Receptor

► GABA$_A$ Receptor

γ-Pak

► Pak2

δ-Opioid Receptor

► Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function

ζ

► CD3ζ

κ-Opioid Receptor

► Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function

μ-Opioid Receptor

► Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function

A

A1 (BCL2-Related Protein A1), BFL-1, BCL2L5 (BCL-2 like 5)

▶ BCL-2 Family

AbaA (*Aspergillus nidulans*)

▶ Tead

ABC1 (for ABCA1) and ABC2 (for ABCA2)

▶ ABCA Transporters

ABCA

▶ ABCA Transporters

ABCA Transporters

Esther E. Biswas-Fiss[1,2], Stephanie Affet[1] and Malissa Ha[1]
[1]Department of Bioscience Technologies, Program in Biotechnology, JSHP, Thomas Jefferson University, Philadelphia, PA, USA
[2]Department of Molecular Biology, University of Medicine and Dentistry of New Jersey, SOM, Stratford, NJ, USA

Synonyms

ABCA; ABC1 (for ABCA1) and ABC2 (for ABCA2); ABCR and rim protein (for ABCA4); ATP-binding cassette transporter subfamily A

Historical Background

ATP-binding cassette (ABC) transporters represent members of a transmembrane protein superfamily that bind and hydrolyze ATP to mediate the transport of a wide array of substrates across extra- and intracellular membranes in organisms ranging from prokaryotes to man. Forty-eight ABC genes have been identified to date, which have been additionally divided into seven subfamilies ABCA through ABCG (Dean et al. 2001; Higgins 2001; Kaminski et al. 2006; Albrecht and Viturro 2007). This entry will focus on the ABCA subfamily consisting of 12 members uniformly present in humans and most vertebrate species: ABCA1-ABCA13, with the exception of ABCA11 which appears to be a pseudogene. Four additional members of the ABCA family, ABCA14-17, have been identified in rodents, however, as yet no orthologs have been described in humans, and as a result will not be discussed here. Within the ABCA subfamily, the transporters ABCA5, ABCA6, ABCA8, ABCA9, and ABCA10 share significant sequence homology and form a gene cluster locus on chromosome 17q (Table 1); these five transporters are sometimes referred to as the "ABCA6-like transporters." The importance of ABCA proteins are underscored by the fact that loss of function mutations in their genes are linked to a number of inherited diseases, such as

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

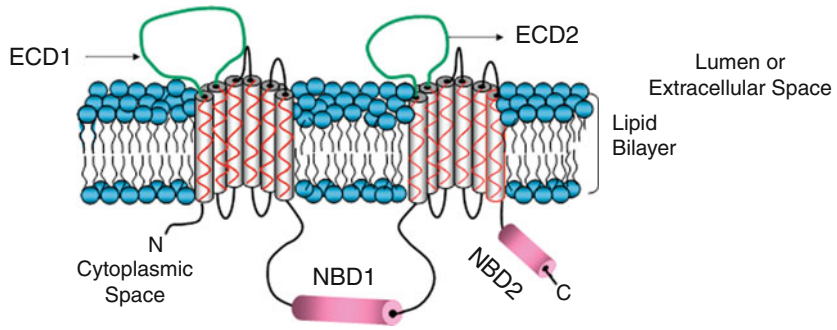
ABCA Transporters, Table 1 ABCA subfamilies and associated characteristics

Gene	Locus	Length (aa)	Expression	Functional significance	Associated diseases
ABCA1	9q31.1	2,261	Ubiquitous	Cholesterol homeostasis	Tangier disease
ABCA2	9q34	2,436	Brain	Neuronal associated lipid transport; drug resistance in cancer	Alzheimer's
ABCA3	16p13.3	1,704	Lung (alveolar type II cells)	Pulmonary surfactant secretion	Fatal surfactant deficiency, interstitial lung disease
ABCA4	1p22.1	2,273	Rod and cone photoreceptors	Transports retinal	STGD, AMD, CRD, RP
ABCA5	17q24	1,642	Skeletal muscle, kidney, and liver	Intracellular trafficking	Unknown
ABCA6	17q24	1,617	Ubiquitously liver, lung, heart, brain, and ovaries	Macrophage lipid transport	Unknown
ABCA7	19p13.3	2,146	Spleen, thymus, and bone marrow	Lipid transport	Unknown
ABCA8	17q24	1,581	Heart, liver, and muscle	Demonstrated to exert an ATPase-dependent drug transport function	Unknown
ABCA9	17q24	1,624	Heart, but found throughout the body	Macrophage lipid homeostasis	Unknown
ABCA10	17q24	1,543	Muscle, heart	Lipid transport	Unknown
ABCA12	2q34	2,595	Keratinocytes, placenta, skin, testis, and fetal brain	Lipid trafficking	Harlequin ichthyosis, lamellar ichthyosis type 2
ABCA13	7p12.3	5,058	Trachea, testis, and bone marrow		Schizophrenia and bipolar disorder

Tangier disease (ABCA1), Stargardt macular dystrophy (ABCA4), and Harlequin ichthyosis (ABCA12). An underlying theme in ABCA-associated diseases is defects in the transport of specific lipid-based substances. This entry will discuss ABCA subfamily protein structure and membrane topology, cellular function and disease associations, the intersection of ABCA transporters with cell signaling pathways, and future directions of ABCA research.

Common Structural Features of ABCA Subfamily Members

The prototypical mammalian ABCA transporter consists of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) encoded by a single polypeptide chain (Fig. 1). The nucleotide-binding domains (NBDs) are responsible for hydrolyzing ATP to provide energy to drive the conformational change required to transport substrates across extra- or intracellular membranes (Kaminski et al. 2006; Albrecht and Viturro 2007). The nucleotide-binding domains are the most highly conserved regions among members of the ABC transporter family. Each nucleotide-binding domain contains two sequence motifs, the Walker A (GXXGXGK(T/S) and Walker B ((R/K)XXXXGXXXXLhhhhD) motifs ("h" representing a hydrophobic amino acid), which are common to the general category of nucleotide-binding proteins. In ABCA proteins, the NBD1 and NBD2 domains are followed by conserved 80-residue signature sequences specific for the ABCA subfamily that contains the consensus sequence S/T-S/T-h-D/E-D/E. The TMDs are believed to participate in substrate translocation, and thus share little homology with other members of the subfamily. Structural topology varies significantly between the eight subclasses of ABC transporters. Topologically in the ABCA subfamily, each half transporter contains a transmembrane domain comprised of six membrane-spanning helices, followed by a soluble domain containing a NBD. In addition, each ABCA half transporter possesses a large extracellular loop, which is a characteristic unique to this subfamily (Fig. 1). The ABCA subfamily has some of the largest ABC proteins identified to date, one of which (ABCA13) is over 5,000 amino acids long and greater than 570 kDa in predicted molecular mass (Kaminski et al. 2006; Albrecht and Viturro 2007) (Table 1).



ABCA Transporters, Fig. 1 *Predicted structural organization of ABCA proteins and their important functional domains*. Pictorial representation showing the cytoplasmic nucleotide-binding domain (NBD), extracellular domain (ECD), and transmembrane domain (TMD) of a typical ABCA protein

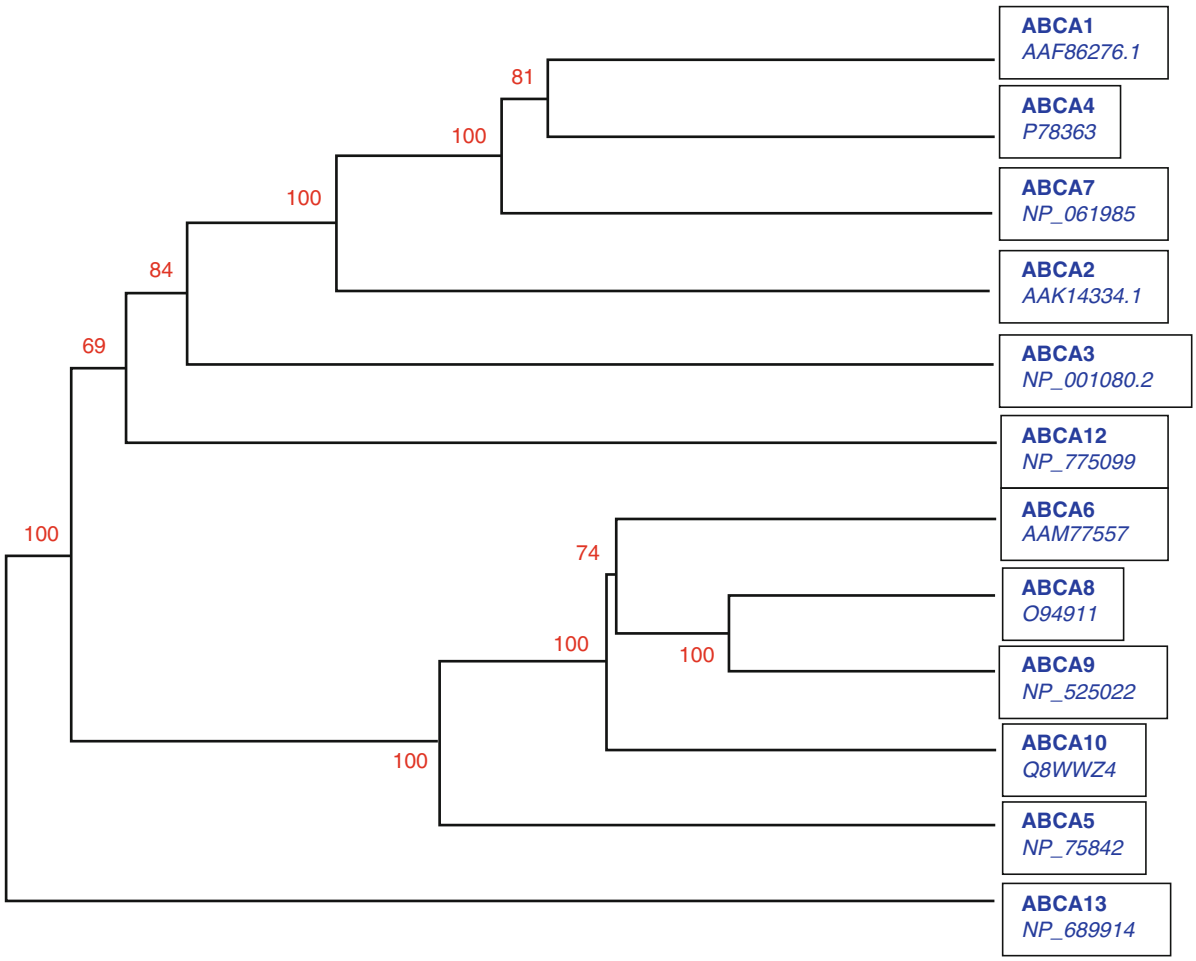
Unifying Themes Among ABCA Subgroups

The ABCA subgroup of 12 structurally related "full-size" transporters are responsible for the transport of a variety of physiologic lipid compounds in an ATP-dependent manner. A few subgroups have particular similarities between them; for example, ABCA12 and ABCA3 are exclusively localized to secretory intracellular lipid storage compartments such as lamellar granules in keratinocytes and lamellar bodies in pneumocytes. Besides cellular location, several subgroups have close sequence homology with one another. ABCA9 displays the highest amino acid sequence identity with ABCA8 (78%), ABCA6 (68%), ABCA10 (71%), and ABCA5 (53%) (Ordovas 2000; Kaminski et al. 2006; Albrecht and Viturro 2007) Fig. 2. The detailed mechanism of transport has yet to be determined for any of the mammalian ABCA transporters, so it is not yet know if that will prove to be a unifying feature of this group. Collectively, ABCA transporters are associated with a wide range of heritable disorders, display distinct tissue distribution and regulation of gene expression, yet together they underscore the critical importance of lipid homeostasis in human health and disease.

ABCA Subgroups

ABCA1 – The ABCA1 gene is expressed in a large number of human tissues with the highest levels present in the placenta, liver, lung, adrenal glands, and fetal tissues. The gene is localized to chromosome 9q31 and spans 50 exons. The 149kb ORF encodes a 2,261 aa polypeptide with a predicted molecular mass of 254 kDa (Table 1). ABCA1 has been identified to be a major regulator of HDL metabolism and mutations in this gene have been shown to be responsible for Tangier disease (Ordovas 2000). ABCA1 is believed to orchestrate cellular phospholipid and cholesterol active transport. It is required for optimal lipidiation of ApoA-1 in nascent HDL biogenesis and promotes the unidirectional efflux of cholesterol and phospholipids from the cell (Fitzgerald et al. 2010). To date, over 50 disease-associated mutations have been identified in the ABCA1 gene (Fasano et al. 2005). Individuals heterozygous for ABCA1 mutations are at increased risk for familial high-density lipoprotein deficiency (FHD), atherosclerotic cardiovascular disease (ACVD), and perhaps age-related macular degeneration (AMD) (Yu et al. 2011). Molecular diagnostic testing is increasingly available to aid in the diagnosis of ABCA1-related diseases.

In executing its cellular function, ABCA1 interacts with ApoA-I protein, oligomerizing to form a homotetramer which leads to the development of a high-affinity lipid-binding site. ApoA-I appears to interact with ABCA1 through its extracellular domains. Interaction with ApoA-I activates the phosphatidylcholine pathway by upregulating phosphatidylcholine synthase (Iatan et al. 2011). ▶ Interferon-gamma decreases ABCA1 expression levels in a JAK/STAT-regulated manner (Fitzgerald et al. 2010). Recent studies suggest that ABCA1-related ACVD could be treated using peroxisome proliferator-activated receptors (PPARs) and LSR agonists which have been shown to influence ABCA1 gene expression (Wakino and Itoh 2010).



ABCA Transporters, Fig. 2 *Phylogenetic tree of human ABCA subfamily transporter genes*. Full-length amino acid sequences were aligned using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and phylogenetic analysis was performed utilizing the PHYLIP software (http://evolution.genetics.washington.edu/phylip.html). Bootstrap values (%) out of 100 iterations are indicated at each branch point

ABCA2 – ABCA2 is highly expressed in the cells of the ovary, nervous (CNS), and hematopoietic systems. Functionally, it is associated with lipid transport and drug resistance in cancer cells, including tumor stem cells. Cellular immunolocalization reveals co-localization of ABCA2 with late endolysosomes and trans-Golgi organelles where it appears to function in cholesterol homeostasis and LDL metabolism (Mack et al. 2006). The gene is localized to chromosome 9q34.3 and spans 48 exons. The 149kb ORF encodes a 2,436 aa polypeptide with a predicted molecular mass of 270 kDa. Alternative splicing of the first exon to the second results in two variants, 1A and 1B.

Studies have indicated a relationship between ABCA2 and both early onset and late onset/sporadic Alzheimer's disease. In vitro studies showed that; ABCA2 is highly expressed in human neuroblastoma cells and co-localizes with beta-amyloid; overexpression of ABCA2 increased amyloid precursor protein (APP) protein levels. A single-nucleotide polymorphism in human ABCA2 has been found to be associated with early development of this disease (Mace et al. 2005). ABCA2 is a cholesterol responsive gene along with ABCA1 and ABCA7. Micro RNA-mediated downregulation of the ABCA2 gene product has been observed in untreated colon cancer suggesting a possible therapeutic manipulation of gene expression in the future.

ABCA3 – The ABCA3 protein is highly expressed in the lung and has been localized primarily to the

outer, or limiting, membrane of the lamellar bodies of type II alveolar structures. The ABCA3 gene is localized to chromosome 16p13.3 and spans 33 exons. The ORF encodes a 1704 aa polypeptide with a molecular mass of 191 kDa (Table 1). Consistent with glucocorticoid-responsive regulation of gene expression, expression of ABCA3 increased more than 30-fold following stimulation of fetal lung explants with dexamethasone, cAMP, and isobutylmethylxanthine (Takahashi et al. 2005; van der Deen et al. 2005; Kaminski et al. 2006).

ABCA3 has been shown to play an important role in the formation of pulmonary surfactant which lowers the surface tension at the air–liquid interface thus preventing the collapse of the alveoli. A wide range of mutations (>150) within ABCA3 have been linked to fatal respiratory distress syndrome in the neonatal period, and with interstitial lung disease in older infants, children, and adults (Wert et al. 2009). ABCA3 appears to function to import surfactant phospholipids, such as phosphatidylcholine (PC) and phosphatidylglycerol (PG), from the cytosol into the lamellar body and is thought, therefore, to be important for lamellar body biogenesis. ▶ STAT3, activated by ▶ IL6, regulates ABCA3 expression and influences lamellar body formation in alveolar type II cells (Matsuzaki et al. 2008). The *C. elegans* protein, ced-7, is homologous to human ABCA3. Ced-7 functions in the engulfment of cell corpses during programmed cell death, although it is not known if mammalian ABCA3 also performs a similar function.

ABCA4 – ABCA4 (previously referred to as ABCR or the rim protein) is a retina-specific member of the ABCA subfamily. It was the first ABCA transporter to be causatively linked to genetic disease (Allikmets et al. 1997). The ABCA4 gene spans nearly 150 kb and contains at least 50 exons (Table 1). It encodes a 2,273 amino acid protein which is localized in the retina along the rims of rod and cone photoreceptor outer segment disk membranes. ABCA4 may act as an ATP-dependent flippase that translocates N-retinylidene-phosphatidylethanolamine (-PE) from the lumen to the cytoplasmic side of the disk membrane (Biswas-Fiss 2008; Tsybovsky et al. 2010; Pollock and Callaghan 2011). This view is supported by the observations that ABCA4 indeed binds the retinoid N-retinylidene-PE with high affinity (Molday et al. 2006). Recently, the ECD2 domain of ABCA4 was shown to bind all-*trans* retinal specifically (Biswas-Fiss et al. 2010). Efflux of all-*trans* retinal is required for the continued recycling of retinoids released from photobleached rhodopsin as part of the visual transduction cycle. Studies conducted with ABCA4 (+/−) mice support the hypothesis that lack of ABCA4 transport activity may lead to the accumulation of toxic all-trans retinal derivatives (lipofuscin) in the rod and cone photoreceptors. Accumulation of lipofuscin leads to apoptosis of the supporting retinal pigment epithelium and, eventually, the photoreceptors themselves (Mata et al. 2001).

To date >500 sequence variations in the ABCA4 gene have been identified which are linked to four macular degenerative diseases including Stargardt disease (STGD), cone-rod dystrophy (CRD), autosomal retinitis pigmentosa type 19 (RP19), and age-related macular degeneration (AMD) (Tsybovsky et al. 2010; Pollock and Callaghan 2011). The mutations are found throughout the entire reading frame of the gene and significant progress has been made in determining their biochemical significance (Biswas-Fiss 2008; Tsybovsky et al. 2010; Pollock and Callaghan 2011). ABCA4-related diseases vary in their age of onset and speed of progression – yet they all eventually lead to blindness. Genotyping of patients through microarray and DNA sequencing is currently available, and the future holds promise for some in the form of gene therapy. The challenge today is to correlate the numerous ABCA4 mutations with the broad range of clinical phenotypes and to understand how this translates to ABCA4 protein dysfunction at a molecular level. Development of a transport assay would be a major step forward toward accomplishing this goal.

ABCA6-Like Transporters – Five closely related members of the human ABCA subfamily form a compact cluster on chromosome 17q24.2–3, comprising the genes for ABCA5, 10, 6, 9, 8 (in that order) (Table 1). The common chromosomal location of these ABCA6-like transporters and their overall high peptide sequence homology strongly supports the hypothesis that these transporters evolved from a common ancestral gene (Dean et al. 2001). The exact cellular function of ABCA6-like transporters remains unclear. Recent studies suggest that ABCA6, ABCA9, and ABCA10 may play a role in the connective tissue disorder pseudoxanthoma elasticum (PXE) (Schulz et al. 2006). The finding that the genes for ABCA6, 9, and 10 are regulated by cholesterol in human macrophages suggests their potential involvement in lipid

transport processes in these cells (Takahashi et al. 2005; Kaminski et al. 2006; Fitzgerald et al. 2010). Immunohistochemical studies revealed that ABCA5 is highly expressed in cardiomyocytes of the heart, oligodendrocytes and astrocytes of the brain, alveolar type II cells of the lung, and Leydig cells of the testis. Much remains to be understood about this subgroup of ABCA transporters, including their disease associations, transported ligands, and the physiological significance of their clustering at the 17q24 locus.

ABCA7 – The ABCA7 transporter gene was initially cloned from human macrophages in which it is subject to regulation by cholesterol influx and efflux via the sterol regulatory-binding element 2 (Tanaka et al. 2011). The ABCA7 gene contains 46 exons and spans nearly 32 kb being localized to chromosome 19p13.3. The ORF encodes a 2,146 amino acid protein with a predicted molecular weight of 235 kDa. Its expression levels are regulated in a manner opposite that of ABCA1 protein, which is upregulated in the presence of sterols. ABCA7 is highly expressed in myelo-lymphatic tissues with highest expression in peripheral leukocytes, thymus, spleen, and bone marrow; however, it was also found in platelets and keratinocytes (Takahashi et al. 2005; Kaminski et al. 2006; Fitzgerald et al. 2010). ABCA7 shows the greatest homology with ABCA1 (55%) and ABCA4 (49%) transporters. ABCA7 has been implicated in the regulation of ceramide and phospholipid export from the macrophages of human microgila (Wang et al. 2003; Takahashi et al. 2005; Kim et al. 2008). The precise physiological role of ABCA7 remains uncertain. Tanaka and coworkers observed identity between the ECD1 domain (residues 195 and 352) of ABCA7 and a Sjögren's syndrome autoantigen (Tanaka et al. 2003). They determined that ABCA7 encodes the autoantigen SS-N and plasma cells infiltrating salivary glands of patients with Sjögren's syndrome were immunoreactive against a monoclonal antibody derived from the ABCA7 ECD1.

ABCA7 mediates the formation of HDL when exogenously transfected and expressed; however, no endogenous effect of ABCA7 in HDL formation has been found. Recently, ABCA7 has been linked to phagocytosis regulated by sterol regulatory element-binding protein 2. Coupled with the observation that HDL apolipoproteins stabilize ABCA7 against calpain-mediated degradation, ABCA7 may represent a link between sterol homeostasis and the host-defense response infection, inflammation, and apoptosis (Tanaka et al. 2003). Further research is required to delineate the specific substrate(s) of ABCA7 and its precise physiological function in these processes.

ABCA11 – ABCA11 is considered a pseudogene as no functional "ABCA11" gene been found in *Homo sapiens* (Kaminski et al. 2006).

ABCA12 – ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with the transport of lipids in lamellar granules (LG) to the apical surface of granular layer keratinocytes. ABCA12 localizes throughout the entire Golgi apparatus to LGs at the cell periphery, mainly in the granular layer keratinocytes. ABCA12 was first identified in human placenta yet is highly expressed in the skin, testis, and fetal brain (Annilo et al. 2002; Kaminski et al. 2006; Wenzel et al. 2007). The gene is composed of 53 exons and maps to chromosome 2q35. ABCA12 mutations are known to underlie the three main types of autosomal recessive congenital ichthyoses: harlequin ichthyosis (HI), lamellar ichthyosis, and congenital ichthyosiform erythroderma (Albrecht and Viturro 2007). Fifty-two mutations have been identified to date. Studies have shown that the nature and severity of mutations in the ABCA12 gene accounts for the resulting clinical phenotype: Harlequin ichthyosis (HI), which is a severe form of congenital ichthyosis, is typically fatal in the first few days of life, while a second phenotype is a milder form manifesting as lamellar ichthyosis type 2 (Kaminski et al. 2006). Ceramide was reported to upregulate ABCA12 expression via PPAR (peroxisome proliferator-activated receptor) delta-mediated signaling pathway, providing a substrate-driven, feed-forward mechanism for regulation of this key lipid transporter.

ABCA13 – ABCA13 represents the largest ABC transporter comprising 5,058 amino acids (Prades et al. 2002). In normal tissues, the highest mRNA expression of the ABCA13 full-length mRNA was found in human trachea, testis, and bone marrow. Prades et al. (2002) determined that the ABCA13 gene contains 62 exons and spans more than 450 kb. Exons 17 and 18 are large, containing 4,779 and 1,827 bp, respectively. Research has found that ABCA13 shares a common genomic region on chromosome 7p12.3 with the locus linked to the T-cell tumor invasion and metastasis, INM7 (Prades et al. 2002). With this information and with the high expression levels in

leukemic cell lines, it has been suggested that ABCA13 may play a possible role in hemato-oncological pathologies (Kaminski et al. 2006). More recently, human genetic data suggest variants in the ABCA13 locus are associated with schizophrenia, bipolar disorder, and depression (Knight et al. 2009). Despite these insights into the physiological role of ABCA13, further studies are required to determine the exact functional role and physiological substrates of this transporter.

Summary

The ABCA subfamily form an intriguing group of transporters whose function broadly relates to lipid homeostasis. Although in several instances, such as with the ABCA6-like subgroup, the exact cellular function and mechanism of action remain unknown, their important physiological role is underscored by the often severe diseases that result from mutations in their genes. Prospects for future research relate to the degree in which each transporter is understood. For relatively well-characterized transporters such as ABCA1, ABCA3, and ABCA4, a significant amount is known about the prospective ligand a well-established link between a given monogenic disorder and mutations in the transporter-encoding gene exist. For these transporters, current and future research aimed at understanding the genotype–phenotype correlation (biochemical as well as clinical) is important, so that more accurate prognoses and specific therapies may be implemented. Ultimately, the development of transport assays, which can analyze the actual transport event itself, must be developed in order to determine the precise effect of a given mutation on transporter function. In the case of less well-understood transporters, such as the ABCA6-like subgroup, fundamental research is required to determine the nature of the transport ligand and how this relates to human health and disease. Finally, defining the role of protein–protein interactions in ABCA subgroup mechanism of action is necessary in order to determine the synergistic relationships between the other members of the ABCA and/or ABC protein family as well as to define a given ABCA proteome. Certainly, it can be said that the roadmap of ABCA disease-related mutations provide scientists with a wealth of clues to uncover the secrets of this important class of ABC transporters.

References

Albrecht C, Viturro E. The ABCA subfamily–gene and protein structures, functions and associated hereditary diseases. Pflugers Arch. 2007;453(5):581–9.

Allikmets R, Shroyer NF, et al. Mutation of the stargardt disease gene (ABCR) in age-related macular degeneration. Science. 1997;277(5333):1805–7.

Annilo T, Shulenin S, et al. Identification and characterization of a novel ABCA subfamily member, ABCA12, located in the lamellar ichthyosis region on 2q34. Cytogenet Genome Res. 2002;98(2–3):169–76.

Biswas-Fiss EE. Biochemical Defects in the nucleotide binding domains of the retina specific ABC transporter delivery, Humana Press, Totowa, NJ; 2008.

Biswas-Fiss EE, Kurpad DS, et al. Interaction of extracellular domain 2 of the human retina-specific ATP-binding cassette transporter (ABCA4) with all-trans-retinal. J Biol Chem. 2010;285(25):19372–83.

Dean M, Rzhetsky A, et al. The human ATP-binding cassette (ABC) transporter superfamily. Genome Res. 2001;11(7):1156–66.

Fasano T, Bocchi L, Pisciotta L, Bertolini S, Calandra S. Denaturing high-performance liquid chromatography in the detection of ABCA1 gene mutations in familial HDL deficiency. J Lipid Res. 2005;46:817–22.

Fitzgerald ML, Mujawar Z, et al. ABC transporters, atherosclerosis and inflammation. Atherosclerosis. 2010;211(2):361–70.

Higgins CF. ABC transporters: physiology, structure and mechanism–an overview. Res Microbiol. 2001;152(3–4):205–10.

Iatan I, Bailey D, et al. Membrane microdomains modulate ligand binding activity of oligomeric ABCA1 and Apoa-I-mediated lipid removal: molecular evidence that ApoA-I interaction with abca1 activatesthe phosphatidylcholine biosynthesis pathway. J Lipid Res. 2011;52(11):2043–55.

Kaminski WE, Piehler A, et al. ABC A-subfamily transporters: structure, function and disease. Biochim Biophys Acta. 2006;1762(5):510–24.

Kim WS, Weickert CS, et al. Role of ATP-binding cassette transporters in brain lipid transport and neurological disease. J Neurochem. 2008;104(5):1145–66.

Knight HM, Pickard BS, et al. A cytogenetic abnormality and rare coding variants identify ABCA13 as a candidate gene in schizophrenia, bipolar disorder, and depression. Am J Hum Genet. 2009;85(6):833–46.

Mace S, Cousin E, et al. ABCA2 is a strong genetic risk factor for early-onset Alzheimer's disease. Neurobiol Dis. 2005;18(1):119–25.

Mack JT, Beljanski V, et al. The ATP-binding cassette transporter ABCA2 as a mediator of intracellular trafficking. Biomed Pharmacother. 2006;60(9):587–92.

Mata NL, Tzekov RT, et al. Delayed dark-adaptation and lipofuscin accumulation in abcr+/− mice: implications for involvement of ABCR in age-related macular degeneration. Invest Ophthalmol Vis Sci. 2001;42(8):1685–90.

Matsuzaki Y, Besnard V, et al. STAT3 regulates ABCA3 expression and influences lamellar body formation in alveolar type II cells. Am J Respir Cell Mol Biol. 2008;38(5):551–8.

Molday RS, Beharry S, et al. Binding of N-retinylidene-PE to ABCA4 and a model for its transport across membranes. Adv Exp Med Biol. 2006;572:465–70.
Ordovas JM. ABC1: the gene for Tangier disease and beyond. Nutr Rev. 2000;58(3 Pt 1):76–9.
Pollock NL, Callaghan R. The lipid translocase, ABCA4: seeing is believing. FEBS J. 2011;278(18):3204–14.
Prades C, Arnould I, et al. The human ATP binding cassette gene ABCA13, located on chromosome 7p12.3, encodes a 5058 amino acid protein with an extracellular domain encoded in part by a 4.8-kb conserved exon. Cytogenet Genome Res. 2002;98(2–3):160–8.
Schulz V, Hendig D, et al. Mutational analysis of the ABCC6 gene and the proximal ABCC6 gene promoter in German patients with pseudoxanthoma elasticum (PXE). Hum Mutat. 2006;27(8):831.
Takahashi K, Kimura Y, et al. ABC proteins: key molecules for lipid homeostasis. Med Mol Morphol. 2005;38(1):2–12.
Tanaka AR, Abe-Dohmae S, et al. Effects of mutations of ABCA1 in the first extracellular domain on subcellular trafficking and ATP binding/hydrolysis. J Biol Chem. 2003;278(10):8815–9.
Tanaka N, Abe-Dohmae S, Iwamoto N, Yokoyama S. Roles of ATP-binding cassette transporter A7 in cholesterol homeostasis and host defense system. J Atheroscler Thromb. 2011;18:274–81.
Tsybovsky Y, Molday RS, et al. The ATP-binding cassette transporter ABCA4: structural and functional properties and role in retinal disease. Adv Exp Med Biol. 2010;703:105–25.
van der Deen M, de Vries EG, et al. ATP-binding cassette (ABC) transporters in normal and pathological lung. Respir Res. 2005;6:59.
Wakino S, Itoh H. Anti-atherosclerotic effects by PPARgamma and its ligands through the activation of reverse cholesterol transport system. Nihon Rinsho. 2010;68(2):217–23.
Wang N, Lan D, et al. ATP-binding cassette transporter A7 (ABCA7) binds apolipoprotein A-I and mediates cellular phospholipid but not cholesterol efflux. J Biol Chem. 2003;278(44):42906–12.
Wenzel JJ, Piehler A, et al. ABC A-subclass proteins: gatekeepers of cellular phospho- and sphingolipid transport. Front Biosci. 2007;12:3177–93.
Wert SE, Whitsett JA, et al. Genetic disorders of surfactant dysfunction. Pediatr Dev Pathol. 2009;12(4):253–74.
Yu Y, Reynolds R, et al. Association of variants in the LIPC and ABCA1 genes with intermediate and large drusen and advanced age-related macular degeneration. Invest Ophthalmol Vis Sci. 2011;52(7):4663–70.

ABCR and Rim Protein (for ABCA4)

► ABCA Transporters

AC9

► ADCY9 (Adenylyl Cyclase 9)

Acidic FGF

► FGF (Fibroblast Growth Factor)

ACIX

► ADCY9 (Adenylyl Cyclase 9)

ACK1

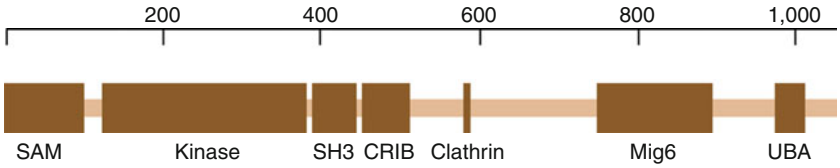
Takaya Satoh
Department of Biological Science, Graduate School of Science, Osaka Prefecture University,
Sakai, Osaka, Japan

Synonyms

Activated Cdc42Hs-associated kinase 1; CD38 negative kinase 2; Proline-rich tyrosine kinase 1; Pyk1; Tnk2

Historical Background

The tyrosine kinase ACK1 (activated Cdc42Hs-associated kinase 1) was first identified as a specific target of the small GTPase Cdc42 (Manser et al. 1993). ACK1, a related tyrosine kinase Tnk1, and the non-tyrosine kinase protein Mig6 (Gene 33/receptor-associated late transducer) constitute a family of proteins with conserved domain structures. In addition to the kinase catalytic domain, various domains and amino acid sequence motifs, which are responsible for the interaction with diverse signal transducing proteins, are found in ACK1 (Fig. 1). Several types of splice variants (including a protein previously designated ACK2 [activated Cdc42Hs-associated kinase 2]) are present. Two orthologues of the mammalian ACK1 gene, *DACK* and *DPR2*, exist in the *Drosophila melanogaster* genome. In *Caenorhabditis elegans*, two orthologues encode ACK family protein tyrosine kinases, ARK-1 and B0302.1.



ACK1, Fig. 1 Domain structure of ACK1. Domains found in the mouse ACK1 protein (NCBI accession: NP_058068) are schematically shown. SAM, sterile alpha motif domain; Kinase, tyrosine kinase catalytic domain; SH3, Src homology 3 domain; CRIB, Cdc42/Rac interactive binding domain; Clathrin, clathrin-binding motif; Mig6, Mig6 homology domain; UBA, ubiquitin-association domain. The upper scale indicates the amino acid position number

Regulation of ACK1 Activity

ACK1 is a multidomain non-receptor tyrosine kinase expressed in a variety of tissues in mammals. In addition to the N-terminally located kinase catalytic domain, ACK1 contains a membrane-targeting sterile alpha motif domain, a Src homology 3 (SH3) domain, a Cdc42/Rac interactive binding domain, a clathrin-binding motif, a Mig6 homology domain, a proline-rich domain (which overlaps with the clathrin-binding motif and the Mig6 homology domain), and a ubiquitin-association domain (Fig. 1). These domains are responsible for recognition and interaction of diverse binding partners (Mahajan and Mahajan 2010).

Originally, ACK1 was identified as a specific target of Cdc42: The active GTP-bound, but not inactive GDP-bound, Cdc42 interacts with the Cdc42/Rac interactive binding domain of ACK1. Thus, Cdc42 is expected to have a crucial role in the regulation of ACK1. Although co-expression of Cdc42 in cells results in tyrosine phosphorylation of ACK1, the binding of Cdc42·GTP to purified ACK1 was found to be insufficient for stimulating its tyrosine kinase activity in vitro (Yang et al. 1999; Yokoyama and Miller 2003). Given that many signaling molecules interact with ACK1, Cdc42 may not be a unique upstream regulator, but rather may collaborate with other regulatory proteins to modulate the activity of ACK1 in the cell.

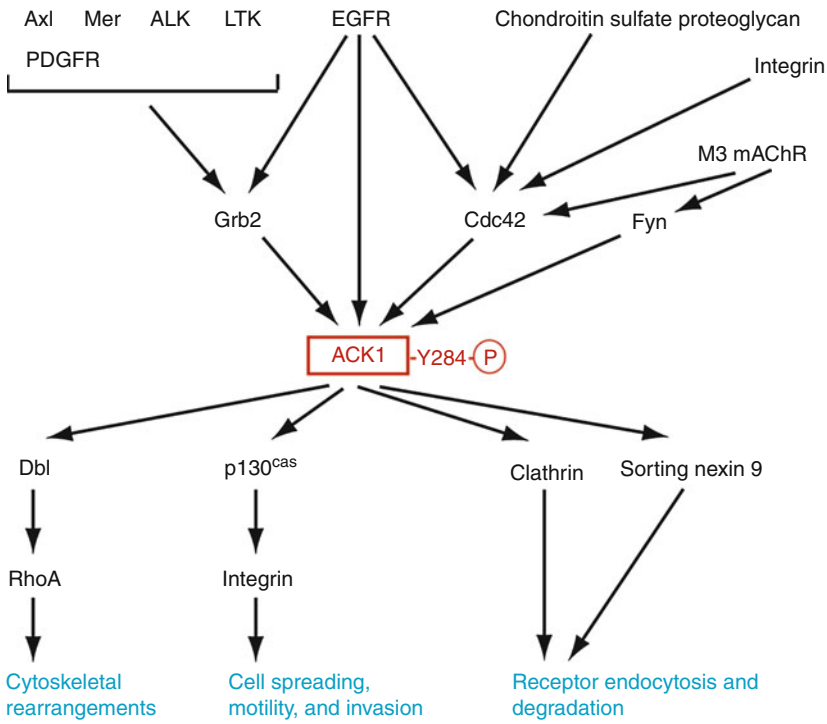
Many protein kinases are catalytically activated through conformational change of the activation loop triggered by its phosphorylation. Therefore, ACK1 kinase activity may also be modulated by phosphorylation of the activation loop. One tyrosine (Y284, all residue numbers hereafter are based on the mouse ACK1 sequence [NCBI accession: NP_058068]), which exists within the activation loop of ACK1, was identified as a primary autophosphorylation site (Yokoyama and Miller 2003). Comparison of tertiary structures of phosphorylated and unphosphorylated activation loops of ACK1, unexpectedly, revealed that ACK1 adopts an activated conformation independent of phosphorylation of Y284 (Lougheed et al. 2004). Therefore, the activation loop of ACK1 is not autoinhibitory, although it may have another role in the regulation of catalysis.

Recently, the intramolecular association between the N-terminal kinase catalytic domain and the C-terminal Mig6 homology domain, which may suppress the kinase activity, has been revealed (Prieto-Echagüe et al. 2010). In fact, amino acid changes in kinase catalytic (E346K) and Mig6 homology (F836A) domains disrupt this interaction, and markedly increase the kinase activity (Prieto-Echagüe et al. 2010). In addition, the former mutation has been characterized as a constitutively activated mutant in cancer cells (see below).

Although the underlying mechanism remains totally unknown, the tyrosine phosphorylation level of ACK1 significantly increased in response to temperature shift-down to 25°C and hyperosmotic shock (Satoh et al. 1996).

Role of ACK1 in Signaling Triggered by Cell-Surface Receptors

The role of ACK1 in growth factor receptor signaling has been well documented (Fig. 2). Epidermal growth factor (EGF) treatment of cells induces rapid and transient tyrosine phosphorylation of ACK1 (Satoh et al. 1996; Galisteo et al. 2006). In addition, ACK1 binds to the adaptor protein Grb2 through its N-terminal SH3 domain, which is enhanced by EGF stimulation (Satoh et al. 1996). The binding of ACK1 to Grb2 is required for EGF-dependent tyrosine phosphorylation of ACK1 (Kato-Stankiewicz et al. 2001).



ACK1, Fig. 2 Role of ACK1 in signaling triggered by cell-surface receptors. ACK1-mediated signaling pathways downstream of various receptors are shown. EGFR, epidermal growth factor receptor; mAChR, muscarinic acetylcholine receptor; PDGFR, platelet-derived growth factor receptor

Tyrosine-phosphorylated ACK1 in turn phosphorylates and activates its downstream target Dbl, leading to the induction of transient cytoskeletal rearrangements through RhoA (Ras homologue A) (Kato-Stankiewicz et al. 2001; see below). Mig6 binds to the EGF receptor and inhibits EGF signaling, sharing its EGF receptor-binding domain with ACK1. Thus, it is anticipated that ACK1 also binds to the EGF receptor directly, and negatively regulates downstream signaling. In addition to the EGF receptor, diverse receptor tyrosine kinases, such as Axl, Mer, ALK, LTK, and the platelet-derived growth factor receptor interact with ACK1 (Galisteo et al. 2006; Pao-Chun et al. 2009). The binding of ACK1 to Axl, ALK, LTK is mainly mediated by Grb2, having a role in ligand-induced down-regulation (Pao-Chun et al. 2009). In *C. elegans*, ARK-1 also physically interacts with SEM-5 (a *C. elegans* orthologue of Grb2), inhibiting LET-23 (a *C. elegans* orthologue of the EGF receptor)-mediated ovulation.

Melanoma chondroitin sulfate proteoglycan is a cell-surface antigen that stimulates integrin-$\alpha_4\beta_1$-mediated adhesion and spreading of melanoma cells. Clustering of this antigen induces the activation of Cdc42 and ACK1, leading to tyrosine phosphorylation of $p130^{Cas}$, a key molecule for the induction of tumor cell motility and invasion (Eisenmann et al. 1999). Therefore, ACK1 may play an important role in the regulation of melanoma chondroitin sulfate proteoglycan-dependent melanoma cell migration and invasion. ACK1 is also activated upon cell attachment to fibronectin in a Cdc42-dependent manner, suggesting a role in outside-in signaling of integrins (Yang et al. 1999; Galisteo et al. 2006). Stimulation of the M3 muscarinic acetylcholine receptor also triggers the activation of ACK1. Neither the increase in intracellular Ca^{2+} nor the activation of protein kinase C is required for this ACK1 activation. Instead, Cdc42 and the tyrosine kinase Fyn may be involved.

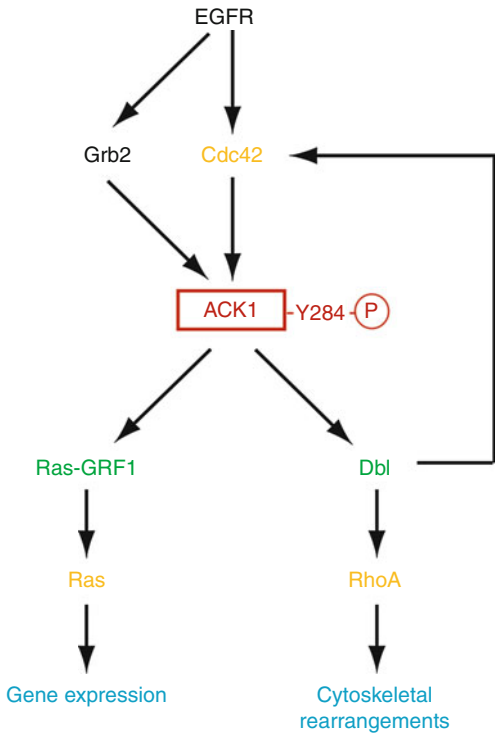
Beyond regulating downstream signaling cascades, ACK1 participates in the regulation of ligand-induced endocytosis and degradation of the receptor. ACK1 possesses a conserved clathrin-binding motif, which in fact interacts with the N-terminal head region of the clathrin heavy chain (Teo et al. 2001; Yang et al. 2001). Activated Cdc42 negatively regulates this interaction. When overexpressed, ACK1 competes with AP-2 for the binding to clathrin, and thus suppresses clathrin-mediated endocytosis of the transferrin receptor. Furthermore, ACK1 directly binds to sorting nexin 9 (SH3PX1) through the interaction between the

proline-rich domain of ACK1 and the SH3 domain of sorting nexin 9, thereby facilitating endocytosis of the EGF receptor. EGF-induced degradation of the EGF receptor is mediated by ubiquitination. ACK1 binds to the ubiquitinated EGF receptor through its ubiquitin-association domain, thereby regulating ligand-induced EGF receptor degradation (Shen et al. 2007). In addition, ACK1 is ubiquitinated by E3 ubiquitin ligases Nedd4-1 and Nedd4-2 and subjected to degradation along with the EGF receptor in response to EGF stimulation.

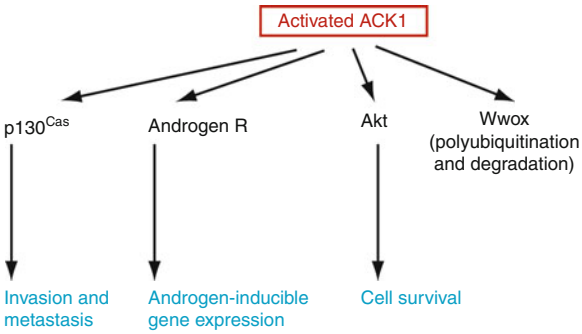
ACK1 as a Link Between Small GTPases

Dbl is the first-identified guanine nucleotide exchange factor (GEF) for Rho family GTPases. Upon tyrosine phosphorylation of Dbl by activated ACK1, GEF activity of Dbl toward the Rho family is significantly increased (Kato et al. 2000). Thus, ACK1 may act as a link between Rho family GTPases, forming a positive feedback loop of Cdc42 signaling (Fig. 3). Tyrosine-phosphorylated Dbl also activates RhoA, leading to cytoskeletal rearrangements (Kato-Stankiewicz et al. 2001).

Another GEF whose activity is regulated by ACK1 is Ras-GRF1 ($CDC25^{Mm}$ (mammalian homologue of cell division cycle 25)) (Kiyono et al. 2000). Ras-GRF1 targets both Ras and Rac1 through its CDC25 homology and Dbl homology domains, respectively. The latent GEF activity toward Rac1 is induced by G protein βγ subunits and ▶ Src-dependent tyrosine phosphorylation of Ras-GRF1 although its Ras GEF activity remains unchanged. In contrast, co-expression of activated ACK1 causes tyrosine phosphorylation of Ras-GRF1 and promotes its GEF activity toward Ras. Through this mechanism, ACK1 links Cdc42 to Ras. Tyrosine phosphorylation of Ras-GRF1 by ACK1, on the other hand, does not induce Rac1 GEF activity. Taken together, different tyrosine kinases including ACK1 phosphorylate different residues of Ras-GRF1, leading to selective induction of GEF activity toward Ras or Rac1.



ACK1, Fig. 3 ACK1 as a link between small GTPases. ACK1-mediated signaling pathways that link Ras and Rho family GTPases are shown. Small GTPases are shown in yellow, and GEFs are shown in green. EGFR, epidermal growth factor receptor



ACK1, Fig. 4 ACK1 in Cancer. Signaling pathways downstream of activated ACK1 in cancer cells are shown. Androgen R, androgen receptor; Wwox, WW domain-containing oxidoreductase

ACK1 in Cancer

Several lines of evidence support the notion that ACK1 is intimately associated with cancer (Fig. 4). Overexpression of a dominant-negative mutant of ACK1 or small interfering RNA-mediated knockdown of ACK1 inhibited v-Ras-transformed cell survival and growth, suggesting a crucial role of ACK1 in cell transformation (Nur-E-Kamal et al. 2005). ACK1 is also involved in the regulation of $p130^{Cas}$-mediated

signaling, such as spreading of melanoma cells (Eisenmann et al. 1999) and migration of breast epithelial cells. Thus, ACK1 may have a critical role particularly in invasion and metastasis of cancer cells. In fact, overexpression of ACK1 in tumor cells enhances invasive phenotypes in vivo and in vitro (van der Horst et al. 2005). Furthermore, amplification of the *ACK1* gene identified in human primary tumors correlates with poor prognosis (van der Horst et al. 2005).

The involvement of ACK1 in prostate cancer progression has been well described. Expression and tyrosine phosphorylation levels of ACK1 are elevated in clinical specimens of androgen-independent prostate cancer (Mahajan and Mahajan 2010; van der Horst et al. 2005). A constitutively activated ACK1 mutant, when expressed in a human prostatic adenocarcinoma cells, remarkably promotes anchorage-independent growth and tumor formation in nude mice (Mahajan and Mahajan 2010). Transgenic mice expressing this constitutively activated ACK1 in the prostate indeed develop prostatic intraepithelial neoplasia (Mahajan and Mahajan 2010).

One mechanism for ACK1-dependent promotion of prostate tumorigenesis is negative regulation of the proapoptotic tumor suppressor WW domain-containing oxidoreductase (Mahajan and Mahajan 2010). Activated ACK1 associates with and tyrosine phosphorylates WW domain-containing oxidoreductase, leading to its polyubiquitination followed by degradation.

Another mechanism is tyrosine phosphorylation of the androgen receptor by ACK1 (Mahajan et al. 2007). Activation of the androgen receptor may be important in androgen-independent progression of prostate cancer. Activated ACK1 phosphorylates two tyrosine residues in the transactivation domain of the androgen receptor, leading to the recruitment of the androgen receptor to the androgen-responsive enhancer and subsequent androgen-inducible gene expression in the absence of androgen. Thereby, activated ACK1 promotes androgen-independent growth of prostate xenograft tumors (Mahajan et al. 2007). On the other hand, ACK1 is required for tyrosine phosphorylation and activation of the androgen receptor by ligand-activated HER2 as evidenced by the effect of knockdown and the treatment with a specific inhibitor, dasatinib (Mahajan et al. 2007; Liu et al. 2010). Therefore, ACK1 activation downstream of cell-surface receptor tyrosine kinases such as HER2 may be a crucial event in prostate cancer cells. Another ACK1-specific inhibitor, termed AIM-100, also suppresses phosphorylation and transcriptional activation of the androgen receptor (Mahajan and Mahajan 2010).

The activation of the pro-survival protein kinase Akt has been implicated in a variety of human cancers. It has been well documented that Akt activation occurs through specific phosphorylation of serine and threonine residues downstream of ▶ phosphatidylinositol 3-kinase. Recently, a novel signaling mechanism whereby ACK1 directly regulates Akt by phosphorylating a tyrosine residue located in the kinase domain (Mahajan and Mahajan 2010). Upon tyrosine phosphorylation, Akt is translocated to the plasma membrane and then activated. This mechanism, in fact, has become relevant to human cancers. The expression level of Akt that is phosphorylated by ACK1 is significantly increased in breast cancers, and is correlated with the severity of disease progression.

Four somatic missense mutations were identified in ACK1 in various cancers: Mutations in the N-terminal sterile alpha motif domain (R34L and R99Q) were identified in lung adenocacinoma and ovarian mucinous carcinoma, respectively, a mutation in the kinase catalytic domain (E346K) was found in ovarian endometrioid carcinoma, and a mutation in the SH3 domain (M409I) was identified in gastric adenocacinoma. All of these mutations increase ACK1 kinase activity, suggesting that somatic mutations may represent a mechanism for oncogenic activation of ACK1 (Prieto-Echagüe et al. 2010). Several somatic and germ-line mutations were detected also in tumor cell lines. Among them, a mutation in the ubiquitin-association domain (S1002N) renders ACK1 unable to bind ubiquitin, and maintains the epidermal growth factor receptor level after stimulation. Thereby, this ACK1 mutant may contribute to prolonged mitogenic signaling in cancer cells.

Summary

ACK1 is a non-receptor tyrosine kinase originally identified as a target of the small GTPase Cdc42. ACK1 interacts with diverse signal transducing proteins through multiple domains. The kinase activity of ACK1 is suppressed by the intramolecular interaction between the catalytic domain and the C-terminal Mig6 homology domain. Upstream signals are believed to

induce conformational change of inactive ACK1, leading to its activation. ACK1 functions downstream of a variety of receptor tyrosine kinases such as the EGF receptor. In addition, other types of cell-surface receptors, including melanoma chondroitin sulfate proteoglycan and integrins, employ ACK1 as a transducer of signals that regulate cell motility. ACK1 is also responsible for ligand-induced endocytosis of cell-surface receptors through the binding to the clathrin heavy chain and sorting nexin 9. ACK1 is ubiquitinated by E3 ubiquitin ligases and subjected to degradation. ACK1 acts as a link between small GTPases by phosphorylating GEFs, such as Dbl and Ras-GRF1. Gene amplification and mutational activation of ACK1 are closely associated with human cancers. Particularly, the involvement of ACK1 in prostate cancer is well addressed. ACK1 phosphorylates the androgen receptor and the serine/threonine kinase Akt, leading to their activation. Overall, ACK1 is a key regulator of cell-surface receptor signaling in various types of cell responses including cytoskeletal rearrangements and transcriptional activation. Once excessively activated, ACK1 causes unregulated proliferation and survival, properties characteristic of cancer cells. Thus, ACK1 may be a promising therapeutic target of cancers.

References

Eisenmann KM, McCarthy JB, Simpson MA, Keely PJ, Guan JL, Tachibana K, Lim L, Manser E, Furcht LT, Iida J. Melanoma chondroitin sulphate proteoglycan regulates cell spreading through Cdc42, Ack-1 and p130^{cas}. Nat Cell Biol. 1999;1:507–13.

Galisteo ML, Yang Y, Ureña J, Schlessinger J. Activation of the nonreceptor protein tyrosine kinase Ack by multiple extracellular stimuli. Proc Natl Acad Sci USA. 2006;103:9796–801.

Kato J, Kaziro Y, Satoh T. Activation of the guanine nucleotide exchange factor Dbl following ACK1-dependent tyrosine phosphorylation. Biochem Biophys Res Commun. 2000;268:141–7.

Kato-Stankiewicz J, Ueda S, Kataoka T, Kaziro Y, Satoh T. Epidermal growth factor stimulation of the ACK1/Dbl pathway in a Cdc42 and Grb2-dependent manner. Biochem Biophys Res Commun. 2001;284:470–7.

Kiyono M, Kato J, Kataoka T, Kaziro Y, Satoh T. Stimulation of Ras guanine nucleotide exchange activity of Ras-GRF1/CDC25^{Mm} upon tyrosine phosphorylation by the Cdc42-regulated kinase ACK1. J Biol Chem. 2000;275:29788–93.

Liu Y, Karaca M, Zhang Z, Gioeli D, Earp HS, Whang YE. Dasatinib inhibits site-specific tyrosine phosphorylation of androgen receptor by Ack1 and Src kinases. Oncogene. 2010;29:3208–16.

Lougheed JC, Chen RH, Mak P, Stout TJ. Crystal structures of the phosphorylated and unphosphorylated kinase domains of the Cdc42-associated tyrosine kinase ACK1. J Biol Chem. 2004;279:44039–45.

Mahajan K, Mahajan NP. Shepherding AKT and androgen receptor by Ack1 tyrosine kinase. J Cell Physiol. 2010;224:327–33.

Mahajan NP, Liu Y, Majumder S, Warren MR, Parker CE, Mohler JL, Earp HS, Whang YE. Activated Cdc42-associated kinase Ack1 promotes prostate cancer progression via androgen receptor tyrosine phosphorylation. Proc Natl Acad Sci USA. 2007;104:8438–43.

Manser E, Leung T, Salihuddin H, Tan L, Lim L. A non-receptor tyrosine kinase that inhibits the GTPase activity of p21^{cdc42}. Nature. 1993;363:364–7.

Nur-E-Kamal A, Zhang A, Keenan SM, Wang XI, Seraj J, Satoh T, Meiners S, Welsh WJ. Requirement of activated Cdc42-associated kinase for survival of v-Ras-transformed mammalian cells. Mol Cancer Res. 2005;3:297–305.

Pao-Chun L, Chan PM, Chan W, Manser E. Cytoplasmic ACK1 interaction with multiple receptor tyrosine kinases is mediated by Grb2: an analysis of ACK1 effects on Axl signaling. J Biol Chem. 2009;284:34954–63.

Prieto-Echagüe V, Gucwa A, Craddock BP, Brown DA, Miller WT. Cancer-associated mutations activate the nonreceptor tyrosine kinase Ack1. J Biol Chem. 2010;285:10605–15.

Satoh T, Kato J, Nishida K, Kaziro Y. Tyrosine phosphorylation of ACK in response to temperature shift-down, hyperosmotic shock, and epidermal growth factor stimulation. FEBS Lett. 1996;386:230–4.

Shen F, Lin Q, Gu Y, Childress C, Yang W. Activated Cdc42-associated kinase 1 is a component of EGF receptor signaling complex and regulates EGF receptor degradation. Mol Biol Cell. 2007;18:732–42.

Teo M, Tan L, Lim L, Manser E. The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors. J Biol Chem. 2001;276:18392–8.

van der Horst EH, Degenhardt YY, Strelow A, Slavin A, Chinn L, Orf J, Rong M, Li S, See LH, Nguyen KQ, Hoey T, Wesche H, Powers S. Metastatic properties and genomic amplification of the tyrosine kinase gene ACK1. Proc Natl Acad Sci USA. 2005;102:15901–6.

Yang W, Lin Q, Guan JL, Cerione RA. Activation of the Cdc42-associated tyrosine kinase-2 (ACK-2) by cell adhesion via integrin β_1. J Biol Chem. 1999;274:8524–30.

Yang W, Lo CG, Dispenza T, Cerione RA. The Cdc42 target ACK2 directly interacts with clathrin and influences clathrin assembly. J Biol Chem. 2001;276:17468–73.

Yokoyama N, Miller WT. Biochemical properties of the Cdc42-associated tyrosine kinase ACK1. Substrate specificity, authphosphorylation, and interaction with Hck. J Biol Chem. 2003;278:47713–23.

Activated Cdc42Hs-Associated Kinase 1

► ACK1

Activators of G-Protein Signaling (AGS)

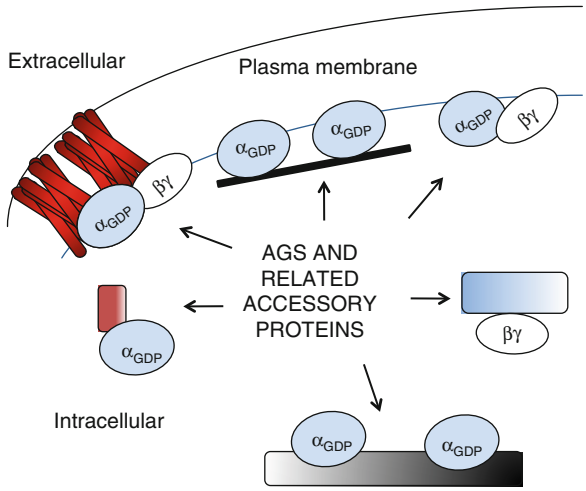
Joe B. Blumer and Stephen M. Lanier
Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

Synonyms

DYNLT1/Tctex-1 (AGS2); FNDC1/fibronectin type III domain containing 1 (AGS8); GNAO /Gαo/guanine nucleotide binding protein/alpha activating activity polypeptide O (AGS10); GPSM1/G-protein signaling modulator 1 (AGS3); GPSM3/G-protein signaling modulator 3/G18/NG1 (AGS4); GPSM2/G-protein signaling modulator 2/LGN/mPINS (AGS5); MITF/microphthalmia-associated transcription factor (AGS13); PSMD4/Rpn10/proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (AGS9); RAS D1/DEXRAS1 (AGS1); RGS 12/regulator of G-protein signaling 12 (AGS6); TFE3/transcription factor binding to IGHM enhancer 3 (AGS11); TFEB/transcription factor EB (AGS12); TRIP13/16E1BP/thyroid hormone receptor interactor 13 (AGS7).

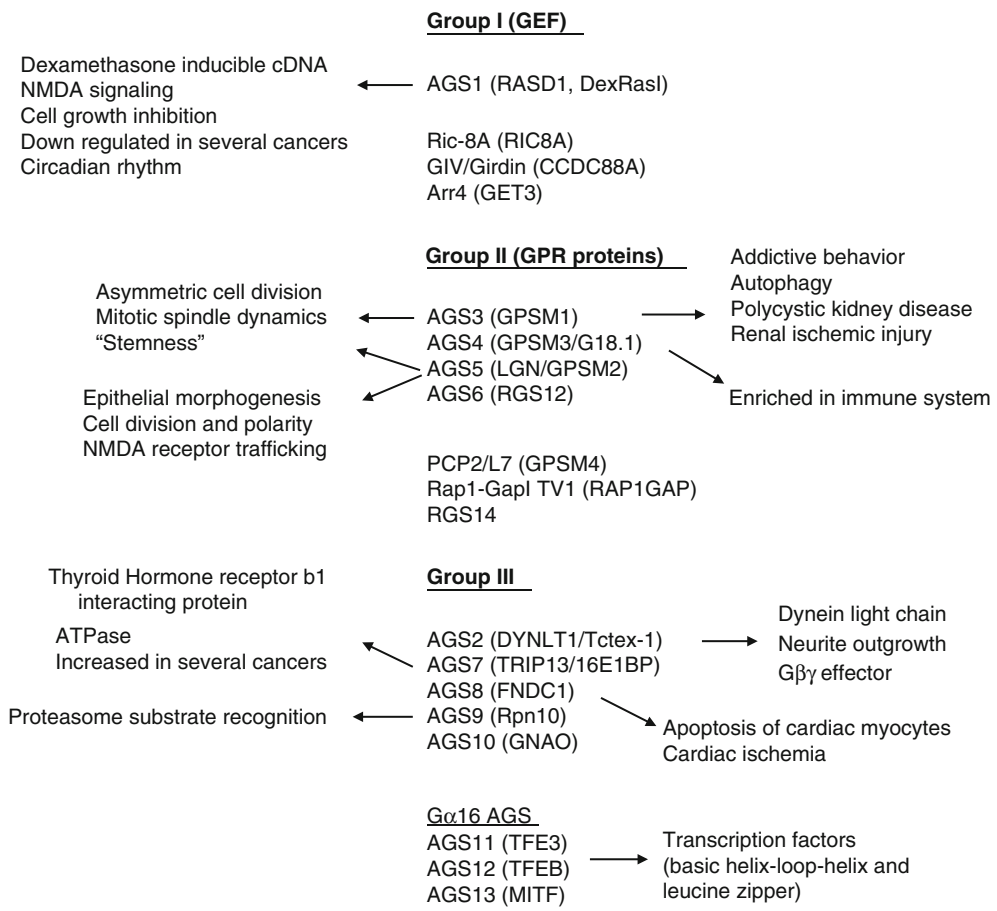
Historical Background

Activators of G-protein signaling (AGS) proteins define a group of proteins identified in a yeast-based functional screen of mammalian cDNA libraries as receptor-independent activators of the G-protein signaling cascade. Activators of G-protein signaling are one subgroup of accessory proteins for G-protein signaling systems. Accessory proteins are generally defined as proteins other than the core triad of receptor (R), G-protein, and effector (E) that regulate the efficiency and/or specificity of signal transfer from G-protein coupled receptors (GPCRs) to G-proteins, segregate a signaling complex to microdomains of the cell, regulate the basal activity of the system, and/or provide alternative modes of signal input to G-protein signaling systems that operate independent of a typical GPCR. Such accessory proteins may influence G-protein signaling systems operating at the cell cortex or at intracellular locations (Fig. 1).



Activators of G-Protein Signaling (AGS), Fig. 1 *Schematic diagram indicating points of influence of accessory proteins on G-protein signaling systems.* AGS and related proteins may serve as alternative binding partners for G-protein subunits and also regulate signal transfer at multiple points within the broader G-protein signaling system (The figure is a modification of the concept figure presented by Sato et al. 2006)

The concept of accessory proteins resulted from a confluence of several independent lines of investigation (see Blumer et al. 2007, 2011; Gonczy 2008; Knoblich 2010; Sato et al. 2006; Willard et al. 2004). These lines of investigation included cell-specific differences in signal transfer from R to G, the partial purification of a putative non-receptor G-protein activator from extracts of NG108-15 cells, identification of G-protein subunits in intracellular organelles, identification of unexpected binding partners for G-protein subunits, and the identification of non-receptor proteins that could influence the activation state of G-proteins eventually leading to the development of a functional yeast-based screen for mammalian entities that activated G-protein signaling in the absence of a receptor (Sato et al. 2006; Cismowski and Lanier 2005; Cismowski et al. 1999; Takesono et al. 1999). Interspersed with these biochemical approaches was the realization that there were changes in signal processing through G-protein signaling systems that occurred independent of any obvious changes in receptor number or G-protein expression levels, suggesting additional undefined regulatory mechanisms. Another line of investigation evolved out of the study of asymmetric cell division in *Drosophila melanogaster* neuroblasts and sensory organ precursor cells in parallel with the *Caenorhabditis elegans* embryo.



Activators of G-Protein Signaling (AGS), Fig. 2 *Functional roles of different Activators of G-protein signaling*. This figure includes proteins that were not isolated in the yeast-based functional screen as AGS proteins, but were identified in other protein interaction or functional screens and shown to exhibit biological activity consistent with their inclusion as Group 1 (RIC8A, CCDC88A, GET3) or Group II (GPSM4, RAP1GAP, RGS14) AGS proteins (Modified from Blumer et al. 2007)

Thus, a confluence of biochemical, cell biology, and model organism data indicated unexpected modes of regulation for heterotrimeric G-proteins associated with previously unknown functional roles for this signaling system.

The discovery of AGS proteins involved the development of a functional readout that would allow rapid screening of mammalian cDNAs for their ability to activate G-protein signaling in the absence of a receptor. The initial reports of the discovery of AGS proteins using the yeast-based functional screen (Cismowski et al. 1999) included three AGS proteins with each defining a distinct group of AGS proteins (Fig. 2) (Cismowski et al. 1999; Takesono et al. 1999). AGS1 behaved as a guanine nucleotide exchange factor for Gαi/Gαo. AGS3 is the prototype for Group II AGS proteins. AGS2 defined Group III AGS proteins, which generally interact with Gβγ. In the yeast-based functional platform, Group I and II AGS proteins were functionally active in yeast strains expressing Gαi2, Gαi3, but not Gα16 or Gαs whereas the activity of Group III AGS proteins was independent of the type of Gα expressed. Subsequent screening of different mammalian cDNA libraries generated from different tissues exposed to different challenge paradigms or exhibiting specific pathologies yielded 13 distinct mammalian cDNAs that exhibited bioactivity in this functional platform (Fig. 2). AGS11-13, which are actually transcription factors, were identified in the functional screen using yeast expressing Gα16, but

there is not currently enough information to determine how they should be subclassified within the larger group of AGS proteins (Sato et al. 2011).

Group I AGS Proteins

To date, AGS1 is the only cDNA isolated in the yeast expression cloning system exhibiting functional activity consistent with its classification as a guanine nucleotide exchange factor (GEF). Initially isolated as a dexamethasone-inducible cDNA (Kemppainen and Behrend 1998), AGS1 (RASD1) appears to inhibit cell growth (Vaidyanathan et al. 2004) and is downregulated in various cancers (Dalgin et al. 2007; de Souza Rocha Simonini et al. 2010; Furuta et al. 2006; Nojima et al. 2009). AGS1 is also reported to interact with Gβγ (Hiskens et al. 2005). Additional non-receptor GEFs such as ▶ Ric-8A and ▶ Ric-8B (Miller et al. 2000; Tall et al. 2003), GIV/Girdin (Le-Niculescu et al. 2005), and Arr4 (Lee and Dohlman 2008) have been identified by other functional and protein interaction screens, and these proteins would also fit the definition of Group I AGS proteins.

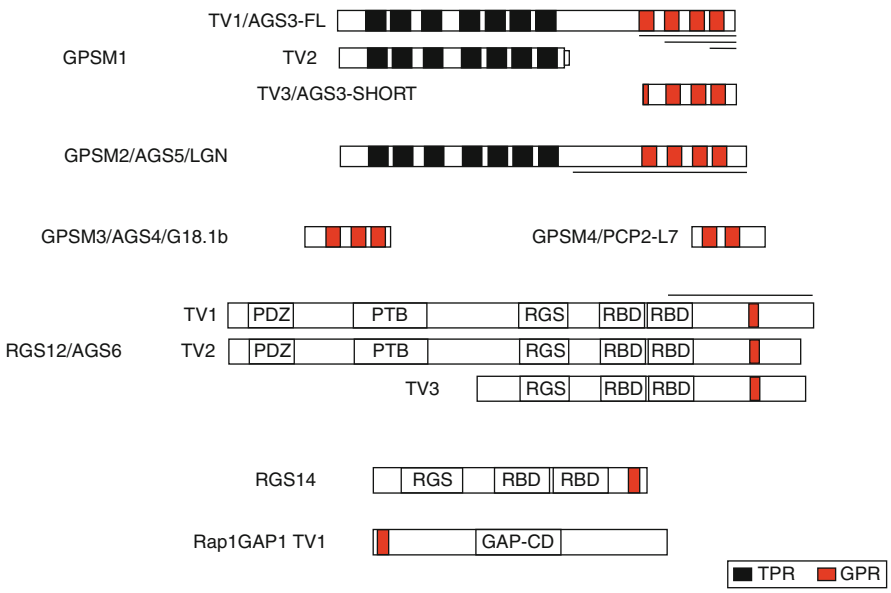
Group II AGS Proteins

Group II AGS proteins are characterized by the presence of one to four, evolutionarily conserved, G-protein regulatory (GPR) or GoLoco motifs (Takesono et al. 1999; Siderovski et al. 1999), which are 20–25-amino acid cassettes that interact with Gαi/o-GDP and Gαt-GDP. Although the core residues within GPR motifs are conserved, individual GPR motifs may differ in their relative affinities for different Gαi/o and ▶ Gαt family members. The GPR motif defines a novel, totally unexpected mechanism for regulation of the activation-deactivation cycle of heterotrimeric G-proteins with potentially broad conceptual implications. In contrast to Group I and III AGS proteins, each member of the Group II AGS proteins has a shared structural motif (GPR/GoLoco) (Fig. 3).

The genes encoding AGS3, AGS4, and AGS5 were named G-protein signaling modulator (GPSM) 1, 3, and 2, respectively, by the HUGO Gene Nomenclature Committee. There are at least three additional GPR proteins in mammals, Pcp2/L7 (GPSM4), RGS14, and Rap1Gap (Transcript Variant 1, isoform a, Rap1GapII), which to date have not been identified in any of the yeast-based functional screens described above, likely reflecting variations in cDNA representation in the libraries used for screening or other factors which affect their activity in the yeast screen. In silico screens based on conserved GPR consensus sequences also identified a putative GPR motif in the protein WAVE1/Scar, although it is not yet known if the motif actively engages Gα-GDP in the context of WAVE1 function in the cell (Song et al. 2006).

Proteins with GPR motifs can be further subgrouped based on the number of GPR motifs they contain. One subset (AGS3, AGS4, AGS5, and Pcp2/L7) has 2–4 GPR motifs, whereas a second subset (AGS6/RGS12, RGS14, Rap1Gap) contains a single GPR motif. Within the first subset, AGS3 and AGS5 share a similar domain architecture and are ~60% identical at the amino acid level with an amino-terminal tetratricopeptide repeat (TPR) domain and four carboxyl-terminal GPR motifs. AGS4 and Pcp2/L7, as well as the AGS3 variant AGS3-short, have 2–3 GPR motifs and lack any other clearly defined protein interaction domains. Within the second subset (AGS6/RGS12, RGS14, and Rap1Gap), in addition to their single GPR motif, each of the three proteins contain a GTPase-activating (GAP) domain in addition to other protein interaction or regulatory motifs. Thus, AGS6/RGS12 and RGS14 have an interesting arrangement of a GPR domain, which can dock $G\alpha_{GDP}$ independently of Gβγ, and a GAP domain which accelerates GTP hydrolysis on the transition state of activated Gα, thus acting to terminate $G\alpha_{GTP}$-based signaling. Although conceptually this is a very interesting combination of domains with completely different, but perhaps complementary, effects on the nucleotide-bound state of Gα, the functional roles of these intramolecular domains within AGS6/RGS12 and RGS14 on the integration of G-protein signaling are not yet fully understood. The GAP domain in Rap1Gap does not regulate heterotrimeric Gα subunits but rather targets the ras-related small GTPase Rap1.

GPR motifs serve as docking sites for Gαi-GDP free of Gβγ and this GPR-Gα signaling module is regulated by both cell surface G-protein coupled receptors and non-receptor GEFs (▶ RIC-8, GIV/Girdin), which promote exchange of GDP for GTP and dissociation or rearrangement of Gα and the GPR motif in a manner



Activators of G-Protein Signaling (AGS), Fig. 3 *Schematic representation of Group II AGS proteins*. Where indicated, the *lines* underneath the schematic representation of individual entities correspond to the coding region actually isolated in the initial yeast-based functional screen (The figure is reproduced from Blumer et al. 2011)

analogous to that observed for G-protein-coupled receptor-mediated regulation of Gαβγ (Garcia-Marcos et al. 2011; Tall and Gilman 2005; Thomas et al. 2008; Vellano et al. 2011). The GPR-Gα signaling module plays a central role in asymmetric cell division in multiple organisms and system adaptation (Fig. 1) (Blumer et al. 2007; Gonczy 2008; Knoblich 2010). Determination of the X-ray crystal structure of the GPR/GoLoco peptide-Gαi1 complex provides a structural basis for understanding interaction of the motif with GαGDP (Bosch et al. 2011; Kimple et al. 2002). Structure-activity studies have revealed specific amino acids in the GPR motif that influence interaction with Gα (Peterson et al. 2002) and a key residue in Gα that is required for interaction with GPR motifs (Bosch et al. 2011).

Mechanistically, proteins with GPR motifs may impart biological activity by influencing subunit interactions to promote or sustain dissociation of Gαβγ heterotrimers in the absence of nucleotide exchange. In the context of the G-protein activation-deactivation cycle, the GPR protein may also influence subunit interactions by binding $G\alpha_{GDP}$ prior to reassociation with Gβγ. In either situation, alterations in Gβγ-regulated effector pathways would be expected. The $G\alpha_{GDP}$-GPR complex itself is actually suggested to be a putative "bioactive" entity (Gonczy 2008). The Gα-GPR signaling module can also function as a discreet signaling system that is regulated by non-receptor guanine nucleotide exchange factors and perhaps G-protein-coupled receptors. Interplay between the Gα-GPR and the classical heterotrimer Gαβγ signaling modules offers additional interesting mechanisms for signal integration (Blumer et al. 2011).

A nonsense mutation in GPSM2, which truncates the reading frame of the protein, is associated with nonsyndromic hearing loss likely as a result of altered planar cell polarity in the auditory system (Walsh et al. 2010; Yariz et al. 2011). Renal AGS3 (GPSM1) is markedly elevated in polycystic kidney disease and in response to renal injury (Nadella et al. 2010; Regner et al. 2011). In cell culture, AGS3 traffics into the aggresome pathway and it is also a central player in regulating autophagy (Garcia-Marcos et al. 2011).

Group III AGS Proteins

The mechanisms by which the Group III AGS proteins activate G-protein signaling in the yeast functional screen and function in mammalian signaling systems are not yet fully understood. As more information becomes available, it is likely that members of this loosely defined Group III will exhibit different mechanisms of action in terms of their ability to lead to the end readout of Gβγ-dependent growth in the yeast functional screen. One mechanism by which the

Group III proteins may act is by an interaction with Gβγ and/or heterotrimer to influence subunit interactions in a way that there is productive effector engagement. AGS2 (tctex1/DYNLT1) is actually a light chain for the cytoplasmic motor protein dynein and regulates neurite outgrowth through its interaction with Gβγ and dynein (Sachdev et al. 2007). AGS7 (TRIP13) was identified as a thyroid receptor–interacting protein and AGS8 (FNDC1) promotes apoptosis of cardiac myocytes. AGS9 (Rpn10) is a component of the 26S proteasome.

Summary

The discovery of AGS and related proteins altered our basic concepts of G-protein signaling. First, Gα and Gβγ are processing signals within the cell distinct from their role as transducers for cell surface receptors and such signals involve previously unrecognized functional roles for heterotrimeric G-protein subunits. Secondly, Gα and Gβγ may complex with alternative binding partners independent of the classical Gαβγ heterotrimer, providing a distinct signaling pathway with its own set of activators and effectors.

AGS and related accessory proteins or signal regulators are intimately involved in generating signaling diversity in ways that are not yet fully recognized. Such accessory proteins have evolved to provide a mechanism for cells to adapt acutely and for a longer-term to physiological and pathological challenges without altering the major components of the signaling core itself. AGS proteins and related entities play unexpected and important functional roles in a number of systems and impact a number of signaling pathways that influence cell growth and survival. Rapidly accumulating data from disease tissue profiling and genomic-based technologies indicate that selected AGS proteins may serve as biomarkers for specific diseases and their altered expression or function in disease states suggests candidate signaling modules for therapeutic targeting.

Central questions in the field are as follows: What regulates the subcellular location of AGS proteins? What regulates the interaction of AGS proteins with Gα or Gβγ? What is downstream of the GPR-Gα signaling module? Are there pathologies associated with polymorphisms in AGS proteins? Are AGS proteins candidate therapeutic targets? How did the different "G-switch" modules (e.g., Gα-GPR, Gαβγ) and their regulation by non-receptor and cell surface receptors evolve in response to different evolutionary pressures in terms of signal processing?

References

Blumer JB, Smrcka AV, Lanier SM. Mechanistic pathways and biological roles for receptor-independent activators of G-protein signaling. Pharmacol Ther. 2007;113:488–506.

Blumer JB, Oner SS, Lanier SM. Group II activators of G-protein signalling and proteins containing a G-protein regulatory motif. Acta Physiol (Oxford) 2011; in press.

Bosch DE, Kimple AJ, Sammond DW, Muller RE, Miley MJ, Machius M, et al. Structural determinants of affinity enhancement between GoLoco motifs and G-protein alpha subunit mutants. J Biol Chem. 2011;286:3351–8.

Cismowski MJ, Lanier SM. Activation of heterotrimeric G-proteins independent of a Gprotein coupled receptor and the implications for signal processing. Rev Physiol Biochem Pharmacol. 2005;155:57–80.

Cismowski MJ, Takesono A, Ma C, Lizano JS, Xie X, Fuernkranz H, et al. Genetic screens in yeast to identify mammalian nonreceptor modulators of G-protein signaling. Nat Biotechnol. 1999;17:878–83.

Dalgin GS, Holloway DT, Liou LS, DeLisi C. Identification and characterization of renal cell carcinoma gene markers. Cancer Inform. 2007;3:65–92.

de Souza Rocha Simonini P, Breiling A, Gupta N, Malekpour M, Youns M, Omranipour R, et al. Epigenetically deregulated microRNA-375 is involved in a positive feedback loop with estrogen receptor alpha in breast cancer cells. Cancer Res. 2010;70:9175–84.

Furuta J, Nobeyama Y, Umebayashi Y, Otsuka F, Kikuchi K, Ushijima T. Silencing of peroxiredoxin 2 and aberrant methylation of 33 CpG islands in putative promoter regions in human malignant melanomas. Cancer Res. 2006;66:6080–6.

Garcia-Marcos M, Ear J, Farquhar MG, Ghosh P. A GDI and a GEF regulate autophagy by balancing G protein activity and growth factor signals. Mol Biol Cell. 2011;22:673–86.

Gonczy P. Mechanisms of asymmetric cell division: flies and worms pave the way. Nat Rev Mol Cell Biol. 2008;9:355–66.

Hiskens R, Vatish M, Hill C, Davey J, Ladds G. Specific in vivo binding of activator of G protein signalling 1 to the Gbeta1 subunit. Biochem Biophys Res Commun. 2005;337:1038–46.

Kemppainen RJ, Behrend EN. Dexamethasone rapidly induces a novel ras superfamily member-related gene in AtT-20 cells. J Biol Chem. 1998;273:3129–31.

Kimple RJ, Kimple ME, Betts L, Sondek J, Siderovski DP. Structural determinants for GoLoco-induced inhibition of nucleotide release by Galpha subunits. Nature. 2002;416:878–81.

Knoblich JA. Asymmetric cell division: recent developments and their implications for tumour biology. Nat Rev Mol Cell Biol. 2010;11:849–60.

Lee MJ, Dohlman HG. Coactivation of G protein signaling by cell-surface receptors and an intracellular exchange factor. Curr Biol. 2008;18:211–5.

Le-Niculescu H, Niesman I, Fischer T, Devries L, Farquhar MG. Identification and characterization of GIV, a novel G alpha is interacting protein found on COPI, ER-Golgi transport vesicles. J Biol Chem. 2005;280:22012–20.
Miller KG, Emerson MD, McManus JR, Rand JB. RIC-8 (Synembryn): a novel conserved protein that is required for G(q)alpha signaling in the *C elegans* nervous system. Neuron. 2000;27:289–99.
Nadella R, Blumer JB, Jia G, Kwon M, Akbulut T, Qian F, et al. Activator of G protein signaling 3 promotes epithelial cell proliferation in PKD. J Am Soc Nephrol. 2010;21:1275–80.
Nojima M, Maruyama R, Yasui H, Suzuki H, Maruyama Y, Tarasawa I, et al. Genomic screening for genes silenced by DNA methylation revealed an association between RASD1 inactivation and dexamethasone resistance in multiple myeloma. Clin Cancer Res. 2009;15:4356–64.
Peterson YK, Hazard 3rd S, Graber SG, Lanier SM. Identification of structural features in the G-protein regulatory motif required for regulation of heterotrimeric G proteins. J Biol Chem. 2002;277:6767–70.
Regner KR, Nozu K, Lanier SM, Blumer JB, Avner ED, Sweeney Jr WE, et al. Loss of activator of G-protein signaling 3 impairs renal tubular regeneration following acute kidney injury in rodents. FASEB J. 2011;25:1844–55.
Sachdev P, Menon S, Kastner DB, Chuang JZ, Yeh TY, Conde C, et al. G protein beta gamma subunit interaction with the dynein light-chain component Tctex-1 regulates neurite outgrowth. EMBO J. 2007;26:2621–32.
Sato M, Blumer JB, Simon V, Lanier SM. Accessory proteins for G proteins: partners in signaling. Annu Rev Pharmacol Toxicol. 2006;46:151–87.
Sato M, Hiraoka M, Suzuki H, Bai Y, Kurotani R, Yokoyama U, et al. Identification of transcription factor E3 (TFE3) as a receptor-independent activator of G{alpha}16: Gene regulation by nuclear G{alpha} subunit and its activator. J Biol Chem. 2011;286:17766–76.
Siderovski DP, Diverse-Pierluissi M, De Vries L. The GoLoco motif: a Galphai/o binding motif and potential guanine-nucleotide exchange factor. Trends Biochem Sci. 1999;24:340–1.
Song KS, Peterson YK, Freidman A, Blumer JB, Sato M, Lanier SM. Identification and characterization of a G-protein regulatory motif in WAVE1. FEBS Lett. 2006;580:1993–8.
Takesono A, Cismowski MJ, Ribas C, Bernard M, Chung P, Hazard 3rd S, et al. Receptor-independent activators of heterotrimeric G-protein signaling pathways. J Biol Chem. 1999;274:33202–5.
Tall GG, Gilman AG. Resistance to inhibitors of cholinesterase 8A catalyzes release of Galphai-GTP and nuclear mitotic apparatus protein (NuMA) from NuMA/LGN/Galphai-GDP complexes. Proc Natl Acad Sci USA. 2005;102:16584–9.
Tall GG, Krumins AM, Gilman AG. Mammalian Ric-8A (synembryn) is a heterotrimeric Galpha protein guanine nucleotide exchange factor. J Biol Chem. 2003;278:8356–62.
Thomas CJ, Tall GG, Adhikari A, Sprang SR. Ric-8A catalyzes guanine nucleotide exchange on G alphai1 bound to the GPR/GoLoco exchange inhibitor AGS3. J Biol Chem. 2008;283:23150–60.
Vaidyanathan G, Cismowski MJ, Wang G, Vincent TS, Brown KD, Lanier SM. The Ras-related protein AGS1/RASD1 suppresses cell growth. Oncogene. 2004;23:5858–63.
Vellano CP, Shu FJ, Ramineni S, Yates CK, Tall GG, Hepler JR. Activation of the regulator of G protein signaling 14-Galphai1-GDP signaling complex is regulated by resistance to inhibitors of cholinesterase-8A. Biochemistry. 2011;50:752–62.
Walsh T, Shahin H, Elkan-Miller T, Lee MK, Thornton AM, Roeb W, et al. Whole exome sequencing and homozygosity mapping identify mutation in the cell polarity protein GPSM2 as the cause of nonsyndromic hearing loss DFNB82. Am J Hum Genet. 2010;87:90–4.
Willard FS, Kimple RJ, Siderovski DP. Return of the GDI: the GoLoco motif in cell division. Annu Rev Biochem. 2004;73:925–51.
Yariz KO, Walsh T, Akay H, Duman D, Akkaynak AC, King MC et al. A truncating mutation in GPSM2 is associated with recessive nonsyndromic hearing loss. Clin Genet. 2011; in press.

ACTR

► Steroid Receptor Coactivator Family

ADAP

Ana Kasirer-Friede
Department of Medicine, University of California, La Jolla, San Diego, CA, USA

Synonyms

Fyn-binding protein (FYB); SLP-76 adapter-binding protein of 130 kDa (SLAP-130)

Historical Background

The adhesion and degranulation promoting adapter protein (ADAP) is a large protein which is alternatively spliced to produce a 120/130 kDa isoform, and is expressed in T cells, proB cells, and myeloid cells. ADAP was first cloned in 1997 by two independent labs that were working to elucidate signaling pathways induced by engagement of the T cell receptor. In early studies, the kinase, Fyn-T, was found to be an important mediator of mature, peripheral T cell activation. In order to identify potential substrates of Fyn-T, da Silva et al., used GST–FYN-SH2 domain fusion proteins to

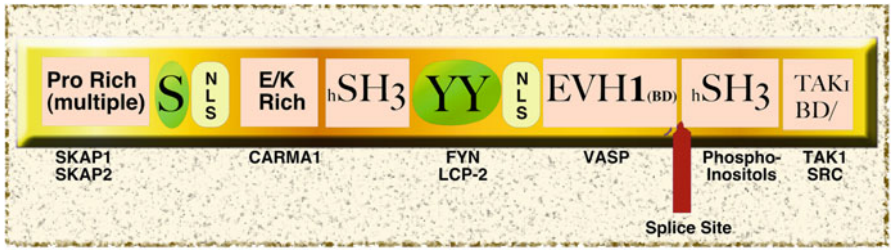
immunoprecipitate a 120/130 kDa phosphorylated protein from a TCR/antiCD3 stimulated T cell line. An antibody developed against this protein was used to screen a Jurkat cell line cDNA library, and led to the cloning of ADAP. In the same year, Musci et al., used the SH2 domain of another protein LCP2 (previously known as SLP-76) that is phosphorylated upon T cell stimulation and modulates T cell function, to precipitate out ADAP from pervanadate-stimulated JA2/SLP-SH2 Jurkat T cells expressing a chimeric surface protein with the extracellular and transmembrane domains from HLA.A2 and the SH2 domain from LCP2. The newly cloned proteins were named Fyn-binding protein, FYB, and SLP-76-associated protein of 130 kDa (SLAP-130) respectively, depending on the precipitating protein used in cloning; however, the name, adhesion and degranulation-promoting adapter protein, ADAP, was adopted in 2001. Another major milestone was achieved with the generation of chimeric and knockout ADAP mice ($ADAP^{-/-}$) by Griffiths et al., and Peterson et al., and helped resolve some of the controversies related to ADAP function that had surfaced using various cell lines (Peterson 2003). In recent years, the elucidation of new ADAP domains and interaction partners has expanded the repertoire of functions attributed to ADAP, that span from actin regulatory roles in lymphocytes, macrophages, and platelets, to mast cell degranulation, T cell proliferation, thymopoiesis, osteoclastogenesis, eosinophil survival, and integrin regulation (Koga et al. 2005;Wang and Rudd 2008).

ADAP Structure and Domains

ADAP fulfills its scaffolding function through a variety of domains that permit assembly of multimolecular complexes to modulate diverse functions (NP_001456.3;GI:42476118; NP_955367.1 GI:40788007). The human ADAP protein is reported to have 783 amino acids (AA) in the 120 kDa, or 803AA in the 130 kDa form. The full-length mouse ADAP is 819AA. The regulation of ADAP is likely to be complex due to the presence of several serine/threonine residues and lysine acetylation sites, two putative nuclear localization sequences (PKKKRKV), and 16 tyrosine phosphorylation sites, 13 of which are concentrated in the region between AA486 and 783 (Sylvester et al. 2010). Figure 1 highlights many of the domains that have been mapped to date. At the amino terminal end is one of four proline-rich domains that mediate association with SH3 domain-containing proteins: monomeric actin-binding protein (mABP, also known as HIP55), and the homologous adapter proteins SKAP1, and SKAP2, with ADAP residues 338–358 minimally required for stabilization of the SKAP proteins. There is a glutamic acid/lysine (E/K) rich region that constitutes a MAGUK binding domain. Downstream of this region is the first of two unusual helically extended SH3 domains (hSH3) that have lost the ability to bind the classical SH3 proline-rich recognition motif, but instead may associate with phospholipids (Heuer et al. 2004). Interposed between the hSH3 domains are the mid-protein tyrosines YEDI and two YDDV sequences (residues 461–464; 595–598; and 651–654), which are recognition sites for SH2 domains of FYN and LCP2 respectively, and a central FPPPP site that constitutes a VASP EVH1 binding domain. In mast cells, ADAP may bind the SH2 domain of the LCP2 family protein, mast cell immunoreceptor signal transducer (MIST). Limited structural data exists for ADAP: Zimmerman et al., have demonstrated that the two cysteines in ADAP that are adjacent to the $hSH3^{N}$ domain can be reversibly oxidized and reduced in vitro, and result in two distinct conformations of the variable arginine threonine loop (Zimmermann et al. 2007). A three-dimensional C-terminal domain NMR structure has been solved by Heuer et al. (2004) (Fig. 2), and depicts the hSH3 domain as a twisted β-sheet structure, with the N-terminal helical portion packed against the open side of the twisted sheet and contacting residues from the β-strands, thus forming an helically extended SH3 domain. In the very carboxy-terminus of ADAP, there are binding sites for TAK1 and SRC. In summary, the combinatorial binding possibilities that may exist depending on cell-specific expression of binding partners are complex and harbor potential for as yet undetermined functions.

ADAP Role in Proliferation and Transcription

Although evidence from studies in T cell lines and the $ADAP^{-/-}$mouse established that ADAP positively modulates T cell proliferation and cytokine production following stimulation of the T cell receptor (TCR), the potential ADAP effector binding partners that could regulate transcriptional events were not known. Medeiros et al. (2007) first described how ADAP may promote T cell proliferation by modulation of NF-KB. In T cells, activation of the NF-KB family of transcription factors and their translocation to the nucleus to transcription target sites is necessary for T cell

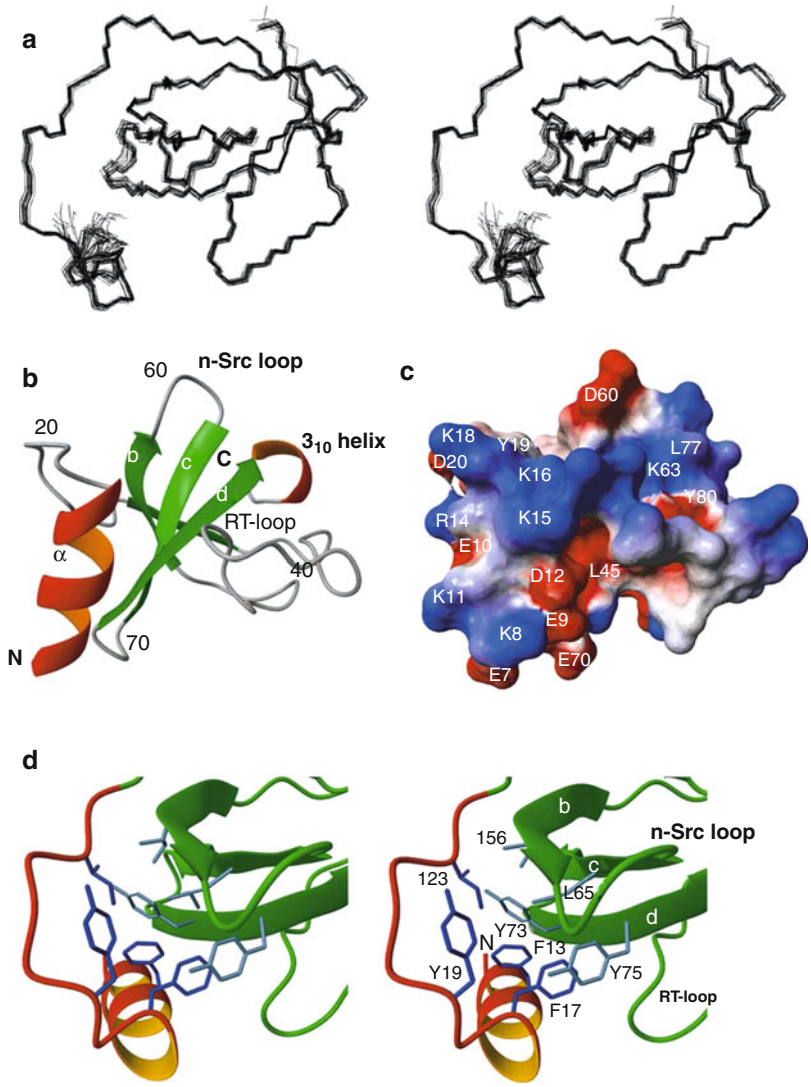


ADAP, Fig. 1 *Structural features of ADAP*. Adhesion and degranulation-promoting adapter protein (ADAP) is a large protein that harbors domains for scaffolding proteins of various classes. Shown here are many of the ADAP domains described to date. The N-terminus domain residues 1–300 support binding of monomeric actin-binding protein (mABP1; also known as HIP55), and the proline-rich regions (Pro-rich) support binding to SH3 domains of the homologous SKAP adapter proteins, SKAP 1 and SKAP2. There are several serine/threonines (S) and tyrosines (Y) that may be phosphorylated and mediate binding to SH2 domains of FYN and LCP-2 (formerly known as SLP-76), an E/K rich region within residues 426–541 for binding the MAGUK domain of CARMA1, two putative nuclear localization sequences (NLS), an FPPPP sequence within 616–624 region that binds the EVH1 domain of VASP, followed by an alternative splice site of 46AA. There are two helically extended SH3 domains (hSH3) that bind phosphoinositols and there is a recognition site for the phosphatase YopH in the C-hSH3 domain. In the very carboxy-terminus end are binding sites for the kinases TAK1 and SRC within -ADAP residues (AA691-708 in murine) and at Y807 respectively. Figure is not drawn to scale

survival. In resting T cells, NF-KB is held in an inhibited state by tight association with IKBα within the IKB family complex in the cytoplasm. T cell stimulation promotes the protein kinase C (PKC)θ dependent assembly of a multiprotein complex that serves to activate IKB. The complex consists of the caspase-like protein MALT1, the adapter Bcl-10, and caspase recruitment domain (CARD) membrane-associated protein1 (CARMA1), which contains a membrane-associated guanylate kinase (MAGUK) domain. Complex assembly results in phosphorylation of IKKα/β, ubiquitination of IKKγ, and degradation of IKBα, thereby freeing NF-KB to translocate to the nucleus. Using wild-type and mutant forms of HA-epitope tagged ADAP constructs expressed in Jurkat cells activated with PMA, Medeiros et al., elegantly showed that ADAP and CARMA1 directly associate and promote assembly of the MALT1/CARMA-1/BCL-10 complex and thereby, relocalization of MALT1 and BCL-10 to the membrane. ADAP and CARMA1 also colocalized at the membrane by way of an association between ADAP E/K rich residues within AA426-546, and the CARMA1 carboxy-terminal MAGUK region. As expected, in ADAP$^{-/-}$ mice, activation of the NF-KB pathway was impaired, and the MALT1/BCL-10 complex was mislocalized. ADAP may additionally drive NF-KB activation by binding the TGFβ-activated kinase1 (TAK1) at ADAP residues AA691-708, recruiting it to PKCθ (Srivastava et al. 2010) and inducing IKKα/β phosphorylation. Recombinant adenovirus constructs and ADAP$^{-/-}$ mice expressing the hCAR receptor (CD21 positive) were used to determine that the two sites on ADAP for TAK1 and CARMA1 appear to operate independently, as only the CARMA1 site is needed for IKKγ ubiquitination. As ADAP has been found to be necessary for thymopoiesis as well, it is possible that ADAP may yet have a further role in proliferation and differentiation, possibly by virtue of the putative nuclear localization signals that have been described but not yet mapped to specific functions.

ADAP Role in Integrin Regulation

Integrins are heterodimeric transmembrane proteins that can undergo conformational changes that enhance their affinity, and their activation state is intimately linked to the adhesive capacity of the cell. Hematopoietic cells, whether involved in immune or hemostatic function, require high affinity and/or high avidity bonds to establish strong and stable cell-matrix interactions in order to withstand hydrodynamic stress as they adhere, migrate, and transmigrate (diapedese). In T cells, integrins are additionally important for conjugate formation with antigen presenting cells (APC). Despite the presence of many integrins on the T cell surface, integrin LFA-1, consisting of subunits αL and β2, plays a particularly important role in T cell function. When transiently adhering to inflamed vessel walls through weak selectin-mediated interactions, integrin LFA-1 undergoes affinity modulation to



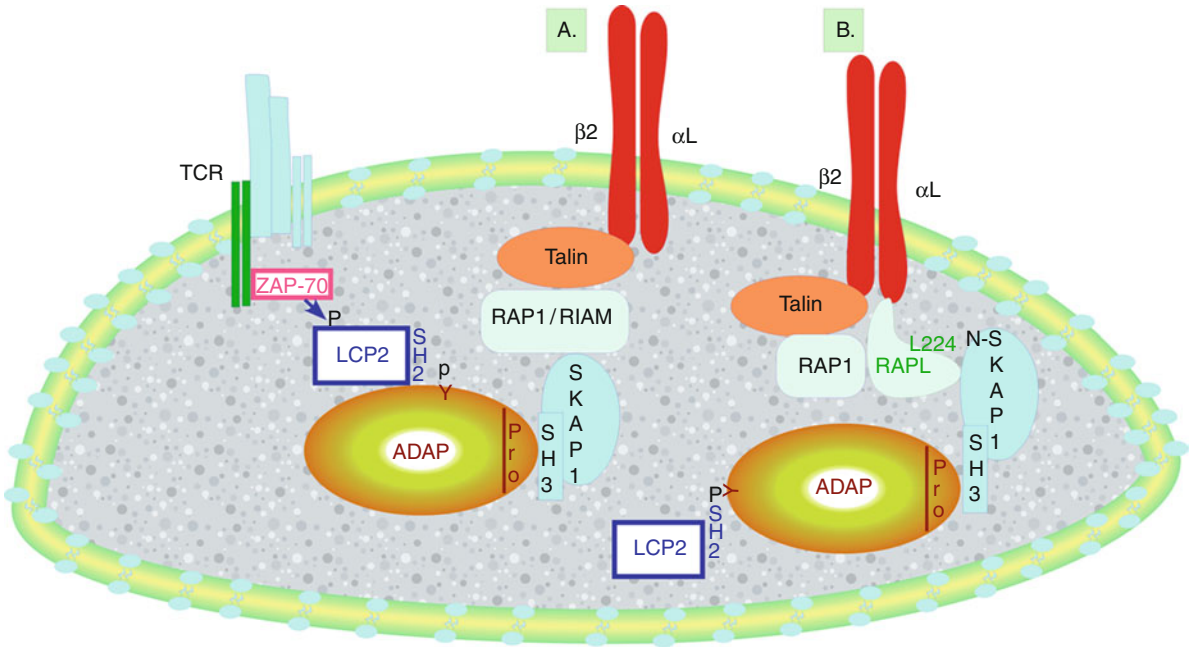
ADAP, Fig. 2 *Solution Structures of the ADAP-hSH3 Domain.* (**a**) Ensemble of the final 20 structures of ADAP-hSH3 domain. Structures are superimposed over the backbone atoms (C^{α}, C′, N) of residues 7–83. (**b**) Ribbon diagram of the lowest energy structure of ADAP-hSH3 domain, produced by the program MOLMOL. Secondary structure elements typical for a regular SH3 fold comprise a β sheet barrel (strands *a–d*), RT loop (between strand *a* and *b*), nSrc-loop (between strand *b* and *c*), and a 3_{10} helix.α : α helix, *a–d*: β strands, *a–d*. (**c**) Electrostatic surface potential calculated with the program MOLMOL highlighting key residues on the surface of the ADAP-hSH3 domain, in particular those of the N-terminal helix. (**d**) Residues at the interface of the helix and the SH3 domain fold. The backbone ribbon belonging to the regular SH3 fold is indicated in *green*, while the ribbon of the N-terminal extension is colored *red*. Side chains of residues of the extended N-terminus and the regular SH3 fold are in *blue* and *pale blue*, respectively. Structures in (**a**) and (**d**) are shown as stereo images. This research was originally published in Structure (Heuer et al. 2004)

mediate stable binding to ICAM 1, 2, or 3, expressed on endothelial cells or on APCs. It was first noted in T cells, that overexpression of ADAP led to enhanced TCR-induced integrin-dependent adhesion, while T cell lines lacking ADAP, and $ADAP^{-/-}$ mice showed deficient TCR-dependent upregulation of adhesiveness. Since the primary driving force for development of ADAP reagents and mice stemmed from interest in T cell physiology, mechanistic insights into ADAP's role in integrin activation have mostly been derived from studies in this cell type (Baker and Koretzky 2008; Jordan and Koretzky 2010; Wang and

Rudd 2008). However, experimental evidence for an ADAP role in integrin affinity modulation in platelets and macrophages is similarly strong. Stimulation of T cells through the TCR and CD28 is known to activate LFA-1 through a signaling cascade where ZAP-70 is recruited to ITAM domains of the TCR and phosphorylated by p59Fyn-T, thereby activating its kinase function to promote phosphorylation of multiple proteins including LCP2, SKAP1, phospholipase Cγ, and ADAP. As ADAP was found to be dispensable for TCR stimulated PLCγ phosphorylation and mitogen-activated protein kinase (MAPK) activation in the ADAP$^{-/-}$ mouse, further investigations focused on downstream events. Several lines of evidence have pointed to a functional partnership between ADAP and LCP2 for upregulation of integrin activation and adhesion. Transfection of Jurkat cells with constructs harboring mutations of ADAP tyrosines in the two YDDV sites that mediate LCP2 binding led to a loss of integrin clustering, T cell APC conjugate formation, and assembly of the peripheral supramolecular activation complex (pSMAC). The discovery that ADAP was constitutively associated with SKAP1, and more recently that ADAP was necessary to stabilize SKAP1 and its homologue SKAP2 in several cells in vitro and in vivo, suggested that SKAP1 may function in conjunction with ADAP and LCP2. Furthermore, it was not clear whether defects in integrin regulation observed in ADAP$^{-/-}$ mice were indeed due to ADAP or SKAP1. This was addressed using the SKAP1 knockout mouse, where ADAP expression is normal. Surprisingly, in SKAP1$^{-/-}$ T cells, similar integrin activation defects were seen to those observed in the ADAP$^{-/-}$ mouse, suggesting that ADAP may function to relocalize SKAP1, but that SKAP1 is the effector molecule of importance for integrin regulation.

Two overlapping models which incorporate ADAP scaffolding of SKAP1 and LCP2 were proposed to explain LFA-1 activation in T cells (Fig. 3) and link to known mediators of integrin activation: talin, Rap1, and the adapter, Rap1-interacting adhesion molecule (RIAM). Talin is a 280 kDa protein consisting of a FERM domain-containing head region that binds to the membrane proximal region and an NPXY motif on the β integrin cytoplasmic tail, to release the heterodimeric α/β subunit clasp and stabilize the open form of the integrin extracellular ligand-binding domain. The talin rod-like tail has binding sites for actin and vinculin, which also binds actin, and thereby reinforces links to the cytoskeleton. Active Rap1 binding to RIAM Ras association domain (RA) and membrane targeting have been shown to activate talin and enhance talin-mediated integrin activation (Shattil et al. 2010). Recently, it was demonstrated by Lee et al. that activation could occur when a 30 residue N-terminal fragment of RIAM directly binds talin to cause it to swing in toward the membrane and access an additional site on the β integrin tail to enhance integrin affinity. The ADAP-SKAP complex is linked to RIAM through a constitutive association between the SKAP1 N-terminal domain and RIAM PH domain. ADAP may thus function as a chaperone for a larger complex to escort effector molecules SKAP1/RIAM and Rap1 to the membrane (Kliche et al. 2006; Menasche et al. 2007). In support of this model, perturbation of the ADAP/SKAP1/RIAM association in turn disrupts recruitment of active Rap1 to the membrane and integrin activation. Activation of Rap1 also increases its affinity for RapL, a molecule often used as an indicator of Rap1 activation state. Interestingly, the alternative model for LFA-1 activation in T cells describes a new interaction of the SARAH RapL carboxy-terminal coiled-coil domain with the SKAP1 N-terminal region. The two molecules bind with a 1:1 stoichiometry as shown by isothermal titration calorimetry (Raab et al. 2010). In this model, the authors propose that an ADAP/SKAP1 module coordinates a Rap-RapL interaction that leads to RapL binding directly to the integrin αL cytoplasmic tail of LFA-1 to modulate integrin activation. It is possible that these models represent separate regulatory units that interface with individual α and β integrin subunits, or they may function together for maximal activation. In contrast, LCP2 and ADAP appear to function independently in integrin activation following stimulation through the cytokine receptor CXCR4 (Horn et al. 2009). Recently, it was noted that the adapter molecule HPK1 may compete with ADAP for LCP2 binding and negatively regulate integrin adhesion (Patzak et al. 2010).

In light of the scaffolding function of ADAP for effectors SKAP1 and LCP2, which were shown to be necessary components of signaling pathways to integrin activation in T cells, a role for ADAP modulation of integrin activation was hypothesized. Although biochemical studies in platelets identified ADAP in large complexes together with SKAP2 and



ADAP, Fig. 3 *The role of ADAP in TCR mediated integrin LFA-1 activation in T cells.* Stimulation through the TCR and CD28 results in binding of ZAP-70 to TCR ITAM domains and activation of its kinase activity, to phosphorylate LCP2. This induces the recruitment of several proteins, including ADAP, which is constitutively associated with SKAP1. (**a**) SKAP1 has been shown to bind the Rap1 binding protein, RIAM. Together, these are recruited to the membrane by ADAP to activate talin, which causes the splaying of the cytoplasmic tails of the integrin and a conformational change to upregulate LFA-1 affinity for its ligands. In an alternative model (**b**), an ADAP/SKAP1 module binds RapL L224 through the SKAP1 N-terminus and is necessary for the assembly of the Rap1/RapL complex and its recruitment to the membrane. Previously, RapL was shown to bind directly to the αL tail of integrin LFA-1 and regulate lymphocyte adhesion

LCP2 following stimulation with a collagen mimetic agonist, no mechanistic data was derived and no direct link between ADAP and activation of αIIbβ3, the platelet integrin crucial for platelet aggregation and spreading, was examined. In the course of investigating signaling pathways between the platelet adhesion receptor GPIb-IX-V and αIIbβ3 activation, Kasirer-Friede et al., found that ADAP underwent SRC-family kinase dependent phosphorylation upon GPIb-IX-V stimulation that occurred independently of intracellular calcium elevations. Subsequent studies using $ADAP^{-/-}$ mice clearly demonstrated a role for ADAP in αIIbβ3 activation downstream of several platelet agonists. The in vivo consequences of an ADAP deficiency were evidenced by increased rebleeding in these mice when challenged by tail vein transection, and by abnormal thrombus formation in the carotid artery upon chemical injury. However, unlike T cells, platelets do not express SKAP1, which is central to both T cell integrin activation models, and mice with a deletion of the homologous SKAP2, which leaves ADAP expression intact, showed no defects in αIIbβ3 integrin activation, or thrombus formation. Overexpression of ADAP in mast cells also enhances integrin activation-dependent adhesion to fibronectin. Similarly, ADAP has been implicated in macrophage, eosinophil, and pre-osteoclast development and/or functions where integrin activation is allegedly important. Nevertheless, sufficient variation exists in cell-specific expression of T cell pathway constituents that a simple unified mechanism to link ADAP to integrin activation is not feasible, and further studies are needed to identify relevant signaling pathways between upstream agonists and integrin activation that connect ADAP with appropriate binding partners in the context of diverse cellular environments.

ADAP Regulation of the Cytoskeleton

Reorganization of the actin cytoskeleton in response to stimulus is necessary for interaction of T cells with antigen presenting cells (APC's), phagocytosis by macrophages, and platelet spreading. Microscopic studies

have revealed a consistent association between ADAP and actin, or between ADAP and actin polymerization regulatory proteins in hematopoietic cells exhibiting a spectrum of distinct cell morphologies and underlying cytoskeletal networks (Peterson 2003; Wang and Rudd 2008). These results are supported by co-immunoprecipitation studies, and suggest that ADAP may be found in large molecular complexes associated with LCP2, which binds exchange factors and adapters that promote lamellipodial extension, and with NCK, Arp2/3, and VASP, which more proximally regulate actin polymerization. Furthermore, ADAP was shown to directly interact with VASP by screening a mouse embryonic expression library with antibodies against the VASP EVH1 domain binding motif, and with NCK, by yeast two-hybrid analysis (Sylvester et al. 2010). In T cells, a highly organized region composed of integrins, TCR, and many other signaling and structural proteins is formed between T cells and APCs, and is referred to as the immunological synapse (IS). The ADAP/VASP interaction helps recruit VASP to the IS, and inhibition of the association between ADAP and Ena/VASP impairs T cell polarization and formation of an actin cap at the T cell/APC junction. IS assembly is preceded by microclustering of several molecules including LCP2, ZAP70, and Linker for Activation of T cells (LAT) and their relocalization to the vicinity of the T cell/APC interface at the membrane events that occur independently of integrin clustering. Not surprisingly, primary T cells lacking ADAP are unable to relocalize LCP2 to microclusters. ADAP is additionally subject to dephosphorylation by SHP2 in response to increased cellular redox potential upon TCR stimulation, and may dampen ADAP-mediated cytoskeletal reorganization.

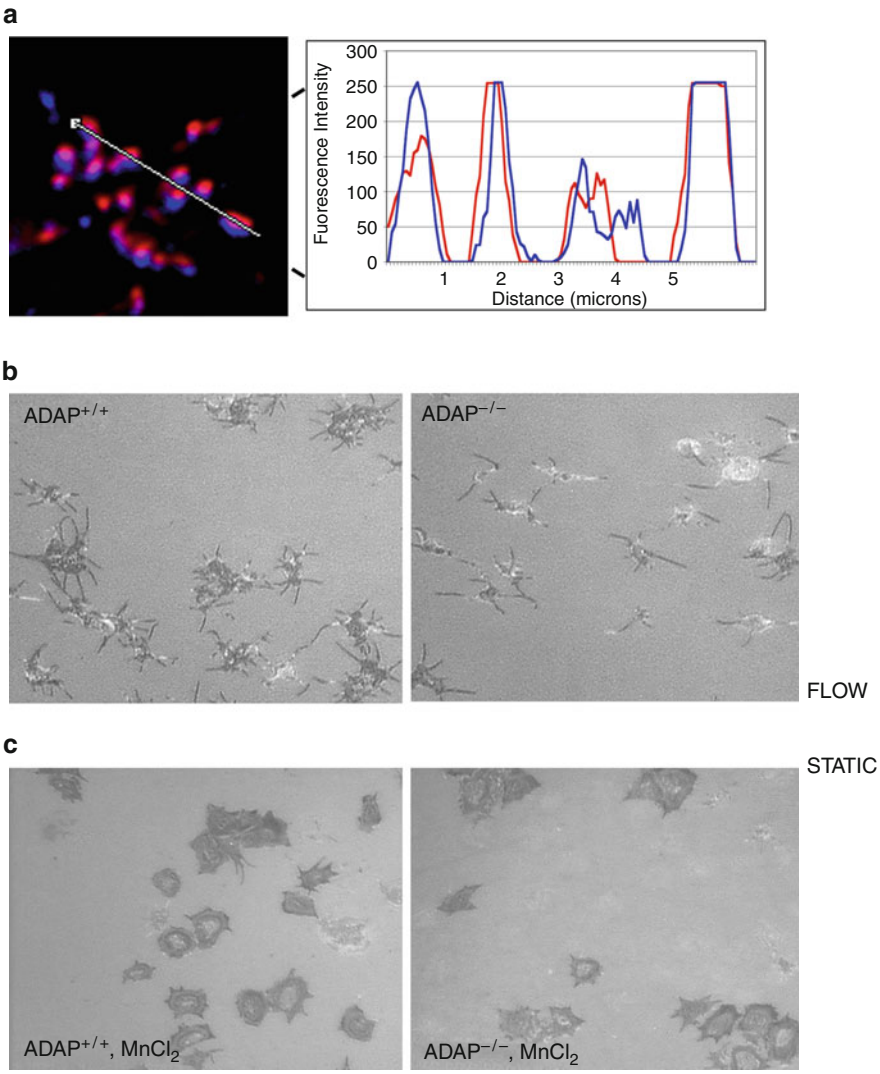
Upon stimulation through the T cell receptor (TCR) by anti-CD3 and CD28 antibodies, the β2 integrin LFA-1 becomes activated and TCRs and LFA-1 form clusters. LFA-1 clusters eventually encircle the TCR clusters to form a highly organized supramolecular activation complex where many signaling molecules also gather. Alternatively, integrins may be directly clustered in cells plated on fibronectin (Baker et al. 2009). Although ADAP is not needed for the immediate actin reorganization and TCR clustering, ADAP is necessary for clustering of LFA-1, a process that is actin dependent, as it is abrogated by cytocholasin D. ADAP regulation of β1 integrin clustering has also been demonstrated in mast cells.

T cell migration is required for T cell mobilization to sites of inflammation, to and within lymph nodes (LFA-1 dependent), and for transmigration through high endothelial venules. ADAP can increase migration in Jurkat T cells (Hunter et al. 2000; Sylvester et al. 2010). The case for an ADAP role in T cell migration is manifold: (1) ADAP expression in T cell blasts increased α4β1-mediated basal migration through fibronectin-coated transwells, and further enhanced migration speeds over those in untransfected cells when stimulated with the CXCR4 ligand, SDF-1α. (2) Mutation of ADAP tyrosines Y755, Y771, and Y780 within the hSH3 domain, to phenylalanine, led to decreased SDF-1α–induced migration of Jurkat cells through a transwell chamber. However, double mutation of the LCP2 binding sites, Y595 and Y651, produced the greatest reduction in SDF-1α induced migration. (3) In contrast, OVA peptide presentation on dendritic cells in lymph node slices decreased T cell mobility, in a manner dependent on an intact binding interface between ADAP/SKAP1 and RapL (Raab et al. 2010).

In platelets, under static, no flow conditions, ADAP is found at the periphery of platelets spread on fibrinogen. However, under shear flow conditions with hydrodynamic shear stresses acting on adhering platelets, the actin cytoskeleton is preferentially organized into actin-rich microclusters together with ADAP and other proteins (Fig. 4a), including VAV, LCP2, VASP, and the focal adhesion constituent, vinculin.

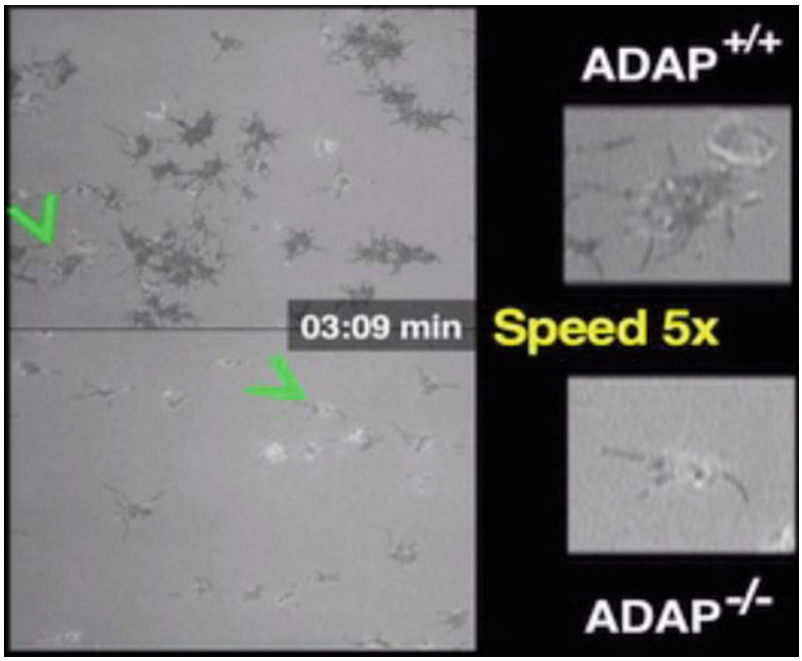
Live videomicroscopy was used to demonstrate that in ADAP$^{-/-}$ platelets, spreading and cluster assembly is greatly reduced under shear flow (Video 1), but not static conditions (Fig. 4b, c), (Kasirer-Friede et al. 2010). Furthermore, the phosphorylation of VAV, which occurs in response to shear stimulation, was almost completely absent in ADAP$^{-/-}$ mouse platelets. This led to a hypothesized role for ADAP in platelet mechanotransduction, mediated at least in part through its regulation of phospho-VAV activation of RAC for lamellipodia formation. ADAP may additionally mediate normal platelet responses under shear flow by additional, thus far unidentified, mechanisms.

In the macrophage, a cell that functions in immune defense by engulfing pathogens, ADAP localizes in membrane ruffles and plaques and at sites of phagocytosis along with actin. ADAP associates with actin regulatory proteins SLP-76, NCK, and VASP as in T cells, as well as with the C-terminal SH3 domain of monomeric actin-binding protein 1 (mAbp1, also



ADAP, Fig. 4 *ADAP in platelet adhesion to fibrinogen under shear flow conditions*. Flow chamber assay of unlabeled platelets adhering from whole blood onto fibrinogen-coated coverslips under shear flow, at a shear rate of 500 s^{-1}. (**a**) Platelets were perfusion fixed after 1.5 min of flow, permeabilized, and stained for F-actin (*red*) and ADAP (*blue*). Actin-rich structures form in $ADAP^{+/+}$ platelets adherent to fibrinogen, in which ADAP is also found. These structures were greatly reduced in $ADAP^{-/-}$ platelets, in which ADAP staining was negative. Furthermore, the normal localization of LCP-2, VAV, VASP, and vinculin to these structures was lost (not shown). A line profile of actin and ADAP fluorescence distribution in representative $ADAP^{+/+}$ platelets indicates almost identical localization. B./C. Identical $ADAP^{+/+}$ or $ADAP^{-/-}$ blood suspensions were either perfused over fibrinogen under shear flow at 500 s^{-1} (**b**) or pre-treated with $MnCl_2$ and gently applied to fibrinogen-coated coverslips within the flow chamber under static conditions (**c**), and continuously imaged by reflection interference contrast microscopy for 3 min. Images shown are after 1.5 min of blood exposure to the matrix. Well spread platelets are observed for both strains under static conditions, but only for $ADAP^{+/+}$ under shear flow, even when cells are treated with MnCl2 (not shown). This research was originally published in Blood (Kasirer-Friede et al. 2010. © the American Society of Hematology)

known as HPK1-interacting protein; HIP55). Thus, its high connectivity places ADAP in a strong position to modulate cytoskeletal dynamics. The pathogenic bacterial family *Yersinia* species has evolved mechanisms that interfere with the ability of macrophages to intercept infection, by injecting the phosphatases, YopH and YopE (Yuan et al. 2005). Interestingly, these proteins target amongst others, SKAP2 and ADAP. YopH binds ADAP C-terminal SH3 domain and central phosphotyrosines, leading to their



ADAP, Video 1 Platelet adhesion and spreading visualized by reflection interference contrast microscopy. $ADAP^{+/+}$ and $ADAP^{-/-}$ platelets adhere and spread onto immobilized fibrinogen from blood under shear flow conditions. A field of cells is shown for both strains (*left panels*), and a representative platelet (*arrowhead*) is highlighted and tracked from the time of capture for at least 1.5 min (*right panels*). Note that dark regions, depicting close surface contacts, grow rapidly in $ADAP^{+/+}$ platelets (*top*) but are greatly reduced in $ADAP^{-/-}$ platelets (*bottom*). This research was originally published in Blood (Kasirer-Friede et al. 2010. © the American Society of Hematology)

dephosphorylation and reduced phagocytosis (de la Puerta et al. 2009). ADAP/SKAP2 can also complex with macrophage inhibitory receptor SHPS-1 and SHP-1 via the SKAP2 SH3 domain, and mice lacking SHP-1 show increased p120/ADAP phosphorylation. In macrophage-like pre-osteoblasts, which also express ADAP, integrin-dependent tyrosine phosphorylation of ADAP occurs, and ADAP knockdown retards migration and progression to the multinucleate stage (Koga et al. 2005). Taken together, this suggests that in macrophages, the phosphorylation state of ADAP may be one of the factors regulating its interactions with actin and actin regulatory proteins.

Summary

Although ADAP was only cloned a little over a decade ago, studies have repeatedly detailed an important role for this protein in regulation of proliferation, adhesion, and cellular development. Although studies thus far have provided important insights into how ADAP interfaces with its binding partners, more work is required to identify novel regulatory mechanisms for the as yet "unassigned" residues such as serines and lysines that may be subject to post-translational modifications within individual cellular environments to effect cell-specific function. New studies likely will continue to expand the repertoire of ADAP binding partners, and increase the understanding of its regulation by virtue of the numerous and diverse domains within the molecule. In the coming era, ADAP mutations may be mapped to specific disease states and ADAP domains may be therapeutically targeted. This is already becoming a reality. ADAP has been implicated in hemostasis and thrombus formation, and in CD8 dependent homeostasis in small intestinal epithelium. As T cell invasion is an important component of allograft rejection, two studies have investigated the role of ADAP in this process. Using $ADAP^{-/-}$ mice, the lack of ADAP expression was found to significantly ameliorate heart allograft rejection, and synergistically ameliorate intestinal allograft rejection in the presence of additional costimulation blockade using anti CD40L

antibodies (Tian et al. 2010). Therefore, it will be interesting to see how basic research studies on ADAP can be translated into health benefits for human patients.

References

Baker RG, Hsu CJ, Lee D, Jordan MS, Maltzman JS, Hammer DA, Baumgart T, Koretzky GA. The adapter protein SLP-76 mediates "outside-in" integrin signaling and function in T cells. Mol Cell Biol. 2009;29(20):5578–89.

Baker RG, Koretzky GA. Regulation of T cell integrin function by adapter proteins. Immunol Res. 2008;42(1–3):132–44.

de la Puerta ML, Trinidad AG, del Carmen RM, Bogetz J, Sanchez Crespo M, Mustelin T, Alonso A, Bayon Y. Characterization of new substrates targeted by Yersinia tyrosine phosphatase YopH. PLoS ONE. 2009;4(2):e4431.

Heuer K, Kofler M, Langdon G, Thiemke K, Freund C. Structure of a helically extended SH3 domain of the T cell adapter protein. ADAP. 2004;12:603–610.

Horn J, Wang X, Reichardt P, Stradal TE, Warnecke N, Simeoni L, Gunzer M, Yablonski D, Schraven B, Kliche S. Src homology 2-domain containing leukocyte-specific phosphoprotein of 76 kDa is mandatory for TCR-mediated inside-out signaling, but dispensable for CXCR4-mediated LFA-1 activation, adhesion, and migration of T cells. J Immunol. 2009; 183(9):5756–67.

Hunter AJ, Ottoson N, Boerth N, Koretzky GA, Shimizu Y. Cutting edge: a novel function for the SLAP-130/FYB adapter protein in beta 1 integrin signaling and T lymphocyte migration. J Immunol. 2000;164(3):1143–7.

Jordan MS, Koretzky GA. Coordination of receptor signaling in multiple hematopoietic cell lineages by the adaptor protein SLP-76. Cold Spring Harb Perspect Biol. 2010;2(4): a002501.

Kasirer-Friede A, Ruggeri ZM, Shattil SJ. Role for ADAP in shear flow-induced platelet mechanotransduction. Blood. 2010;115(11):2274–82.

Kliche S, Breitling D, Togni M, Pusch R, Heuer K, Wang X, Freund C, Kasirer-Friede A, Menasche G, Koretzky GA, Schraven B. The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. Mol Cell Biol. 2006; 26(19):7130–44.

Koga S, Yogo K, Yoshikawa K, Samori H, Goto M, Uchida T, Ishida N, Takeya T. Physical and functional association of c-Src and adhesion and degranulation promoting adaptor protein (ADAP) in osteoclastogenesis in vitro. J Biol Chem. 2005; 280(36):31564–71.

Medeiros RB, Burbach BJ, Mueller KL, et al. Regulation of NF-kappaB activation in T cells via association of the adapter proteins ADAP and CARMA1. Science. 2007;316:754–758.

Menasche G, Kliche S, Chen EJ, Stradal TE, Schraven B, Koretzky G. RIAM links the ADAP/SKAP-55 signaling module to Rap1, facilitating T-cell-receptor-mediated integrin activation. Mol Cell Biol. 2007;27(11):4070–81.

Patzak IM, Konigsberger S, Suzuki A, Mak TW, Kiefer F. HPK1 competes with ADAP for SLP-76 binding and via Rap1 negatively affects T-cell adhesion. Eur J Immunol. 2010; 40(11):3220–5.

Peterson EJ. The TCR ADAPts to integrin-mediated cell adhesion. Immunol Rev. 2003;192:113–21.

Raab M, Wang H, Lu Y, Smith X, Wu Z, Strebhardt K, Ladbury JE, Rudd CE. T cell receptor "inside-out" pathway via signaling module SKAP1-RapL regulates T cell motility and interactions in lymph nodes. Immunity. 2010;32(4):541–56.

Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol. 2010; 11(4):288–300.

Srivastava R, Burbach BJ, Shimizu Y. NF-kappaB activation in T cells requires discrete control of IkappaB kinase alpha/beta (IKKalpha/beta) phosphorylation and IKKgamma ubiquitination by the ADAP adapter protein. J Biol Chem. 2010;285(15):11100–5.

Sylvester M, Kliche S, Lange S, Geithner S, Klemm C, Schlosser A, Grossmann A, Stelzl U, Schraven B, Krause E, Freund C. Adhesion and degranulation promoting adapter protein (ADAP) is a central hub for phosphotyrosine-mediated interactions in T cells. PLoS ONE. 2010;5(7):e11708.

Tian J, Rodriguez-Barbosa JI, Pabst O, Roemermann D, Foerster R, Beckmann J, Hoffmann MW. ADAP deficiency combined with costimulation blockade synergistically protects intestinal allografts. Transpl Int. 2010;23(1):71–9.

Wang H, Rudd CE. SKAP-55, SKAP-55-related and ADAP adaptors modulate integrin-mediated immune-cell adhesion. Trends Cell Biol. 2008;18(10):486–93.

Yuan M, Deleuil F, Fallman M. Interaction between the Yersinia tyrosine phosphatase YopH and its macrophage substrate, Fyn-binding protein, Fyb. J Mol Microbiol Biotechnol. 2005; 9(3–4):214–23.

Zimmermann J, Kuhne R, Sylvester M, Freund C. Redox-regulated conformational changes in an SH3 domain. Biochemistry. 2007;46(23):6971–7.

ADAP1

Benjamin J. Gosney and Venkateswarlu Kanamarlapudi
Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales, UK

Synonyms

ARF GAP with dual pleckstrin homology (PH) domains; CENTA1; Centaurin alpha; GC1L; GCS1L; p42IP4; PIPBP

Historical Background

ADAP1 (ARF GAP with dual pleckstrin homology (PH) domains 1) was originally identified as a 42 kDa

protein that binds with high affinity to phosphatidylinositol (3,4,5)-triphosphate (PIP_3) or its inositol head group, inositol 1,3,4,5-tetrakisphosphate (IP_4). Hence, it was originally named PIP_3/IP_4-binding protein (PIP3BP/p42IP4) and has also been called Centaurin-α1 (Hammonds-Odie et al. 1996; Stricker et al. 1997; Tanaka et al. 1999). It has been renamed ADAP1, to more accurately describe the structure and function of this protein (Kahn et al. 2008). The membrane lipid PIP_3 and the water-soluble ligand IP_4 both serve as signal transducers that link extracellular stimuli to numerous intracellular signaling cascades required for cell growth, multiplication, differentiation, and death (Vanhaesebroeck et al. 2001; Hanck et al. 2004). ADAP1 contains an amino-terminal zinc-finger motif with an ADP-ribosylation factor (ARF) GTPase-activating protein (GAP) homology domain and two adjacent pleckstrin homology (PH) domains (one in the middle (N-PH) and the other at the carboxy terminus (C-PH)) that are required for binding to PIP_3. It has high sequence similarity to the Centaurin-α2 protein, with 58% amino-acid identity and 75% similarity (Hanck et al. 2004; Whitley et al. 2002). The *ADAP1* gene is located on human chromosome 7 (Hanck et al. 2004).

Protein Function and Regulation of Activity

ADAP1 has both GAP domain-dependent and -independent effects. The rat homolog was shown to have a role in dendritic density and branching in a GAP domain-dependent manner. Dendritic lamellipodia and filopodia have been implicated in dendritic branching and spine formation, and the same study demonstrated that ADAP1 plays a part in their formation; this effect was again dependent on the GAP domain (Moore et al. 2007). In HeLa cells, human ADAP1 prevents cortical actin reorganization, which was shown to be a consequence of its GAP activity toward the ARF6 small GTPase (Venkateswarlu et al. 2004). Additionally, it has been shown that co-transfection of HEK293 with ADAP1 and the β2 adrenoceptor leads to a decrease in receptor internalization and an increase in $G\alpha_s$ signaling (Lawrence et al. 2005); this effect was GAP domain dependent.

Cell spreading is a GAP domain-independent effect of ADAP1. Intriguingly, expression of a ΔGAP mutant, which lacks the GAP domain, increased cell spreading, suggesting that the interaction of ADAP1 with ARF6 may decrease this effect. This function might be linked to its co-localization with the ARF5 and ARF6 small GTPases (Thacker et al. 2004). Other studies unconcerned with its GAP activity found that ADAP1 induced the downstream activation of ERK1/2 and Elk-1 by a ▶ PI3K-dependent pathway (Hayashi et al. 2006), and infection of chick embryonic fibroblasts with retrovirus encoding ADAP1 led to anchorage-independent proliferation at an increased rate through AP-1 activation (Chanda et al. 2003), suggesting that ADAP1 could have oncogenic potential. Mitochondria isolated from neuroblastoma cells that overexpress ADAP1 showed significantly decreased Ca^{2+} capacity and lag-time for Ca^{2+} retention, suggesting that ADAP1 is involved in the regulation of mitochondrial Ca^{2+} transport (Galvita et al. 2009).

In unstimulated PC12 cells, ADAP1 localizes to the cytoplasm and nucleus. Upon stimulation of PC12 cells with EGF, ADAP1 translocates to the plasma membrane. This has been shown to be dependent on a PI3K-induced increase of PIP_3 at the plasma membrane, which binds to the PH domains of ADAP1 (Venkateswarlu et al. 1999; Venkateswarlu and Cullen 1999). The translocation was shown to be abrogated in HEK293 by pretreatment with thrombin, an effect that was exacerbated by increasing the intracellular IP_4 pool using LiCl (Sedehizade et al. 2005). As thrombin acts through a Gq-linked G- protein-coupled receptor (GPCR), it is possible that other Gq-linked GPCRs may have a similar effect.

All PKC isoforms (PKCα, λ, μ, and ζ) bind to and phosphorylate ADAP1 at Ser 87 in the GAP domain and Thr 276 in the C-PH domain (Zemlickova et al. 2003).

KIF13B is a kinesin motor protein that was shown to inhibit ADAP1 GAP activity but also relocate ADAP1 to the leading edge of the cell (Venkateswarlu et al. 2005; Kanamarlapudi 2005). This finding raises the possibility that KIF13B both temporally and spatially regulates the activity of ARF6.

As mentioned above, ADAP1 is an inositol phosphate–binding protein which binds to its ligands, the membrane-bound PIP_3 and the cytosolic IP_4, with similar affinities (Stricker et al. 1997; Tanaka et al. 1999). It also binds to phosphatidylinositol(3,4)-bisphosphate (PI(3,4)P_2) (Rao et al. 1999; Kalscheuer et al. 2009).

KIF13B was identified as an ADAP1-binding partner in a yeast two-hybrid screen. KIF13B contains an N-terminal motor domain, a large stalk domain at the center, and the CAPGLY domain (a putative microtubule interacting sequence) at the C terminus. ADAP1 directly interacts with the stalk domain of KIF13B through its GAP domain and this interaction is essential to maintain its localization at the leading edges of the cell. Moreover, KIF13B negatively regulates the GAP activity of ADAP1 in vivo. Together, these studies suggest that an ADAP1–KIF13B complex may provide a means for concentrating ADAP1 at the leading edges of the cell, where it regulates ARF6 activity (Venkateswarlu et al. 2005; Kanamarlapudi 2005). Moreover, in neurons, the ADAP1–KIF13B complex transports PIP_3 to the neurite ends, and thus regulates the establishment of neuronal polarity (Horiguchi et al. 2006).

All PKC isoforms (PKCα, λ, μ, and ζ) bind to ADAP1 in vitro by their cysteine-rich C1 domain, as demonstrated through affinity chromatography and GST pull-down assays. This interaction results in phosphorylation of Centaurin-α1 at Ser 87 and Thr 276 (Zemlickova et al. 2003).

ADAP1 has been shown to bind to the scaffolding protein RanBPM in vitro and in vivo by the SPRY domain of RanBPM and the ARF GAP domain of ADAP1. The association between these two proteins is inhibited by the ADAP1 ligand IP_4 (Haase et al. 2008).

ADAP1 also associates with casein kinase I (CKI) isoforms α, δ, ε, λ1, λ2, and λ3 but there is no evidence that this interaction modulates the activity of either ADAP1 or CKI, so it is difficult to assign a function to this protein–protein interaction. It has been suggested that ADAP1 may act as an adaptor protein for CKI lipid binding (Dubois et al. 2002). It is also possible that the ADAP1–CKI interaction has a role in the etiology of Alzheimer's disease due to an established role for CKI in disease pathology and an increased expression of ADAP1 in patients with Alzheimer's disease (Reiser and Bernstein 2002).

ADAP1 binds to the metalloendopeptidase NRDc (N-arginine dibasic convertase; also known as nardilysin), although the functional consequences of this interaction are not known (Stricker et al. 2006). A functional interaction is suggested by anatomical co-localization. In the human brain, considerable overlap was found between ADAP1 and nardilysin (NRDc). In the hypothalamic paraventricular nucleus (PVN), about two-thirds of ADAP1-immunoreactive neurons co-express NRDc, whereas 20% of the NRDc-containing neurons did not co-stain for ADAP1 (Bernstein et al. 2007). The proteins ADAP1 and nardilysin dissociate from each other in the presence of IP_4, but not of $Ins(1,4,5)P_3$ (Stricker et al. 2006). The ligand interaction of ADAP1 is stereospecific (Stricker et al. 1996).

In a study investigating nuclear-located binding proteins for ADAP1, nucleolin (which is also involved in the pathology of Alzheimer's disease (Reiser and Bernstein 2004)), the DNA-binding protein Pur-α, and the cerebellar leucine-rich acidic nuclear protein (LANP) were found to be ADAP1-interacting proteins. At lower concentrations, RNA polymerase II transcription cofactor 4 (mediator of RNA polymerase II transcription, subunit 8 (Med8)), the splicing factor arginine/serine-rich 2 (SFRS2), the pre-mRNA splicing factor SF3, the β2 subunit of the adaptor-related protein complex 3, and the protein similar to ribosomal protein S9 were shown to interact with ADAP1 (Dubois et al. 2003).

In vitro binding between ADAP1 and actin has also been demonstrated (Thacker et al. 2004). Actin binding is mediated through its PIP_3-binding PH domain, similar to that shown for Bruton's tyrosine kinase (BTK).

ADAP1 expression is upregulated in neurons in the brain of patients with Alzheimer's disease (Reiser and Bernstein 2002, 2004). In rats, after acoustic and electric fear stimulation (startle response), the mRNA and protein levels were downregulated within 2 h in the amygdala, hypothalamus, and cingulate/retrosplenial cortex. *ADAP1* mRNA decreased by about 50% for about 24 h (Reiser et al. 2004).

There were characteristic changes of *ADAP1* expression during development in the rat brain. mRNA levels of *ADAP1* were quantified at 7, 14, and 21 days of age, as well as in various brain regions of adult rats, including the cerebellum, cortex, striatum, thalamus, hypothalamus, olfactory bulb, hippocampus, and tectum (superior and inferior colliculus) (Aggensteiner and Reiser 2003).

Major Sites of Expression and Subcellular Localization

ADAP1 is abundantly expressed in the brain. It is also expressed at very low levels in nonneuronal tissues

such as spleen, lung, kidney, peripheral blood leukocytes, and retina (Hammonds-Odie et al. 1996; Venkateswarlu and Cullen 1999; Stricker et al. 1997). In the brain, *ADAP1* was found in neurons of the cortex, hypothalamus, and hippocampus (Sedehizade et al. 2002; Moore et al. 2007). Developmental expression analysis of *ADAP1* in rodent brains revealed that its expression is detectable as early as embryonic day 16 and peaks between 2 weeks and 4 weeks postnatally (Aggensteiner and Reiser 2003; Moore et al. 2007).

In the rat brain, high levels of developmentally regulated *ADAP1* expression are found in cerebellum, hippocampus, cortex, and thalamus. In adults, the immunoreactivity is localized in most neuronal cell types and probably also in some glial cells (Kreutz et al. 1997a). Prominent immunoreactivity is found in axonal processes and in cell types with long neurites. In the hypothalamus, a subpopulation of parvocellular neurons in the peri- and paraventricular nuclei is heavily labeled. This is confined by strong immunoreactivity in the lamina externa of the median eminence in close proximity to portal plexus blood vessels. Electron microscopy shows that ADAP1 protein is frequently associated with presynaptic vesicular structures (Kreutz et al. 1997a).

In rat, porcine, and bovine retina, *ADAP1* is localized by in situ hybridization in the ganglion cell layer, the inner nuclear cell layer, and the outermost part of the outer nuclear cell layer (Kreutz et al. 1997b). Wide-field amacrine and retinal ganglion cells are intensely immunostained. Prominent immunoreactivity in the on/off sublaminae of the inner plexiform layer and in the optic nerve layer indicates a pre- and/or postsynaptic localization of the protein. Moreover, there is significant ADAP1 protein expression in the inner segment of photoreceptors. The end-feet of Müller glial cells in the optic nerve layer are also stained (Kreutz et al. 1997b).

ADAP1 and KIF13B/GAKIN co-localize after transfection of tagged proteins at the tip of growing neurites in PC12 cells. In cultured hippocampal neurons, endogenous ADAP1 is localized in growth cones (Horiguchi et al. 2006).

ADAP1 localizes to the cytoplasm and nucleus (Tanaka et al. 1999; Venkateswarlu et al. 1999). In polarized cells such as neurons, ADAP1 also localizes to dendrites, dendritic spines, and the postsynaptic region (Moore et al. 2007). ADAP1 is also found in mitochondria, where it regulates calcium levels (Galvita et al. 2009).

Phenotypes and Splice Variants

No studies on the phenotypic effects of ADAP1 have been carried out due to the lack of ADAP1-knockout mice. However, knockdown of Centaurin-α1 by short interfering RNA in cultured hippocampal neurons inhibits dendritic branching and length (Moore et al. 2007) and in HEK cells results in a reduction of EGF-stimulated ERK activation (Hayashi et al. 2006). In yeast, the deletion of the gene encoding the ADAP1 homolog, *Gcs1*, hypersensitizes the mutant to sodium fluoride. This effect can be rescued through introduction of human *ADAP1* (Venkateswarlu et al. 1999).

Although there is no evidence yet for the expression of splice variants, northern analysis of rat brain Centaurin-α (renamed as "ADAP") revealed a major transcript at 2.5 kb and a minor one at 4.0 kb (Hammonds-Odie et al. 1996). Both *ADAP* and *ADAP1* were cloned from a rat brain cDNA library (Hammonds-Odie et al. 1996; Aggensteiner et al. 1998). ADAP is highly homologous to ADAP1, but contains a 43 amino-acid C-terminal extension and lacks the N-PH domain due to three frame-shift mutations in the cloned cDNA. However, ADAP has not been detected thus far by RT-PCR or immunoblotting, and therefore it is not yet possible to say whether ADAP is a splice variant of ADAP1 or not (Aggensteiner et al. 1998).

The *ADAP1* clone was corrected later in Thacker et al. (2004). Re-sequencing the original cDNA clone revealed two sequencing errors in the original submission (accession number U51013). Species comparisons with the revised rat sequence indicated that rat ADAP1 is the ortholog of human and murine ADAP1, porcine p42IP4, and bovine PIP3BP.

Immunoblot analysis of endogenous rat brain ADAP1 demonstrated that it migrates as a doublet, but the basis for the difference in molecular mass has not yet been determined (Moore et al. 2007).

Hammonds-Odie and coworkers raised polyclonal antibodies against a 19-amino-acid peptide (AGELRRALLELLTRPGNSR) from the N-terminal end of rat Centaurin-α and against a C-terminal fusion protein. These antibodies are used in Western blotting applications for the detection of Centaurin-α (Hammonds-Odie et al. 1996). The Reiser group has produced its own anti-p42IP4 rabbit polyclonal and mouse monoclonal antibody, raised against a peptide

consisting of amino acids 353–371 of pig ADAP1, and used it to detect human, pig, and rat ADAP1 by Western blot and immunohistochemistry (Stricker et al. 1997, 2003, 2006; Kreutz et al. 1997a; Aggensteiner et al. 1998; Sedehizade et al. 2002; Reiser and Bernstein 2002; Aggensteiner and Reiser 2003).

There are also several anti-Cetaurin-α1 antibodies that are commercially available. By far the most widely used commercially available antibody against ADAP1 is a mouse monoclonal (P421; IgG1 subtype) raised against full-length pig ADAP1. This antibody is available from Santa Cruz Biotech (sc-51836), Abcam (ab10168), and HyTest Ltd (4MA10), and has been used for detection of ADAP1 from human and pig by ELISA and Western blotting (Thacker et al. 2004; Venkateswarlu et al. 2005; Hayashi et al. 2006).

A goat anti-ADAP1 polyclonal antibody, raised against the synthetic peptide CQEYAVEAHFKHKP, which corresponds to amino acids 362–374 of human ADAP1, is available from Abcam (ab27476) and Everest Biotech (EB06120). This antibody can be used in ELISA, immunoctyochemistry, immunohistochemistry, and Western blotting applications to detect human, rat, and mouse ADAP1 (Moore et al. 2007).

A mouse polyclonal antibody, raised against the full-length human ADAP1 protein, is available from Novus Biologicals (H00011033-B01). This antibody is useful for ELISA, Western blot, and immunofluoresence, and detects human ADAP1. A rabbit anti-ADAP1 antibody generated against GST-fused full-length human ADAP1 is available from Proteintech Group (13911-1-AP) and can be used to detect human and mouse ADAP1 by Western blot and immunohistochemistry.

Summary

ADAP1 (also known as Centaurin-α1/PIP3BP/p42IP4/ADAP1) is a phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3)/IP_4-binding protein that regulates actin cytoskeleton organization and membrane trafficking by acting as a GTPase-activating protein (GAP) of ADP-ribosylation factor 6 (ARF6). The binding is stereospecific for D-inositol (1,3,4,5) P_4 (IP_4) as it does not bind to inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3). It also associates with casein kinase Iα (CKIα), Ran-binding protein in microtubule-organizing center (RanBPM), nardilysin, kinesin motor protein (KIF13B), protein kinase C (PKC), and nucleolin. ADAP1 localization and activity have been shown to be regulated by phosphoinositol 3-kinase (▶ PI3K), protein kinase C (PKC) isoforms, and the kinesin motor protein KIF13B. It is abundantly expressed in brain tissue and is also detected in nonneuronal tissues at very low concentrations. ADAP1 expression is upregulated in neurons in the brain of patients with Alzheimer's disease and is detected in amyloid plaques, indicating that it may be involved in neuronal diseases. ADAP1 is normally localized in the cytoplasm, nucleus, and mitochondria and it translocates to the plasma membrane by binding to PIP_3 produced by agonist-activated PI3K to act as a GAP for ARF6. ADAP1 localizes to dendritic spines and synapses in neurons and can affect differentiation, PIP_3 transport, and vesicle secretion. It has also been implicated in regulation of Ca^{2+} transport in mitochondria. There is no information on ADAP1-null phenotypes in mammals, but due to its putative role in neuronal development, it is possible that loss of ADAP1 may affect the viability of the organism. There are no known polymorphisms of the *ADAP1* gene.

References

Aggensteiner M, Reiser G. Expression of the brain-specific membrane adapter protein p42IP4/centaurin alpha, a Ins (1,3,4,5)P4/PtdIns(3,4,5)P3 binding protein, in developing rat brain. Brain Res Dev Brain Res. 2003;142:114.

Aggensteiner M, Stricker R, Reiser G. Identification of rat brain p42(IP4), a high-affinity inositol(1,3,4, 5)tetrakisphosphate/phosphatidylinositol(3,4,5)trisphosphate binding protein. Biochim Biophys Acta. 1998;1387:1–28.

Bernstein HG, Stricker R, Dobrowolny H, Trübner K, Bogerts B, Reiser G. Histochemical evidence for wide expression of the metalloendopeptidase nardilysin in human brain neurons. Neuroscience. 2007;146:48.

Chanda SK, White S, Orth AP, Reisdorph R, Miraglia L, Thomas RS, DeJesus P, Mason DE, Huang Q, Vega R, Yu DH, Nelson CG, Smith BM, Terry R, Linford AS, Yu Y, Chirn GW, Song C, Labow MA, Cohen D, King FJ, Peters EC, Schultz PG, Vogt PK, Hogenesch JB, Caldwell JS. Genome-scale functional profiling of the mammalian AP-1 signaling pathway. Proc Natl Acad Sci USA. 2003;100:2114.

Dubois T, Howell S, Zemlickova E, Aitken A. Identification of casein kinase I alpha interacting protein partners. FEBS Lett. 2002;517:1–324.

Dubois T, Zemlickova E, Howell S, Aitken A. Centaurin-alpha 1 associates in vitro and in vivo with nucleolin. Biochem Biophys Res Commun. 2003;301:27.

Galvita A, Grachev D, Azarashvili T, Baburina Y, Krestinina O, Stricker R, Reiser G. The brain-specific protein, p42(IP4) (ADAP 1) is localized in mitochondria and involved in regulation of mitochondrial $Ca2^{+}$. J Neurochem. 2009;109:6.

Haase A, Nordmann C, Sedehizade F, Borrmann C, Reiser G. RanBPM a novel interaction partner of the brain-specific protein p42IP4/centaurin alpha-1. J Neurochem. 2008; 105:61.

Hammonds-Odie LP, Jackson TR, Profit AA, Blader IJ, Turck CW, Prestwich GD, Theibert AB. Identification and cloning of centaurin-alpha. A novel phosphatidylinositol 3,4,5-trisphosphate-binding protein from rat brain. J Biol Chem. 1996;271:312.

Hanck T, Stricker R, Sedehizade F, Reiser G. Identification of gene structure and subcellular localization of human centaurin alpha 2, and p42IP4, a family of two highly homologous, Ins 1,3,4,5-P4-/PtdIns 3,4,5-P3-binding, adapter proteins. J Neurochem. 2004;88:2.

Hayashi H, Matsuzaki O, Muramatsu S, Tsuchiya Y, Harada T, Suzuki Y, Sugano S, Matsuda A, Nishida E. Centaurin-alpha1 is a phosphatidylinositol 3-kinase-dependent activator of ERK1/2 mitogen-activated protein kinases. J Biol Chem. 2006;281:320.

Horiguchi K, Hanada T, Fukui Y, Chishti AH. Transport of PIP3 by GAKIN, a kinesin-3 family protein, regulates neuronal cell polarity. J Cell Biol. 2006;174:331.

Kahn RA, Bruford E, Inoue H, Logsdon JM, Nie Z, Premont RT, Randazzo PA, Satake M, Theibert AB, Zapp ML, Cassel D. Consensus nomenclature for the human ArfGAP domain-containing proteins. J Cell Biol. 2008;182:622.

Kalscheuer VM, Musante L, Fang C, Hoffmann K, Fuchs C, Carta E, Deas E, Venkateswarlu K, Menzel C, Ullmann R, Tommerup N, Dalprà L, Tzschach A, Selicorni A, Lüscher B, Ropers HH, Harvey K, Harvey RJ. A balanced chromosomal translocation disrupting ARHGEF9 is associated with epilepsy, anxiety, aggression, and mental retardation. Hum Mutat. 2009;30:1.

Kanamarlapudi V. Centaurin-alpha1 and KIF13B kinesin motor protein interaction in ARF6 signalling. Biochem Soc Trans. 2005;33(Pt 6):1279.

Kreutz MR, Böckers TM, Sabel BA, Hülser E, Stricker R, Reiser G. Expression and subcellular localization of p42IP4/centaurin-alpha, a brain-specific, high-affinity receptor for inositol 1,3,4,5-tetrakisphosphate and phosphatidylinositol 3,4,5-trisphosphate in rat brain. Eur J Neurosci. 1997a;9:10.

Kreutz MR, Böckers TM, Sabel BA, Stricker R, Hülser E, Reiser G. Localization of a 42-kDa inositol 1,3,4,5-tetrakisphosphate receptor protein in retina and change in expression after optic nerve injury. Brain Res Mol Brain Res. 1997b;45:2.

Lawrence J, Mundell SJ, Yun H, Kelly E, Venkateswarlu K. Centaurin-alpha 1, an ADP-ribosylation factor 6 GTPase activating protein, inhibits beta 2-adrenoceptor internalization. Mol Pharmacol. 2005;67:6.

Moore CD, Thacker EE, Larimore J, Gaston D, Underwood A, Kearns B, Patterson SI, Jackson T, Chapleau C, Pozzo-Miller L, Theibert A. The neuronal Arf GAP centaurin alpha1 modulates dendritic differentiation. J Cell Sci. 2007;120(Pt 151):2683.

Rao VR, Corradetti MN, Chen J, Peng J, Yuan J, Prestwich GD, Brugge JS. Expression cloning of protein targets for 3-phosphorylated phosphoinositides. J Biol Chem. 1999; 274:5331.

Reiser G, Bernstein HG. Neurons and plaques of Alzheimer's disease patients highly express the neuronal membrane docking protein p42IP4/centaurin alpha. Neuroreport. 2002;13:1820.

Reiser G, Bernstein HG. Altered expression of protein p42IP4/centaurin-alpha 1 in Alzheimer' disease brains and possible interaction of p42IP4 with nucleolin. Neuroreport. 2004; 15:119.

Reiser G, Striggow F, Hackmann C, Schwegler H, Yilmazer-Hanke DM. Short-term down-regulation of the brain-specific, PtdIns(3,4,5)P3/Ins(1,3,4,5)P4-binding, adapter protein, p42IP4/centaurin-alpha 1 in rat brain after acoustic and electric stimulation. Neurochem Int. 2004;45:1.

Sedehizade F, Hanck T, Stricker R, Horstmayer A, Bernstein HG, Reiser G. Cellular expression and subcellular localization of the human Ins(1,3,4,5)P(4)-binding protein, p42(IP4), in human brain and in neuronal cells. Brain Res Mol Brain Res. 2002;99:128.

Sedehizade F, von Klot C, Hanck T, Reiser G. p42(IP4)/centaurin alpha1, a brain-specific PtdIns(3,4,5)P3/Ins (1,3,4,5)P4-binding protein: membrane trafficking induced by epidermal growth factor is inhibited by stimulation of phospholipase C-coupled thrombin receptor. Neurochem Res. 2005;30:10.

Stricker R, Chang YT, Chung SK, Reiser G. Determination of specificity of a high-affinity inositol 1,3,4,5-tetrakisphosphate binding site at a 42 kDa receptor protein, p42IP4: comparison of affinities of all inositoltris-,-tetrakis-, and -pentakisphosphate regioisomers. Biochem Biophys Res Commun. 1996;228:212.

Stricker R, Hülser E, Fischer J, Jarchau T, Walter U, Lottspeich F, Reiser G. cDNA cloning of porcine p42IP4, a membrane-associated and cytosolic 42 kDa inositol(1,3,4,5) tetrakisphosphate receptor from pig brain with similarly high affinity for phosphatidylinositol (3,4,5)P3. FEBS Lett. 1997;405:224.

Stricker R, Vandekerckhove J, Krishna MU, Falck JR, Hanck T, Reiser G. Oligomerization controls in tissue-specific manner ligand binding of native, affinity-purified p42(IP4)/centaurin alpha1 and cytohesins-proteins with high affinity for the messengers D-inositol 1,3,4,5-tetrakisphosphate/phosphatidylinositol 3,4,5-trisphosphate. Biochim Biophys Acta. 2003;1651:1–223.

Stricker R, Chow KM, Walther D, Hanck T, Hersh LB, Reiser G. Interaction of the brain-specific protein p42IP4/centaurin-alpha1 with the peptidase nardilysin is regulated by the cognate ligands of p42IP4, PtdIns(3,4,5)P3 and Ins(1,3,4,5)P4, with stereospecificity. J Neurochem. 2006;98:2.

Tanaka K, Horiguchi K, Yoshida T, Takeda M, Fujisawa H, Takeuchi K, Umeda M, Kato S, Ihara S, Nagata S, Fukui Y. Evidence that a phosphatidylinositol 3,4,5-trisphosphate-binding protein can function in nucleus. J Biol Chem. 1999;274:712.

Thacker E, Kearns B, Chapman C, Hammond J, Howell A, Theibert A. The arf6 GAP centaurin alpha-1 is a neuronal actin-binding protein which also functions via

GAP-independent activity to regulate the actin cytoskeleton. Eur J Cell Biol. 2004;83:10.
Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem. 2001;70:535.
Venkateswarlu K, Cullen PJ. Molecular cloning and functional characterization of a human homologue of centaurin-alpha. Biochem Biophys Res Commun. 1999;262:119.
Venkateswarlu K, Oatey PB, Tavaré JM, Jackson TR, Cullen PJ. Identification of centaurin-alpha1 as a potential in vivo phosphatidylinositol 3,4,5-trisphosphate-binding protein that is functionally homologous to the yeast ADP-ribosylation factor (ARF) GTPase-activating protein, Gcs1. Biochem J. 1999;340(Pt 2):1.
Venkateswarlu K, Brandom KG, Lawrence JL. Centaurin-alpha1 is an in vivo phosphatidylinositol 3,4,5-trisphosphate-dependent GTPase-activating protein for ARF6 that is involved in actin cytoskeleton organization. J Biol Chem. 2004;279:820.
Venkateswarlu K, Hanada T, Chishti AH. Centaurin-alpha1 interacts directly with kinesin motor protein KIF13B. J Cell Sci. 2005;118(Pt 111):2471.
Whitley P, Gibbard AM, Koumanov F, Oldfield S, Kilgour EE, Prestwich GD, Holman GD. Identification of centaurin-alpha2: a phosphatidylinositide-binding protein present in fat, heart and skeletal muscle. Eur J Cell Biol. 2002;81:4.
Zemlickova E, Dubois T, Kerai P, Clokie S, Cronshaw AD, Wakefield RI, Johannes FJ, Aitken A. Centaurin-alpha(1) associates with and is phosphorylated by isoforms of protein kinase C. Biochem Biophys Res Commun. 2003;307:31.

ADAPT78

▶ Regulator of Calcineurin 1 (RCAN1)

Adaptor Protein Complex 4

▶ AP-4

Adaptor-Related Protein Complex 4

▶ AP-4

Adcy9

▶ ADCY9 (Adenylyl Cyclase 9)

ADCY9 (Adenylyl Cyclase 9)

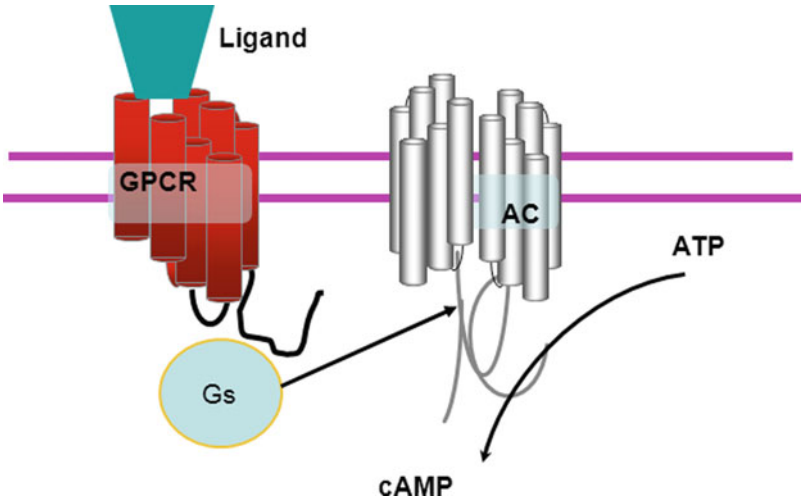
Ferenc András Antoni
Division of Preclinical Research, EGIS Pharmaceuticals PLC, Budapest, Hungary

Synonyms

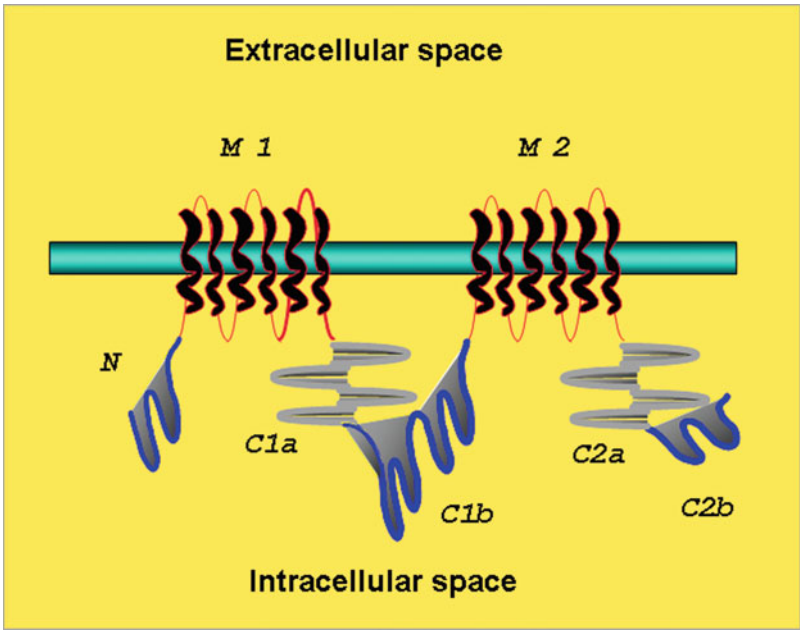
AC9; ACIX; Adcy9; Adenylate cyclase 9; Adenylyl cyclase type 9

Historical Background

Adenylyl cyclase (AC) is the enzyme (EC 4.6.1.1) that converts Mg-ATP to adenosine 3′:5′monophosphate or cyclic AMP and pyrophosphate first reported in 1958 by Sutherland and Rall. Eventually, a membrane-delimited biochemical cascade (Fig. 1) was discovered as the molecular machinery of signal transduction by cell surface receptors *via* heterotrimeric G proteins (Ross and Gilman 1980). The era of gene cloning revealed an unexpectedly complex structure of membrane-bound adenylyl cyclase: a single large polypeptide chain that crosses the plasma membrane 12 times, the catalytic core being formed between two structurally homologous cytoplasmic domains (C1a and C2a) held together by noncovalent interactions (Fig. 2). The crystal structures of these domains in complex with Gsα and the likely mechanisms underlying catalysis have been reported (Tesmer and Sprang 1998). There are nine genes encoding membrane-bound adenylyl cyclases in vertebrates (Antoni 2000; Willoughby and Cooper 2007). Manifestations of the molecular diversity of adenylyl cyclases fall into three principal categories: (1) different allosteric mechanisms for the regulation of cAMP synthesis; (2) variation of tissue distribution through the control of gene expression; and (3) selective intracellular targeting (Antoni 2000; Willoughby and Cooper 2007). However, the biological reasons for the complexity of the structure of the holoenzyme as well as the respective physiological roles of the nine paralogues of membrane-bound adenylyl cyclase remain largely unknown (Antoni 2000; Willoughby and Cooper 2007).



ADCY9 (Adenylyl Cyclase 9), Fig. 1 Basic scheme of the activation of adenylyl cyclase by heptahelical receptors in the plasma membrane. Upon binding of the ligand the heterotrimeric G protein Gs becomes active and the Gsa subunit binds to adenylyl cyclase and induces an increase of catalytic activity



ADCY9 (Adenylyl Cyclase 9), Fig. 2 Blueprint of the structure of membrane-bound adenylyl cyclases. A single polypeptide chain crosses the plasma membrane (in *blue cylinder*) 12 times forming the M1 and M2 transmembrane domains. The N terminus (N) is in the cytoplasm just as the C1 and C2 cytoplasmic domains. On the basis of structure activity studies it is useful to subdivide in the latter into a and b subdomains. The C1a and C2a domains show significant sequence homology and are known to form the catalytic core of the enzyme, which is responsive to G proteins and forskolin. Catalysis requires Mg^{2+} as well as Mn^{2+}. In the case of AC9 the preC1a segment as well as C1b and the C2b domains contain phosphorylation cluster

Protein Structure and Regulation

The primary structure of adenylyl cyclase 9 (AC9) has two notable features when compared with its mammalian paralogues (Paterson et al. 2000). Firstly, the nonconserved cytoplasmic domains C1b and C2b (Fig. 2) are considerably longer, e.g., in the case of C2b 112 amino acid residues for AC9 versus 16 for AC2. Secondly, there is a segment of amino acid residues in all adenylyl cyclases between the predicted sixth intramembrane helix and the relatively conserved C1a domain (pre-C1a) that is highly variable between

AC paralogues; in the case of AC9 it consists of 34 amino acid residues (in other ACs it is 16 or less) and contains a phosphorylation cluster <http://www.phosphosite.org/proteinAction.do?id=8311&showAllSites=true>.

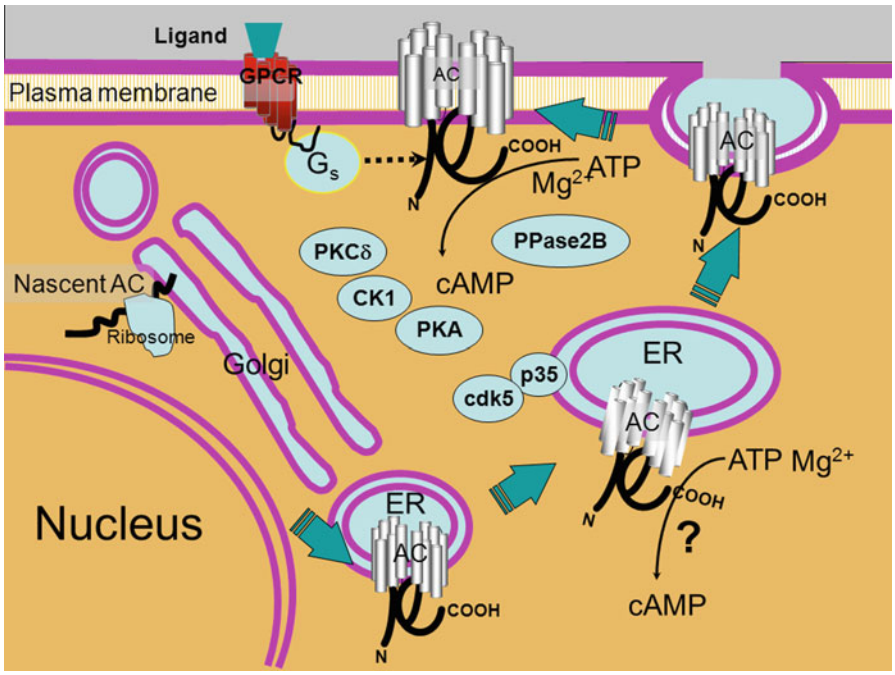
Adenylyl cyclases are subject to three main classes of physiological regulation by (1) heterotrimeric G proteins, (2) calcium ions, and (3) protein phosphorylation. All isoforms of AC, including AC9, are activated by the alpha subunit of the heterotrimeric G protein, G protein alpha s, and are thus stimulated by a variety of heptahelical cell surface receptors (7-TMR). There are apparently no effects of G protein beta/gamma subunits or Gi alpha on AC9 activity in membranes prepared from human embryonic kidney cells (HEK 293) overexpressing human AC9 or Sf9 cells infected with baculovirus encoding AC9 (Premont et al. 1996). In accordance, a putative Giα binding site sequence delineated in AC5 is absent from AC9. However, in intact AtT20 cells where the cAMP response to the activation of 7-TMR is predominantly through AC9 (Antoni et al. 1995), prominent inhibition of cAMP production by somatostatin acting through SST2/5 receptors in a pertussis toxin-sensitive manner has been demonstrated. Moreover, in HEK293 cells stably overexpressing AC9 and the long form of the D2 dopamine receptor, the D2 agonist quinpirole-inhibited isoproterenol evoked cAMP accumulation – an effect blocked by pertussis toxin (Cumbay and Watts 2004). A possible caveat in the latter study is that the expression plasmid for human AC9 used does not code for the entire correct sequence of AC9, as the C-terminal region contains a double frameshift mutation (Paterson et al. 2000). Taken together, the action of Gi-coupled receptors on AC9 is in need of further investigation.

Adenylyl cyclase 9 (AC9) was discovered on the basis of unique sensitivity of the agonist-evoked cAMP response to inhibitors of calcineurin in a pituitary tumor cell line (Antoni et al. 1995). The molecular choreography of the regulation by calcineurin is not known; currently it cannot be excluded that it is indirect, i.e., via a protein phosphatase cascade the main effector of which would be protein phosphatase 1. Pharmacological evidence indicates that activation of protein kinase C inhibits Gsα-mediated activation of AC9 (Cumbay and Watts 2004). Proteomic analysis has revealed at least 12 sites of phosphate incorporation in the isoform-specific segments (preC1a, C1b, and C2b) of human AC9 <http://www.phosphosite.org/proteinAction.do?id=8311&showAllSites=true>. Some of these phosphorylations, especially those in the pre-C1a cluster as well as Ser1307 in the C2b domain, which is an amino acid residue unique to AC9 in primates, have been analyzed in detail in HEK293 cells (Simpson et al. 2006). Specifically, Ser 365 and Ser1307 are constitutively phosphorylated by cyclin-dependent protein kinase 5 in HEK293 cells. Stimulation by cAMP leads to a marked increase in the levels of phosphoSer374 and a reduction in phosphoSer1307. The functional impacts of these changes of phosphorylation await elucidation.

Tissue Distribution and Subcellular Targeting

AC9 mRNA is widely expressed in the body (Paterson et al. 1995; Premont et al. 1996). The brain, heart, skeletal muscle, endocrine organs, the aorta, and the prostate all express high levels of AC9 mRNA whereas the spleen and thymus have low levels. Within the brain all neurons appear to express AC9, with particularly high levels of protein in the hippocampus and the cingulate cortex (Antoni et al. 1998). The promoter region of the *Adcy9* gene has not been analyzed in detail.

It is assumed that ACs are largely localized to the plasma membrane where they would be concentrated and ready for interaction with Gsα. This concept is supported in HEK293 cells and other epithelial cell lines, where transfected ACs, including AC9 (Paterson et al. 2000; Antoni et al. 2006) appear predominantly in the plasma membrane. However, the situation may be different in neurons where the bulk of adenylyl cyclase appears to localize to discrete punctae in the cytoplasm (Antoni et al. 2006). Within nerve cells of the adult rodent brain AC9 appears to be restricted to the somatodendritic compartment and is also found throughout the cytoplasm with no obvious enrichment in the plasma membrane. In this context it is worth recalling a salient structural feature of membrane-bound adenylyl cyclases: the cytoplasmic domains are sufficient for Gsα-mediated cAMP biosynthesis. Thus the cytoplasmic domain of a fully synthesized and folded adenylyl cyclase located in the smooth endoplasmic reticulum is, at least in principle, fully responsive to physiological stimulation provided Mg^{2+}



ADCY9 (Adenylyl Cyclase 9), Fig. 3 Intracellular trafficking of membrane-bound adenylyl cyclase poses important questions. As the cytoplasmic loops of the protein are catalytically active in the presence of ATP and Mg^{2+}, it is plausible that cAMP is produced in the cytoplasm. Indeed, adenylyl cyclases could be targeted to relevant cellular compartments other than the plasma membrane. Data on AC9 indicate that at least four different types of protein kinase, protein kinase C delta (PKCd), protein kinase A (PKA), casein kinase 1(CK1), and cyclin-dependent protein kinase 5/p35 complex (cdk5/p35) phosphorylate this enzyme; moreover the protein phosphatase calcineurin and calyculin A sensitive phosphatases contribute to its regulation. All of these enzymes can be attached to cell membranes. Similarly AC1 and AC8 which are stimulated by Ca^{2+} calmodulin acting on the cytoplasmic domain could provide for propagating cAMP signals that move in concert with intracellular Ca^{2+} waves

and ATP are present in sufficient amounts (Fig. 3). Indeed, it has been reported that upon receptor activation at the cell surface Gsα translocates into the cell interior (Allen et al. 2005). Furthermore, stimulators of V2-type vasopressin receptors that are cell membrane permeant can induce cAMP production *via* mutant V2 receptors that fail to be exteriorized to the cell surface (Robben et al. 2009). This latter finding also implies that functionally active signalosomes containing heptahelical receptor, Gs and adenylyl cyclase can be assembled in intracellular membranes.

Physiological Role and Phenotypes

AC9 is by far the most abundant adenylyl cyclase in the brain, yet relatively little is known about its role in the CNS. AC9 mRNA levels in the hippocampal CA1 field and the dentate gyrus were reduced in aged mice (Mons et al. 2004). Moreover, AC9 mRNA levels in the hippocampus of young adult mice were significantly increased after training sessions in the Morris water maze (fixed hidden platform paradigm) and showed a significant correlation with the level of performance in this test of spatial memory (Mons et al. 2004). While these data indicate that higher AC9 levels are associated with superior cognitive performance, further evidence is required to substantiate this notion.

In the neuroendocrine system, AC9 appears to play an important role in glucocorticoid feedback regulation of the secretion of adrenocorticotropin by the anterior pituitary gland (Antoni et al. 2003).

Although AC9 is not abundant in the immune system, this enzyme is implicated in the response of macrophages to stimuli activating pattern recognition receptors (Alper et al. 2008).

A mutation in the AC9 coding sequence has been identified which is present in 30% of the North American Caucasian and Oriental population and leads to a substantial reduction of receptor-induced cAMP

synthesis (Small et al. 2003). This may potentially contribute to vulnerability to certain polygenic disorders. Intriguingly, a significant association between the same mutation and the familial occurrence of bipolar depression has been reported in a cohort of patients in Japan (Toyota et al. 2002).

Pharmacology and Therapeutic Potential

Adenylyl cyclases have been considered as therapeutic targets for a long time, but potent and selective drugs to modulate these enzymes have remained elusive. The paucity of data on the physiological role of AC9 is also reflected by the dearth of ideas concerning its potential in clinical therapy. Forskolin has no or very little effect on AC9 heterologously expressed in HEK293 or SF9 cells. Moreover, soluble miniproteins constructed from AC9 C1 and C2 domains also fail to respond to this drug (Yan et al. 1998; Haunsø et al. 2003). Yan and coworkers have shown that mutation of a single amino acid, Tyr1082Leu, will render AC9 sensitive to the stimulatory action of forskolin. Another unusual feature of AC9 is that it is largely resistant to inhibition by adenosine analogs (Haunsø et al. 2003) which are "dead-end" inhibitors of all other ACs. Calmidazolium is a very effective (Ki = 5 μM), albeit nonspecific inhibitor of AC9 (Haunsø et al. 2003).

Summary

Adenylyl cyclase 9 is a membrane-bound adenylyl cyclase with unique regulatory properties. While the enzyme is prominently expressed in vital organs of the body such as the brain, the heart, and most endocrine glands, precious little is known about its physiological role and potential involvement in the pathogenesis of human diseases. More work with genetically modified models is required to understand the biological role of the molecular diversity of adenylyl cylcases.

References

Allen JA, Yu JZ, et al. {beta}-Adrenergic receptor stimulation promotes G{alpha}s internalization through lipid rafts: a study in living cells. Mol Pharmacol. 2005;67(5): 1493–504. doi:10.1124/mol.104.008342.

Alper S, Laws R, et al. Identification of innate immunity genes and pathways using a comparative genomics approach. Proc Natl Acad Sci USA. 2008;105(19):7016–21. doi:10.1073/pnas.0802405105.

Antoni FA. Molecular diversity of cyclic AMP signaling. Front Neuroendocrinol. 2000;21:103–32.

Antoni FA, Barnard RJO, et al. Calcineurin feedback inhibition of agonist-evoked cAMP formation. J Biol Chem. 1995;270: 28055–61.

Antoni FA, Palkovits M, et al. Ca^{2+}/calcineurin-inhibited adenylyl cyclase highly abundant in forebrain regions important for learning and memory. J Neurosci. 1998; 18(23):9650–61.

Antoni FA, Sosunov AA, et al. Short-term plasticity of cyclic adenosine 3′,5′-monophosphate signaling in anterior pituitary corticotrope cells: the role of adenylyl cyclase isotypes. Mol Endocrinol. 2003;17(4):692–703.

Antoni F, Wiegand U, et al. Cellular localisation of adenylyl cyclase: a post-genome perspective. Neurochem Res. 2006;31(2):287–95.

Cumbay MG, Watts VJ. Novel regulatory properties of human type 9 adenylate cyclase. J Pharmacol Exp Ther. 2004;310(1):108–15. doi:10.1124/jpet.104.065748 jpet.104.065748 [pii].

Haunsø A, Simpson J, et al. Small Ligands Modulating the Activity of Mammalian Adenylyl Cyclases: A Novel Mode of Inhibition by Calmidazolium. Mol Pharmacol. 2003;63:624–31.

Mons N, Segu L, et al. Effects of age and spatial learning on adenylyl cyclase mRNA expression in the mouse hippocampus. Neurobiol Aging. 2004;25(8):1095–106.

Paterson JM, Smith SM, et al. Control of a novel adenylyl cyclase by calcineurin. Biochem Biophys Res Commun. 1995;214:1000–8.

Paterson JM, Smith SM, et al. Characterisation of human adenylyl cyclase IX reveals inhibition by Ca^{2+}/calcineurin and differential mRNA polyadenylation. J Neurochem. 2000;75(4):1358–67.

Premont RT, Matsuoka I, et al. Identification and characterization of a widely expressed from of adenylyl cyclase. J Biol Chem. 1996;271:13900–7.

Robben JH, Kortenoeven MLA, et al. Intracellular activation of vasopressin V2 receptor mutants in nephrogenic diabetes insipidus by nonpeptide agonists. Proc Natl Acad Sci USA. 2009;106(29):12195–200. doi:10.1073/pnas.0900130106.

Ross EM, Gilman AG. Biochemical properties of hormone-sensitive adenylate cyclase. Annu Rev Biochem. 1980;49:533–64. doi:10.1146/annurev.bi.49.070180.002533.

Simpson J, Morrice N, et al (2006) Regulation of adenylyl cyclase 9 by cyclin-dependent protein kinase 5 (cdk5). Abstract, Bioscience 2006. Portland Press, Glasgow, pp 0449

Small K, Brown K, et al. An Ile to met polymorphism in the catalytic domain of adenylyl cyclase type 9 confers reduced beta2-adrenergic receptor stimulation. Pharmacogenetics. 2003;13(9):535–41.

Tesmer J, Sprang S. The structure, catalytic mechanism and regulation of adenylyl cyclase. Curr Opin Struct Biol. 1998; 8(6):713–9.

Toyota T, Yamada K, et al. Association analysis of adenylate cyclase type 9 gene using pedigree disequilibrium test in bipolar disorder. Mol Psychiatry. 2002;7(5):450–2.

Willoughby D, Cooper DM. Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains. Physiol Rev. 2007;87(3):965–1010. doi:87/3/965 [pii] 10.1152/physrev.00049.2006.
Yan SZ, Huang ZH, et al. Conversion of forskolin-insensitive to forskolin-sensitive (mouse-type IX) adenylyl cyclase. Mol Pharmacol. 1998;53(2):182–7.

Adenine Nucleotide Translocase

▶ ANT

Adenomatous Polyposis Coli

▶ Glycogen Synthase Kinase-3

Adenyl Cyclase

▶ Adenylyl Cyclase

Adenylate Cyclase

▶ Adenylyl Cyclase

Adenylate Cyclase 9

▶ ADCY9 (Adenylyl Cyclase 9)

Adenylyl Cyclase

Carmen W. Dessauer[1] and Rachna Sadana[2]
[1]Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, Houston, TX, USA
[2]Department of Natural Sciences, University of Houston-Downtown, Houston, TX, USA

Synonyms

Adenyl cyclase; Adenylate cyclase

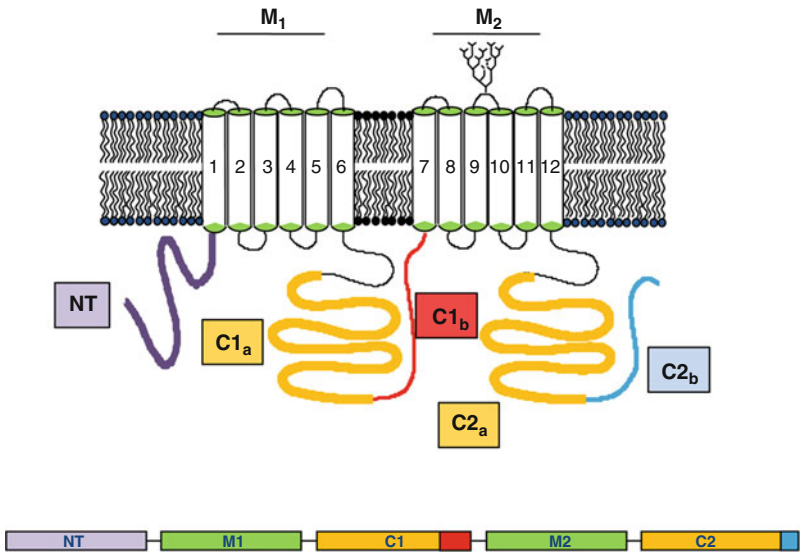
Historical Background

Adenylyl cyclases (ACs), ATP-pyrophosphate lyases, comprise a family of enzymes that catalyze the synthesis of cyclic AMP from ATP. Cyclic AMP (cAMP) was identified in 1957 as the first "second messenger," relaying signals from hormone-bound receptors to protein kinase A (PKA) and other cAMP-sensitive effectors, including cyclic-nucleotide gated channels, cAMP-activated exchange proteins (EPAC), and a subset of phosphodiesterases that degrade cyclic nucleotides. Catalytic activity of AC is regulated in response to activation of G protein-coupled receptors (GPCRs) by a number of hormones and neurotransmitters. Various studies using biochemical and genetic tools have implicated the importance of cAMP in a variety of physiological functions that include but are not limited to oogenesis, embryogenesis larval development, hormone secretion, glycogen breakdown, smooth muscle relaxation, cardiac contraction, olfaction, water homeostasis, and learning and memory (Sadana and Dessauer 2009). The discovery of cAMP and subsequent studies related to the regulation and physiological functions of AC has given rise to four Nobel Prizes in Medicine and remains a highly active area of research (Beavo and Brunton 2002).

Nine mammalian transmembrane ACs have been cloned and characterized since the initial cloning of AC1 in 1989. A tenth "soluble" form of AC (sAC) has also been characterized that lacks transmembrane domains and has distinct regulatory properties. Based upon homology and regulation patterns, membrane-bound ACs are classified into four groups; Group I comprises the Ca^{2+}-stimulated AC 1, 3, and 8; Gβγ-stimulated AC 2, 4, and 7 belong to Group II, Group III contains Giα/Ca^{2+}-inhibited AC5 and 6, while Group IV contains the distantly related forskolin-insensitive AC9.

Topology and Structure

Mammalian transmembrane ACs share a common structure that can be divided into five sections: a variable N-terminus (N), the first transmembrane domain (M1, with six membrane spans), a large cytoplasmic domain (C1), followed by a second transmembrane domain (M2, also with 6 membrane spans), and a second cytoplasmic domain (C2) (Fig. 1).



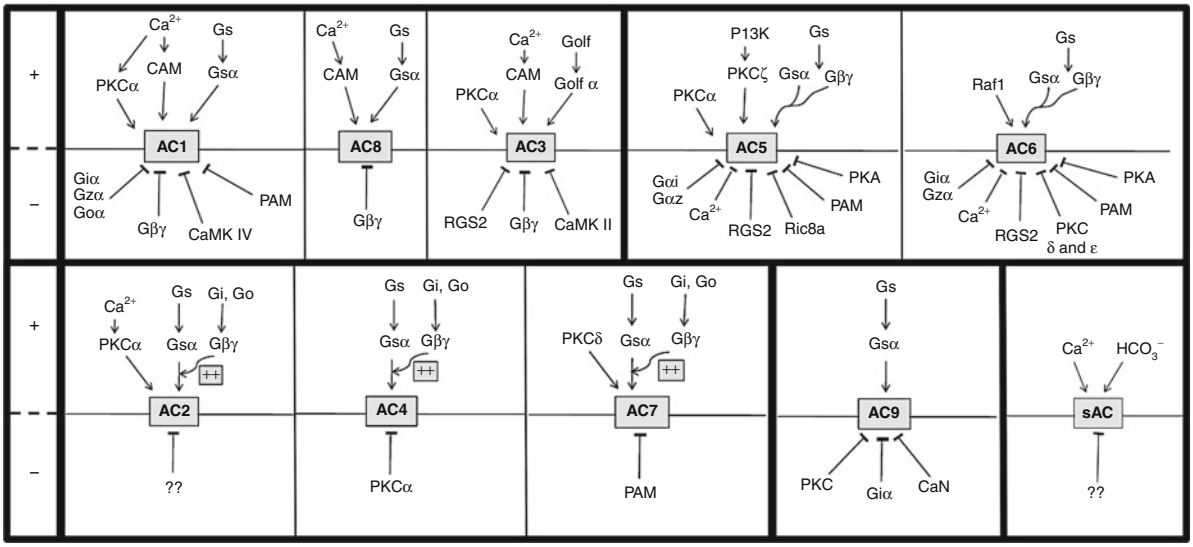
Adenylyl Cyclase, Fig. 1 Topology of membrane-bound ACs

The N-terminal portions of the two cytoplasmic domains (C1a and C2a; ~ 230 aa residues) are roughly 40% identical to each other and are highly homologous among isoforms. The topology of ACs is based largely on prediction programs for membrane spanning regions, whereas knowledge of the catalytic site comes from X-ray structures of a complex containing the C1a/C2 domains bound to the activators forskolin and Gsα. The catalytic site of AC lies at the interface of the cytoplasmic domains (C1a and C2a), forming a pseudo-symmetrical site that is primed for bidirectional regulation. The two related pockets, a substrate binding site and a forskolin site, lie along the domain interface. Although neither cytoplasmic domain has catalytic activity on their own, AC enzyme activity and many forms of regulation can be reconstituted by simple mixing of purified C1 and C2 domains of AC. The active site of ACs share similarities with the active site of DNA polymerases despite the differences in sequence and surrounding structures (Sinha and Sprang 2006).

The "soluble" form of adenylyl cyclase (sAC) has homology to cyanobacterial adenylyl cyclases with several known splice variants. The overall structure of the catalytic core of sAC is highly conserved with the transmembrane ACs, although the primary sequences differ significantly. Unlike transmembrane ACs, it does not respond to the classical G protein mode of activation but is regulated by calcium and bicarbonate levels. The sAC isoform has clear roles in male fertility and sperm motility, in addition to potential roles in acid/base homeostasis and metabolic sensing.

Regulation of ACs by G Proteins

All isoforms of membrane-bound mammalian AC are stimulated by the heterotrimeric G protein Gs (reviewed in (Sadana and Dessauer 2009; Sinha and Sprang 2006; Willoughby and Cooper 2007; Beazely and Watts 2006), Fig. 2). Appropriate agonist-bound, GPCRs activate Gs by catalyzing the exchange of GDP for GTP and facilitating the dissociation of the GTP-bound α-subunit (Gsα) from a complex of G protein α and βγ subunits. The GTP-bound α-subunit of Gs in turn activates AC, increasing the rate of synthesis of cAMP. The intrinsic GTPase activity of the G protein α subunit hydrolyzes bound GTP to GDP, terminating the activation of AC by GTP-Gsα after several seconds. GDP-Gsα then reassociates with Gβγ and awaits a new cycle of activation. This seemingly straightforward pathway is regulated at every step, from the phosphorylation and desensitization of the GPCR to the control of cAMP breakdown by numerous phosphodiesterases. Much of this regulation is targeted directly at the enzyme AC, which serves to integrate a large number of different signaling inputs. Golf,α which is highly homologous to Gsα and expressed in the olfactory



Adenylyl Cyclase, Fig. 2 *Regulatory patterns for AC isoforms*. Group I is represented by AC 1, 8, and 3, Group II contains AC 2, 4, and 7, Group III contains AC 5 and 6, and AC9 is in Group IV. The enzyme sAC is a distinct class of AC and does not respond to direct regulation by heterotrimeric G proteins

system and brain striatum, also stimulates AC. Gsα interacts with the C2 domain of AC to facilitate closure of the active site, increasing the affinity of the C1 and C2 domains for each other.

Select AC isoforms (AC 1, 5, and 6) are directly inhibited by members of the inhibitory class of G proteins, Giα, Gz, Go (Beazely and Watts 2006). This regulation can be dependent on the form of activation. For example, calmodulin-stimulated AC1 is potently inhibited by Gi family members (i, z and o), whereas AC1 is either very weakly inhibited or not at all when stimulated by Gsα- or forskolin. AC 5 and 6 are inhibited by Giα and Gzα. Giα interacts in a cleft formed in the C1 domain of AC5, analogous to the Gsα binding site in C2, and acts in opposition to Gsα. AC9 can also be inhibited by Gi-coupled receptors, which is presumably due to direct inhibition by the Giα subunit.

In addition to the α subunit, Gβγ subunits of heterotrimeric G proteins can regulate AC activity. For example, in Group I ACs 1 and 8 are inhibited by Gβγ, often more potently than the inhibition by Giα. Although Gβγ alone is not able to stimulate Group II ACs (AC2, 4, and 7), it can greatly enhance the activation by Gsα (∼five- to tenfold for AC2). This conditional Gβγ stimulation is the hallmark of the Group II family of ACs. Gβγ also conditionally stimulates AC5 and 6 in the presence of Gsα (∼1.5–2-fold), but not to the same extent as the AC2 family (Fig. 2).

Additional Modes of AC Regulation

Forskolin, a diterpene derived from the root of the Indian plant *Coleus forskohlii*, highly activates all the membrane-bound AC isoforms except AC9. This reagent is a highly useful tool for studying AC and the effects of cAMP. It has also been explored for possible medicinal uses and was originally characterized as a vasodilator. Forskolin binds at the interface of the C1 and C2 domains at a site distinct from the catalytic site (Tesmer et al. 1997) and is either additive or synergistic with regulation by Gsα. AC9 is not activated by forskolin because it lacks a key amino acid residue within this forskolin binding pocket. No physiological analogs of forskolin have been identified that can bind at the forskolin binding pocket to regulate AC activity.

AC activity can also be controlled by calcium, protein kinases, and a number of additional regulatory and scaffolding proteins. Most of this regulation is highly isoform specific. For example, although all AC isoforms are inhibited by high, nonphysiological concentrations of calcium, sub-micromolar calcium concentrations inhibit AC5 and 6 by directly binding to a magnesium ion binding site within the catalytic core. When bound to calmodulin, calcium can activate AC1 and 8, and conditionally stimulate AC3 in the presence of Gsα or forskolin. The calcium/calmodulin binding

sites for AC1 have been mapped to the C1b and C2 domains; whereas the sites on AC8 reside within the N-terminus and C2 domain. Finally, calcium/calmodulin-bound protein kinases can inhibit ACs. AC3 and AC1, but not AC8, are subject to feedback inhibition by calmodulin kinases (CamK) CaMK II and CaMK IV, respectively.

Several protein kinases regulate AC activity by directly phosphorylating AC isoforms. Feedback inhibition of AC 5 and 6 occurs via phosphorylation of the C1b domain by cAMP-activated PKA. This type of regulation is facilitated by A-kinase anchoring proteins (AKAPs) that anchor the regulatory subunit of PKA (Dessauer 2009). AKAPs are scaffolding proteins that bring together a large number of regulatory and downstream effector proteins. They can also bind to a subset of ACs. For example, AKAP79/150 (AKAP5) binds to ACs 2, 3, 5, 6, 8, and 9. The formation of an AKAP79/150-AC-PKA complex facilitates preferential phosphorylation of AC 5/6 by anchored PKA to inhibit AC activity. Much like calcium/calmodulin regulation of AC1, this sets up an important negative feedback loop to temporally regulate cAMP production and downstream signaling. A similar type of regulation also occurs for mAKAP-AC5-PKA complexes that regulate stress-responsive pathways in the heart.

Regulation by protein kinase C (PKC) isoforms is quite complex. Conventional PKCs (α and β; activated by calcium and diacylglycerol) often stimulate AC 2 and 5 and are synergistic with other forms of regulation. The novel PKCδ isoform (activated by only diacylglycerol) displays synergy with Gsα in activating AC7, while the atypical PKCζ stimulates AC5. Conventional or novel PKCs can also inhibit AC activity. AC4 is inhibited by PKCα when stimulated by Gsα, but not under basal or forskolin-stimulated conditions. Gsα-stimulated AC9 is also inhibited by conventional PKCs. The novel PKC δ and ε isoforms inhibit AC6 by phosphorylating several sites within its N-terminus. The sites of PKC phosphorylation on ACs vary between these different isoforms.

Additional kinases that regulate AC activity include ▶ Raf1 that can stimulate AC6 activity. The role of phosphatases in regulating ACs should not be overlooked. Although this has not been greatly examined, AC9 is inhibited by the phosphatase calcineurin (also known as protein phosphatase 2B, PP2B); however, it is unknown whether calcineurin acts directly on AC9. Another phosphatase, PP2A, is scaffolded by AC8, although a direct regulatory role is still unclear.

Finally, a wide range of additional proteins have been reported to regulate specific AC isoform activity including, ▶ regulators of G-protein signaling 2 (RGS2; inhibition of AC 3, 5, and 6), the protein associated with myc (PAM; inhibition of AC 1, 5, 6, and 7), the synaptic vesicle protein snapin (prevents PKC inhibition of AC 6), and the guanine nucleotide exchange protein Ric8a (inhibits AC 5). The functional roles for some of these proteins are further discussed below.

AC Physiological Roles

The physiological roles for ACs have been defined based upon localization and animal studies of genetic ablation or overexpression of an individual AC isoform (reviewed in (Sadana and Dessauer 2009); Table 1). However, since few specific antibodies against individual AC isoforms exist, much of the tissue specificity is based upon the detection of mRNAs for ACs. Some of the early noted functions included roles for AC1 and 8 (primarily expressed in brain) in learning and memory; AC3 (most abundant in olfactory epithelium) in olfaction; and AC5 and 6 (dominant in heart) for cardiac contractility. However, studies of physiological roles for additional AC isoforms have lagged behind, and in some cases no knockout animal models exist (AC 2) or the mice are embryonic lethal for unknown reasons (AC9). The largest difficulty in defining specific roles for ACs stems from the expression of multiple isoforms in any given cell type. For example, cardiac myocytes express AC 5, 6, and 9 (and possibly others) while AC 2, 3, 4, 6, and 7 are readily detected in cardiac fibroblasts. Is the expression of multiple AC isoforms a form of redundancy within the system or does each AC have a specific role(s) in these cell types? The answer may be both. Discussion of specific systems will attempt to address some of this complexity.

In Learning and Memory

Nearly all AC isoforms are expressed in brain but they display distinct roles. Early studies with Drosophila mutants *rutabaga* (deficient in Ca^{2+}/CAM stimulated AC activity whose sequence is closely related to AC1) demonstrated that mutant flies failed to learn to avoid a neutral odor. The role for AC1 in learning and

Adenylyl Cyclase, Table 1 Tissue distribution and physiological functions of individual mammalian AC isoforms

AC isoform	Sites of expression	Physiological functions
AC1	Brain, adrenal medulla, SA node	Learning, memory, synaptic plasticity, opiate withdrawal, pain memory, neuronal excitotoxicity
AC8	Brain, lung, pancreas, testis, adrenal	Learning, memory, synaptic plasticity, opiate withdrawal, stress anxiety, ethanol consumption
AC3	Olfactory epithelium, pancreas, brain, heart, lung, testis, BAT	Olfaction, sperm function, kidney function
AC2[a]	Brain, lung, skeletal muscle, heart	
AC4[b]	Widespread	
AC7	Widespread	Ethanol dependency, immune response
AC5	Heart, striatum, kidney, liver, lung, testis, adrenal, BAT	Cardiac contraction, motor coordination, opiate dependency, pain responses
AC6	Heart, kidney, liver, lung, brain, testis, skeletal muscle, adrenal, BAT	Cardiac contraction and calcium sensitivity, water homeostasis
AC9[b]	Widespread	
sAC	Testis and detected in all tissues	Sperm capacitation, fertilization

[a]Knockout not available
[b]Not fully characterized

memory in mammalian systems correlates well with its expression in brain and the phenotypes of mice deficient in AC1 ($AC1^{(-/-)}$). AC8 is also expressed mainly in brain and shares many regulatory properties of AC1, thus it is not surprising that it also is involved in learning and memory.

The formation of memories relates to the change in connections between neurons in the brain, often referred to as synaptic plasticity. This change is often measured as long-term potentiation (LTP) which is a long-lasting enhancement of neuronal connections, which enhances synaptic transmission or communication. Synaptic plasticity is dependent on calcium, therefore it is not surprising that AC 1 and 8 play such a large role. $AC1^{(-/-)}$ and $AC8^{(-/-)}$ animals show decreased LTP in the mossy fiber bundles of neurons that form connections with the CA3 region of the hippocampus (Wu et al. 1995; Wang et al. 2003). A complete loss of mossy fiber LTP was observed in the double knockout of AC 1 and 8. In contrast, the overexpression of AC1 actually enhanced recognition memory and LTP (Wang et al. 2004). These and many additional studies suggest that AC 1 and 8 are redundant in learning and memory and the formation of fear-related memories. However, there are some distinct roles for AC 1 and 8. For example, AC1 is important for the stability of neuronal circuits in response to activation deprivation, while AC8 is more involved in synaptic plasticity related to anxiety. This correlates well with the high expression of AC8, but not AC1, in the thalamus, habenula, and hypothalamus, regions involved in responses to stress. In summary, although AC 1 and 8 are not necessary for survival, they play clear roles related to learning and memory.

In Pain

Signaling pathways associated with cAMP have long been known to play a crucial role in the processing of painful stimuli (reviewed in (Pierre et al. 2009)). Various AC isoforms (AC 1, 2, 5, 6, and 8) are expressed in spinal cord, but deletion of AC1 and AC5 results in attenuated pain responses. AC1, but not AC8, knockout mice have significantly reduced behavioral responses to acute muscle pain. These findings were confirmed by use of a specific AC1 inhibitor. Chronic muscle inflammatory pain was also significantly reduced in AC1 and the AC1/8 double knockouts, but could be rescued by activating other ACs using forskolin. Thus AC1 plays an important role in acute and chronic muscle pain, although clearly additional ACs are present that can rescue impaired effects.

Mice deficient in AC5 also have attenuated pain responses in acute thermal and mechanical pain tests. They display decreased sensitivity to inflammatory pain and inflammatory visceral pain (induced by injection of sulfate or acetic acid). $AC5^{(-/-)}$ mice also display strongly attenuated mechanical and thermal

allodynia (an exaggerated response to normal stimuli) in neuropathic pain models. Although AC1 and AC5 belong to different families in term of their regulatory properties, they share a common feature in that both are inhibited by PAM, which is upregulated in the spinal cord in response to pain.

In Addiction

The analgesia properties of opiates such as morphine are mediated by Gi-coupled opiate receptors and are related in part to the inhibition of adenylyl cyclase (reviewed in (Watts and Neve 2005) and (Pierre et al. 2009)). Long-term morphine use causes an upregulation of AC signal transduction components (AC 1 and 8, PKA, and ▶ CREB) in regions of the brain associated with drug reinforcement and withdrawal. The deletion of AC 1 and 8 causes a reduction in opiate withdrawal behaviors and the double knockout of AC 1 and 8 displays less morphine-induced hyper-locomotion, and no activation of the cAMP-dependent transcription factor, CREB in the reward response circuitry of the brain (ventral tegmentum). AC1 and AC8 also have some distinct functions during chronic morphine exposure, based upon nonoverlapping patterns of gene expression changes. This difference is also reflected in the addictive properties of ethanol where mice deficient in AC8, but not AC1, had decreased voluntary ethanol consumption.

AC5 also plays an important role in opiate actions. The region of the brain (striatum) that shows the highest μ-opiate receptor levels also contain high levels of AC5. Deletion of AC5 results in a loss of opioid-induced Gi-inhibition of AC activity in striatum. In addition, the major behavioral effects of morphine including locomotor activation, pain relief, tolerance, reward and physiological dependence, and withdrawal symptoms were attenuated in $AC5^{(-/-)}$ mice. Thus AC activity plays important roles in opioid responses and addiction; with AC1 and AC8 having roles in withdrawal, hyper-locomotion, and the learned responses to morphine; whereas AC5 is involved in all major behavioral effects of morphine.

In Motor Functions

Motor functions can be divided into voluntary and involuntary (or reflex) movements. Those non-reflex actions require higher cognitive activity in several brain regions, including the striatum. The striatum region of brain is known to be important for the decision-making of voluntary movements which often requires input from dopamine-releasing neurons. Genetic ablation of AC1 and AC8 did not affect motor coordination. However, $AC5^{(-/-)}$ mice no longer respond to the dopamine D2 antagonist class of antipsychotic drugs, although general motor control is unaltered (reviewed in (Sadana and Dessauer 2009)). This behavior correlates well with the high expression of AC5 in striatum. Other $AC5^{(-/-)}$ models exhibit Parkinson's like motor dysfunction, displaying abnormal coordination, a slowness in the execution of movement (bradykinesia), and locomotor impairment (Iwamoto et al. 2003). Motor coordination can be restored by stimulation of dopamine D2 receptors, while bradykinesia was largely restored by either D1 or D2 stimulation of residual striatal AC activity. Although other Gi-inhibited ACs (AC1 and AC6) are present in striatum, they cannot fully compensate for AC5 function in dopamine-dependent motor coordination.

In Cardiac Functions

As discussed above, all of the AC isoforms except AC8 are expressed in cardiac myocytes or fibroblasts. AC1 is expressed only in sinoatrial mode, where it modulates the I(f) pacemaker current. Two closely related isoforms, AC5 and 6 are the major isoforms expressed in cardiac myocytes and have been the focus of several deletion and overexpression studies (reviewed in (Sadana and Dessauer 2009)). These ACs appear to exert opposite effects on the heart, since cardiac overexpression of AC6 appears to be protective, whereas disruption of type 5 AC prolongs longevity and protects against cardiac stress (Yan et al. 2007). However, some overlapping functions must exist in the heart, as deletion of AC 5 or 6 does not give rise to a complete loss of sympathetic regulation.

The deletion of AC5 results in ~40% decreased isoproterenol- (an agonist of the beta-adrenergic receptors; βAR) and forskolin-stimulated AC activity in cardiac membranes and isolated myocytes. There is also a loss of acetylcholine-mediated (Gi) inhibition and reduced calcium-mediated inhibition of cAMP production in $AC5^{(-/-)}$ heart. Although differences in AC5 deletion strains exist, the loss of AC5 results in decreased isoproterenol-stimulated left ventricular (LV) ejection fraction (e.g., the volume of blood pumped out of the heart with each beat). Effects of AC5 deletion are not limited to sympathetic regulation,

as loss of AC5 also eliminates parasympathetic control of cAMP levels and attenuates baroreflexes that maintain blood pressure. The deletion of AC6 represents a functional double knockout of AC5 and AC6 because of reduced AC5 levels in AC6 $^{(-/-)}$ mice (Tang et al. 2008). As expected, βAR-stimulated cAMP levels were reduced ~80% in these animals with greatly reduced βAR-stimulated LV contractile function and reduced calcium transients. The latter effect on calcium handling is likely due to the loss of AC6, rather than AC5.

When the heart is stressed by chronic activation of cAMP (often due to prolonged beta-adrenergic receptor activation), a decrease in cardiac function is observed (cardiac myopathy). The levels of AC5 are also known to increase under these conditions. Deletion of AC5 protects the heart against chronic βAR stimulation and chronic pressure overload by attenuating the decline in cardiac function and defending against increased apoptosis. AC5 disruption is also protective against age-related cardiac myopathy and gives rise to an increased lifespan as compared to wild-type animals.

The differing roles for AC5 and AC6 may lie in the types of AKAP complexes that they are associated with. For example, AC6 is found in complex with AKAP79/150 in heart. Deletion of AKAP150 results in loss of βAR stimulated calcium transients (Navedo et al. 2008), reminiscent of AC6 deletion phenotypes. In contrast, AC5 is found in complex with mAKAP which is associated with the regulation of cardiac hypertrophy and related stress responses (Dessauer 2009). Another AKAP, Yotiao, is important in mediating sympathetic control of cardiac action potential duration. Yotiao is associated with several AC isoforms including AC9 which is present at lower levels in cardiac myocytes. The role for this latter AC isoform is as yet unknown.

In Olfaction

AC 2, 3 and 4 are expressed in the olfactory system. However, AC3 is the predominant isoform in the olfactory epithelium and is largely responsible for odorant and pheromone detection (reviewed in (Wang et al. 2007)). Odorants interact with G protein-coupled receptors to stimulate adenylyl cyclase via Golf. Genetic deletion of AC3 confirms its role in olfaction, as AC3$^{(-/-)}$ mice suffer from major effects on odorant-induced signaling and are impaired in olfactory-dependent learning and olfaction-based behavioral tests. In addition, AC3$^{(-/-)}$ mice are unable to detect mouse urine or pheromones. AC3$^{(-/-)}$ mice also lack intermale aggressiveness and male sexual behavior. AC3 has also been ascribed a role in spermatozoa function and male fertility. In general the vomeronasal organ expressing AC2 is thought to be responsible for pheromone detection, but as discussed, AC3 are also associated with these functions.

In Immune Responses

The role of cAMP in the immune system is complex since cAMP generation can induce apoptosis as well as cell proliferation, differentiation, and activation of various immune cell types. AC7 is the major isoform that regulates cAMP synthesis in both B and T cells. AC 7 $^{(-/-)}$ mice produce fewer leukocytes and have a high mortality rate upon bacterial infections (Duan et al. 2010), which was attributed to changes in the production of certain serum factors required for regulating AC7 activity. AC7 null mice also produce less antigen-specific antibodies to fight infections even though overall immune responses were hyperactive, as measured by the overproduction of cytokines. The unique regulation of AC7 by Gs- and G12/13-coupled receptors makes it well suited to respond to multiple signals and facilitates its multifaceted roles in regulating both innate and adaptive immune responses.

In Kidney

AC6 is localized in the renal tubule and collecting duct of kidneys (Chien et al. 2010). AC6$^{(-/-)}$ mice had normal glomerular filtration rate but were deficient in water homeostasis (Rieg et al. 2010). AC6-deficient mice drank more water, urinated more, and had low urine solute concentrations. The major protein required for transport of water through the plasma membrane (aquaporin) is also mis-localized, displaying reduced phosphorylation in kidneys of AC6 null mice, consistent with a malfunction in water retention. This phenotype is similar to that of nephrogenic diabetes insipidus and is consistent with a loss of vasopressin-induced cAMP in the inner medullary collecting ducts of AC6$^{(-/-)}$ kidney. AC3 has also been ascribed a role in kidney function. Key components of the olfactory signal transduction machinery (olfactory receptor, AC3, and Golf) are expressed in the renal distal nephron. Unlike AC6,

mice deficient in AC3 suffer from reduced glomerular filtration rate and also display low levels of plasma renin (Pluznick et al. 2009).

Summary

ACs can be found in every cell type and tissue, consistent with the very large role for cAMP in so many physiological processes. In addition to those outlined above, cAMP has important roles in development and differentiation, cell proliferation, neurodegeneration and neurotoxicity, asthma, diabetes, fertilization, and hormone secretion. Many GPCRs that regulate cAMP levels are currently targeted for treating conditions that include asthma, heart failure, diabetes, pain, migraines, peptic ulcer disease, obesity, Schizophrenia, Parkinson's, and nausea (Pierre et al. 2009). The roles for specific AC isoforms in these pathophysiological conditions have sparked much interest in exploring AC as a drug target. For example, AC5 inhibitors are currently being considered for treatment of heart failure (Ho et al. 2010), while an AC1 activator could be a memory-enhancing drug (Pierre et al. 2009). Analogs of forskolin that specifically target an AC isoform or selective P-site inhibitors could also prove useful (Iwatsubo et al. 2006). Finally, the local lipid environment of individual AC isoforms or the macromolecular complexes that link ACs to downstream effectors may play a large role in the specificity of downstream signaling. Thus despite the rich history of cAMP research, much work remains to be done.

References

Beavo JA, Brunton LL. Cyclic nucleotide research – still expanding after half a century. Nat Rev Mol Cell Biol. 2002;3:710–8.

Beazely MA, Watts VJ. Regulatory properties of adenylate cyclases type 5 and 6: a progress report. Eur J Pharmacol. 2006;535:1–12.

Chien C-L, Wu Y-S, Lai H-L, Chen Y-H, Jiang S-T, Shih C-M, et al. Impaired water reabsorption in mice deficient in the type VI adenylyl cyclase (AC6). FEBS Lett. 2010;584:2883–90.

Dessauer CW. Adenylyl cyclase–A-kinase anchoring protein complexes: the next dimension in cAMP signaling. Mol Pharmacol. 2009;76:935–41.

Duan B, Davis R, Sadat EL, Collins J, Sternweis PC, Yuan D, et al. Distinct roles of adenylyl cyclase VII in regulating the immune responses in mice. J Immunol. 2010;185:335–44.

Ho D, Yan L, Iwatsubo K, Vatner D, Vatner S. Modulation of β-adrenergic receptor signaling in heart failure and longevity: targeting adenylyl cyclase type 5. Heart Fail Rev. 2010;15:495–512.

Iwamoto T, Okumura S, Iwatsubo K, Kawabe J, Ohtsu K, Sakai I, et al. Motor dysfunction in type 5 adenylyl cyclase-null mice. J Biol Chem. 2003;278:16936–40.

Iwatsubo K, Okumura S, Ishikawa Y. Drug therapy aimed at adenylyl cyclase to regulate cyclic nucleotide signaling. Endocr Metab Immune Disord Drug Targets. 2006;6:239–47.

Navedo MF, Nieves-Cintron M, Amberg GC, Yuan C, Votaw VS, Lederer WJ, et al. AKAP150 is required for stuttering persistent Ca^{2+} sparklets and angiotensin II-induced hypertension. Circ Res. 2008;102:e1–11.

Pierre S, Eschenhagen T, Geisslinger G, Scholich K. Capturing adenylyl cyclases as potential drug targets. Nat Rev Drug Discov. 2009;8:321–35.

Pluznick JL, Zou DJ, Zhang X, Yan Q, Rodriguez-Gil DJ, Eisner C, et al. Functional expression of the olfactory signaling system in the kidney. Proc Natl Acad Sci USA. 2009;106:2059–64.

Rieg T, Tang T, Murray F, Schroth J, Insel PA, Fenton RA, et al. Adenylate cyclase 6 determines cAMP formation and aquaporin-2 phosphorylation and trafficking in inner medulla. J Am Soc Nephrol. 2010;21:2059–68.

Sadana R, Dessauer CW. Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies. Neurosignals. 2009;17:5–22.

Sinha SC, Sprang SR. Structures, mechanism, regulation and evolution of class III nucleotidyl cyclases. Rev Physiol Biochem Pharmacol. 2006;157:105–40.

Tang T, Gao MH, Lai NC, Firth AL, Takahashi T, Guo T, et al. Adenylyl cyclase type 6 deletion decreases left ventricular function via impaired calcium handling. Circulation. 2008;117:61–9.

Tesmer JJ, Sunahara RK, Gilman AG, Sprang SR. Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsα.GTP-γS. Science. 1997;278:1907–16.

Wang H, Ferguson GD, Pineda VV, Cundiff PE, Storm DR. Overexpression of type-1 adenylyl cyclase in mouse forebrain enhances recognition memory and LTP. Nat Neurosci. 2004;7:635–42.

Wang H, Pineda VV, Chan GC, Wong ST, Muglia LJ, Storm DR. Type 8 adenylyl cyclase is targeted to excitatory synapses and required for mossy fiber long-term potentiation. J Neurosci. 2003;23:9710–8.

Wang Z, Nudelman A, Storm DR. Are pheromones detected through the main olfactory epithelium? Mol Neurobiol. 2007;35:317–23.

Watts VJ, Neve KA. Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. Pharmacol Ther. 2005;106:405–21.

Willoughby D, Cooper DM. Organization and Ca^{2+} regulation of adenylyl cyclases in cAMP microdomains. Physiol Rev. 2007;87:965–1010.

Wu ZL, Thomas SA, Villacres EC, Xia Z, Simmons ML, Chavkin C, et al. Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. Proc Natl Acad Sci USA. 1995;92:220–4.

Yan L, Vatner DE, O'Connor JP, Ivessa A, Ge H, Chen W, et al. Type 5 adenylyl cyclase disruption increases longevity and protects against stress. Cell. 2007;130:247–58.

Adenylyl Cyclase Inhibitory Gi Alpha Subunit

► G Protein α i/o/z

Adenylyl Cyclase type 9

► ADCY9 (Adenylyl Cyclase 9)

Adhesion GPCRs

Christiane Kirchhoff[1] and Ben Davies[2]
[1]Department of Andrology, University Hospital Hamburg-Eppendorf, Hamburg, Germany
[2]Wellcome Trust Centre for Human Genetics, University of Oxford Roosevelt Drive, Oxford, UK

Synonyms

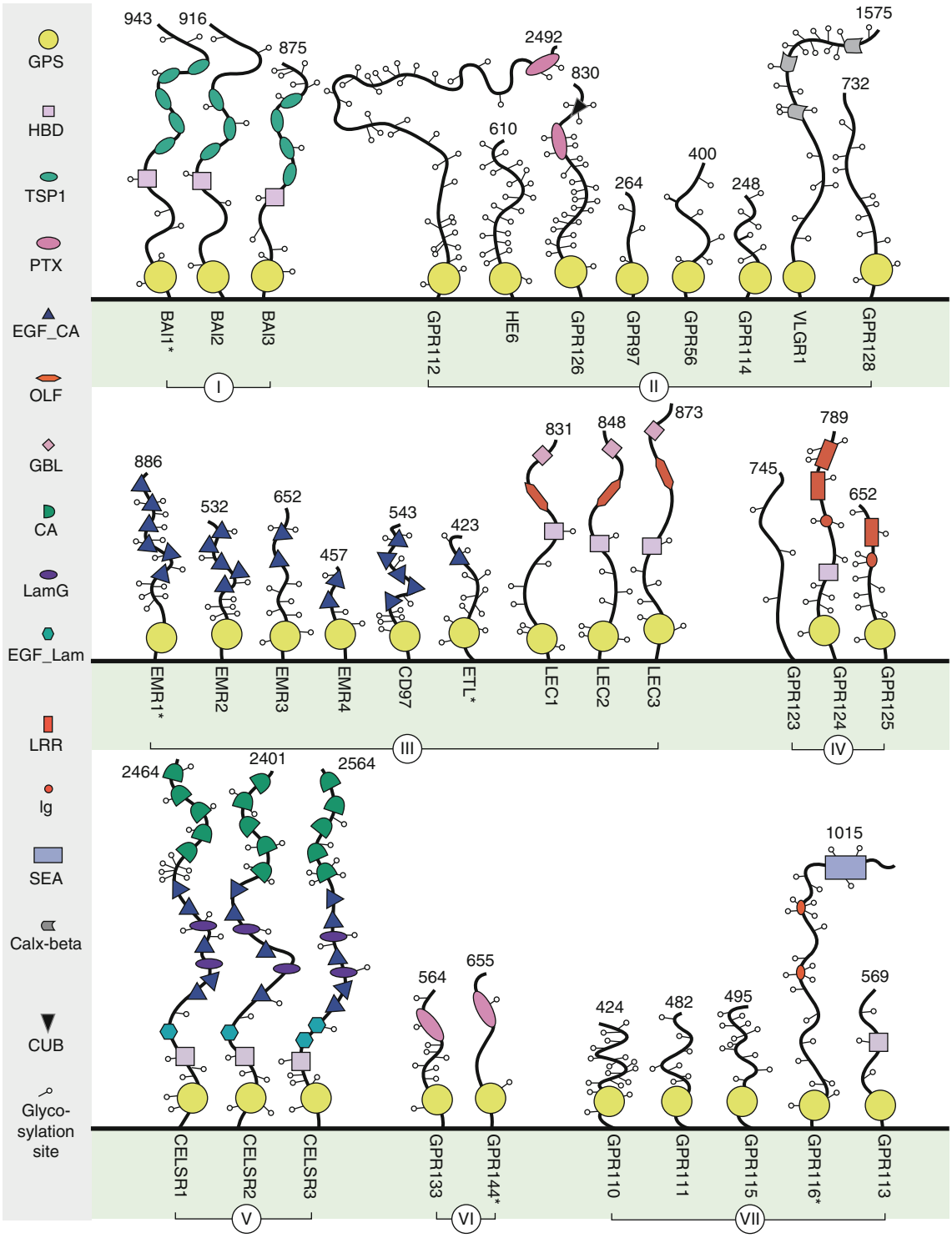
LNB-TM7 receptors

Historical Background

Among the superfamily of G-protein-coupled receptors, the adhesion GPCRs form the second largest family with 33 members in humans (for review, see Bjarnadottir et al. 2007). They are also the most diverse and complex GPCR family, often encoded by very large genes with numerous introns, and comprising highly diverse and variable N-terminal extracellular regions. Despite a remarkable structural diversity and low sequence homology, hydropathy analysis and biochemical data suggest that the adhesion GPCRs share the structural hallmark of all GPCRs – a heptahelical conformation with extracellular N-terminus and intracellular C-terminus. Approximately 150 distinct orthologues have been identified in the animal kingdom to date. The name adhesion GPCRs emphasizes the presence of multiple motifs in their long extracellular N-termini predicting adhesive properties, such as cadherin-, laminin-, lectin-, epidermal growth factor-, olfactomedin-, immunoglobulin-, calcium exchanger β-, thrombospondin-, and mucin-like domains (see Fig. 1).

The Epidermal growth factor Module-containing Receptor EMR1, its mouse homologue F4/80, and CD97, all members of the Epidermal Growth Factor (EGF)-TM7 receptor subfamily, were the first-described adhesion GPCRs (for review, see McKnight and Gordon 1996; 1998). Derived from a single precursor polypeptide, they are expressed at the surface of leukocytes in two parts, (1) a large extracellular domain with variable numbers of modular EGF-like domains and (2) a membrane-spanning domain consisting of seven hydrophobic stretches connected by three intracellular and three extracellular loops. Both parts are associated via a highly glycosylated mucin-like "stalk" which serves as a spacer. The unusual structure of their extracellular regions indicated a function related to cell adhesion and leukocyte migration. Indeed, the ability to bind to extracellular matrix molecules and cellular ligands has been experimentally demonstrated for several members of the EGF-TM7 multigene family (see below).

The subsequent discovery of an increasing number of GPCR-related molecules with a similar domain configuration led to the proposal to reclassify this group of heptahelical membrane proteins as a novel class of receptors (for review, see Stacey et al. 2000). Following the example of the EGF-TM7 receptors, the entire class was termed the LNB-TM7 receptors, where LN stands for their large and complex N-terminal extracellular regions, and B indicates a significant amino acid sequence similarity to G-protein-coupled receptor family B, also named the *Secretin* GPCRs (following the GRAFS classification system, Fredriksson et al. 2003). Progress in molecular biology and bioinformatics techniques led to the sequencing and assembly of whole genomes, and allowed the identification of the full repertoire of human GPCRs. This enabled a phylogenetic analysis based on the complete sequences of the TM7 regions, which demonstrated that the adhesion GPCRs did indeed form a distinct receptor family (Fredriksson et al. 2003). Based on the sequence similarity within their heptahelical regions and intervening loops, the human adhesion GPCRs were further subdivided into different groups or clans (Fig. 1; for review, see Bjarnadottir et al. 2007; Lagerström and Schiöth 2008). Interestingly, this group structure at the same



Adhesion GPCRs, Fig. 1 A schematic presentation of the functional domains found in the N-termini of human adhesion GPCRs (reproduced with permission from Bjarnadottir et al. 2007). GPS = GPCR proteolysis site; HBD = hormone binding domain; TSP1 = thrombospondin type 1 repeat; PTX = Pentraxin doamain; EGF_CA = calcium-binding EGF-like domain; OLF = Olfactomedin-like domain; GBL = galactose binding lectin domain; CA = Cadherin-like

time reflected a functional classification based on the various adhesion motifs present in the N-termini (Fig. 1). Also, receptors within the same phylogenetic clusters showed similar expression profiles in human tissues.

More recently, Schiöth and coworkers suggested that the adhesion GPCRs were an ancient receptor class, and the *Secretin* GPCRs descended from a subgroup during metazoan evolution (Nordström et al. 2009). *Secretin* GPCRs bind polypeptide hormones at a hormone-binding domain (HBD) in their N-termini, and, in response to ligand binding, signal via G-protein activation, predominantly $G_{\alpha S}$. Although several adhesion GPCRs contain a HBD motif in their N-termini as well (compare Fig. 1), no hormone ligands have been identified for this receptor family, and there is only scant evidence of receptor activation and G-protein coupling (see below). Adhesive interactions have been verified for some family members (see below) while the functional role of a majority of adhesion GPCRs is still poorly understood. Thus, their description as LNB-TM7 receptors may still be more correct, although the name adhesion GPCRs now prevails in the literature.

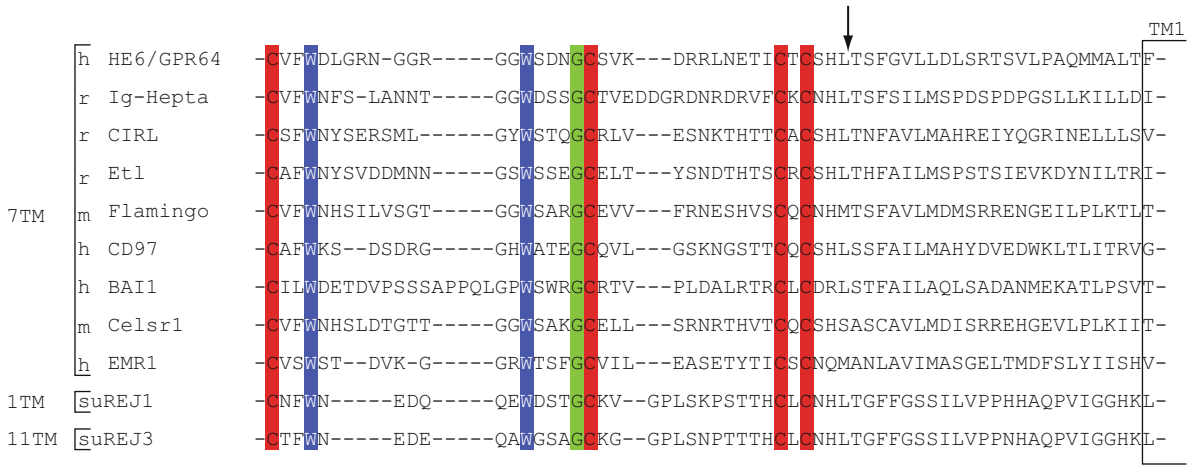
Posttranslational Processing

The adhesion GPCRs are chimeras, composed of a hydrophilic extracellular adhesion part and a hydrophobic membrane-spanning *signaling* part. This chimeric configuration already suggested a dual function, combining interactions with the surrounding extracellular matrix and/or cellular counter receptors with coupling to the intracellular signaling machinery. Different from other GPCRs, however, the "mature" adhesion GPCRs almost invariably consist of two separate subunits which appear to be a defining feature of this receptor class. The two subunits are derived from cleavage at the so-called GPCR proteolytic site (GPS), a characteristic Cys-box-containing motif located in the ectodomain adjacent to the first transmembrane domain (Krasnoperov et al. 1997, 2002; Chang et al. 2003; Lin et al. 2004; Figs. 1 and 2). This site is also involved in the cleavage of other membrane-spanning proteins, but is not found in any other GPCR family in the human and mouse genomes.

The GPS is one of the few conserved motifs present in all the N-termini of adhesion GPCRs, except GPR123 (see Fig. 1). In addition to this site, most adhesion GPCRs share a second homologous stretch in their extracellular domains, a serine-, threonine-, and proline-rich (STP) "stalk," typical of a highly O-glycosylated protein domain. Conservation of these motifs in nearly all adhesion GPCRs suggests an important role. The first functional hint of the GPS motif was revealed when intracellular proteolytic processing of cell surface marker CD97 (cluster of differentiation) was demonstrated (Gray et al. 1996). Translated as a single polypeptide, CD97 cleavage results in the formation of the two subunits. Cloning and Western blot analysis of the lectin-like domain-containing calcium-independent receptor of α-latrotoxin (Krasnoperov et al. 1997; Lelianova et al. 1997), GPR116/Ig-Hepta (Abe et al. 2002) and the GPR64/HE6 receptor (Obermann et al. 2003) has revealed additional "split" receptors. The exact cleavage site was determined for CIRL/Latrophilin/lectomedin receptor 1 (Krasnoperov et al. 2002) and also for the EGF-TM7 receptors (Chang et al. 2003). It is located at homologous sites in the C-terminal part of the GPS domain (see Fig. 2).

The GPS motif is necessary, but not sufficient for cleavage. Rather, cleavage also requires the presence of the "stalk" next to the membrane (Chang et al. 2003; Hsiao et al. 2009). As an example, the ectosubunit of the GPR64/HE6 receptor, which is predominantly expressed in the microvilli of the epididymal duct epithelium (Obermann et al. 2003; Kirchhoff et al. 2008), is characterized by the presence of 20 potential

Adhesion GPCRs, Fig. 1 (continued) (Calcium-dependent adhesion-like) domains; LamG = Laminin G domain; EGF_Lam = EGF-like, fibronectin type III and laminin G domains; LRR = leucine-rich repeats; Ig = Immunoglobulin-like domains; SEA = Sea-urchin sperm protein, enterokinase and agrin module; Calx-beta = motif in Na-Ca exchangers and integrin-beta4; CUB = Domain first found in complement C1r, C1s, uEGF, and bone morphogenetic protein. Subdivision into different groups or clans (Roman numerals I–VII) was based on the sequence similarity within the heptahelical regions and intervening loops. According to a more recent classification, Group III can be further divided into two clusters (Lagerstrom and Schioth 2008). The first cluster contains LEC1-3 and ETL receptors, whereas the second cluster includes EMR1-4 and CD97 receptors



Adhesion GPCRs, Fig. 2 Protein sequence alignment of the juxtamembrane region of adhesion GPCR family members (modified from Abe et al. 2002). Conserved amino acid residues of the GPCR proteolysis site (GPS) are highlighted. The arrow points to the hypothetical cleavage site within the GPS domain. Amino acid sequences obtained from the NCBI database were aligned with the ClustalW program followed by manual adjustment. 7 TM – adhesion GPCRs; h = human; m = mouse; r = rat; 1 TM – suREJ1 = sea urchin Receptor for Egg Jelly 1; 11 TM – suREJ3 = sea urchin Receptor for Egg Jelly 3; TM1 = first amino acid of transmembrane domain 1

N-glycosylation (Asn-Xaa-Ser/Thr) sites and more than 100 potentially O-glycosylated Ser and Thr residues, largely located within the STP region, and most probably forming a hydrophilic mucin-like "stalk" (Obermann et al. 2003). From the large number of glycosylation sites and helix-breaking proline residues, the ectosubunit was predicted to be highly charged and extend from the cell surface like a rod. Similar structural predictions have been made for other adhesion GPCRs, including the EGF-TM7 receptors and the latrophilins (LPHN; synonyms CL/CIRL/Lph). More recently, glycosylation at specific sites of the EGF-TM7 "stalk" has been implicated in cleavage at the GPS (Hsiao et al. 2009).

Proteolytic cleavage is a well-known regulatory mechanism for receptor activation. For example, the G-protein-coupled protease-activated receptors (PARs) signal in response to N-terminal cleavage by extracellular proteases. The new N-terminus then acts as a tethered ligand and binds intramolecularly to the receptor to trigger transmembrane signaling. The mechanism and role of GPS-mediated cleavage seems to be different. Experiments on EMR2, member of the EGF-TM7 family, suggested that it occurs intracellularly in the endoplasmic reticulum (Lin et al. 2004). Whether it could trigger or modulate any signaling events, in analogy to the PAR example, remained unknown. A role in signaling, however, was proposed for GPS cleavage of the Cadherin, EGF LAG seven-pass G-type receptor (CELSR) family members. The extracellular subunits of CELSR2 and CELSR3, including their Cadherin repeats, were proposed to act as a ligand of the seven-transmembrane-spanning part, stimulating calcium release and regulating neurite growth (Shima et al. 2007).

Different from PAR activation, GPS-mediated cleavage is mediated by an autocatalytic reaction rather than by protease activity (Lin et al. 2004). The mechanism is similar to that of N-terminal nucleophile hydrolases, involving the generation and hydrolysis of a (thio)-ester intermediate. Yet, GPS-mediated cleavage appears to be a regulated process, requiring additional modifications like phosphorylation (Kaur et al. 2005) and glycosylation (Hsiao et al. 2009). Endogenous cleavage of the precursor protein may be incomplete; however, full-size, non-cleaved molecules are not normally found in tissues and may not reach the cell surface. Indeed, efficient latrophilin/CIRL receptor trafficking to the cell surface requires proper GPS cleavage (Volynski et al. 2004). Furthermore, deficiency in GPS cleavage caused by mutations has been linked to various human genetic disorders and diseases, including bilateral frontoparietal polymicrogyria (BFPP), and autosomal dominant

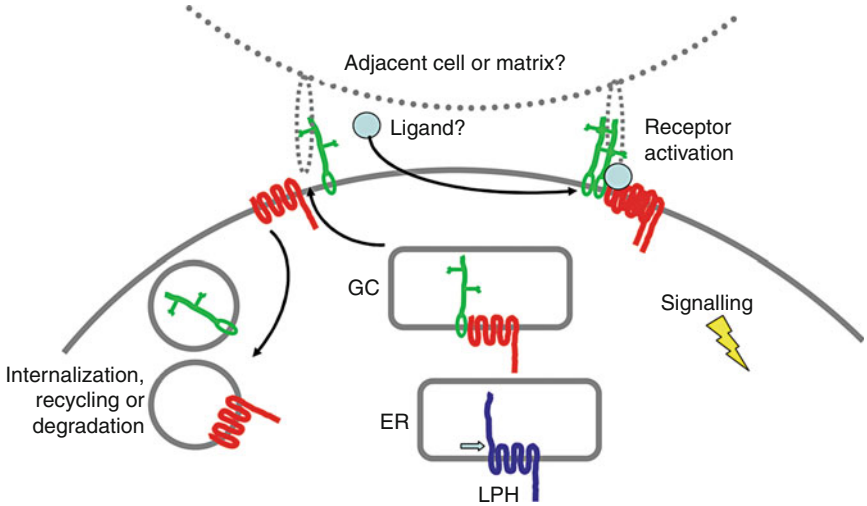
polycystic kidney disease (ADPKD), emphasizing the critical role of this modification (for review, see Yona et al. 2008a).

Surprisingly, GPS-mediated cleavage of adhesion GPCRs does not yield much soluble ectoprotein; rather, both subunits remain frequently tethered at the cell membrane. Observations from different subgroups showed that the extracellular subunits behave as membrane proteins despite a lack of transmembrane regions (Gray et al. 1996; Krasnoperov et al. 1997, 2002; Obermann et al. 2003). Initially, this was thought to be mediated by a non-covalent interaction with the TM7 domain subunit. However, in the case of latrophilin/CIRL and the EGF-TM7 receptors, the two subunits are delivered to different plasma membrane domains, are recycled separately, and can be patched independently by specific antibodies (Volynski et al. 2004; Davies et al. 2007; Silva et al. 2009a). Apparently, both subunits behave as independent membrane proteins, the ectosubunit being anchored in the plasma membrane by an unknown mechanism. Similarly, both GPR64/HE6 subunits were individually solubilized from different parts of the epididymis (Obermann et al. 2003; Davies et al. 2004). In membrane fractions prepared from the proximal part of this organ, the amount of extractable ectosubunit by far exceeded that of the endosubunit. This peculiar observation was in apparent discrepancy with the expected 1:1 stoichiometry of subunits. While the significance of this observation remains unclear, it may reflect different solubilization characteristics of the two subunits, rather than different locations. Despite their independence, however, subunits of latrophilin/CIRL and the EGF-TM7 receptors can reassemble in the plasma membrane to form heterodimers (Davies et al. 2007; Silva et al. 2009a). This observation has led to the concept of a "split personality" receptor (Volynski et al. 2004; see Fig. 3 and below) which may also be applicable to other adhesion GPCRs.

Nevertheless, there are also reports of "shedding" of the extracellular subunit. Increased expression of CD97 at sites of inflammation, for example, is accompanied by detectable levels of soluble extracellular subunit (Gray et al. 1996). The mechanism of this release is not known but might include augmented matrix metalloproteinase activity in a pathological situation. The Brain Angiogenesis Inhibitor 1 (BAI1), on the other hand, seems to release its extracellular subunit in the normal brain. It was inferred that the secreted fragment, named Vasculostatin for its antiangiogenic function, resulted from BAI1 cleavage at the GPS (Kaur et al. 2005). However, the precise cleavage site(s) was not determined. It could be that such "shedding" requires cleavage of the BAI1 molecule at multiple sites (compare Koh et al. 2004) as was shown for other adhesion GPCRs (see below). In summary, GPS cleavage which occurs exactly between the two structurally and functionally different domains is an intrinsic posttranslational modification of many, if not most, adhesion GPCRs. For the family members studied, it occurred largely autocatalytically inside the cell, probably at the endoplasmic reticulum (ER). It is a prerequisite for receptor trafficking and functional expression at the cell surface; however, it may not be a common step in signaling.

Soluble fragments may also be generated by alternative mRNA splicing, providing a mechanism for the generation of soluble ligand or receptor desensitization (for review, see Yona et al. 2008a). For a number of adhesion GPCRs, there is evidence for additional proteolytic cleavage events which might lead to the release of N-terminal fragments. CIRL/Latrophilin can be cleaved in vivo by an unknown protease in the remaining short N-terminal stretch of the seven-transmembrane-spanning endosubunit to yield a small 15 amino acid residue oligopeptide (Krasnoperov et al. 2009). While the majority of receptor molecules remained membrane-bound after cleavage at the GPS (see above), this additional cleavage resulted in the formation of soluble subunits. It was assumed that the soluble fragments contained the non-covalently bound 15-mer at their C-terminal ends (Krasnoperov et al. 2009).

Results by Koh et al. (2004) indicated that the extracellular region of brain-specific angiogenesis inhibitor 1 (BAI1) was cleaved at three sites, and that the BAI1-thrombospondin type 1 repeat fragment was the core extracellular fragment for BAI1's antiproliferative activity. A specifically complex processing was described for Ig-Hepta/GPR116 (Fukuzawa and Hirose 2006), an adhesion GPCR of unknown function which is abundantly expressed in lung and kidney (Abe et al. 2002). These authors further suggested involvement of some fragment(s) in cellular signaling (Fukuzawa and Hirose 2006). GPS-independent cleavage was also described for GPR126/DREG/VIGR (Moriguchi et al. 2004), an adhesion GPCR which



Adhesion GPCRs, Fig. 3 Proposed scheme of latrophilin/CIRL (LPH) processing, activation, and internalization (modified from Volynski et al. 2004). LPH is cleaved intracellularly in the ER. Proteolysis is required for the delivery of the mature, "split" receptor protein to the cell surface. Further posttranslational processing in the Golgi complex (GC) involves glycosylation and may endow the N-terminal fragment with an own membrane anchor; however, the structure of this anchor is currently unknown. On the cell surface, the two subunits behave as independent membrane proteins. Agonist binding to the ectosubunit promotes its association with the endosubunit and or receptor dimerization, presumably leading to the activation of endosubunit-mediated cell signaling pathways. Internalization of subunits may be independent processes.

was recently shown to be essential for myelination in Schwann cells (Monk et al. 2009). Besides cleavage at the GPS, the protein was further cleaved in the middle of the extracellular domain, generating a soluble fragment containing the CUB (for complement C1r/C1s, Uegf, Bmp1) and pentraxin (PTX) domains (Moriguchi et al. 2004). This processing step was inhibited by an inhibitor of furin but not of matrix metalloproteinases. It was speculated that the subfragment could play a role as a secreted ligand (Moriguchi et al. 2004).

It is unknown whether non-GPS cleavage is a common phenomenon of adhesion GPCRs, and its functional significance remains unclear. Members of other GPCR families, including the V2 receptor and endothelin B receptor, undergo a ligand-induced proteolysis to produce peptides with possible bioactivity. Similarly, the liberated fragments of adhesion GPCRs might be a physiological ligand; alternatively, the cleavage might unmask a hidden ligand binding site, or may activate the receptor (for review, see Yona et al. 2008a). For latrophilin, it was suggested that the non-GPS cleavage(s) served to release defective receptor protein from the cell membrane and/or, in conjunction with GPS cleavage, control cell surface expression (Krasnoperov et al. 2009). Finally, regulated intramembrane proteolysis of membrane receptors produces C-terminal fragments that function inside the cell, even in the nucleus. For most adhesion GPCRs, however, it is as yet unclear whether such C-terminal fragments are generated, probably due to the fact, that the appropriate assays have not been performed.

Ligand Binding

In the absence of endogeneous ligands, a first key concept of ligand binding and activation came from studies involving an exogenous agonist, α-latrotoxin. It is a component of the black widow spider venom, and an activating ligand (=agonist) of CIRL/latrophilin (Krasnoperov et al. 1999). α-latrotoxin binding occurs as two sequential steps: (1) the toxin interacts with the ectodomain with medium affinity followed by (2) an interaction with either the first transmembrane domain, with the membrane lipids, or with both. As a result α-latrotoxin penetrates into the lipid bilayer. The second step increases the affinity of the interaction and requires a longer time. The first site

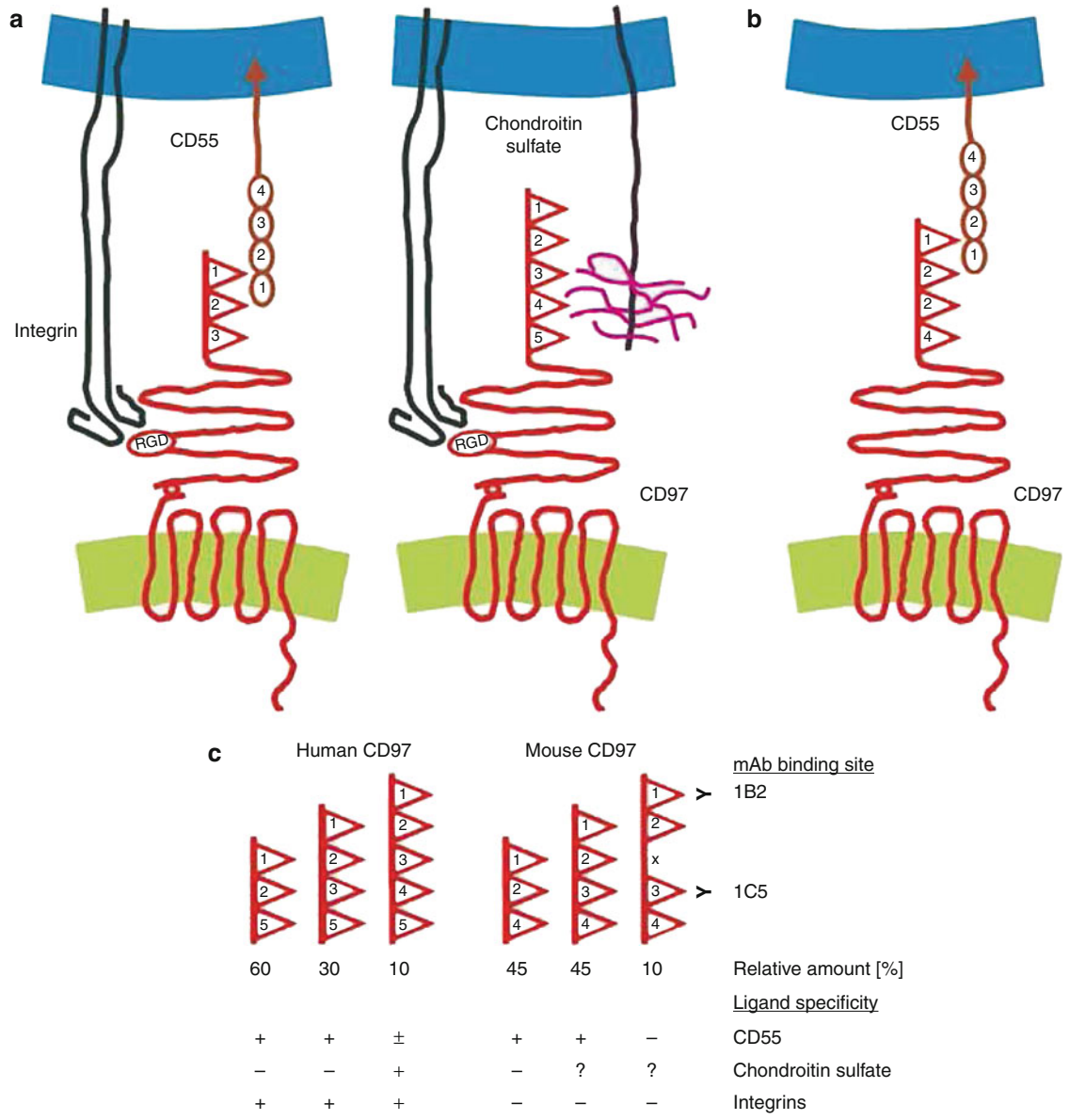
of agonist binding encompassed an area comprising the HBD, stalk, and GPS domain while the N-terminal rhamnose lectin-like (RBL) and olfactomedin-like motifs were *not* required (Krasnoperov et al. 2002). Also, the extracellular loops of the heptahelical domain were apparently not necessary to stabilize the complex with α-latrotoxin (Krasnoperov et al. 1999). It should, however, be kept in mind that this mechanism of interaction may be completely different from the interaction with a putative endogenous CIRL ligand. The recent identification of a latrophilin-associated synaptic surface protein ("Lasso") may be the first step to elucidate the interaction with a physiological ligand (Silva et al. 2009b).

The α-latrotoxin receptor is highly conserved among metazoa; in *Caenorhabditis elegans* the homologous lat-1 is required to coordinately orient cells along a two-dimensional plane lying orthogonal to the axis of apical-basal polarity. Interestingly, different from its mammalian homologue, the extracellular RBL domain of lat-1 was absolutely required for receptor function; constructs lacking the RBL domain but retaining the HBD, GPS, and TM7 domains did not show any biological activity in the *C. elegans* model (Langenhan et al. 2009). This result is consistent with an essential role of the RBL domain in ligand binding; however, results concerning the structure of this domain argued strongly against a carbohydrate ligand for lat-1.

Concerning the binding of endogenous ligands, members of the EGF-TM7 clan are probably the best studied group. Their ligands are large cell-surface proteins and/or components of the extracellular matrix (see below), justifying the name adhesion GPCRs. Still, even for this subfamily it remains unclear whether ligand binding of ectosubunits leads to the activation of endosubunit-mediated cell signaling. CD97, the prototypic EGF-TM7 receptor on leukocytes, was the first receptor for which a cellular ligand, CD55 (also termed decay-accelerating factor, DAF), was demonstrated (Hamann et al. 1996). CD55/DAF is a GPI-anchored molecule expressed by all blood cells and cells in contact with blood and tissue fluid. CD97 binds CD55/DAF via the N-terminal EGF-like domain region (Hamann et al. 1996, 1998; Lin et al. 2001). This was not unexpected as the EGF-like short consensus repeats are the most characteristic structural elements in the extracellular domain, and are common modules used in cell adhesion and chemotaxis. As a result of alternative mRNA splicing, CD97 exists as three major isoforms that contain between three and five EGF-like domains (compare Fig. 4).

All human CD97 isoforms bind CD55/DAF, albeit with different affinities, the smallest isoform binding the ligand with the highest affinity (Hamann et al. 1996, 1998; Lin et al. 2001). The functional consequences of CD97-CD55 binding have not been fully elucidated, and G-protein-mediated signal transduction has not been demonstrated (see below). The largest isoform of human CD97 also interacts with the glycosaminoglycan chondroitin sulfate B (CS; Stacey et al. 2003; Kwakkenbos et al. 2005; compare Fig. 4). Ligand specificity for CS is shared by EMR2, whose EGF domain region is highly similar to that of CD97, but not by other family members. Indeed, only marginal sequence differences in the EGF-like domains result in dramatic differences in their affinity toward the ligand. EMR2, differing from CD97 by only three amino acids within the EGF domains, binds CD55 with a K_d at least an order of magnitude weaker than that of CD97 (Lin et al. 2001). Finally, a third ligand of human CD97 was identified by demonstrating that integrin α5β1 (very late antigen [VLA]-5) and possibly also integrin αvβ3 binds the Arg-Gly-Asp (RGD) motif in the stalk region (Wang et al. 2005; compare Fig. 4). The ability of CD97 isoforms, and also other EGF-TM7 receptors, to interact with such a diverse range of ligand types is based on their variable numbers and sequences of N-terminal EGF-like domains (compare Fig. 4). The first two EGF domains of CD97 (but not EMR2) bind CD55 (decay-accelerating factor), while the fourth EGF domain of both CD97 and EMR2 interacts with CS (Kwakkenbos et al. 2005).

The majority of such receptor–ligand interactions at the cell surface appear to be weak and transient. Lin and coworkers developed a screening strategy which is specifically suited to identify low-affinity ligands for the EGF-like short consensus repeat domains of EGF-TM7 receptors (Lin et al. 2005). In brief, recombinant expression constructs were engineered encoding the variable EGF-like domains coupled to a mouse Fc fragment and a biotinylation signal sequence. These constructs were then expressed, purified, and biotinylated. Finally, the biotinylated proteins were coupled in a specific orientation to avidin-coated fluorescent beads to screen for ligand-bearing cells or tissues (Lin et al. 2005). Based on these multivalent



Adhesion GPCRs, Fig. 4 Cartoon representation of CD97 interacting with its cellular ligands (reproduced with permission from: Hamann J, Veninga H, de Groot DM et al. CD97 in leukocyte trafficking. In: Yona S, Stacey M, eds. Adhesion-GPCRs: Structure to Function. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2010: epub ahead of print http://www.landesbioscience.com/curie/chapter/ 4547/.). (**a**) At the cell surface, CD97 is expressed as a non-covalently associated heterodimer consisting of an extracellular chain and a membrane-spanning chain. The two chains result from autocatalytic processing of a single CD97 propeptide. Alternative splicing generates isoforms with 3, 4, or 5 EGF domains. Shown here are the smallest and the largest isoform. While EGF domains 1 and 2 interact with CD55, EGF domain 4, which only is present in the largest isoform, binds chondroitin sulfate. Integrins bind a RGD motif in the "stalk" region of human CD97. (**b**) Mouse CD97 has a similar structure but the maximum number of EGF domains is only 4. Shown here is the middle isoform. In the largest isoform of mouse CD97, the EGF domains 2 and 3 are separated by 45 amino acids. (**c**) Comparison of binding characteristics of human and mouse CD97 isoforms. Depicted is the composition of the EGF domain region, the relative amount of transcripts present in leukocytes and the ligand specificity. In humans, affinity for CD55 correlates inversely with the number of EGF domains. An interaction of EGF domain 3 of mouse CD97 (the counterpart of EGF domain 4 in humans) with chondroitin sulfate still needs to be proven. The binding site of monoclonal antibodies recognizing specific EGF domains in mouse CD97 is indicated

protein probes, putative cell surface ligands were also identified for other members of the EGF-TM7 clan (Stacey et al. 2001, 2002, 2003).

GPR56, also known as TM7XN1 (7-transmembrane protein with no epidermal growth factor-like NH2-terminal domains 1) functions in tumor cell adhesion and has a role in the development of neural progenitor cells. The amino-terminal domain contains a large number of possible N- and O-linked glycosylation sites similar to mucin-like proteins, but no further adhesion motifs (compare Fig. 1). Tissue transglutaminase 2 (TG2), an integrin-binding adhesion co-receptor for fibronectin, was proposed as a candidate ligand (Xu et al. 2006). Ubiquitously expressed in tissue and tumor stroma, it localizes mainly in the cytoplasm; yet recent reports suggest its presence also at the cell surface, and in the extracellular matrix. TG2 was reported to specifically bind to GPR56 in the mouse (Xu et al. 2006), but not in the human (Nien-Yi Chiang and Hsi-Hsien Lin, personal communication). The significance of this species difference is not known. Although TG2 seemed to interact with the N terminus of mouse GPR56, it is not clear whether it indeed functions as a traditional ligand. As no specific adhesion motifs have been recognized within the N-terminal domain of GPR56, a specific site of interaction remains unknown.

Brain angiogenesis inhibitor 1 (BAI1) is expressed on glial cells within the brain, but also on monocytes and macrophages. Its surface expression is dramatically downregulated in many glioblastomas, consistent with its ability to inhibit angiogenesis and tumor growth in vivo. The large extracellular domain contains one HBD motif and five tandem copies of thrombospondin type 1 repeats (TSRs) (compare Fig. 1). The latter seem to interact with phosphatidylserine (PS), and cells that expressed BAI1 have been shown to selectively engulf a synthetic substrate containing PS. Genetic manipulation of the BAI1 extracellular domain showed that the TSRs were essential for the recognition of PS on apoptotic cells, and direct binding to lipid overlays showed stereospecific binding to PS as well (Park et al. 2007). Also, soluble TSRs derived from BAI1 were shown to inhibit macrophage engulfment of apoptotic thymocytes both ex vivo and in vivo. The adhesive Arg-Gly-Asp (RGD) motif, also present in BAI1, was dispensable under these conditions. Given previous data supporting a role for thrombospondin 1 as a bridging molecule that recognizes apoptotic cells, it may be assumed that PS is a novel ligand for BAI1 that promotes the engulfment activity of cells (see below).

Despite long-standing efforts, no further ligands have as yet been identified for any other members of the adhesion GPCR family. Unavailable 3D structures, heterogeneity of ectodomains, and/or ambiguous relationships within the family make an *in silico* ligand prediction difficult and error-prone. Although phylogenetically related to the *Secretin* GPCRs (Nordström et al. 2009), the peptide hormone ligands of the latter receptor family do not provide a clue. Finally, the idea needs to be considered that orphan adhesion GPCRs may have ligand-independent functions. The concept of ligand-independent receptor activation is connected with the observation that some orphan receptors can heterodimerize with structurally unrelated GPCRs which have identified ligands, thereby regulating functions of the latter, while other receptors were shown to be constitutively active without any ligand.

Reassembly of Subunits and Receptor (Hetero-)Oligomerisation

According to the common and well-established paradigm of GPCR activation, ligand binding leads to conformational changes in the heptahelical domain and cytoplasmic tail, thereby activating intracellular signaling pathways. The adhesion GPCRs may be assumed to follow this paradigm. However, the two-subunit configuration with seemingly independent ecto- and endosubunits ("split receptors"; Fig. 3) and receptor dimerisation/ oligomerisation are specific complications which may explain the difficulties in identifying intracellular signaling pathways. Several models are suitable to explain experimental observations concerning subunit interaction and receptor activation (according to Davies et al. 2007; Silva et al. 2009a; Yi-Shu Huang and Hsi-Hsien Lin, personal communication):

(a) The mature adhesion GPCR molecule exists as a stable heterodimer. After GPS-cleavage in the ER, the two subunits remain non-covalently bound at the cell surface. Upon ligand binding by the ectosubunit, conformational changes are induced in the non-covalently bound endosubunit, followed by signal transduction into the cell.

(b) GPS cleavage, possibly in conjunction with additional processing steps, creates two independent molecular entities at the cell surface. Upon ligand binding, the ectoprotein changes its conformation and is enabled to reassociate with the endoprotein, followed by signal transduction into the cell.
(c) Ectoprotein and endoprotein are independent membrane receptors, each of them binding different ligands. The anchoring mechanism holding the ectoprotein in place is unknown. Upon ligand binding, both membrane proteins are independently capable of signal transduction.
(d) Ectoprotein and endoprotein are independent membrane proteins. Upon ligand binding, signal transduction into the cell is performed by the endoprotein, while the ectoprotein is shed from the cell surface, possibly serving different biological functions.

In the case of CIRL/latrophilin, reassembly of the subunits occurs upon binding of α-latrotoxin to the N-terminal subunit, as suggested in scenario (b); (compare Fig. 3). Subsequently, an intracellular signal is generated via the C-terminus. Thus, the two CIRL/latrophilin fragments do in fact interact; however, the proportion of truly associated, rather than simply colocalized, fragments is difficult to assess (Volynski et al. 2004; Silva et al. 2009a). In *C. elegans*, constructs expressing an N-terminal lat-1 fragment tethered to the cell surface by a single transmembrane helix instead of the GPS-TM7 regions were not able to complement the lat-1 mutant phenotype (Langenhan et al. 2009). Thus, both subunits seem to be necessary for signaling. The adhesive and signaling properties of Celsr/flamingo, that is, functions of the endo- and ectosubunits, appear to be separated in signaling processes during zebrafish gastrulation (Carreira-Barbosa et al. 2009) as suggested in model (c). This contrasts with results obtained for the lat-1 receptor.

Data presented by Volynski et al. (2004) indicate that agonist-induced dimerisation/ oligomerization of the endoprotein accompanies latrophilin signaling (compare Fig. 3). Similarly, EMR2 is constitutively expressed as a dimer, and the dimerization is mediated exclusively by the heptahelical part (Davies et al. 2007). Interestingly, these adhesion GPCRs can also form hybrid receptor complexes by cross-interaction of heterogeneric receptor subunits. Heterodimerisation between different, but closely related members of the EGF-TM7 family resulted in the modulation of expression and ligand binding properties (Davies et al. 2007). Again, the dimerization seemed to be mediated by the TM7 region and did not involve the posttranslational GPS autoproteolysis. Most recently, "promiscuous" interactions between the endosubunits of completely unrelated receptors were observed, and these cross-complexes seemed to be functionally active, although the in vivo amounts were relatively small (∼10%; Silva et al. 2009a).

Coupling to G Proteins

Activated heptahelical receptors interact with and activate heterotrimeric guanine nucleotide–binding proteins (G proteins) at the inner side of the cell membrane. This association is so well established that the term "G protein-coupled receptor" (GPCR) is used as a synonym. Still, only limited data are available which unequivocally demonstrate G protein-coupling for the adhesion GPCRs. CIRL/Latrophilin/lectomedin receptor 1 (LEC1/LPHN2) was the first receptor of this group shown to bind to G proteins (Lelianova et al. 1997), specifically Gαq/11 and Gαo (Rahman et al. 1999). The interaction was found to be strong and functional; it was disrupted by conditions that allow G protein activation and dissociation from the receptor (Rahman et al. 1999). The latrophilin–G protein complex was stable in the presence of GDP but dissociated when incubated with GTP, suggesting a functional interaction. Like other receptors, CIRL/Latrophilin was able to activate more than one G protein subtype. As revealed by colocalization studies, it interacted with Gαq/11 and Gαo, but not with Gαs, Gαi, or Gαz, indicating that coupling is specific and not promiscuous. The {alpha}-subunits of Gq and G11 are almost ubiquitously expressed and couple to {beta}-isoforms of phospholipase C (PLC). Activation leads to the production of inositol-1,4,5-trisphosphate causing subsequent release of Ca2+ from intracellular Ca2+ stores (for review, see Silva et al. 2009b). Despite these reports, G protein-coupled signaling of latrophilin/LEC1 remains controversial. A review by Foord et al. (2002) reported that α-latrotoxin did not activate latrophilin/LEC1 in the classical sense as it is still effective when the transmembrane domains of the

receptor had been removed. This may be explained by the fact that the toxin itself has the ability to form membrane pores, and that these pores are permeable to cations, especially Ca2+, bypassing any signaling that can be triggered by the receptor. Thus, many questions concerning CIRL signaling remain in the absence of its endogenous ligand(s).

G protein-coupling was also reported for GPR56 (Little et al. 2004; Iguchi et al. 2008), but again no final conclusion can be drawn regarding a general principle of signaling. Mass spectrometry screening in retinoic acid–differentiated NT2 teratocarcinoma cells suggested that GPR56 specifically associates with Gαq/11 as part of a larger complex with tetraspanins CD9 (Tspan29) and CD81 (Tspan28) (Little et al. 2004). These authors at the same time reported a lack of Gαq/11 association for CD97. In an overexpression system, Iguchi et al. (2008) observed that GPR56 signaled via a Gα12/13 and Rho pathway. GPR56-mediated intracellular Ca2+ mobilization, on the other hand, was not observed. G proteins G12 and G13 are often activated by receptors which also couple to Gq/G11. A well-established downstream effector of G alpha 12/13-mediated signaling is the monomeric GTPase RhoA, which is a regulator of actin stress fibers and assembly of focal adhesions, gene transcription, and control of cell growth. Indeed, ectopic expression of GPR56 in NIH3T3 cells induced F-actin accumulation in a Gα12/13- and Rho-dependent manner (Iguchi et al. 2008). The transcriptional activation and actin reorganization were found to be inhibited by an RGS domain of the p115 Rho-specific guanine nucleotide exchange factor (p115 RhoGEF RGS) and a dominant negative form of Rho (Iguchi et al. 2008).

Monk et al. (2009) proposed that DREG/Gpr126 drives the differentiation of promyelinating Schwann cells by elevating intracellular cAMP levels. Since many GPCRs induce the production of cAMP by adenylate cyclase activation, the logical supposition would be that Gpr126 may likewise act as an upstream effector of adenylate cyclase. Still, it remains to be demonstrated that Gpr126 actually associates with a G protein complex, or that the regulation of cAMP by Gpr126 is direct. Similarly, a recent study showed that targeted mutation of the very large G protein-coupled receptor 1 (VLGR1), also known as MASS1 or GPR98, resulted in an increase in the expression and in the redistribution of adenylate cyclase 6 in stereocilia of the cochlea (Michalski et al. 2007). The restricted adenylate cyclase immunostaining just nearby the ankle-link molecular complex (ALC) in wild-type mice and its spreading out along the stereocilia in knockout mice argue in favor of a functional coupling between Vlgr1 and adenylate cyclase 6. Vlgr1 is thus expected to activate the Gαs subunit, which in turn activates cAMP-dependent signaling pathways via adenylate cyclase and protein kinase A (PKA) activation. A-kinase anchoring proteins (AKAPs) are believed to localize PKA to the GPCR-associated molecular complex. Again, however, additional studies showing specific interactions are clearly warranted.

For some other adhesion GPCRs, G protein-coupling is described in the patent literature. Summarizing such patent data, Foord et al. (2002) reported that CD97, EMR1, and HE6/GPR64 will activate the G-proteins Gαs/Gq when overexpressed in Xenopus melanophores (C. Jayawickreme, personal communication). These data seem to be at odds with the reported lack of Gαq/11 association for CD97 by Little et al. (2004). However, the signaling properties of GPCRs may depend on the cellular context. According to Foord et al. (2002), CD55/DAF interaction with CD97 (see above) did not appear to induce any G-protein signaling. Thus, whether signaling after EGF-TM7 ligand interaction occurs via classical heterotrimeric G proteins or uses alternative G-protein-independent signaling pathways remains to be seen. Increasing evidence indicates that many heptahelical receptors, including the adhesion GPCRs, signal through G protein-independent pathways, involving JAK/STATs, Src-family tyrosine kinases, GRKs/β-arrestins, and PDZ domain-containing proteins (see below).

Interactions with Other Proteins

Apart from G protein-coupling, GPCRs can associate with a variety of interacting proteins, such as the β-arrestins, PDZ(PSD95/Dlg/ZO-1), SH2, and polyproline-binding proteins. These GPCR interacting proteins (GIPs) may help to create signaling specificity by engaging additional signaling pathways or localizing signaling events to specific subcellular sites.

The predominant target of GIPs is the intracellular C-terminus of GPCRs. PDZ domain proteins, first discovered in the postsynaptic density 95 (PSD95), disk-large (Dlg) and zona occludens-1 (ZO-1) proteins, constitute the largest protein family among the GIPs. They primarily bind a C-terminal S/TXV(L/I) consensus motif, also called a PDZ "ligand." The ability of PDZ domain proteins to bind short and extreme C-terminal sequences offers a way to interact with target proteins without disrupting their overall structure and function. Their predominant function as GIPs is to assemble signaling pathway components into close proximity by recognition of the last four C-terminal amino acids of GPCRs, but they may also regulate the function of their ligands.

A number of adhesion GPCRs express a PDZ recognition motif at their extreme C-terminus, including CD97, BAI1–3, CIRL1/2, VLGR1, as well as GPR123, GPR124, GPR125, and GPR133 (for review, see Bjarnadottir et al. 2007). The consensus motif often is conserved between species, suggesting a functional role. Physical interaction of C-termini with PDZ domain proteins has been described for a several adhesion GPCRs by means of a yeast two-hybrid screen. Using the C-terminus of the receptor molecule as bait, PDZ domain proteins of the Shank family were identified as binding partners of the G protein-coupled α-latrotoxin receptor CL1 (Tobaben et al. 2000; 2002). Shank proteins are multidomain scaffold proteins of the postsynaptic density, connecting neurotransmitter receptors and other membrane proteins with signaling proteins and the actin cytoskeleton (see below). Correspondingly, Kreienkamp et al. (2002) identified the intracellular C-termini of CIRL1 and CIRL2 as interaction partners of the PDZ domain of the proline-rich synapse-associated protein (ProSAP)/somatostatin receptor-interacting protein (SSTRIP) family of postsynaptic proteins (SSTRIP, ProSAP1, and ProSAP2, also known as shank1–shank3, respectively). Shank proteins colocalized with latrotoxin binding GPCR latrophilin 1 (LPHN1; also known as CL1 and CIRL1) at synapses in native brain tissue and may induce clustering of latrophilin 1 in membrane-associated signaling complexes.

The clade of BAI receptors, on the other hand, did not bind to ProSAP/shank (Kreienkamp et al. 2002). Rather, a novel protein was cloned by a similar approach, BAP1 (BAI1-associated protein), which interacts with the cytoplasmic region of BAI1 (Shiratsuchi et al. 1998). The interaction was mediated by the PDZ recognition motif in the carboxy-terminal region of the BAI1 receptor and the PDZ domains of BAP1. BAP1 is a member of the MAGUK (membrane-associated guanylate kinase homologue) family; it possesses a guanylate kinase domain, WW domains, and multiple PDZ domains. The purpose of the interaction is believed to be several fold: (1) the targeting of BAI receptors to their sites of action (e.g., synaptic membranes), (2) anchoring to the actin-based cytoskeleton (see below), and (3) physical association of BAI receptors with elements of the signal transduction machinery (Shiratsuchi et al. 1998).

VLGR1 is involved in the Usher syndrome, an autosomal recessive disorder characterized by combined hearing loss and retinal degeneration. The extreme C-terminus also corresponds to the consensus motif that is recognized as a ligand for the class I subfamily of PDZ domains. Yeast two hybrid and in vitro protein association experiments have shown direct physical interactions between the C-terminus of VLGR1 and the PDZ domain-containing submembrane protein whirlin (Michalski et al. 2007). The large transmembrane protein usherin, the putative transmembrane protein vezatin, and whirlin are colocalized with Vlgr1 at the stereocilia base in developing cochlear hair cells; they are absent in Vlgr1 knockout mice that lack the ankle links (Michalski et al. 2007). The data support the existence of an ankle-link molecular complex (ALC) in the cochlea that includes VLGR1, usherin, vezatin, and whirlin. As all of these proteins bind to myosin VIIa, Michalski et al. (2007) suggested that this actin-based motor protein conveys both transmembrane and submembrane ALC proteins to the stereocilia of the inner ear.

Interactions of adhesion GPCRs and PDZ domain-containing scaffolding molecules with filamentous (F)-actin, as indicated in the examples above, suggest a role in the maintenance and remodeling of the actin cytoskeleton. CIRL-binding ProSAP/shank interacts with fodrin and cortactin–F-actin-binding proteins enriched at cell-matrix contact sites (Kreienkamp et al. 2002). VLGR1 is indirectly connected via its C-terminus to the actin cytoskeleton of stereocilia through the motor domain of myosin VIIa dimers. Similarly, HE6/GPR64 and CD97 colocalize with F-actin in the stereocilia of male excurrent duct

epithelia (Kirchhoff et al. 2008; Veninga et al. 2008). In intestinal epithelial cells, CD97 is located in E-cadherin-based adherens junctions and seems to regulate epithelial strength (Becker et al. 2010). Partial co-staining of CD97 and cortical F-actin indicated that the receptor might be involved in anchoring adherens junction components to the cytoskeletal network. The related EMR2 receptor colocalizes with Rac1 (Yona et al. 2008b), a small Rho-GTPase which regulates actin polymerization. When leukocytes were treated for a short period with small amounts of N-formyl-methionine-leucine-phenylalanine, a peptide chain produced by some bacteria, EMR2 and Rac1 were rapidly translocated to the leading edge and other lamellipodia and colocalized with F-actin (Yona et al. 2008b).

ELMO/Dock180/Rac proteins comprise a conserved signaling module which promotes the internalization of apoptotic cells; ELMO and Dock180 function together as a Guanine nucleotide Exchange Factor (GEF) for Rac, and thereby regulate the phagocyte actin cytoskeleton during engulfment. Using yeast two-hybrid screening to identify upstream ELMO-interacting proteins, Park et al. (2007) identified the BAI1 receptor. Mutational analysis showed that ELMO1 bound to a short alpha-helical stretch within the BAI1 cytoplasmic tail, which was necessary and sufficient for ELMO binding. Furthermore, the formation of a trimeric complex of BAI1–ELMO–Dock180 was associated with enhanced Rac–GTP levels and the greatest increase in apoptotic cell uptake (Park et al. 2007). In addition, the cytoplasmic tail of BAI1 interacts with the Src homology 3 (SH3) domain of a BAI-associated protein 2 (BAIAP2; Oda et al. 1999), also known as the insulin receptor tyrosine kinase substrate of 53 kDa (IRSp53).

GPR56 associates in a complex with Gαq and tetraspanins (Little et al. 2004). Tetraspanin-associated microdomains also connect to the actin cytoskeleton, regulating cell motility and polarity. The C-termini of CD81 and CD9 both possess potential PDZ-domain-binding sites and thus could indirectly link GPR56 to the actin cytoskeleton through the PDZ domains of as yet unknown intracellular proteins. Together with the observation of Rho-dependent actin reorganization during GPR56 signaling (Iguchi et al. 2008), a general role in the dynamic reorganization of the actin cytoskeleton seems likely.

Summary

Although molecular biological and bioinformatics techniques made the identification of all human adhesion GPCRs amenable, the vast majority are still poorly studied orphans with largely unknown structures and functions. Thus, the most important limitation in our current understanding is the persistent paucity of data concerning adhesion GPCR signaling. Being aware of this limitation, the chapter made an effort to list known ligands and to summarize aspects of signal transduction of individual family members in comparison with canonical GPCRs. However, any general characteristics of adhesion GCPR ligands, as well as a generally valid mechanism of receptor docking, ligand-induced activation, signal transduction, and receptor desensitization has remained undiscerned. There are several issues which may account for this shortage. (1) Ligands may be exogenous or may be only expressed in a specific tissue at a particular time under distinct conditions, and a better knowledge of the biology of such receptor/ligand pairs is required before an effort of deorphanization can be undertaken. (2) Orphan adhesion GPCRs may function in the absence of receptor occupation, or some orphans do not activate a signaling cascade alone, but only in conjunction with others. Thus, they may not induce their own second messenger pathway but rather modulate that of others. (3) Some adhesion GPCRs may not stimulate but rather inhibit cell signaling. (4) Although some adhesion GPCRs induce second messenger responses via G proteins, there are indications that others may link to different, perhaps unknown signaling pathways. If this were the case, deorphanization of the remaining will have to wait until these pathways have been defined more clearly. Future challenges in common with other orphan receptors thus are to find endogenous ligands, and to elucidate the general mechanisms underlying the signaling and regulation of receptor desensitization. A unique aspect of the adhesion GCPRs which awaits future elucidation is the significance of their complex posttranslational processing. Many aspects of this processing, including glycosylation, cleavage at the GPS, and the role of "split receptors" subunits remain unclear. The fates of the two subunits and their reassociation at the plasma membrane might provide a variety of different signaling mechanisms which are specific for the adhesion GPCRs.

References

Abe J, Fukuzawa T, Hirose S. Cleavage of Ig-Hepta at a "SEA" module and at a conserved G protein-coupled receptor proteolytic site. J Biol Chem. 2002;277:23391–8.

Becker S, Wandel E, Wobus M, Schneider R, Amasheh S, et al. Overexpression of CD97 in intestinal epithelial cells of transgenic mice attenuates colitis by strengthening adherens junctions. PLoS ONE. 2010;5:e8507. doi:10.1371/journal.pone.0008507.

Bjarnadottir TK, Fredriksson R, Schiöth HB. The adhesion GPCRs: a unique family of G protein-coupled receptors with important roles in both central and peripheral tissues. Cell Mol Life Sci. 2007;64:2104–19.

Carreira-Barbosa F, Kajita M, Morel V, Wada H, Okamoto H, Martinez Arias A, Fujita Y, Wilson SW, Tada M. Flamingo regulates epiboly and convergence/extension movements through cell cohesive and signalling functions during zebrafish gastrulation. Development. 2009;136:383–92.

Chang GW, Stacey M, Kwakkenbos MJ, Hamann J, Gordon S, Lin HH. Proteolytic cleavage of the EMR2 receptor requires both the extracellular stalk and the GPS motif. FEBS Lett. 2003;547:145–50.

Davies B, Baumann C, Kirchhoff C, Ivell R, Nubbemeyer R, Habenicht UF, Theuring F, Gottwald U. Targeted deletion of the epididymal receptor HE6 results in fluid dysregulation and male infertility. Mol Cell Biol. 2004;24:8642–8.

Davies JQ, Chang GW, Yona S, Gordon S, Stacey M, Lin HH. The role of receptor oligomerization in modulating the expression and function of leukocyte adhesion-G protein-coupled receptors. J Biol Chem. 2007;282:27343–53.

Foord SM, Jupe S, Holbrook J. Bioinformatics and type II G-protein-coupled receptors. Biochem Soc Trans. 2002;30:473–9.

Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol. 2003;63:1256–72.

Fukuzawa T, Hirose S. Multiple processing of Ig-Hepta/GPR116, a G protein-coupled receptor with immunoglobulin (Ig)-like repeats, and generation of EGF2-like fragment. J Biochem. 2006;140:445–52.

Gray JX, Haino M, Roth MJ, Maguire JE, Jensen PN, Yarme A, Stetler-Stevenson MA, Siebenlist U, Kelly K. CD97 is a processed, seven-transmembrane, heterodimeric receptor associated with inflammation. J Immunol. 1996;157:5438–47.

Hamann J, Vogel B, Van Schijndel GM, Van Lier RA. The seven-span transmembrane receptor CD97 has a cellular ligand (CD55, DAF). J Exp Med. 1996;184:1185–9.

Hamann J, Stortelers C, Kiss-Toth E, Vogel B, Eichler W, Van Lier RA. Characterization of the CD55 (DAF)-binding site on the seven-span transmembrane receptor CD97. Eur J Immunol. 1998;28:1701–7.

Hsiao CC, Cheng KF, Chen HY, Chou YH, Stacey M, Chang GW, Lin HH. Site-specific N-glycosylation regulates the GPS autoproteolysis of CD97. FEBS Lett. 2009;583:3285–90.

Iguchi T, Sakata K, Yoshizaki K, Tago K, Mizuno N, Itoh H. Orphan G protein-coupled receptor GPR56 regulates neural progenitor cell migration via a G alpha 12/13 and Rho pathway. J Biol Chem. 2008;283:14469–78.

Kaur B, Brat DJ, Devi NS, Van Meir EG. Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor. Oncogene. 2005;24:3632–42.

Kirchhoff C, Osterhoff C, Samalecos A. HE6/GPR64 adhesion receptor co-localizes with apical and subapical F-actin scaffold in male excurrent duct epithelia. Reproduction. 2008;136:235–45.

Koh JT, Kook H, Kee HJ, Seo YW, Jeong BC, Lee JH, Kim MY, Yoon KC, Jung S, Kim KK. Extracellular fragment of brain-specific angiogenesis inhibitor 1 suppresses endothelial cell proliferation by blocking alphavbeta5 integrin. Exp Cell Res. 2004;294:172–84.

Krasnoperov VG, Bittner MA, Beavis R, Kuang Y, Salnikow KV, Chepurny OG, Little AR, Plotnikov AN, Wu D, Holz RW, Petrenko AG. Alpha-latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. Neuron. 1997;18:925–37.

Krasnoperov V, Bittner MA, Holz RW, Chepurny O, Petrenko AG. Structural requirements for alpha-latrotoxin binding and alpha-latrotoxin-stimulated secretion. A study with calcium-independent receptor of alpha-latrotoxin (CIRL) deletion mutants. J Biol Chem. 1999;274:3590–6.

Krasnoperov V, Lu Y, Buryanovsky L, Neubert TA, Ichtchenko K, Petrenko AG. Post-translational proteolytic processing of the calcium-independent receptor of alpha-latrotoxin (CIRL), a natural chimera of the cell adhesion protein and the G protein-coupled receptor. Role of the G protein-coupled receptor proteolysis site (GPS) motif. J Biol Chem. 2002;277:46518–26.

Krasnoperov V, Deyev IE, Serova OV, Xu C, Lu Y, Buryanovsky L, Gabibov AG, Neubert TA, Petrenko AG. Dissociation of the subunits of the calcium-independent receptor of alpha-latrotoxin as a result of two-step proteolysis. Biochemistry. 2009;48:3230–8.

Kreienkamp HJ, Soltau M, Richter D, Böckers T. Interaction of G-protein-coupled receptors with synaptic scaffolding proteins. Biochem Soc Trans. 2002;30:464–8.

Kwakkenbos MJ, Pouwels W, Matmati M, Stacey M, Lin HH, Gordon S, Van Lier RA, Hamann J. Expression of the largest CD97 and EMR2 isoforms on leukocytes facilitates a specific interaction with chondroitin sulfate on B cells. J Leukoc Biol. 2005;77:112–9.

Lagerström MC, Schiöth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov. 2008;7:339–57.

Langenhan T, Prömel S, Mestek L, Esmaeili B, Waller-Evans H, Hennig C, Kohara Y, Avery L, Vakonakis I, Schnabel R, Russ AP. Latrophilin signaling links anterior-posterior tissue polarity and oriented cell divisions in the C. elegans embryo. Dev Cell. 2009;17:494–504.

Lelianova VG, Davletov BA, Sterling A, Rahman MA, Grishin EV, Totty NF, Ushkaryov YA. Alpha-latrotoxin receptor, latrophilin, is a novel member of the secretin family of G protein-coupled receptors. J Biol Chem. 1997;272:21504–8.

Lin HH, Stacey M, Saxby C, Knott V, Chaudhry Y, Evans D, Gordon S, McKnight AJ, Handford P, Lea S. Molecular analysis of the epidermal growth factor-like short consensus repeat domain-mediated protein-protein interactions: dissection of the CD97-CD55 complex. J Biol Chem. 2001;276:24160–9.

Lin HH, Chang GW, Davies JQ, Stacey M, Harris J, Gordon S. Autocatalytic cleavage of the EMR2 receptor occurs at a conserved G protein-coupled receptor proteolytic site motif. J Biol Chem. 2004;279:31823–32.

Lin HH, Stacey M, Chang GW, Davies JQ, Gordon S. Method for selecting and enriching cells expressing low affinity ligands for cell surface receptors. Biotechniques. 2005;38:696–8.

Little KD, Hemler ME, Stipp CS. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-Galpha q/11 association. Mol Biol Cell. 2004;15:2375–87.

McKnight AJ, Gordon S. EGF-TM7: a novel subfamily of seven-transmembrane-region leukocyte cell-surface molecules. Immunol Today. 1996;17:283-7.

McKnight AJ, Gordon S. The EGF-TM7 family: unusual structures at the leukocyte surface. J Leukoc Biol. 1998;63: 271–80.

Michalski N, Michel V, Bahloul A, Lefèvre G, Barral J, Yagi H, Chardenoux S, Weil D, Martin P, Hardelin JP, Sato M, Petit C. Molecular characterization of the ankle-link complex in cochlear hair cells and its role in the hair bundle functioning. J Neurosci. 2007;27:6478–88.

Monk KR, Naylor SG, Glenn TD, Mercurio S, Perlin JR, Dominguez C, Moens CB, Talbot WS. A G protein-coupled receptor is essential for Schwann cells to initiate myelination. Science. 2009;325:1402–5.

Moriguchi T, Haraguchi K, Ueda N, Okada M, Furuya T, Akiyama T. DREG, a developmentally regulated G protein-coupled receptor containing two conserved proteolytic cleavage sites. Genes Cells. 2004;9:549–60.

Nordström KJ, Lagerström MC, Wallér LM, Fredriksson R, Schiöth HB. The secretin GPCRs descended from the family of adhesion GPCRs. Mol Biol Evol. 2009;26:71–84.

Obermann H, Samalecos A, Osterhoff C, Schröder B, Heller R, Kirchhoff C. HE6, a two-subunit heptahelical receptor associated with apical membranes of efferent and epididymal duct epithelia. Mol Reprod Dev. 2003;64:13–26.

Oda K, Shiratsuchi T, Nishimori H, Inazawa J, Yoshikawa H, Taketani Y, Nakamura Y, Tokino T. Identification of BAIAP2 (BAI-associated protein 2), a novel human homologue of hamster IRSp53, whose SH3 domain interacts with the cytoplasmic domain of BAI1. Cytogenet Cell Genet. 1999;84:75–82.

Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, Klibanov AL, Mandell JW, Ravichandran KS. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature. 2007;450:430–4.

Rahman MA, Ashton AC, Meunier FA, Davletov BA, Dolly JO, Ushkaryov YA. Norepinephrine exocytosis stimulated by alpha-latrotoxin requires both external and stored Ca2+ and is mediated by latrophilin, G proteins and phospholipase C. Philos Trans R Soc Lond B Biol Sci. 1999;354:379–86.

Shima Y, Kawaguchi SY, Kosaka K, Nakayama M, Hoshino M, Nabeshima Y, Hirano T, Uemura T. Opposing roles in neurite growth control by two seven-pass transmembrane cadherins. Nat Neurosci. 2007;10:963–9.

Shiratsuchi T, Futamura M, Oda K, Nishimori H, Nakamura Y, Tokino T. Cloning and characterization of BAI-associated protein 1: a PDZ domain-containing protein that interacts with BAI1. Biochem Biophys Res Commun. 1998;247: 597–604.

Silva JP, Lelianova V, Hopkins C, Volynski KE, Ushkaryov Y. Functional cross-interaction of the fragments produced by the cleavage of distinct adhesion G-protein-coupled receptors. J Biol Chem. 2009a;284:6495–506.

Silva JP, Suckling J, Ushkaryov Y. Penelope's web: using alpha-latrotoxin to untangle the mysteries of exocytosis. J Neurochem. 2009b;111:275–90.

Stacey M, Lin HH, Gordon S, McKnight AJ. LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. Trends Biochem Sci. 2000;25:284–9.

Stacey M, Lin HH, Hilyard KL, Gordon S, McKnight AJ. Human epidermal growth factor (EGF) module-containing mucin-like hormone receptor 3 is a new member of the EGF-TM7 family that recognizes a ligand on human macrophages and activated neutrophils. J Biol Chem. 2001;276:18863–70.

Stacey M, Chang GW, Sanos SL, Chittenden LR, Stubbs L, Gordon S, Lin HH. EMR4, a novel epidermal growth factor (EGF)-TM7 molecule up-regulated in activated mouse macrophages, binds to a putative cellular ligand on B lymphoma cell line A20. J Biol Chem. 2002;277:29283–93.

Stacey M, Chang GW, Davies JQ, Kwakkenbos MJ, Sanderson RD, Hamann J, Gordon S, Lin HH. The epidermal growth factor-like domains of the human EMR2 receptor mediate cell attachment through chondroitin sulfate glycosaminoglycans. Blood. 2003;102:2916–24.

Tobaben S, Südhof TC, Stahl B. The G protein-coupled receptor CL1 interacts directly with proteins of the Shank family. J Biol Chem. 2000;275:36204-10.

Tobaben S, Südhof TC, Stahl B. Genetic analysis of alpha-latrotoxin receptors reveals functional interdependence of CIRL/latrophilin 1 and neurexin 1 alpha. J Biol Chem. 2002;277:6359–65.

Veninga H, Becker S, Hoek RM, Wobus M, Wandel E, van der Kaa J, van der Valk M, de Vos AF, Haase H, Owens B, van der Poll T, van Lier RA, Verbeek JS, Aust G, Hamann J. Analysis of CD97 expression and manipulation: antibody treatment but not gene targeting curtails granulocyte migration. J Immunol. 2008;181:6574–83.

Volynski KE, Silva J-P, Lelianova VG, Rahman MA, Hopkins C, Ushkaryov YA. Latrophilin fragments behave as independent proteins that associate and signal on binding of LTXN4C. EMBO J. 2004;23:4423–33.

Wang T, Ward Y, Tian L, Lake R, Guedez L, Stetler-Stevenson WG, Kelly K. CD97, an adhesion receptor on inflammatory cells, stimulates angiogenesis through binding integrin counter receptors on endothelial cells. Blood. 2005;105:2836–44.

Xu L, Begum S, Hearn JD, Hynes RO. GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. Proc Natl Acad Sci USA. 2006;103:9023–8.

Yona S, Lin HH, Siu WO, Gordon S, Stacey M. Adhesion-GPCRs: emerging roles for novel receptors. Trends Biochem Sci. 2008a;33:491–500.

Yona S, Lin HH, Dri P, Davies JQ, Hayhoe RPG, Lewis SM, Heinsbroek SEM, Brown KA, Perretti M, Hamann J, Treacher DF, Gordon S, Stacey M. Ligation of the adhesion-GPCR EMR2 regulates human neutrophil function. FASEB J. 2008b;22:741–51.

ADP Ribosylation Factor GTPase-Activating Protein 1

▶ ArfGAP1

ADP/ATP carrier

▶ ANT

ADP-Ribosyl Cyclase

▶ CD38

ADP-Ribosylation Factor

▶ ARD1/TRIM23

ADP-Ribosylation Factor-Like 10C

▶ Arl8b

ADP-Ribosylation Factor-Like 8B

▶ Arl8b

aFGF

▶ FGF (Fibroblast Growth Factor)

AIB1

▶ Steroid Receptor Coactivator Family

AIF

Nazanine Modjtahedi and Guido Kroemer
INSERM U848, Institut Gustave Roussy, Villejuif cedex, France
University Paris 11, Orsay, France
Institut Gustave Roussy, Villejuif cedex, France

Synonyms

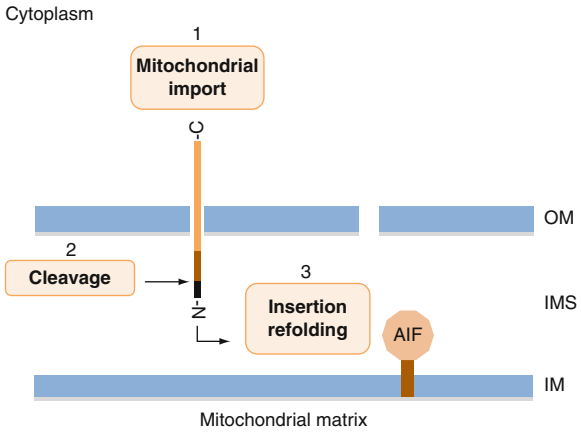
AIFM1 (apoptosis-inducing factor, mitochondrion-associated, 1); PCD8 (programmed cell death 8)

Historical Background

Apoptosis-inducing factor (AIF), which is confined to mitochondria of normal healthy cells, was initially described by Kroemer and coworkers (Susin et al. 1999) as the first caspase-independent death effector. Under conditions of cell death induction, AIF is released from mitochondria and translocated to the nucleus where it contributes to chromatin condensation and DNA fragmentation, two features that are classically associated with apoptosis (Hangen et al. 2010a). Since its initial discovery, the structure of the AIF protein has been resolved and the *AIF* gene has been subjected to genetic manipulations in mice, flies, nematodes, and yeast, revealing the phylogenetically conserved contribution of AIF to cell death, as well as its role in cell survival, proliferation, and differentiation (Hangen et al. 2010a).

AIF Protein Synthesis and Regulation

The *AIFM1* gene resides on human chromosome X (Xq25-Xq26) and is spread out over 16 exons (Susin et al. 1999). The most abundant and ubiquitously expressed AIF transcript (AIF1) is translated in the cytoplasm and imported into the mitochondria of healthy cells with the help of an N-terminal mitochondrial localization signal (MLS) (Fig. 1) (Hangen et al. 2010a). Upon mitochondrial import, the N-terminal part of the MLS is eliminated by a mitochondrial peptidase that cleaves the AIF1 protein after residue M53 (Hangen et al. 2010a).



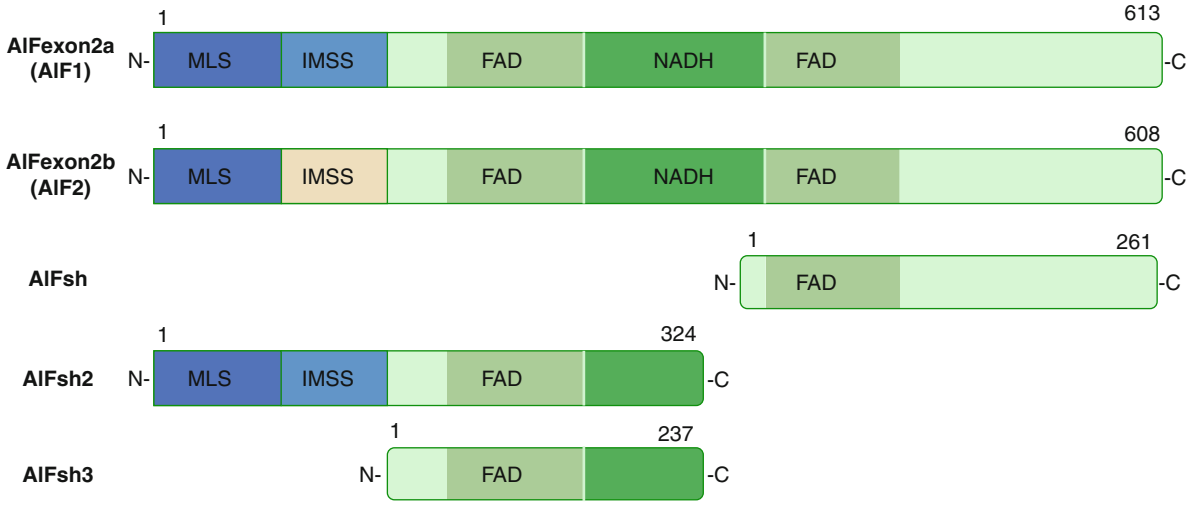
AIF, Fig. 1 Mitochondrial localization of AIF in healthy cells. The scheme delineates: (1) the import of unprocessed full length AIF into mitochondria; (2) the cleavage of its N-terminal MLS by a mitochondrial peptidase; (3) the insertion of AIF into the inner mitochondrial membrane (*IMM*), via its N-terminal transmembrane region. *OM* outer membrane, *IMS* intermembrane space, *IM* inner membrane

The correct targeting of the protein toward the inner mitochondrial membrane is ensured by the C-terminal part of the MLS that functions as an inner membrane sorting signal (IMSS) and harbors a transmembrane (TM) region (residues 66–84) (Hangen et al. 2010a). As the imported and fully processed AIF is inserted into the inner membrane facing the intermembrane space, it adopts its mature folded configuration through the incorporation of its cofactor, flavin adenine dinucleotide (FAD) (Modjtahedi et al. 2006). Analyses of AIF's crystal structures revealed that the flavoprotein AIF bears a similar fold as bacterial nicotinamide adenine dinucleotide (NAD)–dependent oxidoreductases, and contains two FAD-binding segments (residues 128–262 and 401–480) and an NADH-binding domain (residues 263–400) (Fig. 2) (Modjtahedi et al. 2006). Although AIF is able to oxidize NADH and NADPH in vitro, the substrate (s) targeted by its enzymatic activity in vivo remain elusive. Published data indicate that the conformational modifications of AIF must play a tight control over its unknown enzymatic activity via redox-dependent monomer–dimer transitions (Hangen et al. 2010a).

The primary AIF transcript is subjected to tissue-specific alternative splicing (Hangen et al. 2010a) (Fig. 2). The alternative usage of exon 2 (2a or 2b) allows for the production of two splice variants (AIF1 and AIF2). Exon 2a is included in the most abundant AIF isoform (AIF1). The two isoforms exhibit a limited difference, which is confined to the C-terminal part of their MLS (Hangen et al. 2010b). While AIF1 is expressed in almost all tissues, AIF2 mRNA expression is restricted to brain and retina. The modulation of AIF2's expression during brain development and in vitro–induced neuronal differentiation indicates that the expression of this isoform is influenced by differentiation signals (Hangen et al. 2010b). Several additional AIF isoforms have been described (Hangen et al. 2010a). AIFsh is a short variant that is produced from an alternative transcript whose transcriptional start site is located within intron 9 of AIFM1. This variant lacks the N-terminal MLS and the enzymatic domain, but retains the C-terminal domain (which harbors the pro-apoptotic segment). Transfection-enforced expression of AIFsh results in its accumulation in the nucleus and triggers apoptosis (Hangen et al. 2010a). Another short form of AIF (AIFsh2) results from the alternative usage of exon 9b, which contains a stop codon. This isoform maintains the conserved mitochondrial localization and redox function, but lacks the C-terminal pro-apoptotic domain (Hangen et al. 2010a). A third short form of AIF (AIFsh3) lacks the mitochondrial localization signal but otherwise resembles AIFsh2 (Hangen et al. 2010a). Quantitative profiling of mRNA expression and proteomics will be required to assess the precise distribution of each isoform in various tissues.

The Involvement of AIF in Cell Death

Upon mitochondrial outer membrane permeabilization (MOMP) – a feature of most, if not all, apoptotic pathways (Green and Kroemer 2004) – AIF is released from mitochondria and translocates to the nucleus, where it mediates chromatin condensation and DNA degradation (Hangen et al. 2010a). The mitochondrio-nuclear translocation of AIF has been observed during developmental cell death, in cells that die in response to genotoxic agents, in the context of exitotoxicity induced by glutamate or other NMDA receptor agonists, in hypoxia-ischemia followed by reperfusion, in neurodegeneration, and in pathogen exposure. As a result, it has been hypothesized that AIF's lethal activity could regulate a wide range of cell death paradigms (Hangen et al. 2010a). Nonetheless, the genetic deletion or downregulation of murine *Aifm1* revealed

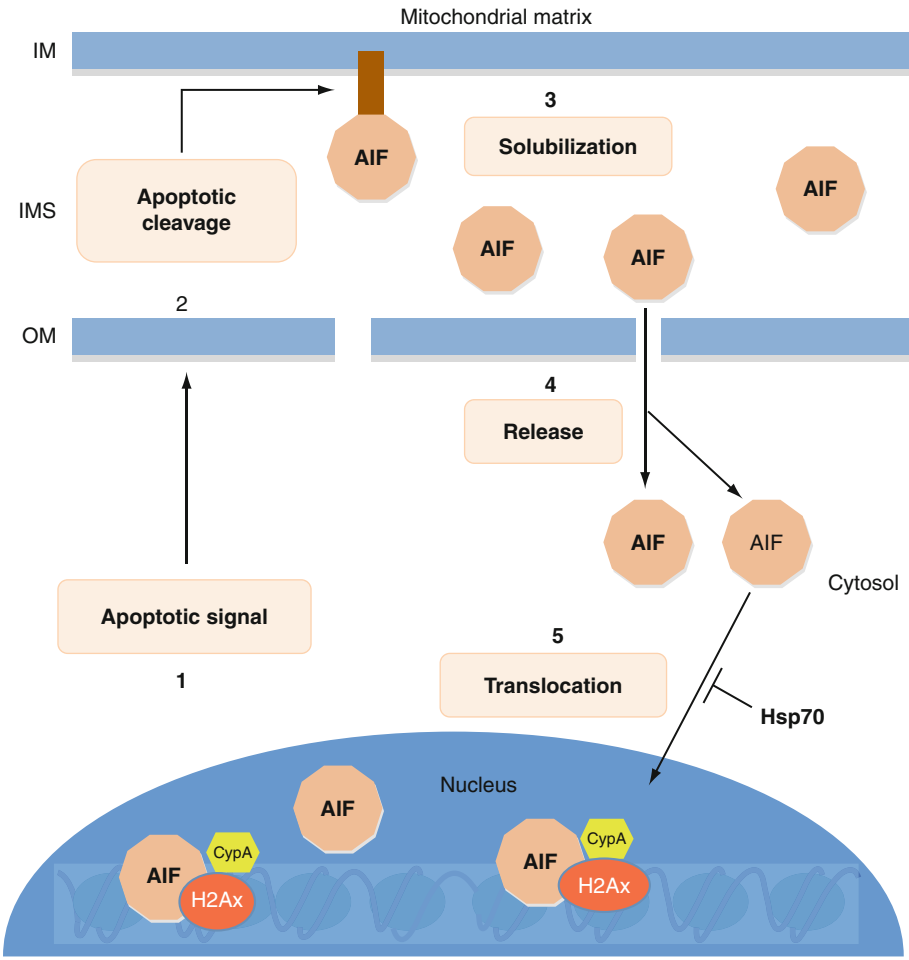


AIF, Fig. 2 Schematic presentation of AIF splice variants. Alternative splice products of human AIF (AIF1, AIF2, AIFsh, AIFsh2, and AIFsh3) are depicted. MLS (*blue*), IMSS (*blue or cream*), FAD-binding domain (FAD; *light green*), NADH-binding domain (NADH; *dark green*), and the C-terminal domain (*green*). Numbers correspond to the first and last amino acids of each variant

that AIF was not a general death effector and that its contribution to cell death depended on the cell type and/or the apoptotic insult (Hangen et al. 2010a). Today, AIF's lethal activity is considered to be required for the programmed death of neurons and photoreceptor cells provoked by excitotoxins, hypoxia-ischemia, hypoglycemia, or withdrawal of trophic support, as in the case of retinal detachment (Hangen et al. 2010a). The neuro-specific death activity of AIF was first suspected from in vitro observations, which implicated AIF in poly (ADP-ribose) polymerase I (PARP-1)–dependent neuronal death (Wang et al. 2009) and then confirmed through the characterization of the mutant Harlequin (Hq) mice, which carries a hypomorphic *Aifm1* mutation that provokes an 80% reduction in the expression level of AIF compared to wild type animals (Klein et al. 2002; Hangen et al. 2010a). For example, in vivo excitotoxic studies using kainic acid–induced seizures revealed that the brains of Hq mice developed less hippocampal damage than wild type animals. In addition, compared to wild type animal, the brain of Hq mouse is more resistant to ischemia-induced damage. Likewise, the prevention of AIF's nucleo-mitochondrial translocation has a neuroprotective effect (Hangen et al. 2010a).

It is generally assumed that the mitochondrial release of AIF requires the proteolytic activity of calcium-dependent cysteine-proteases of the ▶ calpain family that cleave-off the N-terminal transmembrane segment after the leucine 101 (human numbering) and render the protein soluble (Hangen et al. 2010a) (Fig. 3). In neurons that die in response to ischemia or excitotoxicity, it was observed that the proteolytic cleavage of AIF is secondary to the hyperactivation of PARP1, a nuclear DNA repair enzyme involved in the DNA damage response (Schreiber et al. 2006; Hangen et al. 2010a). Other mechanisms of AIF release may exist because the sole accumulation of poly(ADP-ribose) (PAR) polymers, generated by PARP1, may cause the release of AIF from mitochondria of dying neurons without any requirement for AIF proteolysis (Wang et al. 2009). In this latter case, a pool of AIF molecules, which reportedly is associated with the surface of the outer mitochondrial membrane, would be targeted by PAR molecules (Yu et al. 2009).

The lethal activity of AIF is also determined by the nucleic acid binding potential of AIF. Crystal structures of AIF and mutagenesis experiments revealed the existence of positively charged amino acids that are scattered at the surface of the molecule and that are required for the interaction with DNA or RNA and for the induction of nuclear apoptosis by over expressed AIF. Recombinant AIF provokes DNA condensation through direct, sequence-independent interactions with single or double stranded DNA (Hangen et al. 2010a). In addition, AIF interacts with several proteins, in particular cyclophilin A (Artus et al. 2010; Hangen et al. 2010a) and histone H2AX (Artus et al. 2010),

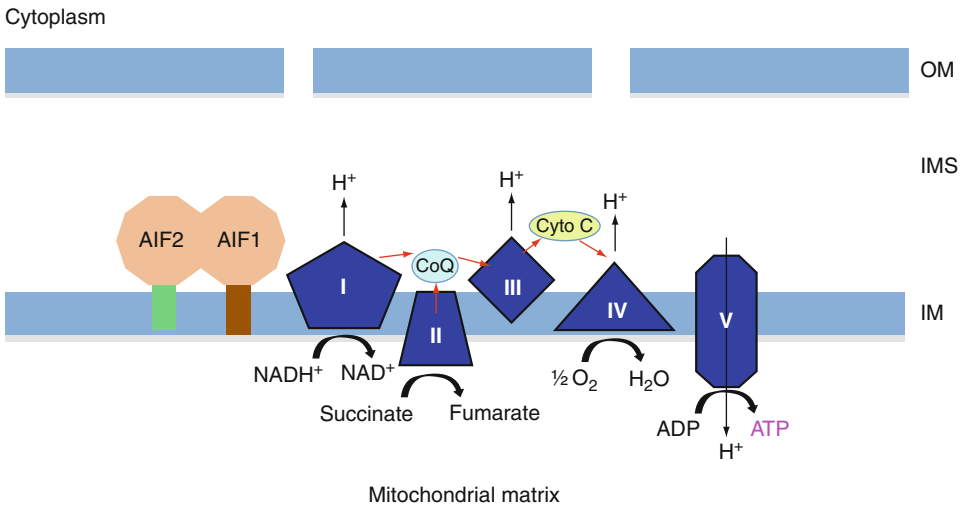


AIF, Fig. 3 Lethal activities of AIF. In cells undergoing programmed death, the activation of AIF lethal function requires: (1) mitochondrial membrane permeabilization in response to lethal signals; (2) the proteolytic activity of a calcium-dependent protease that belongs to calpain family; (3) the solubilization of the membrane-anchored AIF protein; (4) the release of the solubilized protein into the cytosol; and (5) its translocation to the nucleus. The translocation and the chromatin-condensing activity of AIF are positively regulated by its interaction with protein partners, cyclophilin A, and histone H2X

and these interactions are required for the lethal action of nuclear AIF. The RNA binding activity of AIF that was revealed in vitro requires also further investigation (Hangen et al. 2010a).

The Involvement of AIF in Cell Survival, Proliferation, and Differentiation

AIF is a bifunctional flavoprotein. Indeed, in addition to its lethal function within the nucleus of dying cells, AIF plays a vital role in healthy cells, likewise by its impact on mitochondrial bioenergetics (Hangen et al. 2010a). Initially, the revelation of a significant homology between the internal, non-apoptotic, segment of AIF and bacterial NADH-oxidases hinted toward the possibility that AIF could fulfill a non-apoptotic enzymatic function in healthy cells (Hangen et al. 2010a). Later, the phenotypic characterization of Harlequin (Hq) mouse, a model of late-onset neurodegeneration, was instrumental in highlighting the vital non-apoptotic activity of AIF and its impact on cell survival, proliferation, and differentiation (Hangen et al. 2010a). Hq mice develop ataxia and blindness due to an age-associated, progressive loss of terminally differentiated cerebellar and retinal neuron

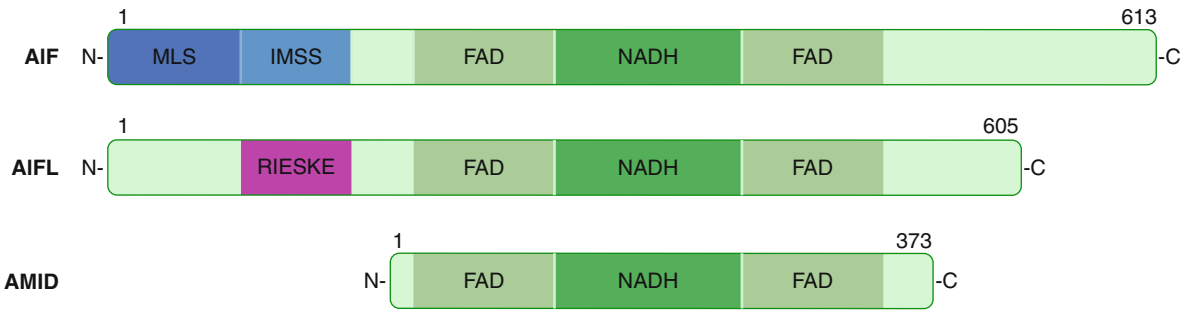


AIF, Fig. 4 Regulation of mitochondrial respiratory chain complexes by AIF. In the mitochondria of healthy cells, the respiratory chain complex I activity depends on the presence of AIF. Schematic presentation of electron transfer activity (*red arrow*) from respiratory chain complexes I to V (*blue*). The proton gradient (H^+) produced by the activity of complexes I, III, and IV is used by complex V for ATP synthesis. Mitochondrial AIF1 (brown transmembrane segment) and AIF2 (green transmembrane segment) both allow for the normal function of complex I subunits. *CoQ* coenzyme Q, *Cyt c* cytochrome *c*

(Klein et al. 2002). Moreover, signs of oxidative stress are detected in the degenerating organs and in dying neurons of Hq animal (Hangen et al. 2010a). All the neurodegenerative traits of Hq mouse are secondary to a retroviral insertion in the first intron of *Aifm1*, which causes an 80% reduction in AIF expression (Klein et al. 2002; Hangen et al. 2010a).

All attempts to create AIF null mice by homologous recombination were unsuccessful because AIF is indispensable for cell survival during embryogenesis (Hangen et al. 2010a). Conditional genetic deletion of *AIFm1* has been possible by targeting specific mouse organs. The specific deletion of AIF in the prospective midbrain and cerebellum revealed that AIF is necessary for cell-type specific neurogenesis in the developing brain (Ishimura et al. 2008). A major defect in cortical development and reduced neuronal survival was observed when *AIFm1* was specifically lost in the telencephalon (Cheung et al. 2006). The conditional deletion of *AIFm1* in muscle and liver has an important impact on whole-body metabolism. Compared to control littermates, muscle- and liver-specific AIF mutant mice are resistant to diet-induced obesity and diabetes (Pospisilik et al. 2007). With aging, mutant mice with muscle-specific loss of AIF develop severe skeletal muscle atrophy and a dilated cardiomyopathy before becoming lethargic around the age of 5 months (Joza et al. 2005).

Biochemical analyses of Hq mice or mice with organ-specific AIF defects revealed that the deletion or depletion of AIF led to a major dysfunction of the mitochondrial respiratory chain (Hangen et al. 2010a) (Fig. 4). Among the five multi-protein complexes that constitute the respiratory chain, complex I is the most reduced by AIF deficiency. Biochemical studies revealed that the observed dysfunction was due to a post-transcriptional loss of complex I protein subunits (Hangen et al. 2010a). Occasionally, a dysfunction of complexes III, IV, or V could also be detected in specific cells or tissues lacking AIF (Hangen et al. 2010a). The global downregulation of AIF in Hq mice also provokes a complex I dysfunction that is limited to those tissues that exhibit degenerative changes (Hangen et al. 2010a). Although the molecular basis for the tissue-specificity of this manifestation is not understood, there is a clear correlation between the downregulation of AIF in degenerating Hq organs, the progressive aggravation of complex I dysfunction, and the phenotypic evolution. Thus, Hq mice constitute a valuable, tissue-specific model of complex I deficiency (Hangen et al. 2010a). In the past, we proposed that AIF was necessary for the maintenance or for the assembly of the mitochondrial respiratory chain complex I subunits (Hangen et al. 2010a). The mechanistic exploration of these effects requires the identification and characterization of the substrates



AIF, Fig. 5 AIF homologs. Schematic presentation of the functional domains of human AIF, AMID, and AIFL. Important domains are the FAD-binding domain (*green*), the NADH-binding domain (*dark green*), the C-terminal domain (*light green*), and the Rieske domain (*fuchsia*)

affected by the enzymatic activity of AIF. In addition to its redox-active domain, AIF could harbor additional functional segments that regulate the structure and/or stability of the inner mitochondrial membrane. This is suggested by the fact that the loss of AIF in the telencephalon entails a degenerative phenotype accompanied by fragmentation of the mitochondrial network and aberrant cristae in cortical neurons (Cheung et al. 2006). The potential inner membrane-stabilizing activity of AIF may reside in its transmembrane segment. This possibility is supported by the recent description of a brain-specific isoform of AIF (AIF2) that is produced through the alternative usage of exon 2 (Fig. 2) and differs from the ubiquitously expressed AIF (AIF1) only within its transmembrane region (Hangen et al. 2010b) (Fig. 4). Both AIF1 and AIF2 localize to the same mitochondrial subcompartment and are similar in their capacity to regulate the stability of complex I subunits, yet differ in their membrane anchorage capacity and in their effects on mitochondrial morphology (Hangen et al. 2010b).

AIF Homologs

AIF is the founding member of the AIF family of proteins, whose members share structural and functional features (Fig. 5). This family has two additional members in humans. AIFL, which is ubiquitously encoded by *AIFM3* located on chromosome 22 (GeneCards; http://www.genecards.org), is a 605 amino acid protein that localizes to mitochondria but lacks a manifest MLS (Modjtahedi et al. 2006). The main homology between AIF and AIFL resides in their shared pyridine nucleotide-disulfide oxidoreductase domain (Modjtahedi et al. 2006) (Fig. 5). AMID (also called PRG3), which is encoded by *AIFM2* located on chromosome 10 (GeneCards) is the third member of the family (Fig. 5). No mitochondrial localization sequence (MLS) was found at the N-terminus of AMID, but mutagenesis experiments suggest the existence of an internal MLS that targets a fraction of AMID to mitochondria while another fraction is found in the cytosol (Modjtahedi et al. 2006). AMID is transcriptionally activated by ▶ p53, and its expression is downregulated in tumors (Modjtahedi et al. 2006). AMID binds DNA in a sequence-independent manner, and its enzymatic activity is affected by this interaction (Gong et al. 2007). Moreover, instead of FAD, AMID uses the cofactor 6-hydroxy FAD for its oxidoreductase activity (Marshall et al. 2005). *Saccharomyces cerevisiae* AIF1P (Ynr074cp), which was initially characterized based on its AIF-like pro-death activities, is phylogenetically equidistant from human AIF, AIFL, and AMID (Modjtahedi et al. 2006).

The Implication of AIF in Disease

When exploring two male infant patients born from monozygotic twin sisters and unrelated fathers, Ghezzi et al. (2010) discovered that a specific mutation in AIFM1 can cause a severe early-onset progressive mitochondrial encephalomyopathy. This pathogenic AIF mutation consists in the deletion of three base pairs coding for arginine residue 201 of the precursor protein. The expression of the pathogenic allele negatively affects OXPHOS. The biochemical examination of fibroblasts from both patients revealed an important

defect in CIII and CIV that was partially corrected by the over expression of recombinant wild type AIF or by continuous culture of mutant fibroblasts in the presence of riboflavin, the precursor of FAD. Muscle biopsies from both patients revealed a severe loss of mitochondrial DNA that could be responsible for the combined multi-complex (CI, CIII, and CIV) dysfunction (Ghezzi et al. 2010). Molecular modeling of the mutant AIF, as well as in vitro experiments realized by Ghezzi et al. (2010), indicate that pathogenic AIF protein is unstable and possesses altered enzymatic properties compared to the unmutated AIF protein. Moreover, the deletion of arginine 201 enhanced the DNA-binding capacity of the mutant AIF and rendered cells more sensitive to death stimuli (Ghezzi et al. 2010).

Summary

Although AIF is not a universal cell death effector, it plays an important role in the programmed death of neurons and photoreceptors triggered by excitotoxins, hypoxia-ischemia, hypoglycemia, or the withdrawal of trophic support. The molecular bases for the tissue-specific and signal-dependent lethal action of AIF are largely elusive and await the exploration of mouse models that allow for the conditional and tissue-specific deletion of each splice variant of AIF. It is conceivable that the mitochondrial dysfunction generated by the release and the nuclear translocation of AIF, in certain circumstances, may influence the cellular response to lethal signals. The creation of mouse models carrying knock-in mutations in each of the functional domains of AIF, including those involved in the regulation of respiratory chain complexes, will be instrumental for understanding the vital and lethal roles of AIF, including that of AIF in human mitochondrial diseases.

References

Artus C, Boujrad H, Bouharrour A, Brunelle MN, Hoos S, Yuste VJ, et al. AIF promotes chromatinolysis and caspase-independent programmed necrosis by interacting with histone H2AX. EMBO J. 2010;29(9):1585–99.

Cheung EC, Joza N, Steenaart NA, McClellan KA, Neuspiel M, McNamara S, et al. Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. EMBO J. 2006;25(17):4061–73.

Ghezzi D, Sevrioukova IF, Invernizzi F, Lamperti C, Mora M, D'Adamo P, et al. Severe X-linked mitochondrial encephalomyopathy associated with a mutation in apoptosis-inducing factor. Am J Hum Genet. 2010;86:1–11.

Gong M, Hay S, Marshall KR, Munro AW, Scrutton NS. DNA binding suppresses human AIF-M2 activity and provides a connection between redox chemistry, reactive oxygen species, and apoptosis. J Biol Chem. 2007;282(41):30331–40.

Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004;305(5684):626–9.

Hangen E, Blomgren K, Benit P, Kroemer G, Modjtahedi N. Life with or without AIF. Trends Biochem Sci. 2010a;35(5):278–87.

Hangen E, De Zio D, Bordi M, Zhu C, Dessen P, Caffin F, et al. A brain-specific isoform of mitochondrial apoptosis-inducing factor: AIF2. Cell Death Differ. 2010b;17(7):1155–66.

Ishimura R, Martin GR, Ackerman SL. Loss of apoptosis-inducing factor results in cell-type-specific neurogenesis defects. J Neurosci. 2008;28(19):4938–48.

Joza N, Oudit GY, Brown D, Benit P, Kassiri Z, Vahsen N, et al. Muscle-specific loss of apoptosis-inducing factor leads to mitochondrial dysfunction, skeletal muscle atrophy, and dilated cardiomyopathy. Mol Cell Biol. 2005;25(23):10261–72.

Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, et al. The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature. 2002;419(6905):367–74.

Marshall KR, Gong M, Wodke L, Lamb JH, Jones DJ, Farmer PB, et al. The human apoptosis-inducing protein AMID is an oxidoreductase with a modified flavin cofactor and DNA binding activity. J Biol Chem. 2005;280(35):30735–40.

Modjtahedi N, Giordanetto F, Madeo F, Kroemer G. Apoptosis-inducing factor: vital and lethal. Trends Cell Biol. 2006;16(5):264–72.

Pospisilik JA, Knauf C, Joza N, Benit P, Orthofer M, Cani PD, et al. Targeted deletion of AIF decreases mitochondrial oxidative phosphorylation and protects from obesity and diabetes. Cell. 2007;131(3):476–91.

Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol. 2006;7(7):517–28.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 1999;397(6718):441–6.

Wang Y, Dawson VL, Dawson TM. Poly(ADP-ribose) signals to mitochondrial AIF: a key event in parthanatos. Exp Neurol. 2009;218(2):193–202.

Yu SW, Wang Y, Frydenlund DS, Ottersen OP, Dawson VL, Dawson TM. Outer mitochondrial membrane localization of apoptosis-inducing factor: mechanistic implications for release. ASN Neuro. 2009;1(5):e00021.

aIF6 in Archae

► TIF6 (eIF6)

AIFM1 (Apoptosis-Inducing Factor, Mitochondrion-Associated, 1)

► AIF

A-Kinase Anchoring Protein (AKAP)

Graeme K. Carnegie[1] and John D. Scott[2]
[1]Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, USA
[2]Department of Pharmacology, Howard Hughes Medical Institute, University of Washington School of Medicine, Seattle, WA, USA

AKAPs

AKAPs are a diverse family of scaffold proteins that form multi-protein complexes, integrating 3'-5'-cyclic adenosine monophosphate (cAMP) -signaling with protein kinases, phosphatases, and other effector proteins.

AKAP Historical Background

Early physiology experiments illustrated that stimulation of 3'-5'-cyclic adenosine monophosphate (cAMP) synthesis by different agonists mobilize cAMP-dependent protein kinase; PKA to elicit distinct physiological outputs, even within the same tissue. For example, adrenergic stimulation selectively activates a pool of PKA associated with the particulate fraction of isolated cardiomyocytes, while prostenoids predominantly activate cytosolic PKA. These observations led to the concept of compartmentation of PKA signaling inside cells (reviewed in Steinberg and Brunton 2001). Initial evidence supporting this concept came from experiments demonstrating that type II PKA co-purifies with microtubules as a consequence of protein–protein interactions between regulatory RII subunits of PKA and microtubule-associated protein MAP2 (the first identified AKAP) (Theurkauf and Vallee 1982).

In the order of 40 RII-binding proteins have since been identified (Welch et al. 2010). The majority of these proteins have been identified through the use of the RII overlay technique and a variety of interaction cloning strategies.

The identification and characterization of AKAP family members has allowed the comparison of their primary sequences as well as their subcellular distribution. Almost all AKAPs bind to the RII dimer of PKA through a well-conserved amphipathic α-helical motif (Carr et al. 1991). The majority of known AKAPs bind specifically to the RII holoenzyme, however, several dual specificity AKAPs, which bind to both PKA subtypes, have also been identified. These include the dual-function anchoring proteins D-AKAP1 and 2 (Huang et al. 1997).

Analysis of AKAP subcellular location shows that each AKAP has a unique distribution within a cell type that is generally conferred by a targeting motif (Colledge and Scott 1999).

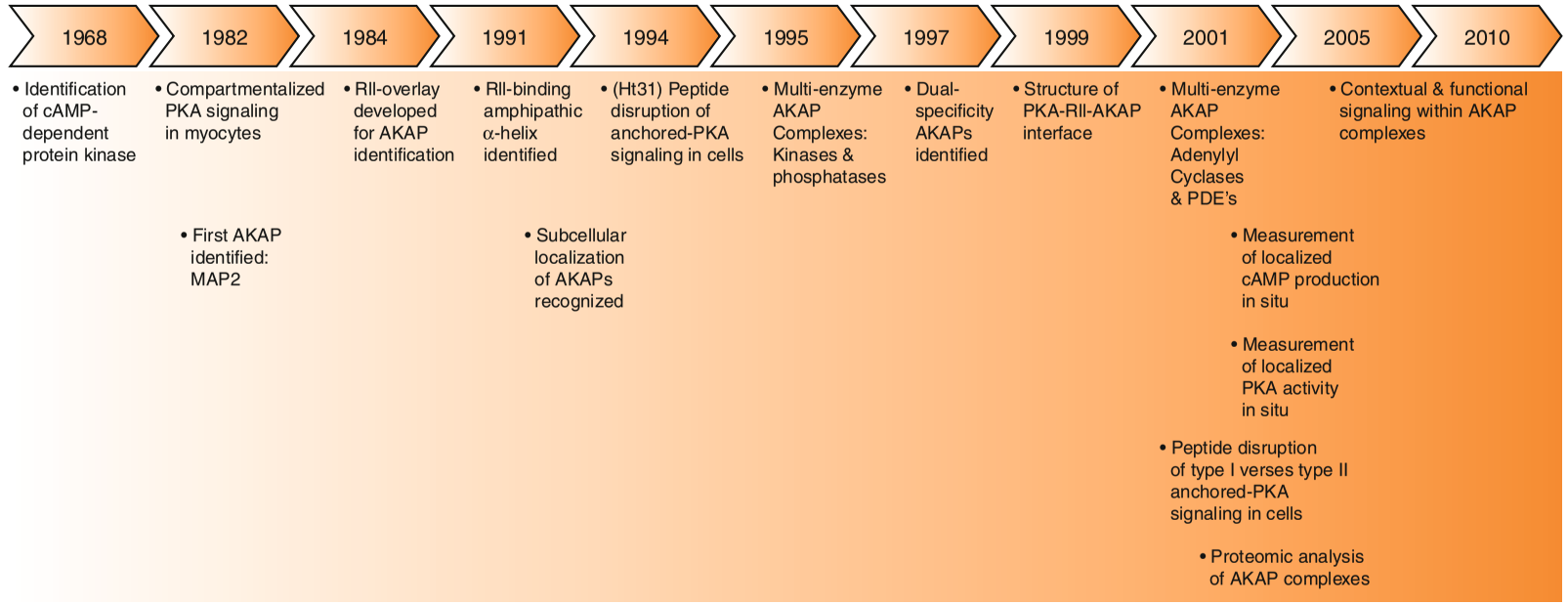
AKAPs are recognized as diverse proteins that assemble multi-protein complexes, to integrate cAMP-responsive events with other signaling processes. For example, AKAP79 binds PKA and also interacts with protein kinase C (PKC) and protein phosphatase 2B (PP2B/calcinuerin) (Klauck et al. 1996). There are now many examples of AKAPs that coordinate enzymes with opposing actions, such as ► adenylyl cyclases and phosphodiesterases.

The historical perspective described here is summarized in Fig. 1. See Smith et al. 2006 for additional details.

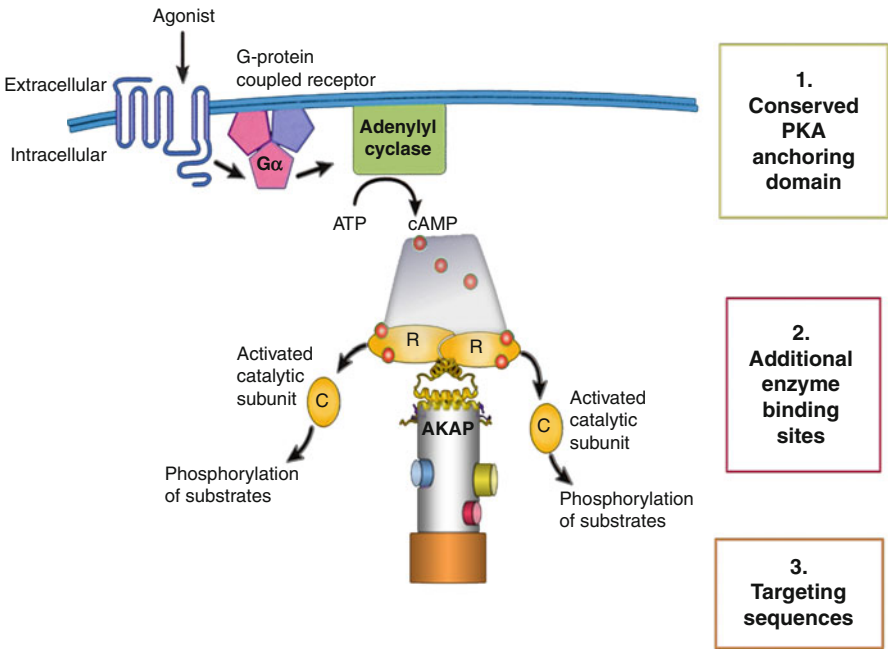
Properties of AKAPs

As depicted in Fig. 2, all members of the AKAP family possess:

1. A conserved protein kinase A (PKA) anchoring domain.
2. Binding sites for additional signaling components. For example, AKAPs act to directly couple PKA to upstream activators of the cAMP cascade (i.e., β-adrenergic receptors and ► adenylyl cyclase), signal terminators (i.e., phosphodiesterases and protein phosphatases), and other elements of signal transduction pathways (i.e., protein kinases, calmodulin, and small molecular weight GTPases).
3. A targeting domain, functioning to compartmentalize signaling complexes to distinct subcellular locations, thereby generating substrate specificity (Colledge and Scott 1999).



A-Kinase Anchoring Protein (AKAP), Fig. 1 *Major discoveries in the study of compartmentalized cAMP signaling and AKAP function*. For further detail, see accompanying text and review by Smith et al. 2006



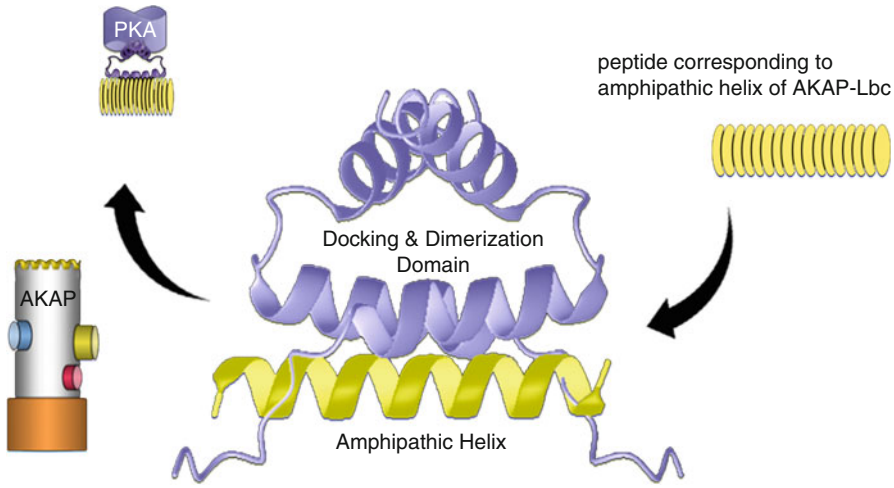
A-Kinase Anchoring Protein (AKAP), Fig. 2 *Properties of AKAPs.* AKAPs regulate the subcellular localization of PKA, thereby generating substrate specificity for PKA. AKAPs have three general properties: (1) AKAPs possess a conserved PKA anchoring domain. (2) AKAPs also bind additional signaling proteins (e.g., other protein kinases, protein phosphatases, phosphodiesterases, adenylyl cyclases, and small G proteins). (3) AKAPs possess targeting sequences directing signaling complexes to discrete subcellular locations

AKAP Nomenclature

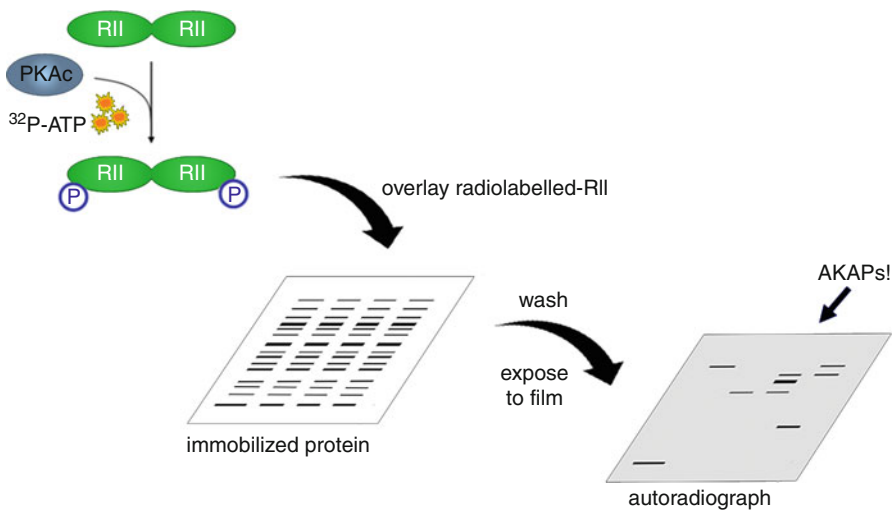
AKAPs have little primary sequence similarity and thus are classified purely on the basis of their ability to bind PKA (Carr et al. 1991). These anchoring proteins were originally named according to their apparent molecular mass determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) or by prediction from the open reading frame: AKAP79, for example, migrates at ~79 kDa by SDS-PAGE. Several AKAPs, such as the muscle-selective mAKAP (originally known as AKAP100) and AKAP-Lbc, were subsequently found to be fragments or smaller transcripts of larger genes and were renamed. More recently identified AKAPs, for example, Gravin, Ezrin, Rab32, WAVE-1, SKIP and cardiac Troponin T retain their original designations. In the context of nucleotide and protein database nomenclature, AKAPs are numbered sequentially (e.g., AKAP79 is termed "AKAP5"). More recent AKAPs with different names, such as Ezrin, Rab32, WAVE-1, and cTnT have not been included in this classification. See Pidoux and Tasken 2010 and Welch et al. 2010 for comprehensive tables of AKAPs (with gene nomenclature committee names).

Techniques for Identification of AKAPs and Disruption of AKAP-Mediated Protein Kinase a Signaling

Most AKAPs contain a recognizable hallmark sequence (approximately 20 amino acid residues, predicted to form an amphipathic helix) that forms a binding site for the R subunits. Structural studies indicate that the hydrophobic face of this region fits into a binding pocket formed by the N-terminal regions of the RII dimer of PKA (Newlon et al. 1997). Cellular delivery of a peptide or related derivatives (cell-soluble stearated forms, or plasmid-based expression) originally based on the RII-binding region in AKAP-Lbc has become a standard means to establish whether anchored pools of PKA participate in various cAMP signaling events by disrupting PKA-RII anchoring inside cells (see Fig. 3). The utility of this peptide as a disruptor of PKA anchoring was first demonstrated in studies showing that perfusion of this peptide into cultured hippocampal neurons disrupts the localized phosphorylation of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptor by anchored PKA. The functional consequence of this disruption was to decrease the responsiveness of the ion channel to synaptic signals (Rosenmund et al. 1994).



A-Kinase Anchoring Protein (AKAP), Fig. 3 *Study of AKAP function by peptide-mediated disruption of protein kinase A anchoring*. The AKAP-amphipathic helix-PKA-RII interaction can be disrupted in vivo by introduction of a competing peptide (originally called Ht31). The disruptor peptide will bind to PKA-RII, thereby displacing PKA from an AKAP inside a cell. Mislocalization of PKA by displacement from an AKAP may lead to uncoupling of site-specific PKA signaling. This has been demonstrated in the processes of channel and receptor regulation, insulin secretion, sperm motility, and oocyte maturation



A-Kinase Anchoring Protein (AKAP), Fig. 4 *Identification of AKAPs by RII overlay*. Purified PKA regulatory subunit RII is radiolabeled with γ-[^{32}P]-ATP and overlayed on to a membrane with immobilized protein. Following incubation with RII and extensive washing of the membrane, AKAPs are identified by autoradiography

The high affinity interaction between AKAPs and the RII subunit dimer of PKA also underlies the RII overlay procedure (shown in Fig. 4), which has been used extensively, with great success, to identify AKAPs.

AKAP Function

AKAPs have been implicated in diverse physiological processes (reviewed in Carnegie et al. 2009), including reproduction and development, learning and memory, cardiac function, and diseases such as cancer and diabetes.

AKAPs, and Reproduction and Development

AKAPs function in the regulation of motility, sperm capacitation, the acrosome reaction, and oocyte maturation. As oocytes undergo meiosis, a change in PKA localization is observed (Rawe et al. 2004), through the expression and localization of different AKAPs. Thus PKA activity is specifically targeted to specific sites and substrates in the oocyte.

Gravin (*AKAP12*) has been implicated in embryogenesis, regulating cell migration through inhibition of a Rho/ROCK/myosin II pathway (Weiser et al. 2007).

AKAPs, and Learning and Memory

One of the first physiological roles identified for AKAPs was the synchronization of synaptic signaling events that underlie learning and memory. AKAP79 (*AKAP5)* (or the mouse ortholog, AKAP150) can regulate synaptic plasticity by coordinating PKA, PKC, and PP2B/calcineurin at the post-synaptic membrane (Klauck et al. 1996). Phosphorylation of channel subunits modulates synaptic efficiency either by regulating the conductance of ion channels or by regulating surface expression of the channel complex.

WAVE-1 (Wiskott–Aldrich syndrome, verprolin-homology domain containing protein) is another AKAP with defined neuronal functions; WAVE-1 null mice display defects in hippocampal learning and memory (Soderling et al. 2003). Expression of the WAVE-1 isoform is restricted to the central nervous system where it functions to organize protein networks involved in the regulation of the actin assembly and synaptic plasticity. WAVE-1 is likely to exist in many different protein complexes, relating to its spatiotemporal function. For example, the RII-binding region of this protein overlaps with the actin-binding domain, whereas the C-terminal region of the protein interfaces with the Arp 2/3 complex, a constellation of actin-related proteins that control changes in cytoskeletal shape.

Cardiac AKAPs

As depicted in Fig. 5, several AKAPs have been identified in the heart. These "cardiac" AKAPs have been implicated in the regulation of cytoskeletal proteins and cardiac ion channels, that coordinate excitation-contraction (EC) coupling. Thus AKAPs mediate cardiac inotropy, chronotropy, and lusitropy. For example, the long splice variant of *AKAP7*; AKAP15/18δ targets PKA to phospholamban, which is a critical regulator of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Lygren et al. 2007), leading to the effects of adrenergic stimulation on calcium reuptake.

AKAP79 (*AKAP5)* also plays a role in the regulation of Ca^{2+}, specifically by targeting PKCα to the L-type Ca^{2+} channel in arterial myocytes. Recent studies demonstrate that AKAP150 null mice were found to lack persistent Ca^{2+} sparklets and have lower arterial wall intracellular calcium and decreased myogenic tone. These null mice were hypotensive and did not develop angiotensin II–induced hypertension (Navedo et al. 2008).

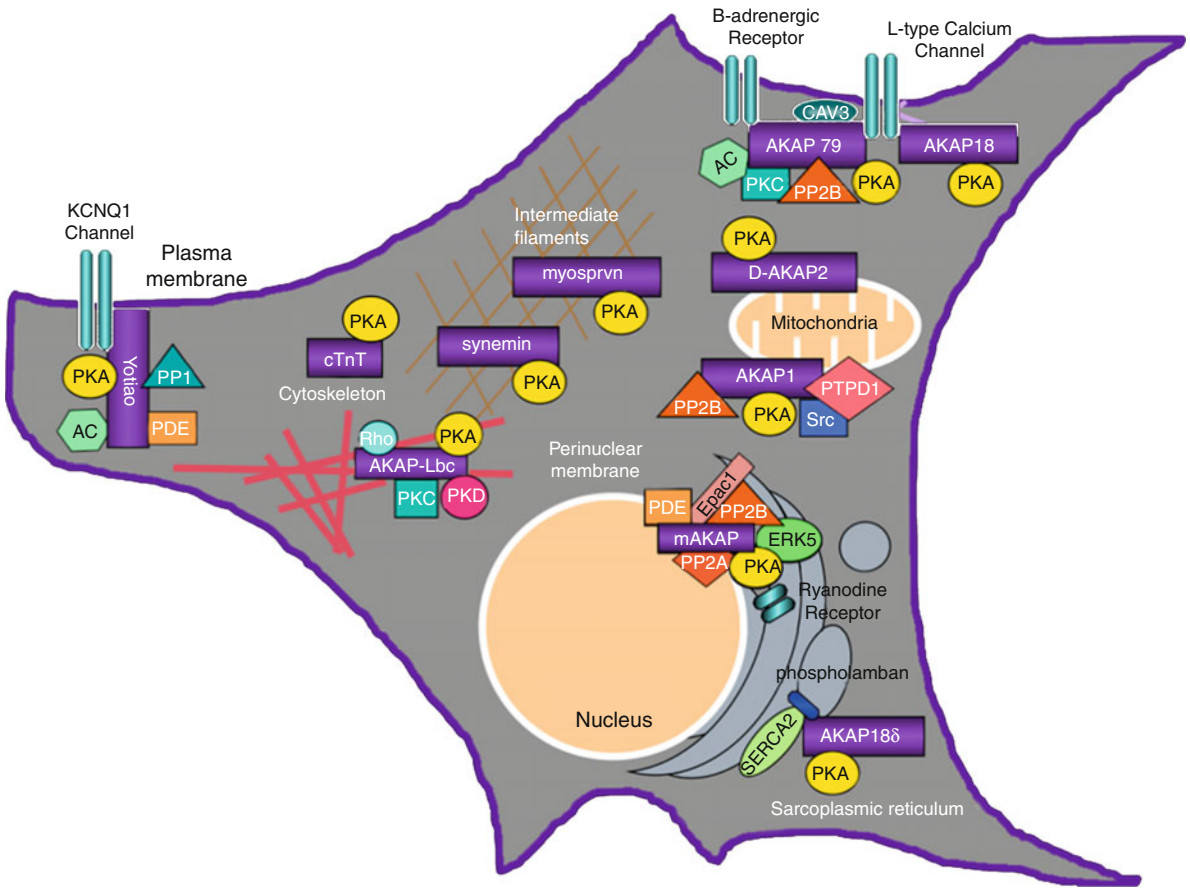
Interestingly, a mutation in Yotiao (*AKAP9*) has been identified in patients with familial long-QT syndrome (LQTS) (Chen et al. 2007). Long QT syndrome (LQTS) is a congenital disorder characterized by a prolongation of the QT interval on ECG and often, ventricular tachyarrhythmias, which may lead to cardiac arrest. Yotiao forms a macromolecular complex (targeting PKA, PP1, and PDE4D3) with the slowly activating cardiac potassium channel I_{Ks}, which is critical for repolarization of the ventricular action potential in the heart. Mutations in either yotiao or the I_{Ks} channel subunits that disrupts their interaction cause a reduction in PKA-mediated phosphorylation of the channel, leading to prolonged ventricular action.

The muscle-specific A kinase–anchoring protein (mAKAP; *AKAP6*) is highly expressed in cardiac tissue and localized to the perinuclear membrane and junctional sarcoplasmic reticulum where it is in close proximity to a variety of substrates such as L-type Ca^{2+} channels and the ryanodine receptor (RyR), thereby functioning in the regulation of contractility. mAKAP scaffolds multiple signaling molecules including PKA, PDE4D3, Epac1, ERK5, PP2A, and PP2B (calcinuerin). mAKAP has also been implicated in cardiac hypertrophy through ERK5 and calcineurin signaling (Bauman et al. 2007).

AKAP-Lbc (*AKAP13*) also plays a role in the induction of hypertrophy, through integration of multiple signal transduction components including Rho and ▶ PKD (Appert-Collin et al. 2007; Carnegie et al. 2008).

AKAPs and the Immune System

cAMP-PKA signaling is well established as a potent negative regulator of T-cell immune function. Prostaglandin E_2 (PGE2) and other ligands promote the production of cAMP, which in turn activates PKA to inhibit TCR-induced T-cell proliferation. Type I PKA is the predominant PKA isoform in T cells and plays a prominent role in immunomodulation. Studies examining the role of RIIα in the immune system in vivo show that T-cell development, homeostasis, and the generation of a cell-mediated immune response are not altered in RIIα null mice. Recently, the dual specificity



A-Kinase Anchoring Protein (AKAP), Fig. 5 *Cardiac AKAPs*. AKAPs are important in the regulation of cardiac function. AKAP18, AKAP79, and yotiao function in channel regulation, while myospryn, synemin, and cTnT act to target PKA to actin filaments, regulating contractility. mAKAP, AKAP121, and AKAP-Lbc play a role in pathological cardiac hypertrophy

AKAP ezrin has been identified, acting to target type I PKA to the TCR-CD3 complex present at membrane microdomains (lipid rafts) in T cells. Targeting of PKA by ezrin facilitates the phosphorylation and activation of the tyrosine kinase Csk. In turn, ▶ Csk negatively regulates Lck tyrosine kinase activity and T-cell receptor activation (Mosenden and Tasken 2011).

AKAPs and Disease

Several SNPs have been identified in patients with different diseases. For example, mutations identified in the gene encoding the AKAP pericentrin (*PCNT*) were demonstrated to cause Seckel syndrome (Rauch et al. 2008). Seckel syndrome is a disorder associated with defective ATR-dependent DNA damage signaling, resulting in a marked reduction of brain and body size. While the mechanism underlying this disorder is not fully understood, the authors demonstrated that collectively, these mutations result in the loss of expression of all mammalian *PCTN* isoforms.

Cancer

Gravin (*AKAP12*) is down-regulated in a number of tumor types including prostate, ovarian, and breast cancer and is associated with metastatic progression of these tumors, providing evidence supporting the role of gravin as a tumor suppressor (Gelman 2002). The observed role of gravin in embryogenesis also supports a tumor supressor function for this AKAP. It is thought that gravin may act to inhibit the migratory movements observed in some tumors that may cause cell invasion and metastasis.

A truncated form of AKAP-Lbc (*AKAP13*) missing both N- and C-terminal regulatory sequences was originally identified as an oncogene from myeloid leukemia patients.

Diabetes

A role for AKAPs in the regulation of hormone (GLP-1)-mediated insulin secretion was first identified in studies using the PKA-AKAP disruptor peptide (Lester et al. 1997). Results demonstrate that insulin secretion can be regulated by the reversible phosphorylation of β-cell proteins through the AKAP79 targeted effects of PKA and PP2B. More recently, AKAP18α or γ has also been implicated in the regulation of glucose-stimulated insulin secretion (Josefsen et al. 2010).

Summary

AKAP-mediated kinase anchoring is acknowledged as a vital means to synchronize spatial and temporal aspects of signal transduction. In addition, AKAPs are now regarded as signaling nodes that integrate a variety of intracellular signals to modulate a plethora of cellular processes. With the extensive molecular, biochemical, and cellular characterization of many AKAPs, in combination with large functional genomic screens and the study of tissue or whole animal models, the future of this field likely lies in precisely defining how specific AKAPs play their part in maintaining normal physiology and what happens to AKAP signaling complexes in disease states.

References

Appert-Collin A, Cotecchia S, Nenniger-Tosato M, Pedrazzini T, Diviani D. The A-kinase anchoring protein (AKAP)-Lbc-signaling complex mediates alpha1 adrenergic receptor-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA. 2007;104(24):10140–5.

Bauman AL, Michel JJ, Henson E, Dodge-Kafka KL, Kapiloff MS. The mAKAP signalosome and cardiac myocyte hypertrophy. IUBMB Life. 2007;59(3):163–9.

Carnegie GK, Soughayer J, Smith FD, Pedroja BS, Zhang F, Diviani D, Bristow MR, Kunkel MT, Newton AC, Langeberg LK, Scott JD. AKAP-Lbc mobilizes a cardiac hypertrophy signaling pathway. Mol Cell. 2008;32(2):169–79.

Carnegie GK, Means CK, Scott JD. A-kinase anchoring proteins: from protein complexes to physiology and disease. IUBMB Life. 2009;61(4):394–406.

Carr DW, Stofko-Hahn RE, Fraser IDC, Bishop SM, Acott TS, Brennan RG, Scott JD. Interaction of the regulatory subunit (RII) of cAMP-dependent protein kinase with RII-anchoring proteins occurs through an amphipathic helix binding motif. J Biol Chem. 1991;266:14188–92.

Chen L, Marquardt ML, Tester DJ, Sampson KJ, Ackerman MJ, Kass RS. Mutation of an A-kinase-anchoring protein causes long-QT syndrome. Proc Natl Acad Sci USA. 2007;104(52):20990–5.

Colledge M, Scott JD. AKAPs: from structure to function. Trends Cell Biol. 1999;9(6):216–21.

Gelman IH. The role of SSeCKS/gravin/AKAP12 scaffolding proteins in the spaciotemporal control of signaling pathways in oncogenesis and development. Front Biosci. 2002;7:d1782–97.

Huang LJ, Durick K, Weiner JA, Chun J, Taylor SS. D-AKAP2, a novel protein kinase A anchoring protein with a putative RGS domain. Proc Natl Acad Sci USA. 1997;94:11184–9.

Josefsen K, Lee YC, Thams P, Efendic S, Nielsen JH. AKAP 18 alpha and gamma have opposing effects on insulin release in INS-1E cells. FEBS Lett. 2010;584(1):81–5.

Klauck TM, Faux MC, Labudda K, Langeberg LK, Jaken S, Scott JD. Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science. 1996;271:1589–92.

Lester LB, Langeberg LK, Scott JD. Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. Proc Natl Acad Sci USA. 1997;94:14942–7.

Lygren B, Carlson CR, Santamaria K, Lissandron V, McSorley T, Litzenberg J, Lorenz D, Wiesner B, Rosenthal W, Zaccolo M, Tasken K, Klussmann E. AKAP complex regulates Ca2+ re-uptake into heart sarcoplasmic reticulum. EMBO Rep. 2007;8(11):1061–7.

Mosenden R, Tasken K. Cyclic AMP-mediated immune regulation – overview of mechanisms of action in T cells. Cell Signal. 2011;23(6):1009–16.

Navedo MF, Nieves-Cintron M, Amberg GC, Yuan C, Votaw VS, Lederer WJ, McKnight GS, Santana LF. AKAP150 is required for stuttering persistent Ca2+ sparklets and angiotensin II-induced hypertension. Circ Res. 2008;102(2):e1–11.

Newlon MG, Roy M, Hausken ZE, Scott JD, Jennings PA. The A-kinase anchoring domain of Type IIa cAMP-dependent protein kinase is highly helical. J Biol Chem. 1997;272:23637–44.

Pidoux G, Tasken K. Specificity and spatial dynamics of protein kinase A signaling organized by A-kinase-anchoring proteins. J Mol Endocrinol. 2010;44(5):271–84.

Rauch A, Thiel CT, Schindler D, Wick U, Crow YJ, Ekici AB, van Essen AJ, Goecke TO, Al-Gazali L, Chrzanowska KH, Zweier C, Brunner HG, Becker K, Curry CJ, Dallapiccola B, Devriendt K, Dorfler A, Kinning E, Megarbane A, Meinecke P, Semple RK, Spranger S, Toutain A, Trembath RC, Voss E, Wilson L, Hennekam R, de Zegher F, Dorr HG, Reis A. Mutations in the pericentrin (PCNT) gene cause primordial dwarfism. Science. 2008;319(5864):816–9.

Rawe VY, Payne C, Navara C, Schatten G. WAVE1 intranuclear trafficking is essential for genomic and cytoskeletal dynamics during fertilization: cell-cycle-dependent shuttling between M-phase and interphase nuclei. Dev Biol. 2004;276(2):253–67.

Rosenmund C, Carr DW, Bergeson SE, Nilaver G, Scott JD, Westbrook GL. Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. Nature. 1994;368:853–6.
Smith FD, Langeberg LK, Scott JD. The where's and when's of kinase anchoring. Trends Biochem Sci. 2006;31(6):316–23.
Soderling SH, Langeberg LK, Soderling JA, Davee SM, Simerly R, Raber J, Scott JD. Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice. Proc Natl Acad Sci USA. 2003;100(4):1723–8.
Steinberg SF, Brunton LL. Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. Annu Rev Pharmacol Toxicol. 2001;41:751–73.
Theurkauf WE, Vallee RB. Molecular characterization of the cAMP-dependent protein kinase bound to microtubule-associated protein 2. JBC. 1982;257:3284–90.
Weiser DC, Pyati UJ, Kimelman D. Gravin regulates mesodermal cell behavior changes required for axis elongation during zebrafish gastrulation. Genes Dev. 2007;21(12):1559–71.
Welch EJ, Jones BW, Scott JD. Networking with AKAPs: context-dependent regulation of anchored enzymes. Mol Interv. 2010;10(2):86–97.

Alpha E Integrin

Bryan A. Anthony and Gregg A. Hadley
Department of Surgery, The Ohio State University Medical Center, Columbus, OH, USA

Synonyms

Alpha polypeptide; CD103; Human mucosal lymphocyte antigen 1

Historical Background

CD103 (integrin αEβ7) was first identified through the binding of a monoclonal antibody (HML-1, human mucosal lymphocyte antigne-1) to a population of lymphocytes that is preferentially associated with gut epithelium (Cerf-Bensussan et al. 1987). It was later identified that HML-1 bound to CD103 which was expressed predominantly on CD3+ CD8+ T cells, and the vast majority of these cells were found in the intestinal mucosa (Russell et al. 1994). Several functionally distinct epitopes were identified. The HML-1 and αE7-1 epitopes were found to function as costimulatory molecules in lymphocyte proliferative responses to a breast cancer epithelial cell line while the αE7-2 and αE7-3 epitopes did not provide such costimulation (Russell et al. 1994).

It is now clear that CD103 is a classic integrin heterodimer composed of the β7 and αE integrin (CD103) subunits. As described above, early studies identified CD103 as an adhesion molecule expressed exclusively by CD8+ T cells in the gut mucosa; however, subsequent studies revealed that CD103 is also expressed by peripheral CD8+ T cells and diverse leukocyte subsets. Recent studies indicate that CD103 is promiscuously expressed by different leukocyte subsets with known immune functional capabilities including not only CD8+ T cells but also interstitial dendritic cells and regulatory T cells. This review is focused on the mechanisms by which CD103 expression is regulated by these leukocye subsets, and the functional impact of CD103 expression on CD8+ T cells, dendritic cells, and regulatory T cells (Tregs). The therapeutic potential of CD103 blockade is also discussed.

Regulation of CD103 Expression

The precise mechanisms regulating CD103 expression by the different leukocyte subsets remain poorly defined. A leading hypothesis is that leukocytes expressing the CD49d/β7 integrin (i.e., gut homing CD8+ T cells) downregulate CD49d and upregulate CD103 in the presence of bioactive transfoming growth factor beta (TGF-β) to generate CD103 expressing cells. There is also evidence that TGF-β directly induces transcription of the αE gene (Itgae) (Robinson et al. 2001). Regardless of the mechanisms involved, it has been clear that bioactive TGF-β plays a dominant role in regulating CD103 expression since the initial reports on the subject by Kilshaw and Murant nearly 20 years ago (Kilshaw and Murant 1990). A key role for TGF-β in regulating CD103 expression by non-CD8 cells is supported by the observation that conversion of naive T cells into CD4+ CD25+ T regs is dependent on TGF-β activity (Coombes et al. 2007). Similarly, TGF-β induces CD103 expression on CD8 T effectors elicited to allogeneic spleen cells cocultured with TGF-β, and CD103 expression by CD8+ T effectors elicited to allogeneic epithelial cells is blocked by TGF-β neutralizing antibody (Hadley et al. 1997). That TGF-β plays a similar role in vivo is supported by the studies of El-Asady et al. who showed that alloreactive CD8+ T cells

deficient in the ability to respond to TGF-β were unable to upregulate CD103 in a murine model of GVHD (El-Asady et al. 2005). Studies from the tumor immunology field reveal that TGF-β has a profound impact on the capacity of CD8+ T cells to upregulate CD103 expression following reexposure to cognate antigen (Le Floc'h et al. 2007). Thus, while the precise mechansims likely differ among cell types, the existing data on the subject indicate that TGF-β plays a key role in the process. In this regard, it is important to note that bioactive TGF-β is ubiquitous at sites of inflammation due to its role in wound healing processes, suggesting that the overall impact of this poorly characterized cytokine may promote rather than down modulate immune responses as is often tacitly assumed.

CD103+ CD8+ T Cells

The majority of studies characterizing CD103 expression by CD8+ T cells initially focused on its role as a homing molecule for lymphocytes in the mucosal immune system. Indeed, the elegant studies of Cepek et al. established that CD103 recognizes the epithelial-specific ligand, E-cadherin (Cepek et al. 1994), which is highly expressed on gut epithelial layers. However, there is compelling evidence that CD103/E-cadherin interactions also play a key role in promoting the effector function of peripheral CD8 T effector populations. In vitro studies indicate that CD103 is upregulated on alloreactive CD8+ T cells cocultured with allogeneic renal epithelial cells, and that such expression promotes lytic activity to epithelial cell targets (Hadley et al. 1997). Wang et al. showed that CD8+ T cells do not express CD103 at early timepoints following renal transplant; however, CD8+ T cells in the graft acquire CD103 expression over time (Wang et al. 2004). Interestingly, in a vascularized renal transplant model, kidneys rejected before CD103 expression was acquired. If acute rejection was delayed using cyclosporin, CD8+ T cells gained expression of CD103 and promoted long-term renal injury (Yuan et al. 2005). Studies from the tumor immunology field indicate that CD103 also plays an important role in effective lysis of tumor cells expressing E-cadherin. Le Floc'h et al. found that CD103 was required for tumor lysis of E-cadherin expressing tumor cells, and that the CD103/E-cadherin interaction is required for cytotoxic granule localization to the immunological synapse and exocytosis (Le Floc'h et al. 2007). Importantly, however, it is important to recognize that CD8+ T effectors generally express high levels of CD49d/CD18 (leukocyte function-associated antigen-1, LFA-1), and that interaction of this integrin with its ligand CD54 (intercellular adhesion molecule-1, ICAM-1) plays a dominant role in regulating lytic activity with interaction of CD103 with E-cadherin apparently serving a backup role in providing the requisite signaling pathways in the event that CD54 is down modulated or otherwise not present on the target cell. In humans, CD103 expression by peripheral CD8+ T cells is confined to small subset (<1%) of circulating memory-phenotype cells. In mice, CD103 is expressed at low levels by 40–60% of peripheral CD8+ T cells, the exact frequency of which is strain-dependent.

CD103+ Dendritic Cells

Dendritic cells (DCs) are a highly heterogeneous cell type requiring numerous molecules to classify each of a variety of subsets. CD103+ DCs have been described as involved in generating gut homing T cells and Tregs (Coombes et al. 2007; Jaensson et al. 2008). CD103+ DCs are found in the spleen, skin, lung, and gut-associated lymphoid tissue (GALT). Because the lung and the GALT are in contact with many nonpathogenic antigens, it is likely that the microenvironments of the lung and GALT promote an immunomodulatory phenotype to prevent excess nonspecific inflammatory responses. CD103+ DCs are ideally suited for this purpose. There is evidence that GALT-derived CD103+ DCs produce lower levels of anti-inflammatory cytokines when cocultured with TLR agonists, have increased costimulatory molecule expression, and are less efficient phagocytes than their CD103− counterparts (Coombes et al. 2007; del Rio et al. 2010). CD103+ DCs in the lung display similar functional characteristics (del Rio et al. 2010). Moreover, there is evidence that CD103+ DCs induce Treg formation in the presence of TGF-β and retenoic acid (Coombes et al. 2007). Naive T cells that are activated in the gut by CD103+ DCs acquire the expression of the transcription factor forkhead box protein 3 (FoxP3). In addition to a critical role in inducing Tregs, CD103+ DCs play an important role

in generating gut homing CD8+ T cells. CD8+ T cells primed by CD103+ DCs have increased CCR9 and CD49d/β7 expression as compared to CD8+ T cells primed by CD103- DC (Johansson-Lindbom et al. 2005). Lamina propria–derived DCs express higher levels of CD103 than do mesenteric lymph node–derived DCs. Consequently, lamina propria DCs are more potent than mesenteric lymph node DCs in generating gut tropic CD8+ T cells. Although CD103− and CD103+ mesenteric lymph node DCs both activate CD8+ T cells capable of producing interferon gamma, only CD103+ DCs generate gut tropic CD8+ T cells. This phenomenon does not appear to be a product of the gut microenvironment, but rather DC imprinting that occurs prior to DC localization to the gut (Johansson-Lindbom et al. 2005). It is important to note that depletion of CD103-expressing DCs does not compromise host immune responses (Zhang et al. 2009), calling into question the importance of CD103+ DCs in promoting overall immune responses.

CD103+ Tregs

Tregs define an immunomodulatory T cell subset characterized by the capacity to suppress immune responses. There are two populations of CD4+ CD25+ Tregs: natural and adaptive. Natural Tregs are generated in the thymus, while adaptive Tregs are derived in the periphery (Sakaguchi 2005). CD103 is a marker found on a subset of adaptive Tregs (Huehn et al. 2004). It has been shown that CD103+ Tregs have immunosuppressive properties that are equal to, or greater than those of CD103− Tregs. Typically, Tregs are CD4 + CD25+ and express FoxP3; however, there is a population of CD25-CD103+ Tregs that express CTLA-4, suppress T cell proliferation in vitro, and prevent severe colitis in the SCID mouse (Lehmann et al. 2002). There is also a distinct cytokine profile expressed in CD103+ CD25+ Tregs. CD103-CD25+ Tregs secrete levels of IL-4, IL-5, and IL-13 that are similar to Th2 CD4+ T cells, but there is almost no IL-4, IL-5, or IL-13 produced by CD103 + CD25+ Tregs (Lehmann et al. 2002). CD103+ Tregs are also more potent suppressors of T cell proliferation. CD103+ Tregs almost completely prevented naive CD4+ T cell proliferation when nonspecifically stimulated. CD103− Tregs also suppressed proliferation, but to a lesser extent (Lehmann et al. 2002). CD4+ CD25+ CD103+ Tregs also exhibit immunosuppressive properties in vivo. Ongoing chronic graft versus host disease (GVHD) can be suppressed through an in vivo transfer of CD4+ CD25+ CD103+ Tregs. Infusion of CD4+ CD25+ CD103+ Tregs has been shown to reduce the number of alloantibody producing plasma cells in mice with chronic GVHD as well as reduce the number of pathogenic effector T cells in GVHD target organs (Zhao et al. 2008). Moreover, infusion of CD103+ Tregs resulted in twofold lower levels of alloantibody as compared to in vitro activated natural Tregs. Additionally, CD103 defines a population of CD8+ Tregs. CD8+ Tregs can acquire their antigen specificity peripherally and promote systemic tolerance. Injection into the anterior chamber of the eye will generate CD8+ Tregs specific for the injected antigen. CD103 has been shown to be essential for the development and function of the CD8+ Tregs (Keino et al. 2006). Koch et al. characterize CD103+ CD8+ Tregs as phenotypically different from other CD8+ suppressor T cell populations. CD103+ CD8+ Tregs express CD28, but lack FoxP3, CD25, LAG-3, CTLA-4, and GITR (Koch et al. 2008).

Therapeutic Potential of CD103 Blockade

The utility of therapeutic strategies for targeting the CD103 pathway are clouded by promiscuity in CD103 expression and uncertainty regarding its precise function on different cell types. CD103 is regulated by TGF-β, but the activation state of the CD8+ T cells at the time it encounters TGF-β determines the function of the CD8+ T cell (Hadley et al. 1997); consequently TGF-β is not a viable therapeutic target. Targeted disruption of CD103 on CD8 effector T cells prevents intestinal GVHD and prolongs renal allograft survival (El-Asady et al. 2005; Yuan et al. 2005), suggesting that CD103 itself may be the optimal therapeutic target. However, CD103+ Tregs have been shown to ameliorate chronic GVHD (Zhao et al. 2008), and CD103+ DC have been shown to promote Treg development (Coombes et al. 2007), arguing against the therapeutic poential of CD103 blockade. Depletion of CD103-expressing cells in vivo dramatically attenuates CD8+ T cell responses, arguing that CD103+ DC or Tregs do not serve critical immune functions in all immune responses (Zhang et al. 2009). Consequently, the overall role of CD103 in promoting disease

remains controversial, and this ambiguity has hindered the development of therapeutic agents to target the pathway.

Summary

CD103 is a classic integrin heterodimer composed of the β7 and αE integrin (CD103) subunits that recognizes the epithelial cell-specific ligand, E-cadherin. CD103 is expressed by diverse leukocyte populations, and there is increasing evidence that it plays a key role in both promoting and regulating immune responses. Whether or not CD103 provides a viable therapeutic target remains to be determined.

References

Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature. 1994;372:190–3.

Cerf-Bensussan N, Jarry A, Brousse N, Lisowska-Grospierre B, Guy-Grand D, Griscelli C. A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. Eur J Immunol. 1987;17:1279–85.

Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007;204:1757–64.

del Rio ML, Bernhardt G, Rodriguez-Barbosa JI, Forster R. Development and functional specialization of CD103+ dendritic cells. Immunol Rev. 2010;234:268–81.

El-Asady R, Yuan R, Liu K, Wang D, Gress RE, Lucas PJ, Drachenberg CB, Hadley GA. TGF-{beta}-dependent CD103 expression by CD8(+) T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. J Exp Med. 2005;201:1647–57.

Hadley GA, Bartlett ST, Via CS, Rostapshova EA, Moainie S. The epithelial cell-specific integrin, CD103 (alpha E integrin), defines a novel subset of alloreactive CD8+ CTL. J Immunol. 1997;159:3748–56.

Huehn J, Siegmund K, Lehmann JC, Siewert C, Haubold U, Feuerer M, Debes GF, Lauber J, Frey O, Przybylski GK, Niesner U, de la Rosa M, Schmidt CA, Brauer R, Buer J, Scheffold A, Hamann A. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. J Exp Med. 2004;199:303–13.

Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med. 2008;205:2139–49.

Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Forster R, Agace WW. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. J Exp Med. 2005;202:1063–73.

Keino H, Masli S, Sasaki S, Streilein JW, Stein-Streilein J. CD8+ T regulatory cells use a novel genetic program that includes CD103 to suppress Th1 immunity in eye-derived tolerance. Invest Ophthalmol Vis Sci. 2006;47:1533–42.

Kilshaw PJ, Murant SJ. A new surface antigen on intraepithelial lymphocytes in the intestine. Eur J Immunol. 1990;20:2201–7.

Koch SD, Uss E, van Lier RA, ten Berge IJ. Alloantigen-induced regulatory CD8 + CD103+ T cells. Hum Immunol. 2008;69:737–44.

Le Floc'h A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, Chouaib S, Mami-Chouaib F. Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. J Exp Med. 2007;204:559–70.

Lehmann J, Huehn J, de la Rosa M, Maszyna F, Kretschmer U, Krenn V, Brunner M, Scheffold A, Hamann A. Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells. Proc Natl Acad Sci USA. 2002;99:13031–6.

Robinson PW, Green SJ, Carter C, Coadwell J, Kilshaw PJ. Studies on transcriptional regulation of the mucosal T-cell integrin alphaEbeta7 (CD103). Immunology. 2001;103:146–54.

Russell GJ, Parker CM, Cepek KL, Mandelbrot DA, Sood A, Mizoguchi E, Ebert EC, Brenner MB, Bhan AK. Distinct structural and functional epitopes of the alpha E beta 7 integrin. Eur J Immunol. 1994;24:2832–41.

Sakaguchi S. Naturally arising Foxp3-expressing CD25 + CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol. 2005;6:345–52.

Wang D, Yuan R, Feng Y, El-Asady R, Farber DL, Gress RE, Lucas PJ, Hadley GA. Regulation of CD103 expression by CD8+ T cells responding to renal allografts. J Immunol. 2004;172:214–21.

Yuan R, El-Asady R, Liu K, Wang D, Drachenberg CB, Hadley GA. Critical role for CD103 + CD8+ effectors in promoting tubular injury following allogeneic renal transplantation. J Immunol. 2005;175:2868–79.

Zhang L, Moffatt-Bruce SD, Gaughan AA, Wang JJ, Rajab A, Hadley GA. An anti-CD103 immunotoxin promotes long-term survival of pancreatic islet allografts. Am J Transplant. 2009;9:2012–23.

Zhao D, Zhang C, Yi T, Lin CL, Todorov I, Kandeel F, Forman S, Zeng D. In vivo-activated CD103 + CD4+ regulatory T cells ameliorate ongoing chronic graft-versus-host disease. Blood. 2008;112:2129–38.

Alpha Polypeptide

► Alpha E Integrin

Alpha v Integrin

▶ Integrin Alpha V (ITGAV)

Alpha-2-Macroglobulin Receptor (A2MR)

▶ CD91

ALX

▶ FPR2/ALX

ALXR

▶ FPR2/ALX

Androgen Receptor (AR)

Hatice Zeynep Kirli, Martina Tesikova and Fahri Saatcioglu
Department of Molecular Biosciences, University of Oslo, Oslo, Norway

Synonyms

Testosterone/Dihydrotestosterone receptor; *NR3C4* (nuclear receptor subfamily 3, group C, member 4)

Historical Background

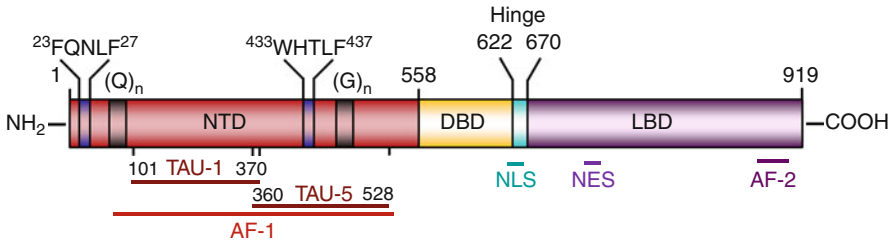
Androgen receptor (AR) mediates the effects of androgens that are responsible for diverse biological functions, such as development and maintenance of the male reproductive system as well as involvement in disease states, such as prostate cancer (for a review, see Brinkmann 2011). AR is a ligand-activated transcription factor (TF) that belongs to the steroid hormone receptor (SHR) family within the nuclear receptor (NR) superfamily of TFs (for a review, see Mangelsdorf et al. 1995). In addition to AR, the SHR family contains the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and the estrogen receptor (ER). SHRs are structurally and functionally related and mediate the action of steroid hormones that affect nearly all aspects of development and homeostasis; they are also implicated in a number of pathological conditions.

The first reports on the protein nature of AR and its isolation from androgen target tissues were published at the end of the 1960s (Fang et al. 1969; Mainwaring 1969; Baulieu and Jung 1970). During the 1970s and 1980s, much effort went into the purification and further characterization of AR from different sources. The human AR cDNA was finally cloned in 1988 and 1989 by several groups just a few years after cloning of the cDNAs of the human SHRs (Chang et al. 1988; Lubahn et al. 1988; Trapman et al. 1988; Tilley et al. 1989). AR is expressed at low to moderate levels in a variety of cell types with high levels present especially in male and female reproductive tissues, adrenal gland, kidney, and skeletal muscle. In addition to androgens, AR can be activated in an androgen-independent manner through other signaling pathways which can significantly contribute to the diversity of AR action.

AR Domains and Function

Similar to other SHRs, AR has four distinct functional domains: N-terminal domain (NTD), DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD). As the names indicate, these domains are responsible for DNA and ligand binding, as well as additional functions, such as dimerization, translocation into and from the nucleus, and interaction with diverse coregulators and TFs (for a review, see Lamb et al. 2001) (Fig. 1). Moreover, there are intramolecular interactions between the NTD and LBD, which are important for AR activity and binding to chromatin (e.g., Ikonen et al. 1997). Several studies found the synthesis of alternatively spliced transcripts in vivo that encode truncated AR isoforms that lack the LBD (for a review, see Dehm and Tindall 2011). Many of these truncated ARs function as constitutively active, ligand-independent TFs which may have implications in disease states.

In the absence of ligand, AR is inactive and exists in the cytosol in a complex with heat shock proteins (HSPs), such as Hsp56, Hsp70, and Hsp90, as well as



Androgen Receptor (AR), Fig. 1 Schematic presentation of the AR protein. Structural and functional domains of AR are shown. The AR N-terminal domain (NTD) contains a ligand-independent activation function (AF-1) with two transactivation units, TAU-1 and TAU-5, containing binding motifs for coregulators. The ^{23}FQNLF^{27} motif contributes to the interaction between the NTD and ligand-binding domain (LBD), whereas the ^{433}WHTLF^{437} motif may influence AR signaling by acting as an autonomous activation domain. The length of the human AR protein can vary due to poly-glutamine ((Q)n) and a poly-glycine ((G)n) stretches of variable lengths in the NTD. The nuclear localization signal (NLS) resides in the flexible hinge region. The LBD harbors a ligand-dependent AF-2 and the nuclear export signal (NES). The numbering of amino acids shown is based on 21 and 23 residues in the poly-glutamine and poly-glycine repeats, respectively

cytoskeletal proteins and other chaperones (for reviews, see Lamb et al. 2001; Brinkmann 2011). Upon ligand binding, AR undergoes a conformational change, dissociates from HSPs and other chaperones, dimerizes, and translocates to the nucleus. Although AR usually acts as a homodimer, it has been shown to form heterodimers with other nuclear receptors including ER, GR, and testicular orphan receptor-4 (TR4). However, the functional consequences of these interactions in vivo are currently not clear. Once in the nucleus, AR binds chromatin in the vicinity of target genes at specific sites known as androgen response elements (AREs), recruits various coregulators, other TFs, and components of the general transcription machinery, and modulates gene expression (Lamb et al. 2001) (Fig. 2).

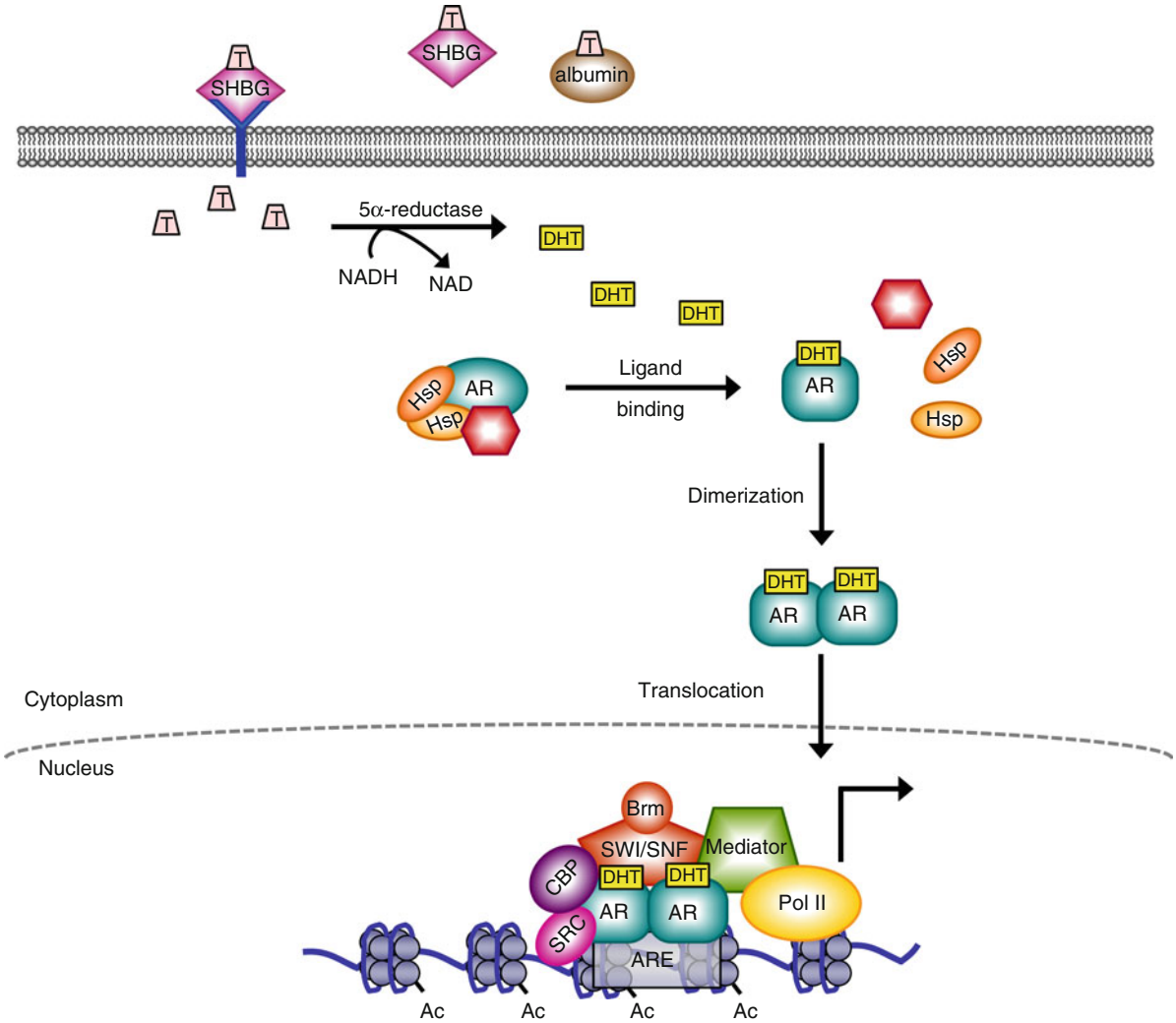
Based on biochemical studies and similar to other TFs, AR has been shown to stably bind its chromatin template in the presence of the ligand. However, development of live cell imaging and advances in green fluorescent protein (GFP) technology have demonstrated dynamic interactions of AR with its specific regulatory sites. Using fluorescence recovery after photobleaching (FRAP) analysis, residence times of these transient interactions were measured on a time scale of seconds and were influenced by the nature of the ligand (Klokk et al. 2007).

In addition to the dynamic, chromatin-binding-dependent transactivation function of AR, liganded AR can regulate transcription independently of its ability to bind DNA. This happens through the direct protein–protein interactions between AR and coregulators and/or TFs that alter their intrinsic transcriptional activities. For example, there is evidence that AR increases the activity of coactivators GR interacting protein-1 (GRIP1), cAMP-response element-binding protein (CBP), and p300, whereas it inhibits activity of other TFs and signaling pathways, such as those involving activator protein-1 (AP1) and nuclear factor-κB (NF-κB) (for reviews, see Kaarbo et al. 2007; Bennett et al. 2010). This greatly increases the repertoire of genes that can be regulated by AR.

Given the importance of androgens in the normal physiology and disease states, several studies have sought to identify genome-wide AR target gene profiles using ChIP-chip and ChIP-seq (e.g., Yu et al. 2010; Massie et al. 2011). These studies started to identify not only the genes that are AR targets, which for example involve different aspects of cell cycle and metabolism, but also other factors that may be important for AR activity, such as FOXA1 as an AR pioneer factor.

In addition to regulation by its cognate ligand, AR is subject to modification by phosphorylation, acetylation, methylation, sumoylation, and ubiquitination (for a review, see Gioeli and Paschal in press). Although the exact consequences of these modifications to AR function in normal physiology and implications for disease states are not yet known, they likely have a role in at least fine tuning of AR function.

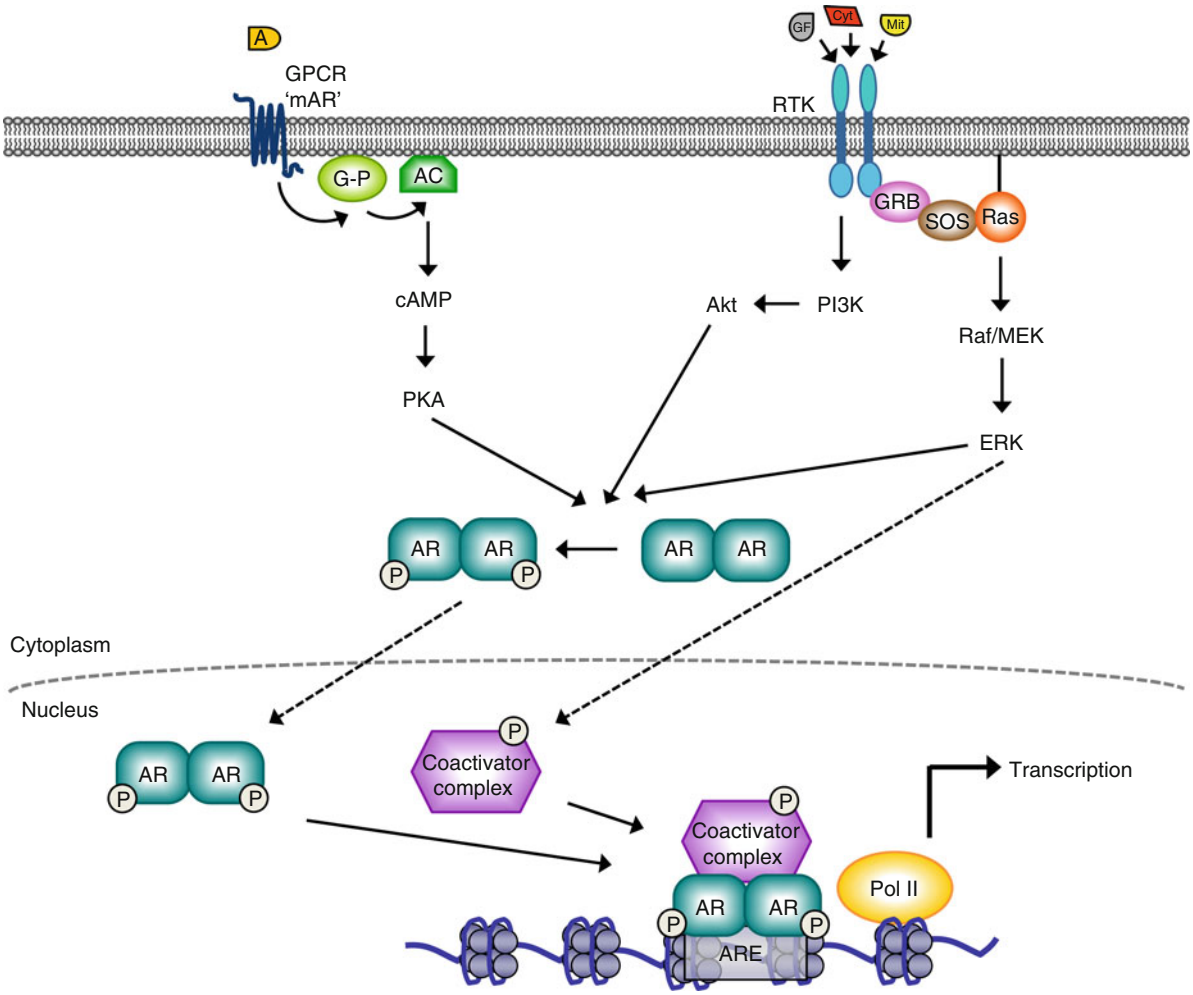
The importance of posttranslational modifications to AR activity is illustrated by modulation of AR action by a number of signaling pathways initiated by growth factors, cytokines, and mitogens (Fig. 3). Several growth factors, such as insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF), and interleukin-6 (IL-6), were found to transactivate AR independently



Androgen Receptor (AR), Fig. 2 AR transcriptional activation. Testosterone (T) circulates in the blood bound to steroid hormone-binding globulin (SHBG) or albumin and enters the cells through diffusion or through a SHBG receptor at the plasma membrane. In some cells, T is converted to the more active metabolite dihydrotestosterone (DHT) through the action of 5α-reductase. Upon ligand binding, AR undergoes a conformational change followed by dissociation from the multi-subunit chaperone complex. It then forms dimers and translocates to the nucleus where it binds androgen response elements (AREs) of target genes and recruits coregulators (some examples of which are shown) and basal transcription machinery, resulting in transcriptional regulation. HSP, heat shock protein; CBP, CREB binding protein; SRC, steroid receptor coactivator; BRM, Brahma; SWI/SNF, a chromatin modifying complex

of androgens (for a review, see Foradori et al. 2008). Such cell surface signals stimulate downstream ▶ mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathways, increasing AR activity as well as the activity and interaction of different coactivators with AR. This increase in activity is achieved, at least in part, through phosphorylation of AR and the coactivators. For example, AR has been shown to be phosphorylated on Ser650 upon EGF stimulation in prostate cancer cells. Similarly, IL-6 stimulation leads to phosphorylation of the AR coactivator SRC-1.

To better understand the molecular mechanisms of AR action, several AR knockout and knockin mouse models have been generated, both systemic and tissue specific (for a review, see Kerkhofs et al. 2009). Use of these models resulted in the identification of specific AR roles in male fertility, prostate development,



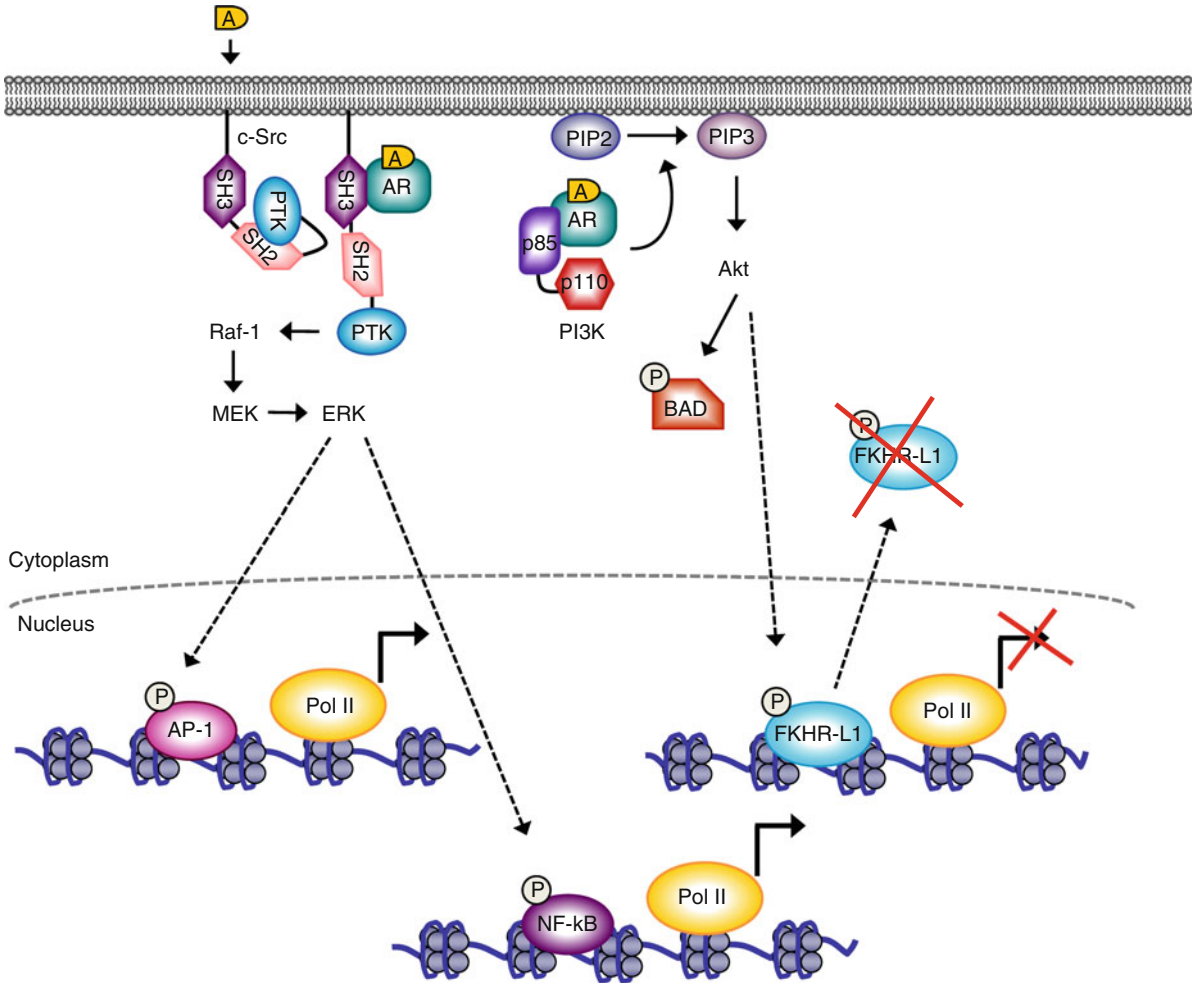
Androgen Receptor (AR), Fig. 3 AR-mediated cytoplasmic and nuclear signaling pathways through activation of plasma membrane receptors. Androgens (A) may activate the cytoplasmic kinase PKA via binding to a G-protein-coupled receptor (GPCR) at the plasma membrane and may thereby change the intracellular levels of second messenger molecules, such as cAMP. This can lead to phosphorylation and nuclear translocation of AR where it can act as a transcription factor. Nonsteroid molecules, such as growth factors (GF), cytokines (Cyt), and mitogens (Mit), arriving at the cell surface activate MAPK (Ras/Raf/MEK/ERK) and PI3K/Akt signaling pathways which similarly phosphorylate and activate the AR, leading to its translocation into the nucleus. ERK may also increase phosphorylation of AR coactivators which may activate them and thus increase AR activity

metabolism and diabetes, immune functions, bone metabolism, and several cancers. These models are facilitating the delineation of the molecular mechanisms of AR function in vivo.

Non-genomic Actions of AR

Over the past two decades, a number of studies have demonstrated non-genomic actions of AR, characterized by rapid cellular response and lack of transcription and translation of androgen-target genes. These activities are initiated at the plasma membrane or in the cytosol, triggering activation of intracellular signaling molecules, such as the extracellular-signal regulated kinase (ERK), protein kinase A (PKA), PI3K/Akt, and protein kinase C (PKC) (for reviews, see Foradori et al. 2008; Bennett et al. 2010) (Fig. 4).

For example, following androgen binding, AR interacts with the p85 regulatory subunit of ▶ PI3K



Androgen Receptor (AR), Fig. 4 Non-genomic actions of AR. Androgen-bound AR interacts with the SH3 domain of the tyrosine kinase c-► Src in the cytosol and activates the MAPK (Raf-1/MEK/ERK) signaling. This then enhances phosphorylation events that activate TFs (e.g., AP-1 or ► NF-κB) and promotes cell survival and growth. Liganded AR can also directly interact with the p85 subunit of ► PI3K and mediate activation of the protein kinase Akt which in turn phosphorylates the cytoplasmic (e.g., BAD) and nuclear (e.g., FKHR-L1) pro-apoptotic proteins which results in their degradation. These events promote cell survival by inhibiting apoptosis. P, phospho; BAD, BCL2-associated agonist of cell death; FKHR-L1, forkhead transcription factor like 1 (also known as FOXO3A)

in the cytosol which then phosphorylates and activates the downstream effector serine-threonine kinase Akt. The AR-mediated phosphorylation of Akt is inhibited by the AR antagonist bicalutamide documenting the direct involvement of AR in this process.

Another example of non-genomic AR action is where androgen-bound AR physically interacts with and activates the tyrosine kinase ► Src blocking its auto-inhibitory effect. This stimulates the ► Src/Raf-1/MEK/ERK-2 signaling cascade. One of the targets of ► Src is the adaptor molecule SH2-containing protein (Shc) which is an upstream regulator of MAPK. ► Src-activated MAPK pathway modulates AR-mediated transcription by phosphorylating AR and p160 family of steroid receptor coactivators (SRCs) and thereby regulates several cellular processes such as cell proliferation, migration, and differentiation.

AR and Disease States

Given the diverse role of androgens in a variety of cell types, perturbances to AR action have been linked to

a number of disease conditions. For example, AR dysregulation has been associated with cancer (e.g., prostate, testicular, colorectal), cardiovascular defects (coronary artery disease), immune diseases (e.g., type I diabetes), metabolic disorders (i.e., obesity and androgen insensitivity syndrome, osteoporosis), neurological conditions (e.g., Alzheimer's disease), and other diseases such as Kennedy's syndrome (for reviews, see Lamb et al. 2001; Brinkmann 2011).

AR has been most widely studied in terms of its involvement in prostate cancer (for a review, see Wang and Tindall 2011). The key role of androgens in prostate cancer genesis and progression was first observed in 1940s and has formed the basis of androgen ablation/castration therapies for prostate cancer. Although initially highly effective, this therapy ultimately fails and the disease progresses to a castration-resistant state which is fatal. It has been shown that one of the key proteins in both the androgen-sensitive and castration-resistant prostate cancer is AR. During this progression, several mechanisms involving AR are activated, such as local prostate cancer tissue androgen production, AR gene amplification, increased mitogen signaling, increased expression of AR coactivators, and increased sensitivity of AR to low androgen levels (for a review, see Wang and Tindall 2011).

Another disease in which dysregulation of AR has a role is spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease (Kumar et al. 2011). This condition involves expansion of the CAG repeat encoding a poly-Q stretch in the AR NTD; whereas 34 or fewer is normal, 38 or more repeats in the poly-Q stretch gives rise to SBMA. The SBMA is characterized by the degeneration of the motor neurons which are located in the spinal cord and bulbar regions that express AR.

A third AR-linked disease, androgen insensitivity syndrome (AIS), is characterized by partial or complete lack of response to androgens in cells which normally would be androgen sensitive (for a review, see Hughes and Deeb 2006). This results in the failure of normal masculinization in 46XY male individuals. In AIS patients, the AR gene is frequently mutated in the DBD and/or the LBD, making AR lose its function. Mutations can range from complete or partial deletions of the AR gene, which are rare, to point mutations and frame shift mutations. These can disrupt the DNA binding, AR expression levels, and ligand binding specificity or efficacy, which results in at least a partial functional loss in AR.

Summary

AR is a ligand-activated TF which mediates the biological effects of androgens in diverse tissues. Upon ligand binding, AR translocates into the nucleus, binds AREs on target genes, and regulates transcription. In addition, AR can interact with other TFs or cofactors, as well as initiating rapid non-genomic cellular responses by direct interactions with several signaling molecules at the plasma membrane or in the cytoplasm. Conversely, AR can also be activated by mitogens, cytokines, and growth factors through signaling pathways which can regulate AR/coactivator activity through posttranslational modifications. Dysregulation of AR expression and/or function has been associated with a variety of disease states, including prostate cancer, SBMA, and AIS. Thus, AR is a prime target for therapy in a number of conditions.

References

Baulieu EE, Jung I. A prostatic cytosol receptor. Biochem Biophys Res Commun. 1970;38(4):599–606.

Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC. Molecular cell biology of androgen receptor signalling. Int J Biochem Cell Biol. 2010;42(6):813–27.

Brinkmann AO. Molecular mechanisms of androgen action – a historical perspective. Methods Mol Biol. 2011;776:3–24.

Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science. 1988;240(4850):324–6.

Dehm S, Tindall DJ. Alternatively spliced androgen receptor variants. Endocr Relat Cancer. 2011;18(5):R183–96.

Fang S, Anderson KM, Liao S. Receptor proteins for androgens. On the role of specific proteins in selective retention of 17-beta-hydroxy-5-alpha-androstan-3-one by rat ventral prostate in vivo and in vitro. J Biol Chem. 1969;244(24):6584–95.

Foradori CD, Weiser MJ, Handa RJ. Non-genomic actions of androgens. Front Neuroendocrinol. 2008;29(2):169–81.

Gioeli D, Paschal BM. Post-translational modification of the androgen receptor. Mol. Cell. Endocr. (in press), doi:10.1016/j.mce.2011.07.004

Hughes IA, Deeb A. Androgen resistance. Best Pract Res Clin Endocrinol Metab. 2006;20(4):577–98.

Ikonen T, Palvimo JJ, Janne OA. Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor

modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem. 1997;272(47):29821–8.
Kaarbo M, Klokk TI, Saatcioglu F. Androgen signaling and its interactions with other signaling pathways in prostate cancer. Bioessays. 2007;29(12):1227–38.
Kerkhofs S, Denayer S, Haelens A, Claessens F. Androgen receptor knockout and knock-in mouse models. J Mol Endocrinol. 2009;42(1):11–7.
Klokk TI, Kurys P, Elbi C, Nagaich AK, Hendarwanto A, Slagsvold T, et al. Ligand-specific dynamics of the androgen receptor at its response element in living cells. Mol Cell Biol. 2007;27(5):1823–43.
Kumar R, Atamna H, Zakharov MN, Bhasin S, Khan SH, Jasuja R. Role of the androgen receptor CAG repeat polymorphism in prostate cancer, and spinal and bulbar muscular atrophy. Life Sci. 2011;88(13–14):565–71.
Lamb DJ, Weigel NL, Marcelli M. Androgen receptors and their biology. Vitam Horm. 2001;62:199–230.
Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science. 1988;240(4850):327–30.
Mainwaring WI. A soluble androgen receptor in the cytoplasm of rat prostate. J Endocrinol. 1969;45(4):531–41.
Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83(6):835–9.
Massie CE, Lynch A, Ramos-Montoya A, Boren J, Stark R, Fazli L, et al. The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. EMBO J. 2011;30(13):2719–33.
Tilley WD, Marcelli M, Wilson JD, McPhaul MJ. Characterization and expression of a cDNA encoding the human androgen receptor. Proc Natl Acad Sci U S A. 1989;86(1):327–31.
Trapman J, Klaassen P, Kuiper GG, van der Korput JA, Faber PW, van Rooij HC, et al. Cloning, structure and expression of a cDNA encoding the human androgen receptor. Biochem Biophys Res Commun. 1988;153(1):241–8.
Wang D, Tindall DJ. Androgen action during prostate carcinogenesis. Methods Mol Biol. 2011;776:25–44.
Yu J, Mani RS, Cao Q, Brenner CJ, Cao X, Wang X, et al. An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. Cancer Cell. 2010;17(5):443–54.

Angiotensin II Receptor Type 2

► Angiotensin Type 2 Receptor

Angiotensin II Type 2 Receptor Interacting Protein (ATIP)

► MTUS1/ATIP

Angiotensin Type 2 Receptor

Søren Paludan Sheikh
Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, University of Southern Denmark, Odense, Denmark

Synonyms

Angiotensin II receptor type 2

Historical Background

The angiotensin II type 2 receptor (AT2R) shares a high degree of homology with its well-known sister gene, the angiotensin II type 1 receptor (AT1R), and is similarly a part of the renin-angiotensin II system (RAS). The RAS is best known for its powerful effects on regulation of blood pressure and body fluid volume. The AT2R is activated after specific binding of the eight amino acid peptide angiotensin II (AngII), which is generated from angiotensin I by the angiotensin-converting enzyme. The AT2R is a member of the family of seven transmembrane receptors, the largest group of cell surface receptors in the body. Like its family members, the AT2R traverses the cell membrane seven times creating an NH_2-terminal and three loops on the extracellular side and a COOH-terminal plus three loops on the intracellular side. The AT2R is present on multiple different cell types, although its expression level is often lower than that of AT1R. The AT2R's biological functions and intracellular signaling partners have been difficult to tease out before the arrival of AT2R-specific agonists and antagonists. In line with the widespread location of the AT2R, it has effects in most biological systems including the central and peripheral nervous system, the cardiovascular system, endocrine systems, renal function, and the reproductive system. Although AT2R expression is generally low in normal adult tissues, it is highly important in development and in injury/repair mechanisms. Thus, the AT2R is highly expressed in the developing fetus and upregulated after adult tissue injury as well as in several pathological conditions. Expression of the AT2R can be modulated by pathological states associated with tissue remodeling or inflammation

such as hypertension, atherosclerosis, myocardial infarction, stroke, and diabetes. Interestingly, the biological effects are often opposite to the AT1R effects. The most characterized AT2R effect being vasodilatation as opposed to the well-characterized AT1R-mediated vasoconstriction. Hence, from a pharmacological perspective, it would be valuable to have specific AT2R agonists, as these could be potential blood pressure–lowering drugs. The AT2R also elicit cardioprotective-, anti-inflammatory-, anti-proliferative effects and it also regulates apoptosis. Accordingly, studies have been performed to understand and map the location and function of the ligand-binding site and the intracellular signaling systems activated by the AT2R. While the receptor does not seem to activate the canonical G proteins (except Gi in some systems), several different signaling molecules appear to bind to the AT2R COOH-terminal including different kinases, phosphatases, and scaffolding molecules.

Receptor Structure, Ligand Binding, and Antagonists

Seven transmembrane (G protein-coupled) receptors for angiotensin (Ang) peptides include the AT1Ra and AT1Rb subtypes and the AT2R, that all bind the octapeptide AngII with subnanomolar affinities. The AT2R also binds the AngIII and Ang-(1-7) peptides with high affinity. In addition, at least two other receptors have been described, the AT4R (that binds AngIII) and the Mas receptor, that binds Ang-(1-7). Although no AT1R crystals have been described, the AT1R is one of the best characterized seven transmembrane receptors. Computer models indicate that the structure is much like the recently crystallized β2-adrenergic receptor (Adrenergic Receptor). The AT2R receptor is much less characterized, but it is thought that it too possesses a similar three-dimensional structure. Regarding the angiotensin receptor ligands, several different peptides exist and there has been some discussion as to which of these peptides actually bind to the different angiotensin receptors in vivo. For example, both the AngII, Ang-(1-7), and AngIII peptides activate the AT2R. AngIII is almost as potent as AngII, at least in the coronary vascular bed. Finally, there has been some data showing that the AT2R can dimerize with itself and with other seven transmembrane receptors, but not with the AT1R.

The best characterized ligand peptide is AngII that in turn is metabolized into several bioactive peptides. Aminopeptidases cleave AngII to Ang-(2-8) (AngIII) and Ang-(3-8) (AngIV). In addition, carboxypeptidases generate Ang-(1-7) from AngI-(1-10) and AngII-(1-8).

Historically, it has been difficult to separate the AT2R actions from the other Ang receptors because of the common main ligand AngII. The advent of *specific AT2R agonists and antagonists* has made it possible to better study the receptor's biological actions. At least three specific AT2R agonists have been described. The AT2R agonist CGP-42112A was the first non-peptide agonist with reasonably high affinity for the receptor, and it has been widely used. Recently, a novel agonist, referred to as compound 21 and which binds the AT2R with almost as high affinity as AngII, has been developed. Compound 21 exhibits a dissociation constant for AT2R of 0.4 nM, while the dissociation constant for the AT1R is >10,000 nM. Compound 21 reduced the mean blood pressure in spontaneously hypertensive rats and induced vasodilatation in isolated arteries via AT2R stimulation (Bosnyak et al. 2010). A third agonist candidate is compound 22 that stimulates neurite outgrowth, phosphorylates p42/p44 (ERK1/2 MAPK), enhances duodenal alkaline secretion in Sprague-Dawley rats, and also lowers the blood pressure in anesthetized rats (Alterman 2010).

Selective AT2R antagonists are equally important. The most widely used antagonist is PD-123319. Among others, this molecule has been reported to block the effects of Ang-(1-7) in many studies, suggesting that Ang-(1-7) also activates the AT2R.

AT2R Signal Transduction

The AT2R signal transduction has been controversial since the receptor does not activate canonical G proteins (except Gi in a reconstituted system). Instead, the AT2R activates a series of kinases, phosphatases, and scaffold proteins including the AT2R receptor-interacting protein (ATIP) family that binds to the intracellular COOH-terminal tail of the receptor. Somewhat indirectly, an array study in cardiac fibroblasts with AT2R blockade (with PD123319) ascribed 24 genes to be specific for AT2R activation and these genes were related to ten different signaling pathways including cAMP/PKA, Ca^{2+}, PKC, PTK, ERK1/2 (MAPK), PI-3 K, NO/cGMP, Rho, NF-kappaB, and JAK-STAT pathways. Specifically, collagen I and tissue inhibitor of metalloproteinase (TIMP)-1 mRNA

levels decreased after AT2R blockade as compared to an AngII-treated group (Jiang et al. 2007).

Several signaling pathways have been studied in more detail (some of these are slightly overlapping), including:

- Activation of protein phosphatases causing protein dephosphorylation.
- Bradykinin/nitric oxide (NO)/cyclic guanosine 3',5'-monophosphate (cGMP) pathway leading to NO release and vasodilatation.
- Stimulation of phospholipase A(2) and release of arachidonic acid.
- Rho inhibition, which may link the AT2R to the pathogenesis of hypertension and vascular proliferative disorders (Guilluy et al. 2008).
- Activation of scaffolding/adapter proteins like the ATIP family that associate with molecular motors on microtubules.
- Increased VEGF secretion that could increase angiogenesis and play a role in cancer and kidney disease.

The ATIP interaction was first discovered using the yeast two-hybrid system. This protein family presently includes six members that all interact with the AT2R: ErbB3, PLZF, CNK1 Na^+/H^+ exchanger NHE6, TIMP-3, and ATIP/ATBP; for review, see (Rodrigues-Ferreira and Nahmias 2010). Interestingly, association with these angiotensin receptor-binding proteins may change the intracellular location of the AT2R and underlie its roles in neuronal differentiation, tumor growth, and vascular remodeling. Using ATIP1 overexpressing mice (ATIP1-Tg), it has been shown that the ATIP1 plays an inhibitory role in the AT2R-mediated vascular remodeling. The ATIP1-Tg mice exhibited reduced neointima formation of the femoral artery after injury as compared to WT mice, which is in line with the perceived reduced AT2R signaling (Fujita et al. 2009). Control experiments confirmed the result by showing reduced NO, ERK1/2 phosphorylation, and TGFβ levels in the overexpression mice.

Finally, an important notion regarding the effect of AngII stimulation: Since the two AT1R and AT2R subtypes compete for available ligand, the AngII in vivo effect depends on the relative expression of the two receptors on the cell surface in a given biological system. Thus, blocking the AT1R with losartan leaves more AngII for AT2R stimulation yielding a "dual effect" because the receptors often have opposite actions. In addition, losartan also inhibits the renin/angiotensin feedback loop that functions as a negative feedback loop; therefore, blocking AT1R increases the production of AngII peptide. For example, the activated AT1R stimulates fibrosis and the AT2R attenuates fibrosis in many models; hence, losartan would inhibit fibrosis both by blocking the AT1R and by more available AngII for binding to unoccupied AT2Rs. In line with this notion, studies on transgenic mice with or without AT2 receptor expression reveal that the effects of AngII on injury-induced pancreatic fibrosis are determined by the balance between AT1R and AT2R signaling (Ulmasov et al. 2009).

The AT2R Is Important in the Central Nervous System (CNS)

Possible AT2R roles in CNS function, which affect learning, memory, satiety, and behavior have been suggested in both humans and rodents. At2r, gene knockout mice (At2r (-/y)) showed significant defects in their spatial memory, exhibited abnormal dendritic spine morphology, and had lost the AngII reduced food-intake response (Ohinata et al. 2008; Pawlowski et al. 2009). In line with these data, intracerebroventricularly administered AngII and AngIII dose-dependently suppressed food intake in mice and their anorexigenic activities were inhibited by an AT2R-selective antagonist (Ohinata et al. 2008).

Interestingly, the AT2R may also play a role in neurological disorders such as Alzheimer's disease, depression, and Parkinson's disease and possess a neuroprotective effect in response to brain injury such as stroke. The AT2R can attenuate inflammation and oxidative stress that have been suggested to be key factors in the pathogenesis and progression of several, if not all, of the here mentioned diseases. It should be noted that the exact molecular dysfunctions behind the progressive neurodegenerative disorder in Parkinson's and Alzheimer's diseases remain unidentified. It has therefore been suggested that AT2R agonists may protect neurons against cell death, and thus would prove beneficial in the treatment of these diseases. In the case of Parkinson's disease, AngII may attenuate dopaminergic cell death. The AT2R agonist can reduce damage after an ischemic insult to the brain. Indeed, centrally administered CGP42112 (an AT2R agonist) exhibits a neuroprotective effect, which is independent of blood pressure in conscious rat models of stroke. This effect is likely enhanced by AT2R-induced

vasodilatation and an increase in blood flow to the ischemic brain zone improving the brain function and limiting the infarct extension.

Furthermore, it is believed that the neuroprotective AT2R effect on stroke plays a role in the beneficial effects of AT1R blockade in stroke. Thus, losartan is superior in stroke treatment to other antihypertensive drugs that are equipotent with respect to their blood pressure–lowering effect. Losartan's effect appears to be blood pressure independent and mediated through a dual action: selective AT1R blockade and indirect AT2R stimulation by leaving AngII for the unoccupied AT2Rs. This dual action is unique to AT1R blockers.

At the cellular level, AT2R signaling and phenotypic effects on nerve cell differentiation, apoptosis, and morphology have been studied extensively. In NG108-15 cells, the AT2R induces neural outgrowth, a process that requires parallel activation of ERK1/2 and Fyn, a member of the ▶ Src family kinases (Guimond et al. 2010).

The ATR2 Elicits Vasodilatation and Cardioprotection in the Cardiovascular System

The AT2R's actions in the cardiovascular system are well characterized and include *relaxation of cardiac and vascular smooth muscle tone* most likely through increased NO production and bradykinin release. This is the classical AT2R effect; however, the AT2R also *affects cellular apoptosis, inhibits proliferation, diminishes atherosclerotic lesions in humans, and limits the actions of the immune system after cardiac injury*. Accordingly, AT2R agonists are under development for potential therapeutic use in hypertension. In addition, AT2R activation suppresses renin biosynthesis and release in renal juxtaglomerular cells, potentially further adding to the blood pressure–lowering effect.

Treatment of Wistar rats with the AT2R agonist compound 21, 24 h after an experimentally induced myocardial infarction, significantly reduced scar size and improved systolic and diastolic ventricular function (Kaschina et al. 2008). At the molecular level, compound 21 exerted anti-apoptotic effects (diminished myocardial infarction-induced Fas-ligand and caspase-3 expression in the peri-infarct zone) and anti-inflammatory effects (decreased serum monocyte chemoattractant protein-1 and myeloperoxidase as well as reduced cardiac interleukin-6, interleukin-1beta, and interleukin-2 expression). Finally, compound 21 treatment reduced phosphorylation of the ERK1/2 and p38 mitogen-activated protein kinases that are both involved in cell survival after myocardial infarction. Along with similar results from other groups, these data strongly suggest that AT2R agonists should be investigated for beneficial effects on systolic and diastolic function after myocardial infarction.

Since the AT1R and AT2R have opposite effects on vascular smooth muscle tone, and yet bind the same ligand, there are several possible explanations for a given experimental result. An interesting variation over this theme may exist, as spontaneous hypertensive rats may have lost the AT2R vasodilatory effect. In the heart, coronary constriction induced by Ang II, Ang III, and Ang-(1-7) is enhanced in spontaneous hypertensive rats as compared to Wistar rats that have normal blood pressure. The defect in the spontaneous hypertensive rats could be explained either by absence of counter-regulatory AT2R-mediated relaxation and/or by a change of the AT2R phenotype from relaxant to constrictor.

Caution is warranted in interpretation of the literature on AT2R effects on the cardiovascular system, since there have been conflicting reports concerning the in vivo effects of AT2R activation on cardiomyocyte apoptosis and hypertrophy. In some studies, the AT2R appears to be required for hypertrophic growth whereas in the work of others there are no effects on cardiac hypertrophy. Similar controversial findings have been reported in atherosclerosis with regard to AT2R effects on plaque morphology and neointima formation.

In an attempt to tease out the cardiovascular phenotype of the different Ang receptors, triple knockout mice lacking the AT1Ra, AT1b, and AT2R have been generated. As expected, ATR1 deletion alone reduced the heart rate and impaired the in vivo pressor response to an AngII bolus injection. In the triple knockout mice, AngII had no effect on blood pressure suggesting that there are no other AngII-sensitive receptors involved in blood pressure regulation (Gembardt et al. 2008).

Interestingly, the AT2R is expressed in human and rodent atherosclerotic lesions, and a series of papers suggest *an atheroprotective action* of these receptors. Chronic Ang-(1-7) administration inhibits the progression of atherosclerosis and improves endothelial function in a mouse model of atherosclerosis (apolipoprotein E-deficient (ApoE(-/-)) mice). These effects

were reversed with either AT2R or Mas receptor blockade. At the molecular level, Ang-(1-7) decreased superoxide production and increased endothelial nitric oxide synthase immunoreactivity, thus improving NO bioavailability as a result of activation of AT2R and/or Mas receptors (Tesanovic et al. 2010). In agreement with these data, AT2R overexpression decreases collagen accumulation in atherosclerotic plaques.

Increasing the complexity of the RAS system, *sex differences in blood pressure regulation* exists. In females, a low AngII dose decreased blood pressure while it had no effect in men. Surprisingly, a high dose increased mean blood pressure more in men than in women. This could be attributed to an enhancement of the AT2R vasodilatory effects in women as underlined by increased AT2R mRNA levels. These data are consistent with the notion that estrogen upregulates AT2R expression. In turn, this could contribute to the lower blood pressure during pregnancy. In this condition, an enhanced AT2R-mediated vascular relaxation pathway involving increased expression/activity of endothelial AT2Rs and increased postreceptor-activated phospho-eNOS have been reported.

Although controversial because of contradictory results, there is accumulation of evidence suggesting that *genetic polymorphisms in the AT2R gene* can modify disease risk in both cancer and cardiovascular diseases. This is an important field to follow in the future because better understanding of the response in different patients can lead to more individually tailored medicine. Several different genetic polymorphisms have been described, although these mutations do often not appear to influence AT2R function. However, one of these, A1675G in the AT2R gene possibly decreases cardiovascular risk and the severity of atherosclerosis by modifying systemic inflammation especially in hypertensive males (Tousoulis et al. 2010).

The AT2R Affects Cancer and Inhibits Inflammation

While the AT1R proinflammatory effects are rather well established, most current evidence suggests the AT2R instead interferes with and *inhibits proinflammatory pathways*, though the AT2R data in inflammation are still rather preliminary and somewhat controversial. However, the principal mechanisms of AT2R putative anti-inflammatory actions support new possibilities in AT2R research and potential utilization of AT2R stimulation as a novel pharmacological concept in anti-inflammation as reviewed in (Rompe et al. 2010). Expression of AT2R protein is generally upregulated in injury animal models such as experimentally induced stroke, myocardial infarction, and neointima lesions, and AT2R activation interferes with multiple inflammatory pathways. The agonist compound 21 inhibits key proinflammatory cytokines and nuclear factor kappaB. Compound 21 dose-dependently (1 nM–1 μM) reduces tumor necrosis factor-alpha-induced interleukin 6 levels in primary human and murine dermal fibroblasts. After myocardial infarction in the mouse, compound 21 also decreases serum MCP-1 and myeloperoxidase as well as cardiac interleukin expression (IL-6, IL-1beta, and IL-2).

In general, the AT2R has effects that theoretically could *play a role in cancer development* including anti-proliferative, anti-angiogenic, and pro-apoptotic effects. In fact, AT2R expression seems to be upregulated in different types of tumor tissues, including mammary, prostate, and pancreatic cancer. However, the literature contains conflicting reports about the AT2R effect in cancer, so at this stage, it is not entirely clear if blocking or stimulating the receptor would inhibit tumor development.

Regarding prostate cancer, several reports show that AT2R activation induces apoptosis of cancer cells. AT2R overexpression in prostate cancer cells using an adenoviral vector induced apoptosis even without addition of AngII, suggesting that AT2R is constitutively active in this situation. The cell death–signaling pathway was evidenced by increased terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling staining and was dependent on activation of p38 mitogen-activated protein kinase, caspase-8, caspase-3, and p53 activation. AT2R overexpression also induced inhibition of proliferation, a significant reduction of S-phase cells, and an enrichment of G1-phase cells indicating that AT2R is a promising novel target gene for prostate cancer therapy (Li et al. 2009). In other experiments using LL/2 and 3-MCA tumor cells, the AT2R induced the release of vascular endothelial growth factor (VEGF), a soluble pro-angiogenic factor. Blocking this effect revealed a significant decrease in angiogenesis after antagonist (PD123,319) treatment or in AT2R-KO mice suggesting AT2R inhibition would impair tumor development by blocking malignant cell proliferation and tumor angiogenesis (Clere et al. 2010).

These studies thus imply that the effect of AT2R could be different depending on the cancer type and biological setting.

Roles of AT2R in Kidney Disease and Diabetes

It has been appreciated for some time that the RAS acts as a local paracrine system in the kidney with presence of all the known molecular components including angiotensinogen, renin, AngI, angiotensin-converting enzymes, AngII, the AT1R, and the AT2R. Overall, the AT2R is perceived to increase renal blood flow (likely by increasing local NO production), inhibit growth, increase sodium excretion, as well as to participate in fibrosis and remodeling after renal injury. The natriuretic effect is also strongly stimulated by AngIII, further underscoring its role as an AT2R ligand. Interestingly, the AT2R is also important for normal renal development. Inhibition of the AT2R using PD123319 during pregnancy induces malformations in the developing rat kidney.

The AT2R kidney effects on blood pressure and natriuresis add further evidence in favor of using AT2R agonists as antihypertensive agents. However, when reviewing AT2R effects on kidney remodeling and fibrosis, conflicting data appears (Wenzel et al. 2010). One line of data points to a role for the receptor in nephroprotection. AT2R-knockout mice exhibit increased renal injury and mortality in chronic kidney disease suggesting that pharmacological stimulation of the AT2R may positively influence renal pathologies. Other data support the notion that the AT2 receptor mediates pro-inflammatory effects and promotes renal fibrosis and hypertrophy, and consequently blocking the receptor would be the choice of therapeutic intervention.

At the molecular level, the AT2R upregulates slit diaphragm-associated molecules such as nephrin, that is, a key molecule for preventing proteinuria. In a mouse model for proteinuria, AT2R activation improves kidney function by regulating the cell cycle and principal molecular components in the podocyte. Podocytes have important functions in sealing of the glomerular permeability barrier, and therefore the AT2R is considered an interesting pharmacologic target for preventing proteinuria (Kawachi et al. 2009).

One of the most interesting roles of Ang receptors is their involvement in diabetes. Diabetics have more cardiovascular and kidney disease than the background population, and the RAS is considered a highly important pathophysiological component in both diseases. Not only does blocking of the AT1R reduce the development and progression of diabetic nephropathy. This treatment also delays onset of diabetes as compared to other antihypertensive agents even though these reduce the blood pressure to the same extent as AT1R blockers. It has been shown that the beneficial effects on glomerular injury achieved with AT1R blockers are contributed to, not only by blockade of the AT1R, but also by increasing AngII effects mediated through the AT2R (Naito et al. 2010).

The big question is: What is the quantitative significance of AT2R in the establishment of diabetic vascular dysfunction? Although, we do not have the answer to this, it is at least known that the enhanced AT2R and iNOS-induced, NO-mediated vasodilation, impairs AngII-induced contraction in an endothelium-independent manner at the early stage of type 2 diabetes. It is also known that insulin activation of the phosphatidylinositol 3-kinase (PI3K) pathway stimulates glucose uptake in peripheral tissues and NO synthesis in the endothelium. Since AT2R activation increases NO availability in the endothelium, the AT2R activation would contribute to better glucose uptake and delay insulin resistance. Shared insulin and AT2R signaling pathways could underlie the beneficial AT2R effects in metabolic and hypertensive cardiovascular diseases.

Diabetic conditions increase expression of the AT2R in the proximal tubule with a beneficial effect in kidney function and blood pressure regulation. In the hyperglycemic kidney, the AT2R is suggested to regulate key signaling pathways including Akt-mTOR-p70(S6K)and VEGF signaling.

Another interesting mechanism is that the AT2R mediates AngII and high glucose induced decreases in renal prorenin/renin receptor expression, which would protect the kidney in diabetic conditions where increased AngII is harmful. Low renin levels controlled by the AT2R also play a role in nephroprotection in obese Zucker rats.

Gene polymorphisms in the AT2R additionally suggest a link to diabetes, as high RAS activity and the A-allele of the AT2R G1675A polymorphism associate with high risk of severe hypoglycemia in type 1 diabetes. A potential preventive effect of RAS-blocking drugs in patients with recurrent severe hypoglycemia could be interesting to examine (Pedersen-Bjergaard et al. 2008).

Summary

The investigation of AT2R signal transduction and biological effects has increased considerably after the advent of specific, high affinity, and non-peptide AT2R agonists and antagonists that can now be used as tools to identify AT2R-specific effects in the scenario of multiple different Ang receptors. It has become clear that this receptor does not activate classical heterotrimeric G proteins, but rather signals through a series of small G proteins, kinases, phosphatases, and scaffolding molecules that mostly bind to the receptor COOH-terminal. A novel family of scaffolding proteins including ATIP that are especially attached to the AT2R have been identified. These events have lead to increased interest in the AT2R signaling and biology and especially in its putative pathophysiological roles. A long line of recent evidence suggests that pharmacological manipulation of AT2R activity could be clinically applicable in a wide range of diseases especially in hypertension, myocardial infarction, stroke, and diabetes. It has also become clear that the canonical notion that the AT2R antagonizes the effects of the AT1R probably is an oversimplification. Especially since the AT2R also possesses high affinity for Ang-(1-7) and AngIII that do not activate the AT1R. Nevertheless, many of the AT2R effects are counterbalanced by AT1R activation. Solid experimental evidence has demonstrated that AT2R elicits vasodilatation through NO release, and has anti-inflammatory and anti-proliferative effects, and lastly regulates apoptosis in many cell systems. A lot of interest centered on possible shared signaling pathways between insulin and AT2R signaling suggests that AT2R agonist could have beneficial and synergistic effects both in metabolic and cardiovascular disease by a dual mechanism of action including vasodilatation and NO metabolism.

However, effects still remain that we need to understand in more detail including clarification of contradictory results such as the dual roles of AT2R to both inhibit and enhance inflammation, fibrosis, and apoptosis, and we have only begun to investigate the AT2R's role in malignant cells. Furthermore, it will be interesting to follow clinical trials of AT2R agonists and get a preliminary picture of the benefits these drugs may play in future medicine, especially, since we do not yet know which unwanted side effects might turn up.

References

Alterman MJ. Development of selective non-peptide angiotensin II type 2 receptor agonists. Renin Angiotensin Aldosterone Syst. 2010;11(1):57–66. Epub 2009 Oct 30.

Bosnyak S, Welungoda IK, Hallberg A, Alterman M, Widdop RE, Jones ES. Stimulation of angiotensin AT2 receptors by the non-peptide agonist, compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats. Br J Pharmacol. 2010;159(3):709–16. Epub 2010 Jan 28.

Clere N, Corre I, Faure S, Guihot AL, Vessières E, Chalopin M, et al. Deficiency or blockade of angiotensin II type 2 receptor delays tumorigenesis by inhibiting malignant cell proliferation and angiogenesis. Int J Cancer. 2010;127:2279–91.

Fujita T, Mogi M, Min LJ, Iwanami J, Tsukuda K, Sakata A, et al. Attenuation of cuff-induced neointimal formation by overexpression of angiotensin II type 2 receptor-interacting protein 1. Hypertension. 2009;53:688–93. Epub 2009 Feb 16.

Gembardt F, Heringer-Walther S, van Esch JH, Sterner-Kock A, van Veghel R, Le TH, et al. Cardiovascular phenotype of mice lacking all three subtypes of angiotensin II receptors. FASEB J. 2008;22:3068–77. Epub 2008 May 22.

Guilluy C, Rolli-Derkinderen M, Loufrani L, Bourgé A, Henrion D, Sabourin L, et al. Ste20-related kinase SLK phosphorylates ser188 of rhoA to induce vasodilation in response to angiotensin II type 2 receptor activation. Circ Res. 2008;102:1265–74. Epub 2008 Apr 17.

Guimond MO, Roberge C, Gallo-Payet N. Fyn is involved in angiotensin II type 2 receptor-induced neurite outgrowth, but not in p42/p44mapk in NG108-15 cells. Mol Cell Neurosci. 2010;45:201–12. Epub 2010 Jun 25.

Jiang XY, Gao GD, Du XJ, Zhou J, Wang XF, Lin YX. The signalling of AT2 and the influence on the collagen metabolism of AT2 receptor in adult rat cardiac fibroblasts. Acta Cardiol. 2007;62(5):429–38.

Kaschina E, Grzesiak A, Li J, Foryst-Ludwig A, Timm M, Rompe F, et al. Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction? Circulation. 2008;118:2523–32. Epub 2008 Nov 24.

Kawachi H, Han GD, Miyauchi N, Hashimoto T, Suzuki K, Shimizu F. Therapeutic targets in the podocyte: findings in anti-slit diaphragm antibody-induced nephropathy. J Nephrol. 2009;22:450–6.

Li H, Qi Y, Li C, Braseth LN, Gao Y, Shabashvili AE, et al. Angiotensin type 2 receptor-mediated apoptosis of human prostate cancer cells. Mol Cancer Ther. 2009;8:3255–65.

Naito T, Ma LJ, Yang H, Zuo Y, Tang Y, Han JY, et al. Angiotensin type 2 receptor actions contribute to angiotensin type 1 receptor blocker effects on kidney fibrosis. Am J Physiol Renal Physiol. 2010;298:F683–91. Epub 2009 Dec 30.

Ohinata K, Fujiwara Y, Fukumoto S, Iwai M, Horiuchi M, Yoshikawa M. Angiotensin II and III suppress food intake via angiotensin AT(2) receptor and prostaglandin EP(4) receptor in mice. FEBS Lett. 2008;582:773–7. Epub 2008 Feb 5.

Pawlowski TL, Heringer-Walther S, Cheng CH, Archie JG, Chen CF, Walther T, et al. Candidate Agtr2 influenced genes and pathways identified by expression profiling in the developing brain of Agtr2(-/y) mice. Genomics. 2009;94:188–95. Epub 2009 Jun 6.

Pedersen-Bjergaard U, Dhamrait SS, Sethi AA, Frandsen E, Nordestgaard BG, Montgomery HE, et al. Genetic variation and activity of the renin-angiotensin system and severe hypoglycemia in type 1 diabetes. Am J Med. 2008;121:246.e1–8.
Rodrigues-Ferreira S, Nahmias C. An ATIPical family of angiotensin II AT2 receptor-interacting protein. Trends Endocrinol Metab. 2010;21:684–90.
Rompe F, Unger T, Steckelings UM. The angiotensin AT2 receptor in inflammation. Drug News Perspect. 2010;23:104–11.
Tesanovic S, Vinh A, Gaspari TA, Casley D, Widdop RE. Vasoprotective and atheroprotective effects of angiotensin (1-7) in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2010;30:1606–13. Epub 2010 May 6.
Tousoulis D, Koumallos N, Antoniades C, Antonopoulos AS, Bakogiannis C, Milliou A, et al. Genetic polymorphism on type 2 receptor of angiotensin II, modifies cardiovascular risk and systemic inflammation in hypertensive males. Am J Hypertens. 2010;23:237–42. Epub 2009 Dec 3.
Ulmasov B, Xu Z, Tetri LH, Inagami T, Neuschwander-Tetri BA. Protective role of angiotensin II type 2 receptor signaling in a mouse model of pancreatic fibrosis. Am J Physiol Gastrointest Liver Physiol. 2009;296:G284–94. Epub 2008 Nov 25.
Wenzel UO, Krebs C, Benndorf R. The angiotensin II type 2 receptor in renal disease. J Renin Angiotensin Aldosterone Syst. 2010;11:37–41.

Ankyrine-Rich TRP Channel, ANKTM1, TRPA1

► TRP (Transient Receptor Potential Cation Channel)

ANT

Grégory Tufo[1], Lorenzo Galluzzi[2], Guido Kroemer[3,4,5] and Catherine Brenner[1]
[1]INSERM UMR-S 769, Université Paris-Sud XI, Châtenay-Malabry, France
[2]INSERM, U848, Institut Gustave Roussy, Université Paris-Sud XI, Villejuif, France
[3]INSERM U848, Institut Gustave Roussy, Villejuif cedex, France
[4]University Paris 11, Orsay, France
[5]Institut Gustave Roussy, Villejuif cedex, France

Synonyms

Adenine nucleotide translocase; ADP/ATP carrier

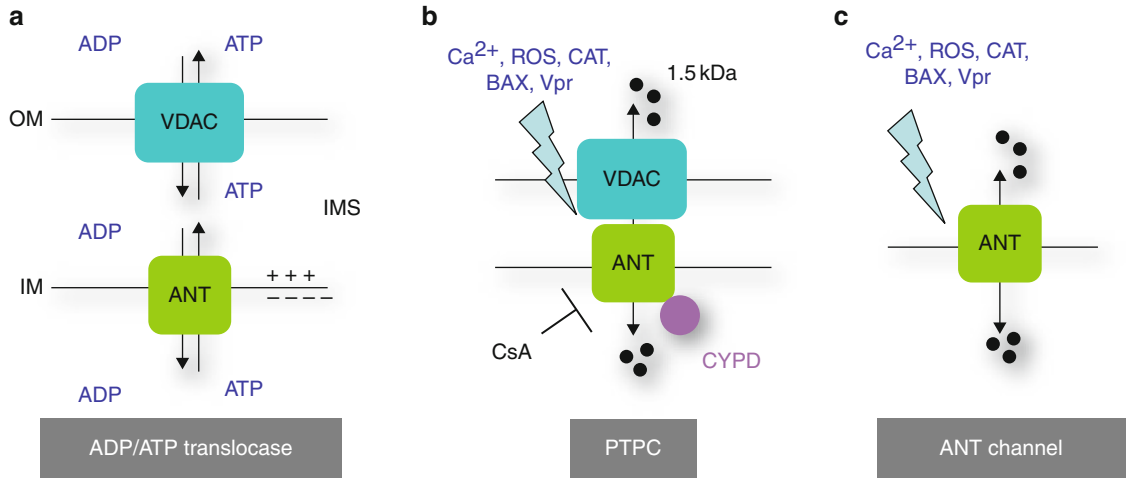
Historical Background

The adenine nucleotide translocase (ANT) belongs to the evolutionarily conserved family of mitochondrial carriers (for a recent review see Palmieri et al. 2010). ANT constitutes one of the most abundant proteins of the mitochondrial inner membrane (IM), in which it is inserted via six transmembrane helices. Both the C- and N-termini of ANT face the mitochondrial matrix. ANT was first characterized as an antiporter that mediates the export of ATP from the mitochondrial matrix to the intermembrane space together with the import of ADP from the intermembrane space to the matrix (Pfaff et al. 1968) (Fig. 1a). This antiporter activity is electrogenic, as it drives the stoichiometric exchange of ATP^{4-} and ADP^{3-}. ANT may also act as a nonselective IM channel, playing a critical role in the permeabilization of mitochondrial membranes that accompanies apoptosis and programmed necrosis (Rück et al. 1998; Brustovetzki et al. 1996; Marzo et al. 1998) (Fig. 1b and c).

Recently, the 3D crystallization of the bovine heart ANT monomer with carboxyatractyloside (CAT) established a structural organization based on a threefold repeat of about 100 amino acids, 6 transmembrane helices with a depression at the intermembrane space surface, and a short mitochondrial carrier sequence (RRRMMM) at the bottom. Each ANT monomer has turned out to associate with several cardiolipin molecules, correlating with functional studies indicating that ANT activity depends on this IM-specific lipid. CAT binding excluded that of adenosine nucleotides (i.e., ADP and ATP), suggesting that the residues of ANT that are involved in the nucleotide binding site are also important for the physical association with CAT. A close-open conformation switch has been suggested to facilitate ADP/ATP antiport and to result from the cooperative binding of the nucleotides. However, the exact molecular and enzymatic mechanisms that account for ADP/ATP exchange by ANT remain elusive.

A Vital Mitochondrial ADP/ATP Carrier

The enzymatic function of ANT as an ADP/ATP exchanger has been first characterized in isolated mitochondria from rodent livers, in yeast, and in native ANT-containing proteoliposomes. These studies have benefited from two specific inhibitors, namely,



ANT, Fig. 1 *Two functions for one protein.* (**a**) ANT is an ADP/ATP translocase inserted in the mitochondrial inner membrane (IM), where it exchanges ATP and ADP between the mitochondrial intermembrane space (IMS) and the mitochondrial matrix. The ADP/ATP exchange rate is dependent on the mitochondrial membrane potential ($\Delta\psi_m$), whose polarity across the IM is indicated with (−) and (+). Moreover, in response to specific stimuli, ANT can function as a nonspecific channel (for molecules with a molecular weight <1.5 kDa), either in association with the voltage-dependent anion channel (VDAC) and cyclophilin D (CYPD) within a multiprotein complex known as permeability transition pore complex (PTPC) (**b**) or on its own (**c**). Calcium (Ca^{2+}), reactive oxygen species (ROS), proteins and peptides (e.g., the pro-apoptotic BCL-2 family member BAX; Vpr, which is encoded by the human immunodeficiency virus 1, HIV-1), as well as small molecules (e.g., carboxyatractyloside, CAT) can all stimulate the pore function of ANT, which in turn can induce mitochondrial membrane permeabilization and hence cell death. Cyclosporine A (CsA) prevents the binding of CYPD to ANT, thereby functioning as a PTPC inhibitor. OM, mitochondrial outer membrane

CAT and bongkrekic acid (BA), which bind different sites within the protein and favor the c- and m-conformation of the protein, respectively (Klingenberg 2008). The efficacy of ANT in exchanging ADP with ATP is moderate, and the relatively high abundance of the carrier at the IM might reflect an adaptation to the high intracellular demand for mitochondrial ATP export (Klingenberg 2008). It is still unclear whether *in cellula* ANT can function independently, as a monomer and/or an homodimer, or whether (at least in some tissues) it requires the physical binding of a partner such as the voltage-dependent anion channel (VDAC), cyclophilin D (CYPD), the mitochondrial phosphate carrier protein (SLC25A3, also known as PIC), the F_OF_1-ATP synthase, and/or the mitochondrial creatine kinase (MTCK1). An association with such mitochondrial proteins might improve the channeling of ATP to hexokinase and of ADP to the F_OF_1-ATP synthase in response to increased ATP demands, and might be particularly relevant for the metabolic adaptation of mitochondria in cancer cells.

The enzymatic characterization of ANT can be carried out with radiolabeled nucleotides. Briefly, isolated mitochondria are loaded with tritiated ATP ([^{3}H]ATP) in a suitable energizing buffer, allowing for oxidative phosphorylation and for the maintenance of a mitochondrial transmembrane potential ($\Delta\psi_m$). Upon the elimination of extramitochondrial nucleotides, ADP/ATP exchange is initiated by addition of exogenous ADP. Then, the import reaction is blocked by CAT, mitochondria are centrifuged, separated from their supernatant, and the exported radioactive ATP is quantified. This methodology has been successfully used to estimate the V_{max} and K_m of ANT from various origins (Pfaff et al. 1968). Alternatively, the addition of an extramitochondrial ATP detection system (containing $NADP^+$, hexokinase, glucose-6-phosphate dehydrogenase, and glucose) to mitochondria suspended in energizing buffer avoids the use of radioactivity and is suitable for the high-throughput screening of molecules that affect ANT antiporter activity (Belzacq-Casagrande et al. 2009).

A Lethal Channel

In response to a number of stimuli, ANT can be converted into a poorly selective cation channel

(Fig. 1b and c). Single-channel current measurements in giant bovine ANT proteoliposomes revealed the reversible opening of a Ca^{2+}-dependent channel (Brustovetsky and Klingenberg 1996). This might be caused by the binding of Ca^{2+} to cardiolipin, a mitochondrial IM lipid that is tightly bound to ANT, in turn triggering a conformational change that results in pore opening. The ANT channel has also been studied upon purification from rat heart, reconstitution of ANT-containing proteoliposomes, and measurement of the release of a fluorescent probe (Rück et al. 1998; Marzo et al. 1998). Several classes of natural or synthetic molecules have been shown to activate or inhibit the channel function of ANT, suggesting that ANT can constitute a pharmacological target (Halestrap and Brenner 2003). In support of this hypothesis, inhibition of ANT by Nelfinavir, a small molecule used in antiretroviral therapy, has been shown to inhibit pathological manifestations linked to massive cell death in mice undergoing septic shock, cerebral ischemia-reperfusion, and fulminant hepatitis (Weaver et al. 2005). Thus, ANT might be targeted to repress cell death signaling in vivo.

Due to similarities in channel conductance and sensitivity to pharmacological inhibitors, ANT has been proposed to constitute the IM channel of the permeability transition pore complex (PTPC), a multiprotein structure assembled at the interface between mitochondrial membranes that is involved in both mitochondrial homeostasis and cell death (Fig. 1b). Of note, despite striking structural similarities, not all ANT isoforms support the formation of the PTPC.

Four ANT Isoforms

The human ANT gene family is composed of four homologues, *ANT1* to *ANT*4. Primary sequence homology ranges from 68% to 88% (Dolce et al. 2005). The expression of distinct ANT isoforms is highly regulated and exhibits tissue specificity, suggesting a differential role for each isoform. Accordingly, the promoter region of *ANT* genes contains elements that can be bound by multiple transcription factors including (though presumably not limited to) members of the OXBOX-REBOX family, GRBOX, SP1, and AP2. ANT1 is mainly expressed in nonproliferating tissues, including skeletal muscle and brain. In contrast, ANT2 is upregulated during growth, and ANT2 constitutes the prevalent ANT isoform that is expressed in hepatocytes, fibroblasts, and lymphocytes. The ANT3 gene is transcribed ubiquitously at a comparatively lower level. In humans, ANT4 can be detected mainly in liver, testis, and brain. In mice, ANT4 is exclusively expressed in embryonic stem cells (Dolce et al. 2005). The genetic invalidation of *Ant1* and *Ant2* in the mouse liver renders mitochondria insensitive to ANT ligands (e.g., CAT). Moreover, the *Ant1*/*Ant2* double knockout enhances the capacity of mitochondria to accumulate Ca^{2+} ions, decreases the probability of PTPC opening, and yet does not compromise cell death, suggesting that ANT1 and 2 are dispensable for apoptosis or that other proteins (e.g., ANT4, other members of the mitochondrial carrier protein family) may substitute for the lethal functions of ANT1 and 2 in their absence. However, genetic strategies to modulate the expression levels of various ANT isoforms in human cancer cell lines revealed that ANT1 and 3 overexpression favors apoptosis (Bauer et al. 1999), whereas increased levels of ANT2 and 4 augment the resistance of cancer cells against death induction (Le Bras et al. 2006; Gallerne et al. 2010).

ANT1 in Cardiopathy

ANT1 has been associated with several cardiac diseases (Dörner and Schultheiss 2007). In particular, five-point mutations of *ANT1* have been identified in patients with autosomal dominant progressive external ophthalmoplegia (adPEO), an adult-onset pathology characterized by weakening of external eye muscles, generalized myopathy, exercise intolerance, and multiorgan disorders including cardiomyopathy. Muscle biopsies of individuals affected by adPEO revealed common intracellular features, including deficient oxidative phosphorylation, scattered ragged red fibers, and multiple mitochondrial DNA deletions. When introduced in yeast, these mutations decrease oxidative growth and lead to diminished ADP/ATP exchange.

Cardiac ischemia/reperfusion and alteration of the myocardium have also been associated with reduced ANT function. It has been speculated that oxidative stress and/or alterations of the lipid metabolism can negatively affect ANT activity, inhibit energy supplies and, therefore, favor PTPC opening and apoptosis.

Surprisingly, transgenic mice that overexpress ANT1 in the heart are protected from cardiac

insufficiency and diabetic cardiomyopathy, correlating with improved mitochondrial ANT functionality and oxidative phosphorylation. In contrast to in vitro studies, no signs of enhanced apoptosis were detected in ANT-transgenic animals. In addition, these animals showed features of athlete's hearts, including enlarged end-diastolic and end-systolic volumes and raised heart rate with unchanged ejection fraction, but no signs of abnormal dilatation.

ANT2 in Cancer

According to the Expressed Sequence Tags database (EST, Unigene), cancer patients often show the deregulated expression of ANT isoforms. In particular, the ANT2 isoform is overexpressed in biopsies from distinct types of cancers as well as in several human tumor cell lines. Changes in the expression levels of ANT2 neither have major effects on cell metabolism and morphology nor negatively affect cell viability or proliferation, suggesting compensatory effects from other ANT isoforms (Le Bras et al. 2006). However, ANT2 overexpression decreases the sensitivity of cancer to some pro-apoptotic stimuli. Accordingly, ANT2 silencing by RNA interference (RNAi) induced apoptosis in breast cancer cells in vitro and tumor regression in mice. Overexpression of ANT4 also protects cancer cells from apoptosis (Gallerne, et al. 2010), but the anti-apoptotic potential of ANT4 in vivo awaits confirmation.

Summary

Mitochondria, which are the cell's powerhouse but also centralize lethal pathways, constitute a crossroad among numerous intracellular signaling pathways. Mitochondrial dysfunction is frequently associated to severe human diseases, including cancer and cardiomyopathies. Some of these pathologies are closely linked to the deregulation of apoptosis as well as that of energy metabolism. The adenine nucleotide translocase (ANT), which possesses two opposite functions (as a vital ADP/ATP antiporter and as a lethal channel), appears to occupy a central position in controlling cell fate. It is now clearly established that both ANT functions can be modulated by pharmacological agents. However, a more detailed knowledge of the specific pathophysiological role of each isoform is required to validate ANT as a potential therapeutic target.

References

Bauer MK, Schubert A, Rocks O, Grimm S. Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis. J Cell Biol. 1999;147(7):1493–502.

Belzacq-Casagrande A, Martel C, Pertuiset C, Borgne-Sanchez A, Jacotot E, Brenner C. Pharmacological screening and enzymatic assays for apoptosis. Front Biosci. 2009; 14:3550–62.

Brustovetsky N, Klingenberg M. Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca2+. Biochemistry. 1996;35(26):8483–8.

Dolce V, Scarcia P, Iacopetta D, Palmieri F. A fourth ADP/ATP carrier isoform in man: identification, bacterial expression, functional characterization and tissue distribution. FEBS Lett. 2005;579(3):633–7.

Gallerne C, Touat Z, Chen ZX, Martel C, Mayola E, Sharaf el dein O, Buron N, Le Bras M, Jacotot E, Borgne-Sanchez A, Lemoine A, Lemaire C, Pervaiz S, Brenner C. The fourth isoform of the adenine nucleotide translocator inhibits mitochondrial apoptosis in cancer cells. Int J Biochem Cell Biol. 2010;42(5):623–9.

Halestrap AP, Brenner C. The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. Curr Med Chem. 2003;10(16):1507–25.

Le Bras M, Borgne-Sanchez A, Touat Z, El Dein OS, Deniaud A, Maillier E, Lecellier G, Rebouillat D, Lemaire C, Kroemer G, Jacotot E, Brenner C. Chemosensitization by knockdown of adenine nucleotide translocase-2. Cancer Res. 2006;66 (18):9143–52.

Marzo I, Brenner C, Zamzami N, Jürgensmeier JM, Susin SA, Vieira HL, Prévost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science. 1998;281(5385):2027–31.

Palmieri F, Pierri CL. Mitochondrial metabolite transport. Essays Biochem. 2010;47:37–52.

Pfaff E, Klingenberg M. Adenine nucleotide translocation of mitochondria. 1. Specificity and control. Eur J Biochem. 1968;6(1):66–79.

Rück A, Dolder M, Wallimann T, Brdiczka D. Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore. FEBS Lett. 1998;426(1):97–101.

Weaver JG, Tarze A, Moffat TC, Lebras M, Deniaud A, Brenner C, Bren GD, Morin MY, Phenix BN, Dong L, Jiang SX, Sim VL, Zurakowski B, Lallier J, Hardin H, Wettstein P, van Heeswijk RP, Douen A, Kroemer RT, Hou ST, Bennett SA, Lynch DH, Kroemer G, Badley AD. Inhibition of adenine nucleotide translocator pore function and protection against apoptosis in vivo by an HIV protease inhibitor. J Clin Invest. 2005;115(7):1828–38.

AP-3

Andrew A. Peden
Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

Historical Background

The cytoplasm of all eukaryotic cells is organized into a complex set of membrane-bound organelles with defined protein and lipid composition. Proteins and lipids of the endocytic and exocytic pathways are transported between these compartments by small vesicles and tubules which pinch off from one compartment and fuse with another and so deliver their contents. The budding of vesicles and tubules from membranes is driven by the recruitment of coat protein complexes from the cytoplasm. Coat complexes have two main functions in this process: First, they select cargo proteins to be packaged into the vesicle, and second, they recruit accessory proteins that help deform the membrane into a bud and bind machinery required for vesicle fission.

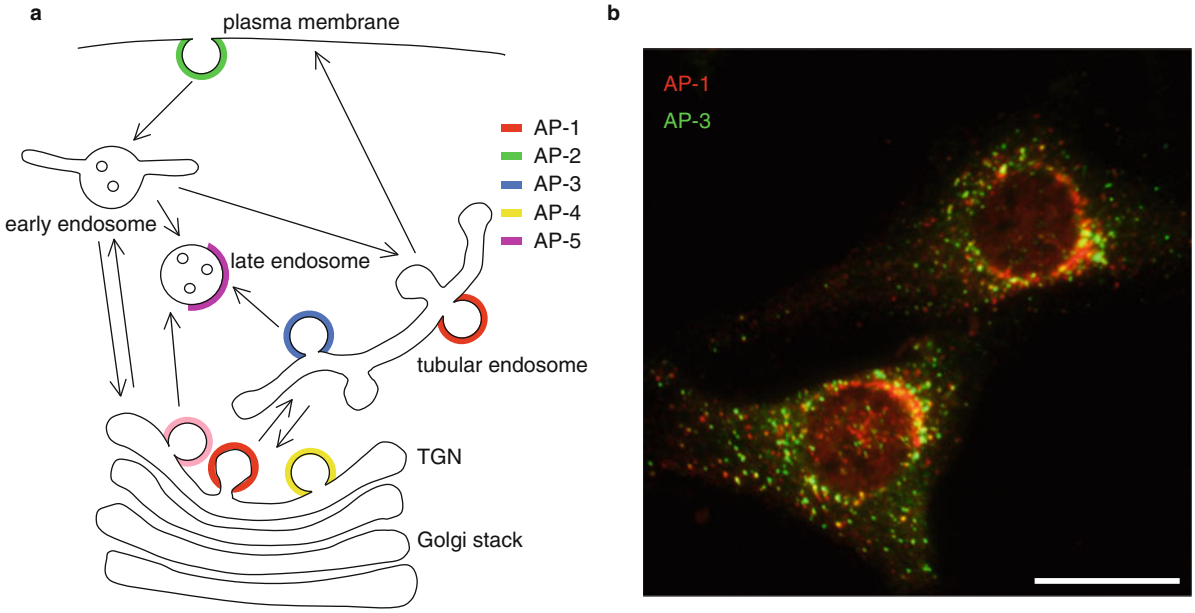
In mammalian cells, there are five related adaptor protein (AP) complexes (AP-1 through 5) (Hirst et al. 2011). Each complex is localized to a specific post-Golgi compartment and is required for the transport of a defined set of cargo molecules (Fig. 1 and Table 3). The AP-1 complex is localized to the trans-Golgi network and tubular endosomes and plays a role in the trafficking of proteins between these compartments such as the mannose 6-phosphate receptors and the R-SNARE, VAMP4. The AP-2 adaptor complex is localized to the plasma membrane and is required for the internalization of cell surface receptors such as the transferrin receptor and the low-density lipoprotein receptor. The AP-3 complex is localized to tubular endosomes and is required for the trafficking of lysosomal proteins such as LAMPI and LIMPII. The ▶ AP-4 complex is localized to the TGN and endosomes and is involved in the trafficking of the amyloid precursor protein. The AP-5 complex is localized to late endosomes. However, what cargo proteins it traffics is not known.

Adaptor protein complexes are heterotetramers consisting of a related set of subunits. The AP-1 complex consists of γ, β1, μ1, and σ1; AP-2 of α, β2, μ2, and σ2; AP-3 of δ, β3, μ3, and σ3; AP-4 of ε, β4, μ4, and σ4; and AP-5 of ζ, β5, μ5, and σ5. Each subunit within the adaptor complex plays a specific role in the process of vesicle budding, for example, the μ subunits bind cargo proteins; the β subunits bind clathrin in the case of β1-3, the structural component of the vesicle required for membrane deformation; and the α/γ type subunits bind accessory proteins that regulate the process.

The first AP complexes to be characterized were AP-1 and AP-2 as they are major components of clathrin-coated vesicles. The next AP complex to be identified was AP-3. The first subunits of the AP-3 complex were isolated by expression cloning in the early 1990s and the subsequent subunits of the complex were identified through their sequence homology to subunits of the AP-1 and AP-2 complexes, reviewed in (Odorizzi et al. 1998).

The first insights into the function of the AP-3 complex came through the discovery that *garnet* fruit flies are missing the δ subunit of the AP-3 complex. *Garnet* flies have reduced pigmentation in their eyes suggesting that the AP-3 complex is involved in the biogenesis of pigment granules, which are a lysosome-related organelle. In support of this hypothesis it was then discovered that the mouse coat color mutants *mocha* and *pearl* have disruption in the β3A and δ subunits respectively, and that people with Hermansky–Pudlak syndrome type 2 are missing the β3A subunit, reviewed in (Odorizzi et al. 1998; Badolato and Parolini 2007). People with HPS type 2 have reduced pigmentation (skin, hair, and eyes), defects in blood clotting, and susceptibility to recurrent infections. The majority of these phenotypes can be explained by defects in melanocytes, platelet dense granules, and lytic granules which are lysosome-related organelles (Badolato and Parolini 2007). In addition to defects in specalized compartments there is also increased trafficking of lysosomal integral membrane proteins over the cell surface in cells isolated from mice or patients lacking functional AP-3 complexes (Table 3) (Dell'Angelica et al. 1999).

This entry will primarily focus on outlining what is currently known regarding the function and regulation of the mammalian AP-3 complex in generic cell types. For detailed reviews regarding the biology of the AP-3 complex in non-vertebrate systems and in specalized cell types such as melanosomes and neurons, see (Raposo and Marks 2007; Dell'Angelica 2009; Danglot and Galli 2007).



AP-3, Fig. 1 *AP complexes are localized to post-Golgi membranes*. (**a**) Diagram showing the primary localization of adaptor complexes within a generic mammalian cell. *Arrows* indicate transport routes between compartments. *TGN* trans-Golgi network. (**b**) A mouse fibroblast stained for the AP-1 (γ) and AP-3 (δ) adaptor complexes. Scale bar 30 μm

AP-3 Complex Subunit Composition, Expression, and Function

The AP-3 complex is a heterotetramer of approximately 320 kDa. The complex consists of two large subunits δ (130 kDa), β3 (120 kDa), a medium subunit μ3 (47 kDa), and a small subunit σ3 (21 kDa) (see Table 1 and Fig. 2) (Simpson et al. 1997). Once the individual subunits of the AP complexes are synthesized they assemble very rapidly into a complex so the levels of individual subunits in the cytoplasm are negligible under normal conditions. Deletion or disruption of either of the large subunits leads to disassembly and degradation of the other subunits of the complex.

Tissue-Specific Expression

There are two isoforms of the β3, μ3, and σ3 subunits. All tissues express δ, β3A, μ3A, σ3A, and σ3B, and neuronal and pancreatic tissues express β3B and μ3B in addition (see Table 1). It has been proposed that there are ubiquitous (δ, β3A, μ3A, σ3A/B) and neuronal (δ, β3B, μ3B, σ3A/B) forms of the AP-3 complex. This hypothesis is partially supported by phenotypic analysis of mice either lacking both complexes (*mocha* mice) or just the neuronal (μ3B) complex, reviewed in Danglot and Galli (2007).

Large Subunits

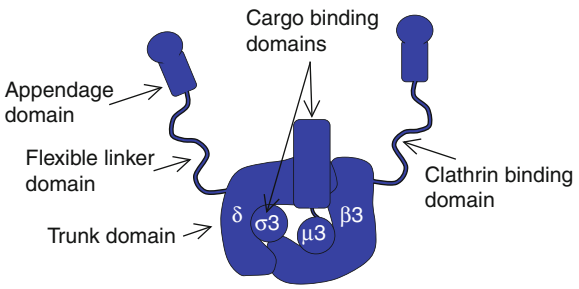
The large subunits (also known as adaptins) of the AP-3 complex have three domains: a folded trunk domain of approximately 70 kDa, a flexible linker domain of around 20 kDa, and a folded appendage domain of 30 kDa (Fig. 2). The trunk domains of δ and β3 consist of α solenoid fold and interact with the σ3 and μ3 subunits respectively. The trunk domain of the δ subunit may also be involved in membrane binding as the comparable region on the α subunit of AP-2 binds phosphatidylinositol 4,5-bisphosphate (PIP_2) (Jackson et al. 2010).

Very little is known about the appendage domains of β3 and δ adaptin. Based on homology they are predicted to have a bi-lobal structure. The appendage domains of α (AP-2) and γ (AP-1) adaptin bind accessory proteins that regulate vesicle budding and fission. At present no appendage domain binding proteins have been identified for AP-3. However, it is likely that they exist because AP-3 complexes lacking the β3 appendage domain are unable to traffic lysosomal proteins efficiently, and *mocha*^{2J} mice that lack the δ appendage domain have a coat color phenotype. Interestingly, the δ ear does not have the conserved platform required for binding accessory proteins found on the α subunit indicating that the δ subunit must bind accessory proteins in an alternate manner.

AP-3, Table 1 AP-3 complex subunits

Subunit name	Official NCBI symbol	Gene ID	Splice isoforms (aa)	Expression	Function within complex
δ	AP3D1	8943	2 (1153 and 1112aa)	Ubiquitous	Binding membranes and accessory proteins?
β3A	AP3B1	8546	1 (1094aa)	Ubiquitous	Binding accessory proteins
β3B	AP3B2	8120	1 (1101aa)	Neuronal and pancreatic tissue	Binding accessory proteins
μ3A	AP3M1	26985	2 (418)	Ubiquitous	Binding cargo, Yxx∅
μ3B	AP3M2	10947	2 (418)	Neuronal and pancreatic tissue	Binding cargo, Yxx∅
σ3A	AP3S1	1176	1 (193aa)	Ubiquitous	Binding cargo, [D/E]xxxL[L/I]
σ3B	AP3S2	10239	1 (193aa)	Ubiquitous	Binding cargo, [D/E]xxxL[L/I]

Information shown for human AP-3 subunits
aa amino acids



AP-3, Fig. 2 *Schematic of the AP-3 adaptor complex*. Structure of the AP-3 complex based on its homology to the AP-2 adaptor complex. Diagram shows subunit interactions and functional domains

Medium Subunits

The main function of the μ3 subunit is to bind cargo molecules that contain tyrosine-based sorting signals (see section "AP-3 Cargo Proteins" and Table 2) (Ohno et al. 1998). The μ3 subunit is predicted to have two folded domains connected via a short linker. The N-terminal domain has a longin type fold and interacts with the other subunits of the complex, and the C-terminal domain binds cargo molecules (see section "AP-3 Cargo Proteins"). μ3A and B have been shown to bind similar cargo molecules using yeast two-hybrid analysis.

Small Subunits

The main known function of the σ3 subunit is to bind cargo molecules that contain dileucine type motifs (see section "AP-3 Cargo Proteins" and Table 2) (Janvier et al. 2003). The σ3 subunits are predicted to be globular and have a longin type fold. Based on the structure of the σ2–dileucine interaction is predicted that σ3 binds dileucine signals using a conserved hydrophobic pocket on its surface (Jackson et al. 2010). σ3A and B are both ubiquitously expressed and at present it is unclear why there are two isoforms. It is possible that having both isoforms may increase the repertoire of cargo molecules the AP-3 complex can bind, or there may be as yet unidentified interacting partners. In addition to binding cargo molecules, σ3A/B have also been shown to be capable of interacting with the appendage domain of the δ subunit, and this interaction may regulate AP-3 recruitment onto membranes (see section "Regulation of the AP-3 Complex").

AP-3 Complex Interacting Proteins

Over the past 15 years, many approaches have been used to identify AP-3 complex interacting partners. These include yeast two-hybrid screens, proteomic studies on immuno-isolated AP-3 complexes, vesicles, and AP-3-coated liposomes. Thus, the number of proposed AP-3 interacting proteins has increased steadily. However, the numbers are relatively low compared to the AP-1 and 2 complexes suggesting that there are more to be discovered. It must be noted that for many of the AP-3 interacting partners it is still not known what domains and/or subunits of the AP-3 complex are involved in mediating the interaction. In the following section, I have outlined some of the known AP-3 interacting proteins. This list is not exhaustive but focuses only on the interacting partners who have had their binding to AP-3 mapped.

AP-3, Table 2 AP-3 interacting proteins

Interacting protein	Interaction motif/domain on binding protein	Interaction domain on AP-3	Method used to show interaction
Clathrin	β-propeller of clathrin's N-terminal domain	Flexible linker domains of β3A (LLDLD) and β3B (LLDLE)	Biochemical "pull down"
ARF1	Switch 1 and switch 2	δ/σ3 subcomplex	Yeast two-hybrid and co-IP
KIF3A	C-terminus (601–702)	β3A hinge (676–902)	Yeast two-hybrid and biochemical pull down
AGAP1	PH domain	δ/σ3 subcomplex	Yeast two-hybrid
BLOC-1 (Dysbindin subunit)	Dysbindin	μ3 or β3B?	Biochemical "pull downs"

A representative list of mammalian AP-3 interacting proteins which have had their binding site mapped

Clathrin

The AP-3 complex has been shown to bind clathrin in vitro and partially colocalize with clathrin in vivo. The β3A/B subunits have a clathrin-binding motif called a clathrin box (L∅x∅[D/E]), where ∅ is a bulky hydrophobic residue and x is any residue (Dell'Angelica et al. 1998). Mutation of this motif abolishes AP-3's interaction with clathrin. Surprisingly, AP-3 is not enriched in purified clathrin-coated vesicles and does not require clathrin to function. Mutation of the clathrin-binding box in AP-3 does not drastically affect lysosomal protein trafficking, and depletion of clathrin from an AP-3 based in vitro budding assay did not inhibit AP-3 budding. Furthermore, a significant fraction of AP-3 positive vesicles and tubules do not label for clathrin. Thus, the role of clathrin in AP-3 function still remains uncertain. Not all adaptor complexes require clathrin to function. For example, AP-4 and AP-5 do not contain clathrin-binding boxes and do not colocalize with clathrin in vivo and yeast AP-3 does not require clathrin for function. At present it is unclear why certain AP complexes require clathrin as a structural scaffold to help generate a vesicle and others do not. It is possible that other proteins may be performing the same function as clathrin. In yeast, it has been proposed that Vps41 may be playing this role for AP-3, and for AP-5 SPG11 may be performing this function.

ARF1 and AGAP1

ARF1 is required for AP-3 recruitment to membranes. The binding domain of ARF1 has been mapped to the trunk domain of the δ/σ3 subcomplex. This interaction has been shown to be modulated by the interaction of the δ-appendage domain with the C-terminal domain of the σ3 subunit (Lefrancois et al. 2004). The ARF1 GAP, AGAP1 also binds to AP-3 (Nie et al. 2003) (see section "Regulation of the AP-3 Complex").

KIF3A

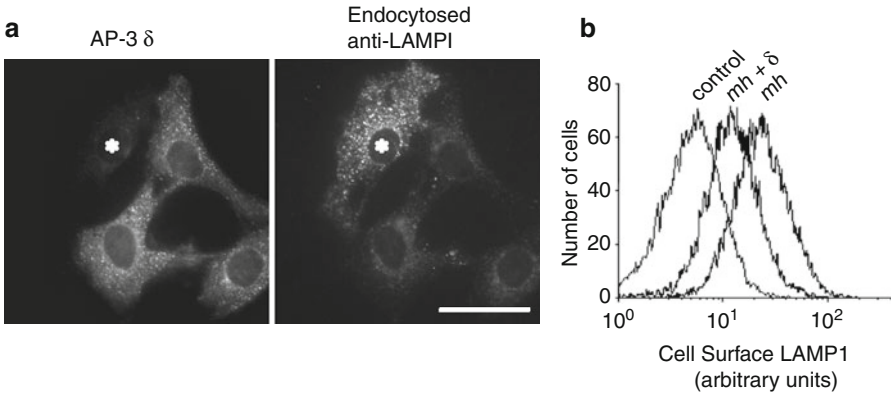
The motor protein KIF3A has been shown to bind to the hinge region of β3A and disruption of this interaction causes a defect in HIV budding (Azevedo et al. 2009). KIF3A is a component of the plus end directed motor Kinesin-2. Kinesin-2 has been shown to play a role in the transport of endosome-derived vesicles.

BLOC-1 Complex

The AP-3 complex interacts with biogenesis of lysosome-related organelles complex-1 (BLOC-1) (Di Pietro et al. 2006). BLOC-1 consists of 8 known subunits: pallidin, cappuccino, muted, snapin, dysbindin, BLOS1, BLOS2, and BLOS3. Disruption of BLOC-1 function leads to defects in the biogenesis of lysosome-related organelles and increased cell surface trafficking of lysosomal proteins. The molecular function of this complex is still unknown. It is likely that the interaction between BLOC-1 and AP-3 occurs through the dysbindin subunit of the complex. However, it remains unclear which subunit or subunits of the AP-3 complex mediate this interaction as μ3 and β3 have both been proposed to bind dysbindin.

AP-3 Cargo Proteins

One of the main functions of an AP complex is to select cargo molecules into forming vesicle (Bonifacino and



AP-3, Fig. 3 *Loss of AP-3 causes increased trafficking of lysosomal proteins over the cell surface*. (**a**) *Mocha* fibroblasts were transfected with the δ subunit of the AP-3 complex and allowed to internalize antibodies to the lysosomal protein LAMPI for 30 min at 37°C. The cells were fixed and stained for δ adaptin and LAMPI. Scale bar 30 μm. *Asterisk marks* cell not expressing δ adaptin. (**b**) *Mocha* (*mh*) fibroblasts were transfected with the δ subunit of the AP-3 complex and the cell surface levels of LAMPI determined using flow cytometry

Traub 2003). Loss of AP-3 complex function leads to increased cell surface trafficking of lysosomal proteins as well as defects in the targeting of certain cargoes to lysosome and lysosome-related organelles (Fig. 3). The AP-3 complex, like the other well-characterized adaptor complexes (AP-1/2), is capable of binding two types of linear motifs found in the cytoplasmic tails of cargo molecules. Studies using yeast two-hybrid assays have shown that the C-terminal domain of the μ3 subunit binds tyrosine-based motifs Yxx∅ (where ∅ is a bulk hydrophobic residue and x is variable but tends to be hydrophilic in nature) (Ohno et al. 1998). Studies using yeast three-hybrid assays have shown that di-leucine-based motifs [D/E]xxxL[L/I] (where x is any residue) bind a hemi-complex formed from the δ/σ3 subunits (Janvier et al. 2003). The binding affinities of both types of motifs for AP complexes are relatively weak and in the μM KD range.

The AP-3 complex can also bind cargo that does not contain either tyrosine or di-leucine-based motifs. For example, the endosomal R-SNARE ▶ VAMP7 and the HIV-GAG protein have shown to interact with the hinge domain of the δ subunit of the complex, and disruption of this interaction alters the trafficking of both proteins. For a detailed list of proposed AP-3 cargo proteins, see Table 3. It must be stated that for many of these proteins it has still to be confirmed whether there is a direct interaction between these proteins and the AP-3 complex. Thus, it is possible that changes observed in their trafficking in AP-3-deficient cells may be indirect.

Regulation of the AP-3 Complex

For AP complexes to function they must continually cycle on and off membranes. This cycle is regulated through a combination of weak μM–KD interactions involving small GTPases, lipids, and cargo proteins. This cycle has been best elucidated for the AP-2 complex (Jackson et al. 2010). The AP-2 complex is initially recruited onto membranes via its interaction with PIP_2. This interaction leads to a conformational switch in the complex that allows the μ2 and σ2 subunits to bind cargo proteins thereby increasing the affinity/avidity of the interaction. In addition to the conformational switch there is also further regulation where the linker domain of the μ2 subunit is phosphorylated, which greatly increases the affinity of the μ2 subunit for binding of cargo molecules. At present very little is known about the regulation of the AP-3 complex. However, it is clear that ARF1 plays a major role in this process.

ARF1, GEFs, and GAPs

The main GTPase-regulating AP-3 membrane association is ARF1 (Table 2). ARF1 is a small GTPase that cycles between an active GTP and an inactive GDP form. In the active form, ARF1 recruits AP-3 onto membranes. Disruption of the GTP cycle using the fungal metabolite Brefeldin A causes ARF1 to accumulate in the GDP locked form and causes AP-3 to dissociate from membranes. Yeast two-hybrid mapping experiments have indicated that ARF1 binds to

AP-3, Table 3 AP-3 cargo proteins

Proposed AP-3 cargo	Interaction motif on cargo	Interaction domain on AP-3	Method used to show interaction	Observed phenotype of cargo protein when interaction with AP-3 is perturbed
LAMPI	GYQTI	μ3	Biocore and yeast two-hybrid	Increased cell surface trafficking in the absence of AP-3
LAMPII	GYEQF	μ3*		Increased cell surface trafficking in the absence of AP-3
LIMPII	ERAPLI	δ/σ3	Biocore and yeast two-hybrid	Increased cell surface trafficking in the absence of AP-3
CD63	GYEVM	μ3	Yeast two-hybrid	Increased cell surface trafficking in the absence of AP-3
CD164/Endolyn	NYHTL	μ3	Yeast two-hybrid	Increased cell surface trafficking in the absence of AP-3
Tyrosinase	EERQPLL	δ/σ3	Biocore, yeast two-hybrid, and biochemical pull down	Altered trafficking in AP-3-deficient melanocytes
ZnT3	C-terminus	?	co-IP	Significant changes in localization and levels in mocha brains
Battenin/CLN3	EEEAESAARQPLI	δ/σ3*	Biochemical pull down	Altered trafficking in mocha and pearl fibroblasts
OCA2	ENTPLL	δ/σ3	Yeast two-hybrid and biochemical pull down	Altered trafficking of transiently expressed OCA2 that can no longer interact with AP-3
HIV-GAG	Matrix fragment	δ-hinge domain	Yeast two-hybrid and biochemical pull down	Altered trafficking to multivesicular bodies when interaction with AP-3 is perturbed
CD1b	SYGNI	μ3	Biocore and yeast two-hybrid	Increased cell surface trafficking and loss of lysosomal localization in the absence of AP-3
VAMP7	VAMP7 longin domain	δ-hinge domain	Yeast two-hybrid and co-IP	Altered trafficking in AP-3-deficient fibroblasts
PI4KIIa	ERQPLL	δ/σ3*	Biochemical pull down and co-IP	Altered trafficking in mocha fibroblasts and changes in distribution in mocha brains
Elastase	YPDA	μ3	Yeast two-hybrid	Altered trafficking/expression in AP-3-deficient neutrophils
High-affinity choline transporter/CHT	?	?		Significant changes in localization and levels in mocha brains
CB_1 cannabinoid receptor	?	?	co-IP	Change in cellular distribution when AP-3 depleted
Chloride Channel/CLC3	?	?		Altered trafficking in mocha fibroblasts and changes in distribution in mocha brains
Chloride Channel/CLC-7	N-terminus (EAAPLL) C-terminus?	δ/σ3*	Biochemical pull down	Increased trafficking over the plasma membrane when sorting signal mutated
Niemann-Pick Type C1/NPC1	?	?		Increased cell surface trafficking in the absence of AP-3
Niemann-Pick Type C2/NPC2	?	?		Decreased levels in the media in AP-3-deficient fibroblasts

IP immunoprecipitation
*Based on the motif found in the cargo protein it is likely to interact with the indicated subunits of the AP-3 complex. However, this has not been formally shown for this cargo

the trunk domain of the δ/σ3 hemicomplex. Further mapping indicated that the C-terminal domain of the σ3 subunit is also important for this interaction (Lefrancois et al. 2004). Interestingly, this C-terminal domain has also been shown to bind the δ appendage domain using three-hybrid mapping and so potentially provides a mechanism for regulating membrane binding. In support of this hypothesis, overexpression

of the appendage domain of δ adaptin caused AP-3 to become cytosolic and increase the trafficking of lysosomal membrane proteins over the plasma membrane.

The activity of ARF1 is regulated by GTP exchange factors and GTPase-activating proteins. It has been shown that ARF1 GEF BIG1 is recruited on to synthetic liposomes enriched in AP-3 and that depletion of BIG1 using siRNA causes the loss of AP-3 from membranes and an increase in the trafficking of lysosomal membrane proteins over the cell surface. The ARF1 GAP, AGAP1 has been shown to bind to AP-3 using the yeast three-hybrid assay (see Table 2). Overexpression of AGAP1 causes AP-3 to dissociate from membranes, and depletion of AGAP1 by siRNA makes AP-3 Brefeldin A insensitive (Nie et al. 2003). In addition, the GAP ARAP1 binds liposomes enriched in AP-3 complexes, and its depletion affects AP-3 binding to membranes (Baust et al. 2008). However, it is not known if ARAP1 directly interacts with AP-3.

PIP Binding

As mentioned earlier it has been shown that AP-2 recruitment to membranes is regulated by PIP_2 binding. The binding site on the trunk domain of the α subunit of AP-2 has been mapped and it is clear that the δ trunk domain of AP-3 also has a basic patch, although not as basic. However, it is not known whether phosphoinositides bind this patch. A good candidate is phosphatidylinositol 3-phosphate (PI3P) as studies using synthetic liposomes have shown that AP-3 preferentially binds to membrane enriched in PI3P. In addition, depletion of the enzyme PI-3KIIIC3, which generates PI3P, affects the recruitment of AP-3 to membranes and increases the trafficking of LAMPI over the plasma membrane (Baust et al. 2008). However, PI3P may not be the only lipid involved in AP-3 recruitment as PI-4KIIα is enriched in AP-3-derived vesicles and it can directly bind AP-3 via its dileucine motif (Craige et al. 2008). Depletion of PI-4KIIα blocks the recruitment of AP-3 to membranes, and like the loss of PI-3KIIIC3 also causes a LAMP1 trafficking defect. At present it is not known whether the binding of AP-3 to phosphoinositides might cause a conformational switch in the complex which allows it to bind cargo. In the case of AP-1, it is thought that this switch may be primarily regulated by the binding of ARF1 and not phosphoinositides.

Phosphorylation

The only subunits of the AP-3 complex to be significantly phosphorylated are β3A and β3B (Faundez and Kelly 2000). These subunits are phosphorylated on serine residues present in acidic patches in their flexible linker regions. In β3A approximately 31% of the hinge residues are acidic and a further 26% are serine residues. The linker regions of β3A and β3B contain many potential casein kinase I and II sites. It has been shown that a casein kinase 1α activity co-purifies with the AP-3 complexes isolated from cytosol. Inhibition of casein kinase activity reduces AP-3-dependent synaptic vesicle budding in vitro. However, it is unclear whether there are other substrates for casein kinase in the budding assay. KIF3A has recently been identified as a β3A linker domain interacting protein and its binding has been shown to be regulated by the phosphorylation state of the hinge domain (Table 2). In vitro assays using yeast extract have suggested that the acidic patches in the β3 linker domain may be substrates for IP7-mediated pyrophosphorylation and the levels of pyrophosphorylation may regulate the interaction (Azevedo et al. 2009).

Summary

Since its discovery over 15 years ago a substantial amount of progress has been made in elucidating the function of the AP-3 complex. It is now known that the complex is involved in the biogenesis of lysosome-related organelles, and loss of the complex in humans causes Hermansky–Pudlak syndrome type 2. The number of cargo molecules the complex traffics and the interacting proteins it binds is steadily increasing. Progress has been made in understanding how the complex is recruited onto membranes. However, several major questions/challenges remain. How many trafficking routes is the AP-3 complex involved in? Does AP-3 have any appendage domain binding proteins? What role does BLOC-1 have in AP-3 function? Why does AP-3 bind clathrin but not require it for its function? How do μ3B and β3B give AP-3 neuronal-specific functions when they appear to bind the same proteins as μ3A and β3A? How do AP-3-binding partners regulate its function? How are all these interactions coordinated within the cell?

References

Azevedo C, Burton A, Ruiz-Mateos E, Marsh M, Saiardi A. Inositol pyrophosphate mediated pyrophosphorylation of AP3B1 regulates HIV-1 Gag release. Proc Natl Acad Sci USA. 2009;106(50):21161–6.

Badolato R, Parolini S. Novel insights from adaptor protein 3 complex deficiency. J Allergy Clin Immunol. 2007;120(4):735–41; quiz 42–3.

Baust T, Anitei M, Czupalla C, Parshyna I, Bourel L, Thiele C, et al. Protein networks supporting AP-3 function in targeting lysosomal membrane proteins. Mol Biol Cell. 2008;19(5):1942–51.

Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem. 2003;72:395–447.

Craige B, Salazar G, Faundez V. Phosphatidylinositol-4-kinase type II alpha contains an AP-3-sorting motif and a kinase domain that are both required for endosome traffic. Mol Biol Cell. 2008;19(4):1415–26.

Danglot L, Galli T. What is the function of neuronal AP-3? Biol Cell. 2007;99(7):349–61.

Dell'Angelica EC. AP-3-dependent trafficking and disease: the first decade. Curr Opin Cell Biol. 2009;21(4):552–9.

Dell'Angelica EC, Klumperman J, Stoorvogel W, Bonifacino JS. Association of the AP-3 adaptor complex with clathrin. Science. 1998;280(5362):431–4.

Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS. Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. Mol Cell. 1999;3(1):11–21.

Di Pietro SM, Falcon-Perez JM, Tenza D, Setty SR, Marks MS, Raposo G, et al. BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. Mol Biol Cell. 2006;17(9):4027–38.

Faundez VV, Kelly RB. The AP-3 complex required for endosomal synaptic vesicle biogenesis is associated with a casein kinase Ialpha-like isoform. Mol Biol Cell. 2000;11(8):2591–604.

Hirst J, Barlow LD, Francisco GC, Sahlender DA, Seaman MN, Dacks JB, et al. The fifth adaptor protein complex. PLoS Biol. 2011;9(10):e1001170.

Jackson LP, Kelly BT, McCoy AJ, Gaffry T, James LC, Collins BM, et al. A large-scale conformational change couples membrane recruitment to cargo binding in the AP2 clathrin adaptor complex. Cell. 2010;141(7):1220–9.

Janvier K, Kato Y, Boehm M, Rose JR, Martina JA, Kim BY, et al. Recognition of dileucine-based sorting signals from HIV-1 Nef and LIMP-II by the AP-1 gamma-sigma1 and AP-3 delta-sigma3 hemicomplexes. J Cell Biol. 2003;163(6):1281–90.

Lefrancois S, Janvier K, Boehm M, Ooi CE, Bonifacino JS. An ear-core interaction regulates the recruitment of the AP-3 complex to membranes. Dev Cell. 2004;7(4):619–25.

Nie Z, Boehm M, Boja ES, Vass WC, Bonifacino JS, Fales HM, et al. Specific regulation of the adaptor protein complex AP-3 by the Arf GAP AGAP1. Dev Cell. 2003;5(3):513–21.

Odorizzi G, Cowles CR, Emr SD. The AP-3 complex: a coat of many colours. Trends Cell Biol. 1998;8(7):282–8.

Ohno H, Aguilar RC, Yeh D, Taura D, Saito T, Bonifacino JS. The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals. J Biol Chem. 1998;273(40):25915–21.

Raposo G, Marks MS. Melanosomes – dark organelles enlighten endosomal membrane transport. Nat Rev Mol Cell Biol. 2007;8(10):786–97.

Simpson F, Peden AA, Christopoulou L, Robinson MS. Characterization of the adaptor-related protein complex, AP-3. J Cell Biol. 1997;137(4):835–45.

AP-4

Shinji Matsuda and Michisuke Yuzaki
Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Synonyms

Adaptor protein complex 4; Adaptor-related protein complex 4

Historical Background

In 1975, coated vesicles, which transport many membrane proteins, were purified and shown to include an ~180-kDa protein named clathrin (Pearse 1975). Later, other molecules involved in clathrin binding to the vesicles were also identified (Keen et al. 1979). In 1987, Keen et al. showed that these molecules formed heterotetrameric complexes, which were termed as adaptor protein complex (AP)-1 and AP-2 (Keen 1987). AP-1 and AP-2 are composed of two large subunits (γ and β1 for AP-1, α and β2 for AP-2), one medium subunit (μ1 for AP-1 and μ2 for AP-2) and one small subunit (σ1 for AP-1 and σ2 for AP-2). Later, another AP complex ▶ AP-3 was identified on the basis of its structural homology to AP-1 and AP-2 (Pevsner et al. 1994; Newman et al. 1995). The ▶ AP-3 complex, which consists of one molecule each of δ, β3, μ3, and σ3 subunits, is involved in protein trafficking and organelle biogenesis within the endosomal–lysosomal system. In 1997, Wang et al. identified a novel human protein termed μ-ARP2, which was homologous to the μ subunits of AP complexes (Wang and Kilimann 1997). Later studies identified three

additional proteins, ε, β4, and σ4, which form a novel adaptor protein complex AP-4, together with μ-ARP2 (renamed μ4) (Dell'Angelica et al. 1999; Hirst et al. 1999).

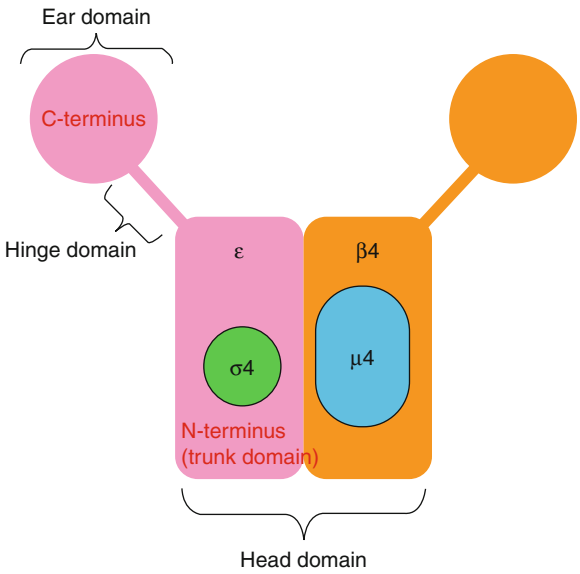
Basic Features

AP-4 is a heterotetrameric protein complex composed of two large subunits (β4 and ε), one medium subunit (μ4), and one small subunit (σ4). Because of the extensive similarities among the four AP complexes, AP-4 is likely to form a structure similar to AP-2 (Fig. 1). The C-terminal domain of two large subunits forms "ear domains," and the "head domain" is composed of the N-terminal domain (trunk domain) of two large subunits and medium and small subunits. The ear and head domains are connected by the flexible "hinge domains" of large subunits. The cargo proteins are thought to be recognized by the μ4 subunit.

Interestingly, AP-4 is found only in the mammals and plants, but the reason why other organisms have lost AP-4 during evolution is unknown. The all four subunits of AP-4 are expressed ubiquitously in almost all mammalian tissues. Immunocytochemical analysis using a Golgi stack maker mannosidase II and trans-Golgi network (TGN) markers TGN38 and furin indicates that the AP-4 complex is associated with the TGN (Dell'Angelica et al. 1999; Hirst et al. 1999; Simmen et al. 2002). Brefeldin A treatment caused dissociation of AP-4 from the TGN, suggesting that the recruitment of AP-4 to TGN membranes is regulated by a member of ADP ribosylation factor family protein (Dell'Angelica et al. 1999; Hirst et al. 1999). Immunoelectron microscopic analysis further indicated that AP-4 localized on the nonclathrin-coated vesicles in TGN (Hirst et al. 1999). Indeed, the hinge domains of the ε and β4 subunits lack clathrin-binding motifs conserved in other adaptor protein complexes. These results suggest that AP-4 is likely to be involved in sorting of nonclathrin-coated vesicles in post-Golgi compartments.

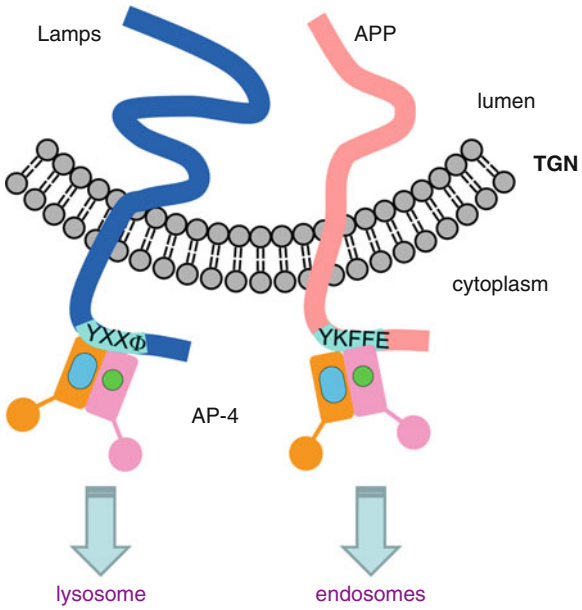
Function of AP-4: Transport to Lysosomes

The YXXØ (where Ø indicates a bulky hydrophobic residue) motif mediates targeting of vesicles at various intracellular transport steps, such as endocytosis, targeting from the plasma membrane to the TGN, targeting to lysosomal compartments, and basolateral sorting in polarized cells. Like μ subunits in other AP complexes, the μ4 subunit of AP-4 binds to the YXXØ



AP-4, Fig. 1 Schematic drawing of the AP-4 complex. AP-4 consists of two large subunits (ε and β4), one medium subunit (μ4), and one small subunit (σ4). The C-terminal domain of large subunits forms ear domains. Head domains are composed of two N-terminal trunk domains of large subunits, medium subunit and small subunit. Ear and head domain are connected by hinge domains of large subunits. The interactions between ε subunit and σ subunit, β4 subunit and μ4 subunit, and ε subunit and β4 subunit have been shown by yeast two-hybrid experiments

motif of several proteins, such as lysosomal membrane proteins Lamp-1 (Igp120) and CD-63 (Lamp-3) (Hirst et al. 1999; Stephens and Banting 1998). Screening of a combinatorial peptide library has shown that μ4 prefers aspartic acid at position Y+1, proline or arginine at Y+2, and phenylalanine at positions Y−1 and Y+3 (Aguilar et al. 2001). A signal that fits this preference is found in Lamp-2. Indeed, Tac chimeral proteins bearing a μ4-specific tyrosine-based sorting signal were targeted to the endosomal–lysosomal system. Nevertheless, knockdown of μ4 has no effect on the localization of these AP-4 interacting proteins to lysosomes (Janvier and Bonifacino 2005). This may be because the interaction between μ4 and the YXXØ motif is very weak (Stephens and Banting 1998) and Lamps are mainly trafficked to lysosomes by endocytosis from the plasma membrane via AP-2 (Janvier and Bonifacino 2005). However, approximately half of the Lamps still reach lysosomes in cells depleted with AP-2, indicating that AP-4 (and other APs) may also be involved in direct transport of Lamps from TGN to lysosomes under certain conditions (Fig. 2).



AP-4, Fig. 2 Schematic drawing of cargo protein recognition at the trans-Golgi network (TGN) and transport to lysosome and endosomes. AP-4 binds to the tyrosine-based sorting motifs (YXXΦ) in certain lysosomal membrane proteins (Lamps) and the sequence YKFFE located in the cytoplasmic region of the Alzheimer's disease amyloid precursor protein (APP) via the μ4 subunit. This association is essential for the transport of these cargo proteins from TGN to lysosome and endosomes

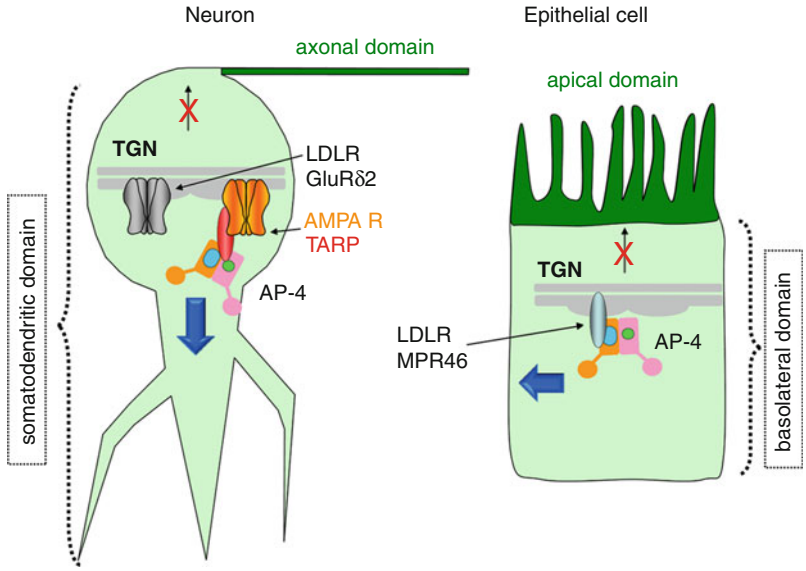
Function of AP-4: Endosomal Targeting of APP

Recently, the μ4 subunit has been reported to interact with the sequence YKFFE located in the cytoplasmic region of the Alzheimer's disease amyloid precursor protein (APP) (Burgos et al. 2010). The YKFFE motif encompasses a YXXØ motif, but it does not bind to μ subunits of other AP complexes. Interestingly, X-ray crystallographic structure determination shows that the YKFFE sequence binds to a site on μ4 that is different from the YXXØ-binding site on the μ2 subunit of AP-2. A mutation in the YKFFE sequence or depletion of μ4 shifted the distribution of APP from endosomes to the TGN, suggesting that AP-4 mediates transport of APP from TGN to endosomes. Impaired transport of APP to endosomes enhanced the generation of pathogenic amyloid β peptide by γ-secretase-catalyzed cleavage of APP. These findings indicate that the interaction between AP-4 and APP regulates APP trafficking at TGN or in the late secretory pathway. Furthermore, they suggest that defects in AP-4 could be a potential risk factor of Alzheimer's disease by producing excessive amyloid β peptide.

Function of AP-4: Polarized Sorting

The plasma membrane of epithelial cells is polarized: they are differentiated into apical and basolateral domains, containing distinctive sets of proteins and lipids. When AP-4 mRNA is disrupted, basolaterally transported proteins, such as the low-density lipoprotein receptor (LDLR) and 46 K cation-dependent mannose-6-phosphate receptor, were distributed nonselectively to both basolateral and apical domains (Simmen et al. 2002). Similarly, Tac chimeral proteins bearing a cytosolic domain of furin were missorted to apical domains. These results indicate that AP-4 mediates basolateral sorting of membrane proteins in epithelial cells (Fig. 3). However, basolateral sorting is also regulated by the AP-1 adaptor protein complex containing the μ1B subunit, which is specifically expressed in most epithelial cells (Ohno et al. 1999). Indeed, many basolateral proteins, such as LDLR and transferrin receptor, are missorted to the apical domain of the epithelial cell line lacking μ1B. Therefore, AP-1B and AP-4 may play complementary and sometimes redundant roles.

Neurons are also highly polarized cells composed of the somatodendritic domain and the axonal domain. The somatodendritic domain receives inputs from other neurons, and the axonal domain transports signals to other cells. Certain neurotransmitter receptors localize in the somatodendritic domain to receive signals from other neurons. For example, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptor is mainly transported to the dendrites where it mediates the majority of excitatory synaptic transmission. Disruption of the gene encoding the β subunit of AP-4 resulted in nonselective distribution of AMPA receptors in axons of hippocampal neurons and cerebellar Purkinje cells both in vitro and in vivo (Matsuda et al. 2008). Similarly, the δ2 subtype of glutamate receptor (▶ GluD2) and LDLR were missorted to axons in AP-4$\beta^{-/-}$ neurons. In contrast, the GluN1 subunit of *N*-methyl-D-aspartate (NMDA) receptors and metabotropic glutamate receptor (mGluR) 1α proteins was selectively targeted to the somatodendritic domain even in the AP-4$\beta^{-/-}$ neurons. These results indicate that AP-4 mediates the somatodendritic transport of AMPA receptors, ▶ GluD2, and LDLR. In addition, because neurons lack AP-1B, certain somatodendritic cargos, such as mGluR1 and GluN1, are likely recognized by unknown adaptor proteins other than AP-4 and AP-1B.



AP-4, Fig. 3 Polarized sorting in neuronal cells and epithelial cells by AP-4. α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors indirectly bind to the μ4 subunit of AP-4 via transmembrane AMPA receptor regulatory proteins (TARPs). LDL receptors (LDLR) and the δ2 glutamate receptor (GluD2) directly bind to μ4. These proteins are sorted to the somatodentritic domain at TGN (*left panel*). In epithelial cells, AP-4 binds to cargo proteins, such as LDLR and 46 K cation-dependent mannose-6-phosphate receptor (MPR46), via the μ4 subunit and regulates basolateral sorting at the TGN (*right panel*)

A motif containing FR and FTF in the C-terminus of the δ2 subtype of glutamate receptors was shown to bind to the μ4 subunit of AP-4 (Yap et al. 2003). In contrast, μ4 does not directly bind to AMPA receptors but associates with transmembrane AMPA receptor regulatory proteins (TARPs), which tightly bind to all subunits of AMPA receptors (Nicoll et al. 2006). An unconventional motif containing phenylalanine and tyrosine (YRYRF) was shown to be essential for the binding of TARP γ3 to μ4 (Matsuda et al. 2008). When TARPs are expressed in hippocampal neurons, they are excluded from axons in wild-type neurons, whereas they are missorted to the axons in AP-4$\beta^{-/-}$ hippocampal neurons. Furthermore, the specific disruption of the interaction between AP-4 and TARPs caused the mislocalization of endogenous AMPA receptors in the axons of wild-type neurons (Matsuda et al. 2008). These results indicate that AP-4 regulates proper somatodendritic-specific distribution of AMPA receptors by binding to TARPs in neurons (Fig. 3).

Human Diseases: AP-4 Deficiency Syndrome

Recently, autosomal recessive loss-of-function mutations in any one of the four subunits of AP-4 are shown to result in similar neurodevelopmental human disorders (Verkerk et al. 2009; Moreno-De-Luca et al. 2011; Abou Jamra et al. 2011). These include five patients in one family lacking the μ4 subunit, four patients in two families with disrupted ε, three patients in one family lacking σ4, and three patients in one family lacking β4. Commonly observed symptoms include an infantile muscular hypotonia that progresses to spastic tetraplegia and hypertonia, leading to inability to walk, severe intellectual disability, absent or markedly delayed speech, stereotypic laughter, and growth retardation. The disruption of any subunit generally destabilizes the entire AP complexes. Indeed, knockdown of μ4 or knockout of β4 results in depletion of the entire AP-4 complex in mice (Matsuda et al. 2008). Thus, these human disorders are now referred to as "AP-4 deficiency syndrome."

As observed in AP-4$\beta^{-/-}$ mice (Matsuda et al. 2008), postmortem brain histology showed irregular thickening of Purkinje cell axons and aberrant localization of ▶ GluD2 in a patient lacking the μ4 subunit (Verkerk et al. 2009). Nevertheless, symptoms of AP-4 deficiency syndrome are generally severer than those observed in AP-4$\beta^{-/-}$ mice. For example, although the mice exhibited a significantly poorer rotorod

performance than wild-type mice, they could walk along a straight line with regular steps throughout their lives. No significant differences in body weight or grip power were observed between the wild-type and AP-4$\beta^{-/-}$ mice. In addition, there seems to be a certain variation in the severity of symptoms in human patients. In a family lacking β4, some patient showed normal speech and could walk independently until the age of 2 (Abou Jamra et al. 2011). Thus, it remains to be determined what causes each symptom in human and what makes difference in phenotypes between human and mice.

Summary

AP-4 is the most recently identified AP complex, whose function has just started to be clarified. It mainly localizes in the TGN and mediates the trafficking of various membrane proteins. It binds to the certain types of tyrosine-based lysosomal trafficking motifs. It also mediates APP trafficking at TGN or in the late secretory pathway. Moreover, in polarized cells, such as the epithelial cells and neurons, AP-4 regulates the polarized sorting of several membrane proteins, such as AMPA receptors and ▶ GluD2. The loss-of-function mutations in any one of the four subunits of AP-4 result in similar neurodevelopmental human disorders called AP-4 deficiency syndrome. It remains to be determined how AP-4 mediated trafficking plays role in the brain development and symptoms in these disorders.

References

Abou Jamra R, Philippe O, Raas-Rothschild A, Eck SH, Graf E, Buchert R, et al. Adaptor protein complex 4 deficiency causes severe autosomal-recessive intellectual disability, progressive spastic paraplegia, shy character, and short stature. Am J Hum Genet. 2011;88:788–95.

Aguilar RC, Boehm M, Gorshkova I, Crouch RJ, Tomita K, Saito T, et al. Signal-binding specificity of the mu4 subunit of the adaptor protein complex AP-4. J Biol Chem. 2001;276:13145–52.

Burgos PV, Mardones GA, Rojas AL, da Silva LL, Prabhu Y, Hurley JH, et al. Sorting of the Alzheimer's disease amyloid precursor protein mediated by the AP-4 complex. Dev Cell. 2010;18:425–36.

Dell'Angelica EC, Mullins C, Bonifacino JS. AP-4, a novel protein complex related to clathrin adaptors. J Biol Chem. 1999;274:7278–85.

Hirst J, Bright NA, Rous B, Robinson MS. Characterization of a fourth adaptor-related protein complex. Mol Biol Cell. 1999;10:2787–802.

Janvier K, Bonifacino JS. Role of the endocytic machinery in the sorting of lysosome-associated membrane proteins. Mol Biol Cell. 2005;16:4231–42.

Keen JH. Clathrin assembly proteins: affinity purification and a model for coat assembly. J Cell Biol. 1987;105:1989–98.

Keen JH, Willingham MC, Pastan IH. Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. Cell. 1979;16:303–12.

Matsuda S, Miura E, Matsuda K, Kakegawa W, Kohda K, Watanabe M, et al. Accumulation of AMPA receptors in autophagosomes in neuronal axons lacking adaptor protein AP-4. Neuron. 2008;57:730–45.

Moreno-De-Luca A, Helmers SL, Mao H, Burns TG, Melton AM, Schmidt KR, et al. Adaptor protein complex-4 (AP-4) deficiency causes a novel autosomal recessive cerebral palsy syndrome with microcephaly and intellectual disability. J Med Genet. 2011;48:141–4.

Newman LS, McKeever MO, Okano HJ, Darnell RB. Beta-NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein. Cell. 1995;82:773–83.

Nicoll RA, Tomita S, Bredt DS. Auxiliary subunits assist AMPA-type glutamate receptors. Science. 2006;311:1253–6.

Ohno H, Tomemori T, Nakatsu F, Okazaki Y, Aguilar RC, Foelsch H, et al. Mu1B, a novel adaptor medium chain expressed in polarized epithelial cells. FEBS Lett. 1999;449:215–20.

Pearse BM. Coated vesicles from pig brain: purification and biochemical characterization. J Mol Biol. 1975;97:93–8.

Pevsner J, Volknandt W, Wong BR, Scheller RH. Two rat homologs of clathrin-associated adaptor proteins. Gene. 1994;146:279–83.

Simmen T, Honing S, Icking A, Tikkanen R, Hunziker W. AP-4 binds basolateral signals and participates in basolateral sorting in epithelial MDCK cells. Nat Cell Biol. 2002;4:154–9.

Stephens DJ, Banting G. Specificity of interaction between adaptor-complex medium chains and the tyrosine-based sorting motifs of TGN38 and lgp120. Biochem J. 1998;335(Pt 3):567–72.

Verkerk AJ, Schot R, Dumee B, Schellekens K, Swagemakers S, Bertoli-Avella AM, et al. Mutation in the AP4M1 gene provides a model for neuroaxonal injury in cerebral palsy. Am J Hum Genet. 2009;85:40–52.

Wang X, Kilimann MW. Identification of two new mu-adaptin-related proteins, mu-ARP1 and mu-ARP2. FEBS Lett. 1997;402:57–61.

Yap CC, Murate M, Kishigami S, Muto Y, Kishida H, Hashikawa T, et al. Adaptor protein complex-4 (AP-4) is expressed in the central nervous system neurons and interacts with glutamate receptor delta2. Mol Cell Neurosci. 2003;24:283–95.

APC

▶ Glycogen Synthase Kinase-3

APKD2

▶ Polycystin-2

APO2L/TRAIL

Luis Martinez-Lostao, Alberto Anel and Javier Naval
Departamento de Bioquímica, Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain

Historical Background

Apo2 ligand/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) was independently identified by two different groups as the third member of the tumor necrosis factor (TNF) superfamily capable of inducing apoptosis in transformed cells while sparing normal cells (LeBlanc and Ashkenazi 2003). Apo2L/TRAIL is a type-II membrane protein, composed of 281 amino acids and its gene, designated as *TNFSF10*, has been located on human chromosome 3 at locus 3q26. Its C-terminal extracellular domain shares significant homology with other members of the TNF superfamily, whereas the N-terminal does not. The polypeptide moiety of the Apo2L/TRAIL monomer has a predicted molecular mass of 32.5 kDa but its mature, fully glycosylated form, has a molecular mass of around 41 kDa. There is a potential cleavage site in the extracellular domain of Apo2L/TRAIL at amino acid position 114 which would generate a soluble form of 24 kDa. Like other members of TNF superfamily, Apo2L/TRAIL is a homotrimer, with each monomer composed of two antiparallel β-sheets. Native Apo2L/TRAIL contains a central Zn atom buried at the trimer interface, which is important for the stability, solubility, and biological activity of the protein (Hymowitz et al. 2000).

Apo2L/TRAIL and Its Receptors

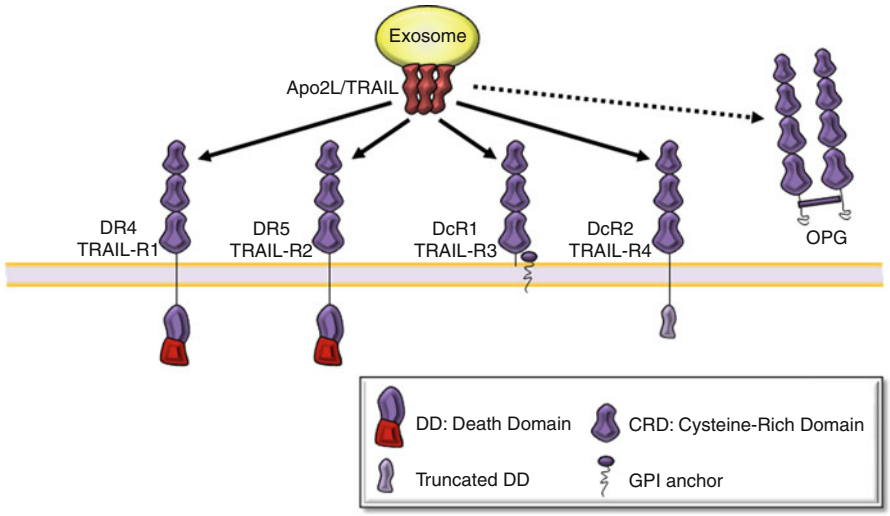
Apo2L/TRAIL can bind to a complex system of receptors with different affinities and distinct signaling outcomes. Five receptors for Apo2L/TRAIL are known in humans, called TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, and TRAIL-R4/DcR2 (Fig. 1) (Ashkenazi and Dixit 1998). Finally, a soluble receptor termed osteoprotegerin (OPG) can also bind to Apo2L/TRAIL.

Only DR4 and DR5 possess a complete death domain (DD) in their intracellular moiety and can transduce apoptotic signals. Apo2L/TRAIL binds to DR4 or DR5 inducing their oligomerization and the subsequent formation of a multiprotein complex called death-inducing signaling complex or DISC (Walczak et al. 1999; Kischkel et al. 2000). DcR1 and DcR2 are two non-apoptotic membrane-bound receptors for Apo2L/TRAIL (Sheridan et al. 1997). DcR1 is a glycosylphosphatidylinositol (GPI)-anchored receptor and lacks a cytoplasmic domain. DcR2 contains a truncated DD, unable to transduce apoptotic signals. DcR1 and DcR2 seem to act as decoy receptors inhibiting Apo2L/TRAIL interaction with death receptors when overexpressed or, alternatively, interfering with the proper assembly of DR4 and DR5 prior to ligand binding. Alternative ways of inhibiting TRAIL apoptotic signaling by DcR1 and DcR2 have been reported. While DcR1 would prevent the assembly of the death-inducing signaling complex (DISC) by titrating Apo2L/TRAIL within lipid rafts, DcR2 would be co-recruited with DR5 within the DISC, thus preventing initiator caspase activation. Anyway, the physiological role of these receptors *in vivo* remains elusive. They might act more as regulatory molecules than decoy receptors.

Finally, Apo2L/TRAIL can bind, but with lower affinity, to a soluble receptor called OPG (LeBlanc and Ashkenazi 2003). The main function of OPG is the binding and modulation of the interaction of receptor activator of NF-κB ligand (RANKL), another TNF superfamily member, with its cell-surface receptor, RANK. RANKL-RANK interaction induces osteoclast activation, differentiation, and bone resorption. Since Apo2L/TRAIL- or DR5-deficient mice (the only TRAIL receptor expressed in mouse) are viable and exhibit normal bone density, it is unlikely that Apo2L/TRAIL may have a role in bone remodeling.

Apo2L/TRAIL Signaling

The initial step of apoptosis induced by Apo2L/TRAIL is the binding of the trimeric ligand to DR4 and/or DR5. Apo2L/TRAIL-DR4 or -DR5 interaction causes clustering of the death receptor followed by that recruiting of adaptor protein Fas-associated death domain (FADD), which in turn promotes the assembly



APO2L/TRAIL, Fig. 1 *Schematic representation of binding of homotrimeric exosome-bound human Apo2L/TRAIL to its receptors.* In humans, Apo2L/TRAIL binds to two pro-apoptotic receptors, TRAIL-R/DR4 (TNFRSF10A) and TRAIL-R2/DR5 (TNFRSF10B), which contain a death domain (DD). Apo2L/TRAIL can also bind to TRAIL-R3/DcR1 (TNFRSF10C), which is a GPI-anchored protein and lacks a cytoplasmic domain, and to TRAILR4/DcR2 (TNFRSF10D) which has a truncated DD. Similar to other TNFRs, TRAIL receptors possess cystein-rich extracellular domains (CRD). Apo2L/TRAIL also binds to the soluble protein osteoprotegerin (OPG)

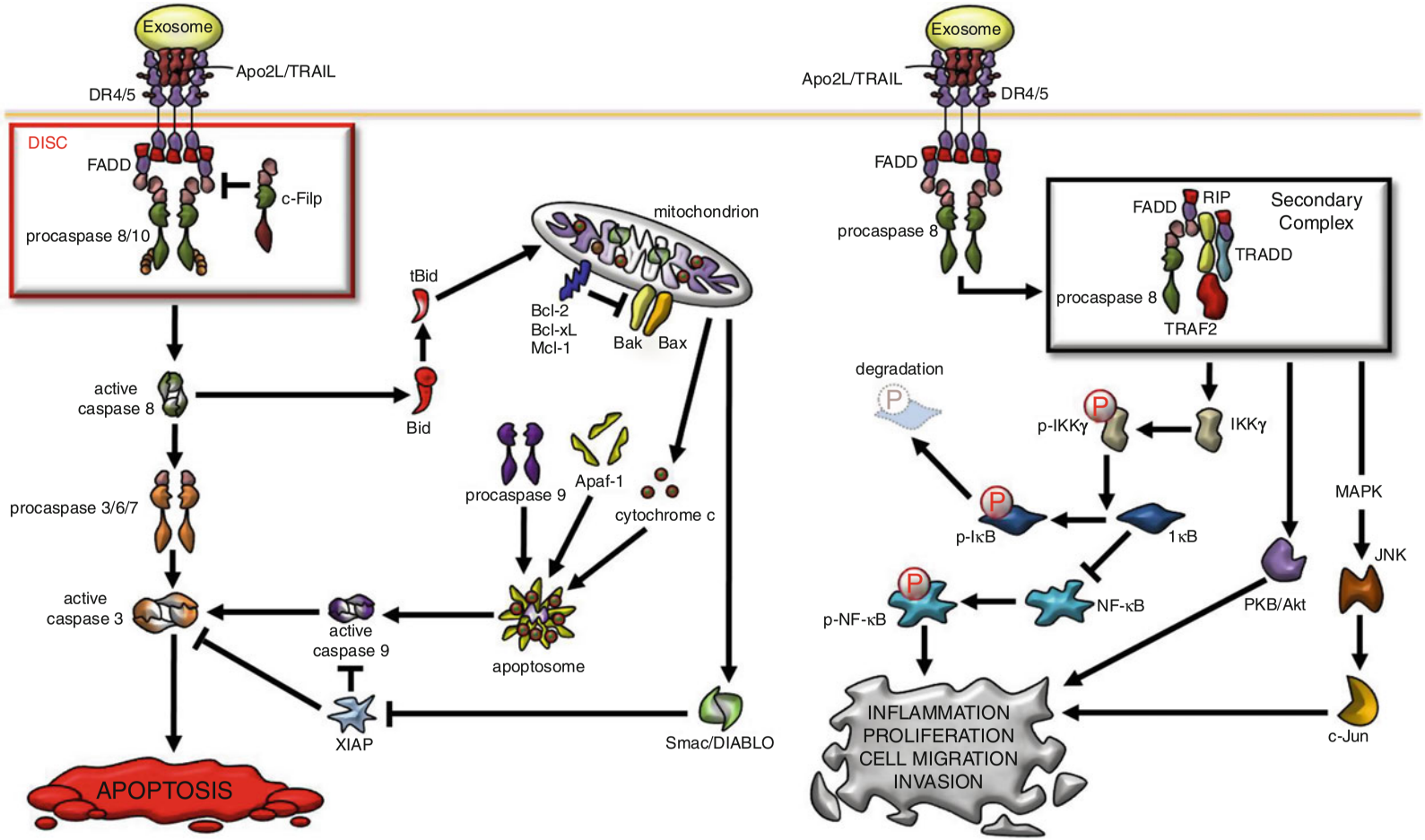
of DISC. The homotypic interaction of FADD with the DRs through their respective DDs exposes the death-effector domain (DED) of FADD and allows it to homotypically bind to procaspase-8 or -10 (Sprick et al. 2000). Recruitment of procaspase-8 to the DISC induces its activation by conformational change followed by autoprocessing and release into the cytosol. Active caspase-8 then cleaves and activates executioner caspases -3 and -7. Caspase-3 in turn cleaves a hundred of cell proteins, including caspase-6, and executes the apoptotic death program (Fig. 2).

DR4 and DR5 receptors may also recruit receptor-interacting protein (RIP) into the receptor complex upon Apo2L/TRAIL binding. RIP phosphorylates IκB kinase (IKK), leading to its degradation. Degradation of IκB promotes phosphorylation of transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Apo2L/TRAIL also activates mitogen-activated protein kinase (MAPK) and protein kinase B (PKB) signaling.

Caspase-8 and -10 are recruited to and activated at DISC with similar kinetics and may function independently of each other. Some authors have reported that caspase-10 may substitute for caspase-8 but, in some cell types, caspase-10 do not restore the sensitivity to Apo2L/TRAIL in caspase-8-deficient cells and so this point remains controversial.

Depending on the cell type, activation of apoptosis through the extrinsic pathway may or not require help from the intrinsic pathway. In type I cells, the DISC-initiated caspase cascade is enough to activate effector caspases and hence the apoptosis induction. By contrast, in type II cells, in which less active caspase-8 seems to be generated, signal amplification through the mitochondrial pathway is needed. The link between the death receptor-triggered extrinsic pathway and the mitochondrial intrinsic pathway is the BH3-only protein, Bid. Upon DISC formation, Bid is cleaved by caspase-8 generating a truncated form of Bid (tBid) that translocates to mitochondrial outer membrane. There, tBid binds to and activates Bax and Bak leading to the release of cytochrome c and other proapoptotic factors from mitochondria. Cytochrome *c*, in the presence of (d)ATP binds to and induces heptamerization of apoptosis protease activating factor 1 (Apaf-1), forming the apoptosome, which recruits and activates by conformational change, procaspase-9. In his turn, activated caspase-9 cleaves executioner caspases (−3 and −7) leading to apoptosis. The mechanism of Apo2L/TRAIL apoptotic signal transduction is similar to that triggered by FasL, another death ligand from the TNF superfamily.

Apo2L/TRAIL apoptotic signaling pathway is regulated at different levels. Not all proteins present in the



APO2L/TRAIL, Fig. 2 *Schematic representation of Apo2L/TRAIL apoptotic and non-apoptotic signaling pathways.* Binding of Apo2L/TRAIL to their respective receptors induces receptor trimerization and formation of the death-inducing signaling complex (DISC), a multiprotein complex containing the adaptor FADD and procaspases-8 and -10. DISC-activated caspases-8 and -10 trigger a caspase cascade by cleaving caspase-3. cFLIP, another protein contained into the DISC can abrogate caspase-8 activation by competing with caspase-8 for the binding to FADD. In some cell types (type I cells), activation of the extrinsic pathway is sufficient to induce Apo2L/TRAIL-induced apoptosis whereas in other cell types (type II), the mitochondrial apoptosis pathway is engaged by the caspase-8 cleaving of proapoptotic Bid protein, leading to release of cytochrome *c* and Smac/DIABLO from mitochondria

DISC are proapoptotic. Cellular FLICE inhibitory protein (cFLIP), which shares high sequence homology with caspase-8 and -10, may inhibit caspase activation at the DISC by competing for binding to FADD. There are a number of splicing variants of cFLIP but only a longer ($cFLIP_L$) and a shorter version ($cFLIP_S$) can usually be detected at protein level. Both isoforms of cFLIP are recruited to the DISC by homotypic DED interactions. The C-terminal part of $cFLIP_L$ consists of two catalytically inactive caspase-like domains whereas the C-terminal portion of $cFLIP_S$ is neither homologous to procaspase-8 nor -10. $cFLIP_S$ can block caspase-8 processing and activation at the DISC. However, the role of $cFLIP_L$ depends on the molecular context: it may function as pro- or anti-apoptotic depending on the stoichiometry of the different cFLIP proteins and that of caspase-8. A recent model combining experimental data and mathematical modelization indicates that $cFLIP_L$, at moderate levels, may facilitate caspase-8 activation at DISC when the intensity of death signal is strong, but if death ligand concentration is low $cFLIP_L$ slows down induction of cell death.

Sensitivity to Apo2L/TRAIL-induced apoptosis may also be modulated by X chromosome-linked inhibitor of apoptosis (XIAP), a endogenous inhibitor of caspases-3, -7 and -9 which prevents inadvertent caspase activation in living cells. Interfering with XIAP expression renders cells highly sensitive to Apo2L/TRAIL bypassing the requirement for mitochondrial amplification in type II cells. Second mitochondrial activator of caspases/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a protein released from mitochondria during apoptosis, binds to and antagonizes XIAP. It has been proposed that the ratios of cFLIP to caspase-8 and XIAP to Smac/DIABLO together may determine whether the cells respond in a type I or type II way to DRs-Apo2L/TRAIL interactions (Fig. 2) (Gonzalvez and Ashkenazi 2010).

Several mechanisms of different nature have been described that may regulate Apo2L/TRAIL signaling. Posttranslational modifications of DR4 and DR5 by glycosylation or palmitoylation seem to be important modulators of the initial events of Apo2L/TRAIL signaling. *O*-glycosylation promoted ligand-stimulated clustering of DR4 and DR5, the first step in DISC formation, essential for recruitment and activation of the initiator caspase-8. Conversely, depletion of certain *O*-glycosylation enzymes attenuates caspase-8 activation at the DISC, whereas overexpression of GALNT14, the *O*-glycosylation-initiating enzyme, enhances the formation of the DISC and sensitizes cells to Apo2L/TRAIL (Wagner et al. 2007). Upon binding of Apo2L/TRAIL to its plasma membrane receptor(s), endocytosis of the ligand-receptor complex usually occurs. Both DR4 and DR5 are rapidly internalized in lipid vesicles after ligation through a dynamin-dependent mechanism. However, contrary to Fas signaling, internalization of the ligand-receptor complex is not required for Apo2L/TRAIL-mediated DISC formation and apoptosis signaling. Recently, ubiquitylation has been shown to be a crucial mechanism which regulates full activation of caspase-8. Death receptor ligation by Apo2L/TRAIL induces polyubiquitination of caspase-8, through an interaction of the DISC with a cullin3 (CUL3)-based E3 ligase (Jin et al. 2009).

Rather than inducing apoptosis, Apo2L/TRAIL can induce survival signals in some cell types. The signaling pathways mainly implicated in Apo2L/TRAIL non-apoptotic signaling are the ▶ NF-κB, MAPK and c-Jun N-terminal kinase (JNK) pathways (Fig. 2) (Falschlehner et al. 2007). Ligand engagement of DR5 quickly leads to formation of the DISC, which signals apoptosis. Subsequently, the primary complex dissociates and multiple proteins including RIP, TNF receptor–associated factor 2 (TRAF2), IKKγ and TNF receptor–associated DD (TRADD), organize into a secondary complex that lacks the ligand and the receptor but contains FADD and caspase-8. This secondary complex may be responsible for the activation of the ▶ NF-κB and MAPK pathways such as JNK and p38. The biological significance of ▶ NF-κB activation by Apo2L/TRAIL is not fully understood but it may serve to restrict apoptosis induction. However, ▶ NF-κB activation could act to increase the threshold of sensitivity to Apo2L/TRAIL-induced apoptosis. Since caspase activation following Apo2L/TRAIL stimulation occurs rapidly, the protection provided by ▶ NF-κB against apoptosis is probably insufficient except in cells that do not activate easily the caspase cascade. Accordingly, ▶ NF-κB activation by Apo2L/TRAIL has been implicated in proliferation, cell migration, and invasion in certain tumor cell lines resistant to Apo2L/TRAIL-induced apoptosis.

It has also been reported that Apo2L/TRAIL can lead to activation of Akt/PKB, a protein kinase typically involved in cell survival and migration. Finally, other studies have shown that Apo2L/TRAIL is able to promote cell proliferation and differentiation though activation of extracellular regulated kinase (ERK), other member of the MAPK family.

In summary, although the physiological relevance of the Apo2L/TRAIL-induced non-apoptotic signaling is not fully established, these observations must be considered in particular in cell types where Apo2L/TRAIL does not induce a rapid apoptosis.

Biological Role of Apo2L/TRAIL

Although the ability of Apo2L/TRAIL to kill certain transformed cells by apoptosis is well established, its physiological role is not fully understood. Studies with mice deficient for Apo2L/TRAIL or its apoptosis-inducing receptor (DR5/TRAIL-R), as well as experiments carried out with Apo2L/TRAIL-blocking agents have led to unraveling diverse functions of Apo2L/TRAIL in vivo. Apo2L/TRAIL deficient mice do not display any overt developmental defects. Similarly, TRAIL-R knockout mice are viable and normally develop indicating that Apo2L/TRAIL signaling in not essential for normal embryonic development (Falschlehner et al. 2009).

The major roles of Apo2L/TRAIL are exerted in the immune system, shaping and regulating the immune response. This was early suggested by the inducible expression of Apo2L/TRAIL in immune cells. At least in human-activated T cells, Apo2L/TRAIL is stored inserted in the inner membrane vesicles of cytoplasmic multivesicular bodies, also known as secretory lysosomes. When T-lymphocytes receive activation signals, native Apo2L/TRAIL is secreted to extracellular medium in the form of microvesicles (exosomes) after fusion of the outer membrane of secretory lysosomes with the plasma membrane. This membrane-bound form of Apo2L/TRAIL displays full pro-apoptotic activity (Anel et al. 2007) (Figs. 1 and 2).

The immunoregulatory role of Apo2L/TRAIL is dependent on two different mechanisms: (1) Apo2L/TRAIL can inhibit IL2-dependent human $CD8^+$ T cell blast proliferation through a cell-cycle arrest in G_2/M and (2) Apo2L/TRAIL is also able to induce apoptosis of $CD8^+$ T cell blasts but, in this case, an additional restimulation is needed. It has also been clearly demonstrated that Apo2L/TRAIL regulates $CD8^+$ T cell memory (Janssen et al. 2005; Anel et al. 2007). The key role of Apo2L/TRAIL in the regulation of T cell responses has been confirmed in Apo2L/TRAIL knockout mice, which are much more susceptible to develop experimentally induced autoimmune diseases, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, than wild-type mice (Lamhamedi-Cherradi et al. 2003). In fact, Apo2L/TRAIL has been proposed to be used as a treatment in several experimental models of those autoimmune diseases with good results, especially in the case of animal models of rheumatoid arthritis. The efficiency of Apo2L/TRAIL for the treatment of this inflammatory disease is greatly improved through its association with liposomes, mimicking its physiological released form in exosomes.

Apo2L/TRAIL is one of the effector arms of natural killer (NK) cells and plays a key role in NK cell-mediated, interferon (IFN)-γ-dependent, suppression of tumor cell growth and prevention of metastasis formation. Indeed, one of the physiological roles of Apo2L/TRAIL seems to be the tumor immune surveillance. TRAIL knockout mice are more susceptible to cell-inoculated and chemically induced tumors as well as to metastasis dissemination than wild-type mice. In addition, TRAIL-deficient mice show a high rate of spontaneous hematological tumors appearing at old age (Zerafa et al. 2005). The low systemic toxicity of Apo2L/TRAIL on normal cells, while exerting a potent pro-apoptotic activity on a variety of human tumors have led to the development of different recombinant versions of Apo2L/TRAIL and agonistic monoclonal antibodies to its signaling receptors (anti-DR4 or anti-DR5). These agents, alone or in combination with chemotherapeutic drugs, provide new and promising approaches to cancer treatment (Ashkenazi et al. 2008).

Summary

The membrane protein Apo2 ligand/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) is a cytokine which interacts with a complex system of membrane receptors and triggers the extrinsic pathway of apoptosis. The ability of Apo2L/TRAIL to kill tumor cells while sparing normal cells makes this cytokine a promising antitumor agent. In fact, numerous clinical trials using recombinant forms or Apo2L/TRAIL or agonistic antibodies are currently underway. During

the last decade, it has become apparent that Apo2L/TRAIL is key in the immune system function, mainly in tumor immune surveillance and in downregulation of immune response. Apo2L/TRAIL exerts immunosuppressive and immunoregulatory functions, important for immune homeostasis, tumor control, and prevention of autoimmunity. Hence, apart from the intended use of Apo2L/TRAIL as a chemotherapeutic agent, a recent body of evidence suggests that this cytokine may also be a promising therapeutic agent for autoimmune diseases. However, distinct aspects of the physiological role of Apo2L/TRAIL in cancer and autoimmunity need to be unraveled to fully understand the importance of this signaling pathway. Among these aspects for future research, it stands out to fully unravel the role of Apo2L/TRAIL in the immune system, as well as to establish the true role of non-apoptotic signaling by Apo2L/TRAIL in vivo.

Further investigations on the mechanism of action of this cytokine will unravel the complexities of its in vivo function and ultimately apply this knowledge to a better treatment of cancer and autoimmune diseases.

References

Anel A, Bosque A, Naval J, Pineiro A, Larrad L, Alava MA, Martinez-Lorenzo MJ. Apo2L/TRAIL and immune regulation. Front Biosci. 2007;12:2074–84. doi:10.2741/2212.

Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science. 1998;281:1305–8. doi:10.1126/science.281.5381.1305.

Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol. 2008;26:3621–30. doi:10.1200/JCO.2007.15.7198.

Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol. 2007;39:1462–75. doi:10.1016/j.biocel.2007.02.007.

Falschlehner C, Schaefer U, Walczak H. Following TRAIL's path in the immune system. Immunology. 2009;127:145–54. doi:10.1111/j.1365-2567.2009.03058.x.

Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene. 2010;29:4752–65. doi:10.1038/onc.2010.221.

Hymowitz SG, O'Connell MP, Ultsch MH, Hurst A, Totpal K, Ashkenazi A, de Vos AM, Kelley RF. A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. Biochemistry. 2000;39:633–40. doi:10.1021/bi992242l.

Janssen EM, Droin NM, Lemmens EE, Pinkoski MJ, Bensinger SJ, Ehst BD, Griffith TS, Green DR, Schoenberger SP. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature. 2005;434:88–93. doi:10.1038/nature03337.

Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR, Ashkenazi A. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell. 2009;137:721–35. doi:10.1016/j.cell.2009.03.015.

Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity. 2000;12:611–20. doi:10.1016/S1074-7613(00)80212-5.

Lamhamedi-Cherradi SE, Zheng SJ, Maguschak KA, Peschon J, Chen YH. Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL−/− mice. Nat Immunol. 2003;4:255–60. doi:10.1038/ni894.

LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ. 2003;10:66–75. doi:10.1038/sj.cdd.4401187.

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P, Ashkenazi A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science. 1997;277:818–21. doi:10.1126/science.277.5327.818.

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, Krammer PH, Walczak H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity. 2000;12:599–609. doi:10.1016/S1074-7613(00)80211-3.

Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, Huw L, Katta V, Cavet G, Hymowitz SG, Amler L, Ashkenazi A. Death-receptor *O*-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med. 2007;13:1070–7. doi:10.1038/nm1627.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med. 1999;5:157–63. doi:10.1038/5517.

Zerafa N, Westwood JA, Cretney E, Mitchell S, Waring P, Iezzi M, Smyth MJ. Cutting edge: TRAIL deficiency accelerates hematological malignancies. J Immunol. 2005;175:5586–90.

Apolipoprotein Receptor (APR)

► CD91

A-Protein

► Recoverin

Arachidonic Acid

▶ Phospholipase A_2

Araf

▶ A-RAF

A-RAF

Jens Rauch[1] and Walter Kolch[1,2]
[1]Systems Biology Ireland, University College Dublin, Dublin, Ireland
[2]UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland

Synonyms

Araf; Araf1; PKS2 (presumably for kinase sequence); RAFA1; v-raf murine sarcoma 3611 viral oncogene homolog

Historical Background

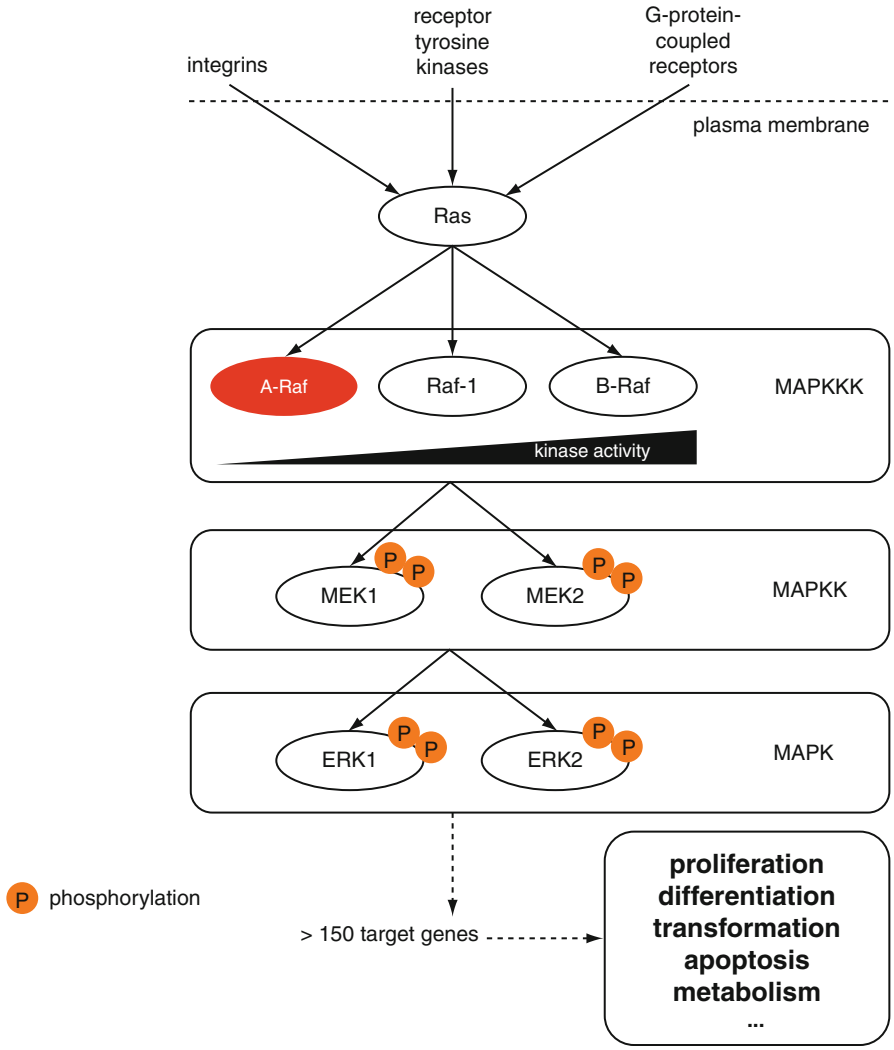
A-Raf is member of the Raf family of serine/threonine protein kinases comprising A-Raf, B-Raf, and ▶ Raf-1. In 1983 and 1986, viral homologues of RAF were isolated during experiments with the aim to identify novel transforming genes. Viral RAF (v-RAF) homologues were encountered in two different viruses, the avian retrovirus Mill Hill 2 (MH2) and the murine sarcoma virus (MSV) 3611. While MH2 contained the avian homologue of v-RAF, 3611-MSV was isolated from a mouse with lymphoma and lung adenocarcinoma. The name RAF derives from the observation that 3611-MSV increased the induction of fibrosarcoma in newborn NSF/N mice (*r*apidly *a*ccelerated *f*ibrosarcoma, or RAF). These initially identified viral RAF (v-Raf) genes originate from the mammalian counterpart of Raf-1 (or ▶ C-Raf) due to homologous recombination. After the discovery of the mammalian Raf-1 gene and initial functional characterization, additional C-RAF homologues were found in mammals, referred to as A- and B-RAF (Daum et al. 1994).

In the 1980s, several groups identified the ARAF gene as a paralogue of Raf-1. Mark et al. used low stringency hybridization techniques on human fetal liver cDNAs in order to identify v-Raf-related sequences and isolated ARAF, which was named PKS at that time (presumably for kinase sequence) (Mark et al. 1986). In the same year, Huleihel et al. isolated the A-Raf cDNA from a murine cDNA spleen library. They could show an 85% homology with Raf-1 and its expression in mouse tissues. Furthermore, incorporation of ARAF into a retrovirus showed that ARAF represents a new proto-oncogene (Huleihel et al. 1986). Later mouse and human ARAF genes were mapped to the X chromosome, and the complete A-RAF mRNA was sequenced from a human T-cell (Daum et al. 1994).

From an evolutionary point of view, there are no Raf kinases in yeasts, and B-Raf seems to be the phylogenetic oldest isoform of the three, which appears in invertebrates. In comparison, mammals possess three Raf isoforms (Raf-1, B-Raf, and A-Raf), with a shared modular structure. In comparison to Raf-1 and B-Raf, A-Raf is by far the least well-understood member of the Raf family (Rauch and Kolch 2010; Wellbrock et al. 2004; Zebisch and Troppmair 2006).

A-RAF and MAPK Signaling

Using genetic and biochemical approaches, A-Raf, like Raf-1 and B-Raf, was shown to be a component of the Ras-Raf-MEK-ERK pathway, also often referred to as the classic mitogen-activated protein kinase (MAPK) cascade. In this pathway Ras activates a three-tiered kinase module where Raf phosphorylates and activates ▶ MEK, and ▶ MEK phosphorylates and activates ERK. This pathway links receptor activation at the plasma membrane to >150 substrates in the cytosol and nucleus, which regulate many fundamental cellular functions such as proliferation, differentiation, transformation, apoptosis, and metabolism (Yoon and Seger 2006) (Fig. 1).



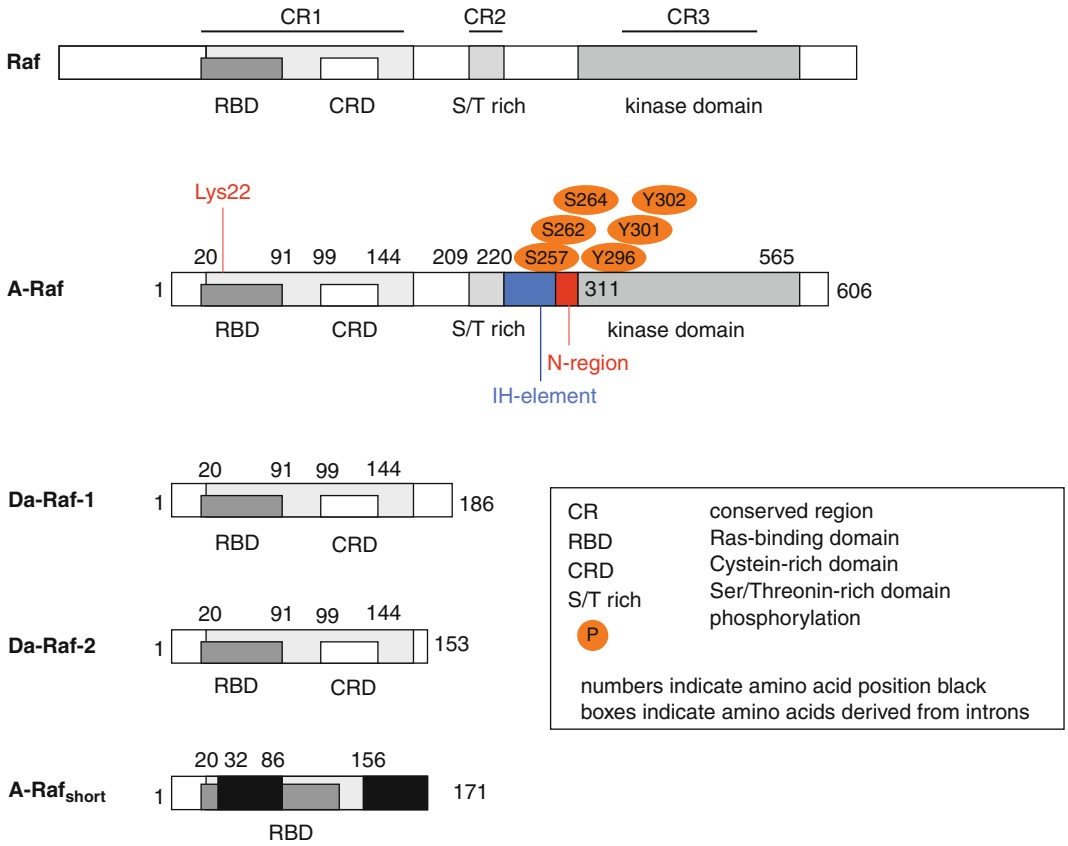
A-RAF, Fig. 1 Canonical Ras-Raf-MEK-ERK pathway

Regulation of Activity

The family of Raf serine/threonine protein kinases shares three conserved regions (CR) (Fig. 2). CR1 contains the Ras-binding domain and a cysteine-rich motif. CR2 features a short cluster of Ser and Thr residues, and CR3 contains the kinase domain. CR1 and CR2 restrain the function of CR3, and activation steps involve the release of this negative interaction as well as posttranslational modifications of CR3. The only bona fide substrates of Raf kinases are MEK1/2.

In general, A-Raf is regulated similar to Raf-1, although important differences have emerged over the years. While binding to active Ras suffices to activate B-Raf, Raf-1, and A-Raf require the presence of both activated Ras and Src, which is thought to phosphorylate tyrosines 301/302 in the negative-charge regulatory (N-) region upstream of the kinase domain (Marais et al. 1997). In B-Raf these tyrosines are replaced by aspartates, whose negative charge substitute for N-region phosphorylation normally induced by Ras binding.

A-Raf has a weak, hardly detectable kinase activity toward MEK. The reasons are (i) a substitution of a critical residue (arginine 22 for lysine) in the A-Raf RBD, which weakens the binding to Ras; and (ii) a non-conserved tyrosine 296 in the A-Raf N-region, whose mutation to glycine increases kinase activity (Baljuls et al. 2007). A-Raf is also positively regulated by phosphorylation (Baljuls et al. 2008). Serine 432 is crucial for the binding of MEK, while phosphorylation of serines 257, 262, and 264 in the



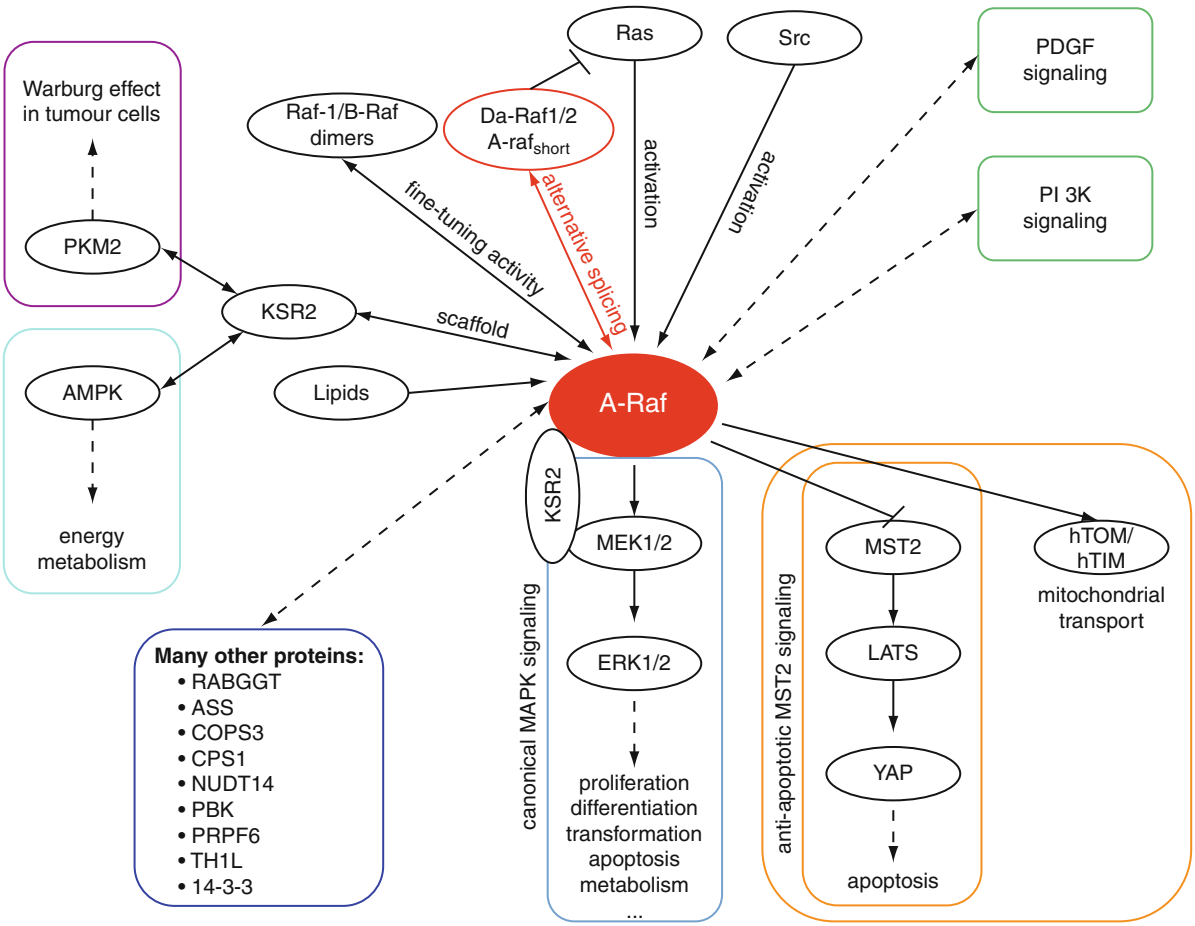
A-RAF, Fig. 2 Domain structure of Raf proteins (*top*). Comparison of domains and phosphorylation sites of the four A-Raf alternative splice forms

*I*soform-specific *H*inge (IH) segment stimulate A-Raf kinase activity. Full phosphorylation of the IH segment accumulates negative surface charges leading to the electrostatic destabilization of the interaction of A-Raf with the inner part of the plasma membrane and release into the cytosol. In addition, A-Raf like Raf-1 can bind lipids, such as phosphatidic acid and phosphoinositides (Johnson et al. 2005). Polyphosphorylated phosphoinositides, such as PI (4,5) and PI(3,4,5) suppress kinase activity. Thus, while sharing Ras as essential activator and MEK as substrate with other Raf isoforms, A-Raf shows unique mechanisms of regulation.

In addition to phophorylation and lipid binding A-Raf activity is also regulated by protein interactions, notably by heterodimerization with other Ras family members (Rushworth et al. 2006). B-Raf–Raf-1 heterodimerization dramatically elevates kinase activity. Heterodimerization is part of physiological Raf activation, but also an important "side effect" of Raf inhibitory drugs in cells harboring mutant Ras where these drugs promote Raf heterodimerization and thereby promote ERK pathway activation (Wimmer and Baccarini 2010). The regulatory subunit of ▶ casein kinase 2 (CK2β) binds and activates A-Raf when co-expressed in insect cells (Hagemann et al. 1997). However, the mechanism and physiological role of CK2β in A-Raf activation in mammalian cells remains to be proven. Other regulatory protein interactions are discussed below.

A-Raf Interacting Proteins

Apart from interacting with components of the ERK pathway, such as Ras, MEK, and other Raf isoforms, A-Raf also interacts with a number of other proteins. We recently summarized these interactions (Rauch and Kolch 2010), and therefore only discussed selected interactions here (Fig. 3).



A-RAF, Fig. 3 Overview about A-Raf interaction partners, cross talk to other signaling pathways, and processes where A-Raf is involved

A-Raf can associate with the p85 regulatory subunit of the phosphatidylinositide 3-kinase (PI3K) via the p85 SH2 domain. The interaction is constitutive and independent of growth factor stimulation or phosphorylation. A constitutively active A-Raf mutant inhibited p85 associated PI3-kinase activity, but it is unclear whether this interaction is a physiological connection between the ERK and ▶ PI3K pathways (Mahon et al. 2005). The hypothesis that A-Raf coordinates different signaling pathways is supported by the observation that A-Raf associates with the platelet-derived growth factor receptor (PDGFR), and specifically suppresses autophosphorylation and phosphorylation of the phospholipase C-γ (PLCγ) docking site, but not the phosphorylation of binding sites for other signaling molecules (Mahon et al. 2005). These functional effects were achieved by expression of an activated A-Raf mutant, while the role of endogenous A-Raf was not examined leaving it unclear whether A-Raf can regulate PDGFR signaling under normal conditions.

A physiological role of A-Raf was shown in the control of apoptosis. A-Raf binds and inhibits the proapoptotic kinase mammalian sterile 20-like kinase (MST2). A-Raf binds to MST2 constitutively, and seems to promote the survival of cancer cells (Rauch et al. 2010). By contrast, Raf-1 binding to MST2 is induced by stress and relieved by mitogens, while B-Raf does not detectably bind MST2 (O'Neill et al. 2004). This differential MST2 binding pattern inversely correlates with the kinase activity toward MEK and the evolution of the Raf family. B-Raf, the oldest member has the strongest MEK kinase activity and little affinity for MST2, while the youngest member, A-Raf, has poor MEK kinase activity but strong capacity to bind and inhibit MST2, suggesting that during evolution the role of Raf has shifted from

activating the ERK pathway to inhibiting the MST2 pathway. Interestingly, both A-Raf and MST2 localize to the mitochondria in tumor cell lines as well as primary tumors (Rauch et al. 2010). A-Raf associates with hTOM and hTIM, two proteins involved in the mitochondrial transport system (Yuryev et al. 2000). The biological function of A-Raf's mitochondrial location and interactions is unknown, but given A-Raf's poor MEK kinase activity the existence of alternative substrates, e.g., at the mitochondria, is likely.

Another intriguing A-Raf interaction partner is Kinase suppressor of Ras 2 (KSR2). In response to TNFα KSR2 recruits A-Raf rather than Raf-1 or B-Raf (Liu et al. 2009), suggesting that KSR2 may redirect A-Raf to the ERK pathway or nucleate other A-Raf specific signaling complexes. Interestingly, KSR2 also binds AMP kinase (AMPK) thereby mediating its stimulatory effects on glucose uptake and fatty acid oxidation (Costanzo-Garvey et al. 2009). The AMPK binding site overlaps the domain responsible for KSR2 membrane association suggesting the interesting possibility that AMPK binding to KSR2 could prevent the membrane translocation required for the efficient activation of the Raf-MEK-ERK module. Thus, metabolic requirements could restrain proliferation signals. A-Raf also may play a direct role in the cross talk between metabolism and proliferation by binding and inhibiting pyruvate kinase M2 (PKM2). PKM2 is the embryonic splice variant of PKM, which is reexpressed in tumors and responsible for the prevalence of anaerobic glycolysis (Warburg effect) typically observed in cancers (Christofk et al. 2008). A-Raf promotes the dimerization and inactivation of PKM2, whereas oncogenic A-Raf elevates the active tetrameric form of PKM (Mazurek et al. 2007). Thus, A-Raf may have a central role in coordinating proliferation via the ERK pathway, cell survival via the inhibition of MST2, and metabolic state in cancer cells via regulation of PKM2 activity.

Many more A-Raf binding partners have been reported, summarized in various interaction databases, e.g., String (http://string.embl.de), but their physiological roles and significance remain unexplored. An interesting dimension is added by the differential subcellular localization of A-Raf, which is found at the membrane, cytosol, and mitochondria. It is likely that A-Raf will engage with different binding partners in different compartments thus increasing the versatility and spatiotemporal coordination of A-Raf signaling.

A-Raf Splice Variants

Alternative splicing occurs in more than 90% of human genes, and greatly expands the information content and versatility of the transcriptome in generating tissue, stage, and development specific gene expression patterns.

For the ARAF1 gene so far, there are three reported alternative splice forms in addition to the wild-type mRNA (Fig. 2). Two of these splice forms, termed DA-Raf1 and DA-Raf2, contain the N-terminal Ras-binding domain, but lack the kinase domain due to preterminal stop codons. Therefore, they are still able to bind to activated Ras, but due to the lack of a kinase domain, act as dominant-negative antagonists of the Ras-ERK pathway (Nekhoroshkova et al. 2009; Yokoyama et al. 2007). Consistent with this functional role, DA-Raf1 promotes myogenic differentiation by binding to Ras and thereby inhibiting activation of the Raf-MEK-ERK pathway (Yokoyama et al. 2007). Similarly, DA-Raf2 binds and colocalizes with ARF6 on tubular endosomes and acts as a dominant effector of endocytic trafficking (Nekhoroshkova et al. 2009).

Recently, A-Raf$_{short}$, a third alternative splice form of the ARAF1 gene was reported. In comparison to DA-Raf1 and DA-Raf2, A-Raf$_{short}$ incorporates intronic sequences, and generates a shortened protein, which lacks the kinase domain. Consequently, A-Raf$_{short}$ acts as a dominant-negative antagonist by binding and blocking activated Ras and thus is a potent inhibitor of ERK signaling and cellular transformation. The expression of A-Raf$_{short}$ is reduced in several cancer entities suggesting that A-Raf$_{short}$ acts as a tumor suppressor protein.

Regulation in Cancer and Other Diseases

Initial studies in mice suggested a highly restricted tissue distribution of A-Raf with highest expression levels observed in epididymis, ovary, and intestine. In the meantime, however, A-Raf was found expressed in most normal tissues, but expression levels seem highly regulated and differ dramatically. While neuronal tissues, for example, express A-Raf only at low levels, the urogenital tract shows a high expression (Luckett et al. 2000). A-Raf mRNA and protein levels are elevated in a number of malignancies. Increased A-Raf mRNA levels were found in peripheral blood mononuclear

cells isolated from two patients with angioimmunoblastic lymphadenopathy with dysproteinemia (Mark et al. 1986). Elevated levels of A-Raf mRNA were also found in pancreatic ductal carcinoma (Kisanuki et al. 2005). In addition, enhanced A-Raf expression was also found in other tumor types, including astrocytic tumors, where high expression of A-Raf negatively correlated with patients' prognosis (Hagemann et al. 2009). Elevated A-Raf expression was also found in a number of head and neck squamous cell carcinomas as well as colon carcinomas.

Several publications addressed the mutational status of the ARAF1 gene. While B-Raf is a well-described target for mutations in human cancers, mutations in A-Raf, like Raf-1, are very rare to nonexistent. This poses the following question: How and why is A-Raf overexpressed in several malignancies although the gene is not altered at the genetic level? One mechanism is an increased expression of A-Raf due to chromosomal aberration. The human ARAF1 gene is situated on the X chromosome, and duplication of X chromosomes was found in testicular germ cell tumors leading to enhanced A-Raf levels. Furthermore, expression of wild-type A-Raf mRNA and protein requires the expression of the splice factor hnRNP H, whose levels are enhanced in several tumors including colon and head and neck cancers (Rauch et al. 2011). High levels of this splice factor ensure the expression of full-length A-Raf protein by suppressing alternative splicing of the *a-raf* mRNA, thus allowing the sufficient production of full-length A-Raf protein to counteract MST2-mediated apoptosis. Low levels of hnRNP H, as found in nonmalignant tissues, cannot suppress alternative splicing of the A-Raf wild-type mRNA, thus favoring the expression of the alternative splice form A-Raf$_{short}$. As mentioned already above, A-Raf$_{short}$ acts as a dominant-negative antagonist of the Ras-MAPK cascade, which seems to keep proliferation and transformation in nonmalignant cells in check.

Mouse Models and Phenotypes

In the 1990s, the first mouse model for the A-Raf gene was reported (Pritchard et al. 1996). It was shown that ablation of the A-Raf gene causes neurological defects. Interestingly, in an inbred mouse background, A-Raf ablation resulted in intestinal and neurological abnormalities. A-Raf knockout mice died 1–3 weeks after birth from megacolon which is reminiscent of Hirschsprung's disease in humans and was caused by a defect in the migration of visceral neurons controlling bowel contractions. In contrast, in an outbred background, A-Raf knockout animals survived to adulthood. In this genetic background, A-Raf ablation did not lead to intestinal abnormalities, but animals displayed a subset of neurological defects. Interestingly, the regulation of ERK and oncogene transformation is not impaired in A-Raf knockout cells. These results, together with the low kinase activity toward MEK suggest that A-Raf does not play a major role in MAPK signaling and that this function is fully compensated by the other two Raf family members. However, A-Raf seems to have a role in the development of the nervous system possibly by regulating neuronal migration. Comparing the knockout phenotypes of all three Raf isoforms in mice indicates that A-Raf and B-Raf may have more specialized functions, while Raf-1 seems to have a more general role in tissue formation.

Summary

What did scientists learn about A-Raf since its discovery in the early 1980s? A simple summary is that Raf proteins have distinct functions, and that A-Raf has more functions than initially expected. Details about its domain, phosphorylation sites, and functions are known in comparison with other Raf proteins and kinases. Furthermore, scientists made headway in deciphering in which pathways A-Raf might play a role. Science now has entered an era where the mapping of the components of signaling networks is rapidly accelerating producing even longer lists faster than ever before. However, these lists are like a telephone book full of names rather than an ordnance survey type of map that connects the names with pathway topologies. Undoubtedly, much more is known about Raf-1 and B-Raf and 25 years of research mainly focused on these two isoforms, as they play a bigger role in MAPK signaling and oncogenesis and are major targets for drug therapies. In comparison, A-Raf on its own seems to play only a minor role in the canonical MAPK pathway. Multiple studies over the last years have shown A-Raf's involvement in other processes, such as energy metabolism, the Warburg effect, mitochondrial transport, and anti-apoptotic signaling. However, it seems that these interactions with other

proteins and processes do not happen at the same time but are rather cell-, tissue-, and time-dependent. Furthermore, A-Raf is found at different localizations of the cell and this spatial regulation is another suitable explanation for the involvement in different pathways. Dissecting these micro-complexes and interacting proteins is a challenging endeavor on the experimental level. To add another level of complexity, three novel alternative splice forms were identified recently with functions that are even antagonistic to the wild-type form.

Despite a lot of research has already been done and had a huge impact on our understanding how A-Raf functions, there are still some unanswered questions:

- A-Raf was shown to bind to a plethora of proteins, which suggests A-Raf's involvement in many other signaling pathways outside the canonical MAPK pathway. However, if there is more than one function in signal transduction, how are these diverse functions coordinated in order to achieve the intended biological outcome and specificity? This question is also relevant for the other members of the Raf family.
- Similarly, A-Raf was found at different subcellular compartments. How does this localized expression impact on signaling pathways and networks?
- What is the impact of dynamic changes in the assembly of A-Raf signaling complexes on biochemical and biological outcome?
- A-Raf was shown to play a role in cancer and other diseases. Is this exploitable for the purpose of therapeutic intervention?

In summary, the current state of analysis of the "A-Raf network" offers a glimpse into a new world. New concepts and tools developed hopefully will widen this glimpse into a window overlooking the whole signaling network.

References

Baljuls A, Mueller T, Drexler HC, Hekman M, Rapp UR. Unique N-region determines low basal activity and limited inducibility of A-RAF kinase: the role of N-region in the evolutionary divergence of RAF kinase function in vertebrates. J Biol Chem. 2007;282(36):26575–90.

Baljuls A, Schmitz W, Mueller T, Zahedi RP, Sickmann A, Hekman M, et al. Positive regulation of A-RAF by phosphorylation of isoform-specific hinge segment and identification of novel phosphorylation sites. J Biol Chem. 2008;283(40):27239–54.

Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature. 2008;452(7184):230–3.

Costanzo-Garvey D, Pfluger P, Dougherty M, Stock J, Boehm M, Chaika O, et al. KSR2 is an essential regulator of AMP kinase, energy expenditure, and insulin sensitivity. Cell Metab. 2009;10(5):366–78.

Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J, Rapp UR. The ins and outs of Raf kinases. Trends Biochem Sci. 1994;19(11):474–80.

Hagemann C, Kalmes A, Wixler V, Wixler L, Schuster T, Rapp UR. The regulatory subunit of protein kinase CK2 is a specific A-Raf activator. FEBS Lett. 1997;403(2):200–2.

Hagemann C, Gloger J, Anacker J, Said HM, Gerngras S, Kuhnel S, et al. RAF expression in human astrocytic tumors. Int J Mol Med. 2009;23(1):17–31.

Huleihel M, Goldsborough M, Cleveland J, Gunnell M, Bonner T, Rapp UR. Characterization of murine A-raf, a new oncogene related to the v-raf oncogene. Mol Cell Biol. 1986;6(7):2655–62.

Johnson LM, James KM, Chamberlain MD, Anderson DH. Identification of key residues in the A-Raf kinase important for phosphoinositide lipid binding specificity. Biochemistry. 2005;44(9):3432–40.

Kisanuki H, Choi YL, Wada T, Moriuchi R, Fujiwara S, Kaneda R, et al. Retroviral expression screening of oncogenes in pancreatic ductal carcinoma. Eur J Cancer. 2005;41(14):2170–5.

Liu L, Channavajhala PL, Rao VR, Moutsatsos I, Wu L, Zhang Y, et al. Proteomic characterization of the dynamic KSR-2 interactome, a signaling scaffold complex in MAPK pathway. Biochim Biophys Acta. 2009;1794(10):1485–95.

Luckett JC, Huser MB, Giagtzoglou N, Brown JE, Pritchard CA. Expression of the A-raf proto-oncogene in the normal adult and embryonic mouse. Cell Growth Differ. 2000;11(3):163–71.

Mahon ES, Hawrysh AD, Chagpar RB, Johnson LM, Anderson DH. A-Raf associates with and regulates platelet-derived growth factor receptor signalling. Cell Signal. 2005;17(7):857–68.

Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. J Biol Chem. 1997;272(7):4378–83.

Mark GE, Seeley TW, Shows TB, Mountz JD. Pks, a raf-related sequence in humans. Proc Natl Acad Sci USA. 1986;83(17):6312–6.

Mazurek S, Drexler HC, Troppmair J, Eigenbrodt E, Rapp UR. Regulation of pyruvate kinase type M2 by A-Raf: a possible glycolytic stop or go mechanism. Anticancer Res. 2007;27(6B):3963–71.

Nekhoroshkova E, Albert S, Becker M, Rapp UR. A-RAF kinase functions in ARF6 regulated endocytic membrane traffic. PLoS One. 2009;4(2):e4647.

O'Neill E, Rushworth L, Baccarini M, Kolch W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science. 2004;306(5705):2267–70.

Pritchard CA, Bolin L, Slattery R, Murray R, McMahon M. Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene. Curr Biol. 1996;6(5):614–7.

Rauch J, Kolch W. A-Raf/v-raf murine sarcoma 3611 viral oncogene homolog. UCSD-nature molecule pages. 2010. doi:10.1038/mp.a000307.01. http://www.signaling-gateway.org/molecule/query?afcsid=A000307. Accessed 6 Apr 2010
Rauch J, O'Neill E, Mack B, Matthias C, Munz M, Kolch W, et al. Heterogeneous nuclear ribonucleoprotein H blocks MST2-mediated apoptosis in cancer cells by regulating a-raf transcription. Cancer Res. 2010;70(4):1679–88.
Rauch J, Moran-Jones K, Albrecht V, Schwarzl T, Hunter K, Gires O, et al. c-Myc regulates A-Raf kinase splicing and ERK pathway activation. Cancer Res. 2011;71(13):4664–74.
Rushworth LK, Hindley AD, O'Neill E, Kolch W. Regulation and role of Raf-1/B-Raf heterodimerization. Mol Cell Biol. 2006;26(6):2262–72.
Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. Nat Rev Mol Cell Biol. 2004;5(11):875–85.
Wimmer R, Baccarini M. Partner exchange: protein-protein interactions in the Raf pathway. Trends Biochem Sci. 2010;35(12):660–8.
Yokoyama T, Takano K, Yoshida A, Katada F, Sun P, Takenawa T, et al. DA-Raf1, a competent intrinsic dominant-negative antagonist of the Ras-ERK pathway, is required for myogenic differentiation. J Cell Biol. 2007;177(5):781–93.
Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors. 2006;24(1):21–44.
Yuryev A, Ono M, Goff SA, Macaluso F, Wennogle LP. Isoform-specific localization of A-RAF in mitochondria. Mol Cell Biol. 2000;20(13):4870–8.
Zebisch A, Troppmair J. Back to the roots: the remarkable RAF oncogene story. Cell Mol Life Sci. 2006;63(11):1314–30.

Araf1

▶ A-RAF

ARAP3

Siân-Eleri Owens and Venkateswarlu Kanamarlapudi
Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales, UK

Synonyms

ARF-GAP, RHO-GAP, ankyrin repeat, and plekstrin homology domains containing protein 3; Centaurin delta 3; Centd3; Drag1; Expressed sequence AI851258.

Historical Background

Small GTPases of the Ras superfamily are binary switches that, by cycling between active GTP-bound and inactive GDP-bound conformations, regulate a wide variety of cellular and developmental events. They are grouped based on the sequence homology and function into five families: Ras, Rho, Ran, Rab, and ARF. The Rho and Arf family small GTPases are well-established regulators of cellular actin rearrangements and vesicular trafficking (Bos et al. 2007). Small GTPases are activated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of small GTPase-bound GDP to GTP, whereas GTPase-activating proteins (GAPs) inactivate small GTPases by stimulating hydrolysis of the small GTPase-bound GTP to GDP (Bos et al. 2007). In general, each small GTPase family has its specific GEFs and GAPs. However, the ARAP protein subfamily, which is composed of ARAP1, ARAP2, and ARAP3, is unique as its members act as GAPs for both Rho and Arf family small GTPases. The ARAP protein subfamily is included in the Arf GAP family in recent consensus nomenclature for the human Arf GAP-domain-containing proteins (Krugmann et al. 2002; Santy and Casanova 2002; Kahn et al. 2008). ARAP3 is a phosphoinositide (PtdIns) 3 kinase and Rap1- regulated GAP for RhoA and Arf6 (Krugmann et al. 2004).

ARAP3 was originally identified as a PtdIns(3,4,5)P_3 second messenger binding protein. It consists of a SAM domain, five PH domains, Rho GAP domain, an Arf GAP domain, and a RA domain (Krugmann et al. 2002). ARAP3 binding to PtdIns(3,4,5)P_3 depends on its amino-terminal tandem PH domains and adjacent sequences (Craig et al. 2010).

Protein Function and Regulation of Activity

The functions of ARAP3 that have been established thus far include regulation of the actin cytoskeleton, lamellipodia formation, and cell spreading (Raaijmakers et al. 2007). ARAP3 has been shown to increase the number of membrane projections, but it only weakly inhibits the migration of HEK293 cells (I et al. 2004). Overexpression of ARAP3 results in a loss of cell adhesion and cell retraction in platelet-derived growth factor (PDGF)-treated pig

aortic endothelial (PAE) cells (Krugmann et al. 2006). ARAP3 overexpression in PAE and NIH3T3 cells inhibits actin stress fiber formation by reducing RhoA activity (I et al. 2004).

ARAP3 Rho GAP activity is necessary for the suppression of cell spreading (I et al. 2004). The RA domain of ARAP3 is essential for the RhoA GAP activity (Krugmann et al. 2004). ARAP3 has also recently been identified as a host protein affecting cellular susceptibility to anthrax toxin (Lu et al. 2004).

It has been hypothesized that ARAP3 is constitutively active in monocytes. This is because unstimulated ARAP3 knockdown THP-1 cells (a monocyte-derived cell line) have increased RhoA activity. Additionally, platelet activating factor (PAF)-mediated inactivation of RhoA is reduced in ARAP3 knockdown THP-1 cells (Nandy et al. 2010).

Stimulation of PC12 cells with growth factors that induce PtdIns(3,4,5)P_3 formation such as EGF results in ARAP3 translocation from cytosol to the plasma membrane of PC12 cells (Krugmann et al. 2002). The dominant negative mutant of ARAP3 (which lacks both RhoGAP and ArfGAP acitivites) has been shown to inhibit significantly NGF- and bFGF-induced neurite outgrowth in PC12 cells, while overexpression of wild-type ARAP3 slightly increases the neurite outgrowth. This result suggests that RhoA inactivation by ARAP3 is required for NGF- and bFGF-induced neurite outgrowth in PC12 cells (Jeon et al. 2010a, b).

ARAP3 Arf and Rho GAP activities are dependent on its N-terminal PH domain binding to PtdIns(3,4,5)P_3 (Krugmann et al. 2004). Translocation of ARAP3 to lamellipodia of PAE cells is dependent on its first PH domain and PtdIns(3,4,5)P_3 produced by agonist-activated PtdIns 3-kinase (Krugmann et al. 2002).

Growth factor stimulation and cell adhesion to fibronectin leads to tyrosine phosphorylation of ARAP3 by Src-family kinases Lyn and Src when they are co-expressed in cells. Incubation with Src-family kinase and PtdIns 3-kinase inhibitors, or Src dominant interfering mutant, results in a decrease of adhesion-induced ARAP3 phosphorylation. Mutation of the two phosphorylation sites, Tyr 1399 and Tyr 1404, increases ARAP3 cellular functions, indicating that ARAP3 may be negatively regulated by the tyrosine phosphorylation. Moreover, both Lyn and Src have been shown to form stable bonds with ARAP3 (I et al. 2004).

ARAP3-dependent RhoA GAP activity is elevated in cells expressing Rap1A (Krugmann et al. 2004). The interaction of ARAP3 with PtdIns(3,4,5)P_3, has been shown to be vital for Rap-GTP to stimulate Rho GAP activity of ARAP3 in vivo, indicating the importance of PtdIns 3-kinase activity for this process (Krugmann et al. 2004). Moreover, Rho and ARF6 activities are increased in both basal and PDGF-stimulated ARAP3-knockdown cells (Krugmann et al. 2006).

Recent studies have shown that none of ARAP3 five PH domains are able to bind to PtdIns(3,4,5)P_3 in isolation. It has been suggested that binding to PtdIns (3,4,5)P_3 involves the formation of a complex mechanism whereby basic residues from two tandem PH domains, the N-terminal SAM domain, and basic residues within ARAP3 synergise in order to bind strongly and specifically to PtdIns(3,4,5)P_3 (Craig et al. 2010).

The small GTPase Rap1 has been shown to interact with the RA domain of ARAP3 and activate its Rho GAP activity (Raaijmakers et al. 2007).

ARAP3 has been shown to bind to the adaptor protein CIN85/CMS via its Pro-Arg motif. CIN85 has been implicated in the internalization of mono-ubiquinated membrane protein (Kowanetez et al. 2004), whereas CMS is involved in cytoskeletal rearrangements (Kirsch et al. 1999). ARAP3 binds to the secondary messenger PtdIns(3,4,5)P_3 through its PH domain(s) (Krugmann et al. 2002). The SAM domain of ARAP3 interacts with the SAM domain of the inositol 5'- phosphatase SHIP2 to form a heterodimer; however, binding of ARAP3 to SHIP2 is not required for SHIP2 activity. The binding of the two proteins seems to be constitutive, as the interaction does not require activation by PtdIns 3-kinase or Rap1. It has been shown that SHIP2, ARAP3, and CIN85/CMS form a mutimeric protein complex (Raaijmakers et al. 2007).

The NMR solution structure of ARAP3 SAM domain shows that it has the classical small five helix bundle. Chemical shift mapping studies have indicated that the interaction of SHIP2-SAM and ARAP3-SAM central regions are involved. ARAP3-SAM binding domain is made up of the carboxy-terminal α 5 helix and adjacent loop regions. It has been hypothesized that SHIP2-SAM and ARAP3-SAM binding is likely via the Mid-Loop/End-Helix Model that is common between SAM-SAM interactions (Leone et al. 2009).

Major Sites of Expression and Subcellular Localization

Studies have shown that ARAP3 expression is ubiquitous, albeit uneven. The strongest expression was detected in leukocytes and in the spleen (Krugmann et al. 2002).

ARAP3 is largely localized within the cytosol of unstimulated cells (Krugmann et al. 2002) and in the F-actin dense membrane ruffles and lamellipodia of some cells (I et al. 2004). ARAP3 binding of PtdIns $(3,4,5)P_3$ through its PH domain results in its translocation to the plasma membrane (Raaijmakers et al. 2007).

Stimulation of cells with growth factors that induce PtdIns$(3,4,5)P_3$ formation such as EGF resulted in ARAP3 translocation to the plasma membrane of PC12 cells. However, stimulation of PAE cells with PDGF, which also induces PtdIns$(3,4,5)P_3$ formation, led to ARAP3 translocation to lamellipodia (Krugmann et al. 2002).

All the subcellular localization studies were performed with exogenously overexpressed ARAP3. Therefore, it is possible that the endogenous protein may have a more specific localization, as is the case for both ARAP1 and ARAP2.

There are now commercially available polyclonal antibodies to ARAP3 raised against a carboxy-terminal peptide (1533–1544 amino acids of Human ARAP3). NZ white rabbits were immunized with purified GST-RAP3 fusion protein (residues 1278–1538) to raise ARAP3 polyclonal antibodies (I et al. 2004). An ARAP3 polyclonal antibody (T-16) is available from Santa Cruz Biotechnology, Inc. that can detect ARAP3 by western blot, immunofluorescence, and ELISA. An ARAP3 polyclonal antibody from Abcam can be used in ELISA. There is also a monoclonal ARAP3 antibody from Abnova (CENTD3 monoclonal antibody (MO3), clone ID6), which can be used for in ELISA and western blot assays.

Phenotypes, Splice Variants, and Disease

Two isoforms have been identified: ARAP3 and $ARAP3^{\Delta SAM}$, which does not possess the N-terminal SAM domain (I et al. 2004).

Knockdown of ARAP3 in PAE cells with RNA interference (RNAi) resulted in the alteration of their phenotype, including changes in cell shape with formation of numerous thin actin stress fibers. The ability of PDGF-stimulated PAE cells to produce lamellipodia and their polarizing ability during wound healing was also reduced upon ARAP3 knockdown (Krugmann et al. 2006).

It has been shown that ARAP3 knockout (KO) in the mice results in embryonic death in mid-gestation due to an endothelial cell-dependent defect in sprouting angiogenesis. Moreover, knock-in mice expressing an ARAP3 mutant (point mutation in its N-terminal PH domain) that cannot bind to PtdIns $(3,4,5)P_3$ showed angiogenesis defects comparable to those of the ARAP3 KO mice. These data suggest the involvement of ARAP3 signaling pathway downstream of PI3Kα in the regulation of embryonic angiogenesis (Gambardella et al. 2010).

Using conditional ARAP3 KO mouse model, it has been shown that ARAP3 regulates neutrophil adhesion-dependent processes. Loss of ARAP3 causes preactivation of neutrophil β2 integrins. ARAP3 deficiency has been shown to increase adhesion-dependent cellular functions such as reactive oxygen species (ROS) production, adhesion, spreading, and granule release in neutrophils. Loss of ARAP3 also interferes with integrin-dependent neutrophil chemotaxis. These studies show that ARAP3 regulates β2 intergrin activity, thereby retaining unstimulated neutrophils in their quiescent state (Gambardella et al. 2011).

An increase in the chemokine PAF in type 2 diabetics has been shown to accelerate atherosclerosis. PAF-stimulation enhances monocyte transendothelial migration *via* Rac-1 activation and RhoA inactivation. PAF-stimulated RhoA inactivation is reversed in ARAP3 knockdown monocytes, indicating that ARAP3 is responsible for PAF-mediated RhoA inactivation (Nandy et al. 2010).

ARAP3 has been shown to be expressed in normal fundic gland mucosa; however its expression in poorly differentiated carcinomas is reduced. Overexpression of ARAP3 has been shown to reduce cell-ECM attachment and cell invasion in vitro in the highly metastatic scirrhous gastric carcinoma cell line (58As9 cells). ARAP3 overexpression was also shown to inhibit peritoneal dissemination of 58As9 cells in vivo. Adhesion to and invasion through the ECM are necessary for peritoneal dissemination of scirrhous gastric carcinoma cells. Since ARAP3

regulates both cell-ECM adhesion and invasiveness, it may be a novel therapeutic target for preventing peritoneal dissemination of scirrhous gastric carcinoma (Yagi et al. 2011).

Summary

The subfamily of ARAP proteins includes three members: ARAP1, ARAP2, and ARAP3, which can act as GAPs for both Arf and Rho family small GTPases. ARAP3 was originally identified as a PtdIns(3,4,5)P_3 secondary messenger binding protein in porcine leukocyte cytosol. It consists of a SAM domain, five PH domains, Rho GAP domain and Arf GAP domain, and a RA domain. ARAP3 activity has been shown to be dependent on its N-terminal PH domain binding to PtdIns(3,4,5)P_3. It is largely localized within the cytosol of unstimulated cells and in the F-actin dense membrane ruffles and lamellipodia of some cells. ARAP3 binding to PtdIns(3,4,5)P_3 through the N-terminal PH domain leads to its translocation to the plasma membrane. It has been shown to play a role in the regulation of the actin cytoskeleton, lamellipodia formation, and cell spreading. ARAP3 also inactivates RhoA in response to NGF and bFGF leading to neurite outgrowth from PC12 cells. It has been shown to inhibit peritoneal dissemination of scirrhous gastric carcinoma cells by regulating cell adhesion and invasion. ARAP3 is tyrosine phosphorylated by Src, which negatively regulates its cellular functions. It forms a multimeric protein complex with CIN85/CMS and SH2-containing inositol phosphatase 2 (SHIP2) but the physiological significance of this interaction is not fully understood. ARAP3 interacts with CIN85/CMS through its proline–arginine motif and with SHIP2 using the SAM domain. It also binds to Rap1 through the RA domain. Studies have shown that ARAP3 expression is ubiquitous, albeit uneven with the strongest expression detected in leukocytes and in the spleen. ARAP3 knockdown with RNAi alters cell shape and reduces PDGF-induced lamellipodia formation in fibroblasts and increases RhoA activity in monocytes. Knockout of ARAP3 in mice results in embryonic death in mid-gestation due to defect in sprouting angiogenesis. ARAP3 inducible knockout studies revealed that it regulates chemotaxis and adehesion-dependent processes in neutrophils.

References

Bos JL, Rehmann H, Wittinghofer A. GEFs and GAPs: critical elements in the control of small G proteins. Cell. 2007;129:865–77.

Craig HE, Coadwell J, Guillou H, Vermeren S. ARAP3 binding to phosphatidylinositol-(3,4,5)-trisphosphate depends on N-terminal tandem PH domains and adjacent sequences. Cell Signal. 2010;22:257–64.

Gambardella L, Hemberger M, Hughes B, Zudaire E, Andrews S, Vermeren S. PI3K signaling through the dual GTPase-activating protein ARAP3 is essential for developmental angiogenesis. Sci Signal. 2010;3:1–9.

Gambardella L, Anderson KE, Nussbaum C, Segonds-Pichon A, Margarido T, Norton L, Ludwig T, Sperandio M, Hawkins PT, Stephens L, Vermeren S. The GTPase-activating protein ARAP3 regulates chemotaxis and adhesion-dependent processes in neutrophils. Blood. 2011;118:1087–98.

I ST, Nie Z, Stewart A, Najdovska M, Hall NE, He H, Randazzo PA, Lock P. ARAP3 is transiently tyrosine phosphorylated in cells attaching to fibronectin and inhibits cell spreading in a RhoGAP-dependent manner. J Cell Sci. 2004;117:6071–84.

Jeon CY, Kim HJ, Morii H, Mori N, Settleman J, Lee JY, Kim J, Kim SC, Park JB. Neurite outgrowth from PC12 cells by basic fibroblast growth factor (bFGF) is mediated by RhoA inactivation through p190RhoGAP and ARAP3. J Cell Physiol. 2010a;224:786–94.

Jeon CY, Kim HJ, Lee JY, Kim JB, Kim SC, Park JB. p190RhoGAP and Rap-dependent RhoGAP (ARAP3) inactivate RhoA in response to nerve growth factor leading to neurite outgrowth from PC12 cells. Exp Mol Med. 2010b;42:335–44.

Kahn RA, Bruford E, Inoue H, Logsdon JM, Nie Z, Premont RT, Randazzo PA, Satake M, Theibert AB, Zapp ML, Cassel D. Consensus nomenclature for the human ArfGAP domain-containing proteins. J Cell Biol. 2008;22:1039–44.

Kirsch KH, Georgescu MM, Ishimaru S. Hanafusa H CMS: an adapter molecule involved in cytoskeletal rearrangements. Proc Natl Acad Sci USA. 1999;96:6211–6.

Kowanetz K, Husnjak K, Höller D, Kowanetz M, Soubeyran P, Hirsch D, Schmidt MH, Pavelic K, De Camilli P, Randazzo PA, Dikic I. CIN85 associates with multiple effectors controlling intracellular trafficking of epidermal growth factor receptors. Mol Biol Cell. 2004;15:3155–66.

Krugmann S, Anderson KE, Ridley SH, Risso N, McGregor A, Coadwell J, Davidson K, Eguinoa A, Ellson CD, Lipp P, Manifava M, Ktistakis N, Painter G, Thuring JW, Cooper MA, Lim ZY, Holmes AB, Dove SK, Michell RH, Grewal A, Nazarian A, Erdjument-Bromage H, Tempst P, Stephens LR, Hawkins PT. Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices. Mol Cell. 2002;9:95–108.

Krugmann S, Andrews S, Stephens L, Hawkins PT. ARAP3 is essential for formation of lamellipodia after growth factor stimulation. J Cell Sci. 2006;119:425–32.

Krugmann S, Williams R, Stephens L, Hawkins PT. ARAP3 is a PI3K- and rap-regulated GAP for RhoA. Curr Biol. 2004;14:1380–4.

Leone M, Cellitti J, Pellecchia M. The Sam domain of the lipid phosphatase Ship2 adopts a common model to interact with Arap3-Sam and EphA2-Sam. BMC Struct Biol. 2009;9:59.
Lu Q, Wei W, Kowalski PE, Chang AC, Cohen SN. EST-based genome-wide gene inactivation identifies ARAP3 as a host protein affecting cellular susceptibility to anthrax toxin. Proc Natl Acad Sci USA. 2004;101:17246–51.
Nandy D, Asmann YW, Mukhopadhyay D, Basu A. Role of AKT-glycogen synthase kinase axis in monocyte activation in human beings with and without type 2 diabetes. J Cell Mol Med. 2010;14:1396–407.
Raaijmakers JH, Deneubourg L, Rehmann H, de Koning J, Zhang Z, Krugmann S, Erneux C, Bos JL. The PI3K effector Arap3 interacts with the PI(3,4,5)P3 phosphatase SHIP2 in a SAM domain-dependent manner. Cell Signal. 2007;19:1249–57.
Santy LC, Casanova JE. GTPase signaling: bridging the GAP between ARF and Rho. Curr Biol. 2002;12:R360–2.
Yagi R, Tanaka M, Sasaki K, Kamata R, Nakanishi Y, Kanai Y, Sakai R. ARAP3 inhibits peritoneal dissemination of scirrhous gastric carcinoma cells by regulating cell adhesion and invasion. Oncogene. 2011;30:1413–21.

ARD1/TRIM23

Joel Moss and Martha Vaughan
Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

Synonyms

ADP-ribosylation factor; Arf-domain protein; Domain protein; Tripartite motif protein 23

Historical Background

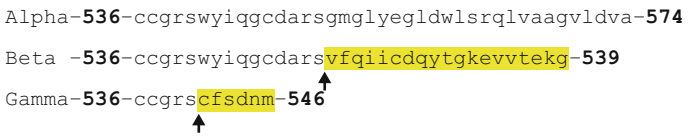
ARD1 was first described in 1993 as the deduced protein product of clones isolated from human and rat genomic cDNA libraries that encoded an about 18-kDa ▶ ADP-ribosylation factor (Arf) sequence at the C-terminus of a 64-kDa molecule (Mishima et al. 1993). Human ARD1 coding region cDNA hybridized with 3.7- and 4.1-kb mRNAs from all rat tissues examined. Both recombinant, full-length ARD1 and its Arf domain (M403-A574), activated cholera toxin ADP-ribosyltransferase activity, at that time a defining characteristic of Arf function, whereas the non-Arf (M1-K402) fragment of ARD1 did not. Using recombinant proteins on nitrocellulose membranes, only the Arf domain (and a recombinant ARF3), not the full-length ARD1, bound radio-labeled GTP (Mishima et al. 1993).

Further investigation of GTPase activity of the same recombinant proteins in solution provided evidence that the N-terminal non-Arf region of ARD1 acted as a GTPase-activating protein (GAP) domain for the Arf moiety (Vitale et al. 1996). Bound GDP dissociated from the Arf domain much more rapidly than it did from the full-length ARD1, but after addition of the non-Arf domain, GDP dissociation was slowed, essentially to that of GDP release from the intact ARD1. Thus, the ARD1 molecule comprises both GDI (GDP-dissociation inhibitor) and GAP domains that can influence biological activity of the Arf domain, although the extent to which these domains regulate its GTPase cycle in cells was not demonstrated.

The first several ARD papers contain more than one comment on its Zn^{2+}-finger structure near the N-terminus, but no mention of a potential E3 ubiquitin ligase activity, which was more widely recognized after ARD1 was described as TRIM23, a protein of the tripartite motif family (Reymond et al. 2001). The TRIM or RBCC structure comprises an N-terminal ring (R) followed by one or two B boxes (B1 and B2 in ARD1) and a predicted coiled-coil (CC) sequence. The B box, apparently found only in this protein family, was suggested as a useful identifier of TRIM molecules. Greatest diversity among TRIM proteins is in regions C-terminal to the CC domains, i.e., the Arf domain in ARD1, where it is compounded by alternative splicing. In addition to the full-length ARD1 initially cloned (Mishima et al. 1993), sequences of two more human ARD1 molecules that differ at the C-terminus (Fig. 1) are reported (Reymond et al. 2001; Venkateswarlu and Wilson 2011).

Regulation of ARD1 Activity

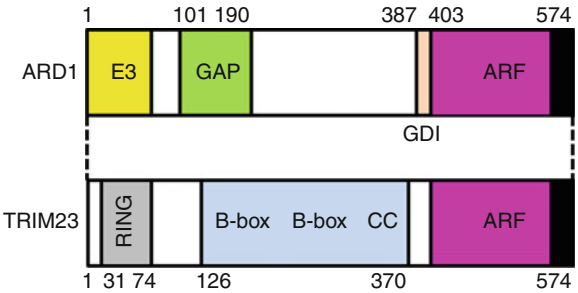
The Arf domain of ARD1 was not a substrate for a partially purified Arf GAP from rat spleen that enhanced GTP hydrolysis by ARFs 1, 3, 5, and 6 as well as Arf-like ARL proteins. Nor did the non-Arf part of ARD1 display GAP activity toward potential substrates other than its own Arf domain



ARD1/TRIM23, Fig. 1 C-terminal sequences of alternatively spliced ARD1α, β, and γ molecules. All three molecules are identical through position 540 in the Arf domain, with identity of α and β through 550. Accession numbers for human ARD1/TRIM23 are: ARD1-alpha (NP_001647), ARD1-beta (NP_150230), and ARD1-gamma (NP_150231)

(Ding et al. 1996). As often cautioned, the extent to which relationships of GAP (and GEF) activities assessed in vitro compare to those in living cells can be difficult to establish.

For more than a decade, limited research on ARD1 treated it simply as an Arf-domain protein of unknown function, even after its E3 ubiquitin ligase activity was described (Vichi et al. 2005). An ARD$^{-/-}$ mouse, generated to provide clues to physiological function(s), has been thus far only a source of murine embryo fibroblasts that proved useful for demonstration of contributions of Arf and E3 ligase actions to the regulation, respectively, of epidermal growth factor receptor (EGFR) degradation and of its own protein level (Meza-Carmen et al. 2011). The recognition of ARD1 as TRIM23, a member of the tripartite motif (TRIM) family of proteins (Reymond et al. 2001), which are critical for rapid innate immune responses to viral infection, introduces what may well be an even more intriguing chapter (Ozato et al. 2008). Relatively recent reports of TRIM23 involvement in NF-kB activation by HCMV (human cytomegalovirus) infection (Poole et al. 2009) and ubiquitination of NEMO (NF-kB essential modulator) catalyzed by TRIM23 (Arimoto et al. 2010) certainly present enormous numbers of intriguing questions to be answered. This entry summarizes, for the most part, findings from the earlier studies that are not yet meaningfully integrated with the important information now emerging. Domain structures as have been described for ARD1 and TRIM23 are aligned in Fig. 2 to facilitate reconciliation of the differently focused publications.



ARD1/TRIM23, Fig. 2 Domain structure of ARD1/TRIM23. The 574-amino-acid molecule contains an E3 ubiquitin ligase ring (*yellow*, E3, ARD1 or *gray*, 31–74, TRIM23) and a C-terminal ARF domain (*pink*, 403–574). At the extreme C-terminus, 34 amino acids (541–574, *black*) are involved in alternative splicing, as shown in Fig. 1. Positions 387–402 act as a GDI for the ARF domain. Intervening positions (126–370, *blue*, in TRIM23) contain B-boxes 1 and 2 plus the C-C region that make this a TRIM protein and overlap specific sequence (*green*) that can inactivate the ARF domain (101–190, GAP). We are grateful for the expert assistance of Dr. Gustavo Pacheco-Rodriguez, who prepared Figs. 1 and 2 and has made important contributions to many ARD1 studies

ARD1: Structure/Function

Structural requirements for functional interaction of the Arf and non-Arf domains of ARD1 were defined in some detail using site-specific mutagenesis and chimeric proteins (Vitale et al. 1997a). Replacement of seven Arf1 amino acids with those in the corresponding positions of the Arf domain in ARD1 enabled it to associate with the non-Arf domain that enhanced its GTPase activity. Further deletion of 15 amino acids from its N-terminus produced an Arf molecule structurally and functionally equivalent to the ARD1 Arf domain. Additional effects of numerous specific amino acid replacements in ARD1 and those of several phospholipids or detergents on interactions and/or activities of the GTP-binding Arf and regulatory non-Arf domains were also reported (Vitale et al. 1997a). Regions in the non-Arf domain responsible for acceleration of GTP hydrolysis by the intrinsic GTPase activity of the Arf domain, plus adjacent sequence necessary to establish the required physical interaction, were also identified. Evidence for interaction of acidic N427 and E428 (plus P432) in the Arf region

with basic R249 and K250 in the non-Arf domain was also obtained as regulated release of bound GDP and control of GTP hydrolysis are equally important for temporal continuity of the Arf domain GTPase cycling.

Structural elements of the ARD1 GDP dissociation-inhibitor (GDI) region were similarly characterized (Vitale et al. 1997b). Although both this action and the enhancement of GTPase catalytic activity involve intramolecular conformational changes, these might well differ in energetic costs and/or the effects on them of environmental changes. Early studies had shown that bound GDPβS was released more slowly from the recombinant Arf domain than from intact ARD1 (Vitale et al. 1996). Experiments with recombinant proteins and/or their fragments more clearly established the discrimination between GDP and GTP ligands. It was further demonstrated that 15 amino acids immediately preceding the Arf domain were responsible for slowing dissociation of GDP, but not GTP, and site-specific mutagenesis revealed importance of the hydrophobic amino acids for stabilization of ARD1-bound GDP (Vitale et al. 1997b). Even as our understanding of structure-function relationships in these actions of the ARD1 molecule continues to improve, their relationships to and integration with ARD1 functions in critical reactions of the innate immune system to viral infection appear to present more new and intriguing questions.

Cellular Localization of ARD1

After multiple attempts to identify endogenous ARD1 at intracellular sites in several tissues, Vitale et al. (1998b) were able to show single protein bands of about 64 kDa using antibodies immunoreactive with different parts of the ARD1 molecule in affinity-purified membranes from human liver presumed representative of lysosome or Golgi structures (Vitale et al. 1998b). Systematic microscopic observations of subcellular distribution of overexpressed EGFP-tagged ARD1 revealed perinuclear fluorescence consistent with Golgi localization in <5% of cells after 4 h, but present in almost 20% by 6 h and approaching 60% after 10 h. At the same time, fluorescence in widely dispersed vesicular structures, apparently lysosomes, was recorded in only <5% at 6 h and 40% of cells at 10 h. Between 12 and 50 h, both vesicular and perinuclear fluorescence was seen in about 60% of cells, suggesting the possibility of ARD1 cycling between Golgi and lysosomal organelles (Vitale et al. 1998b).

In attempts to identify appropriate localization signals in the ARD1 molecule, intracellular distribution of >20 overexpressed ARD1 fragments was evaluated microscopically with particular attention to YXXL motifs in the Arf domain thought to be potentially responsible for Golgi localization (Vitale et al. 2000b). Selective mutation of one or both of the sequences indicated that each could contribute to ARD1 presence in Golgi and that to reach the lysosomes, passage of ARD1 through the Golgi was required. A pentapeptide sequence, KFERQ, had been implicated as a targeting signal for lysosomes. Mutagenesis in 344KTLQQ348 and 369KQQQQ373 sequences of the non-Arf region of ARD1 demonstrated that the latter was critical for lysosomal localization (Vitale et al. 2000a).

ARD1: E3 Ubiquitin Ligase Activity and Arf Domain Function

E3 ubiquitin ligase activity of GST-ARD1 or its RING finger domain (residues 1–110) was demonstrated in vitro using the recombinant proteins plus pure mammalian E1, E2 (usually UbcH6), ATP, and ubiquitin (Vichi et al. 2005). Activity was abolished by deletion or mutation of the ARD1 ring structure. Like activities of the Arf and GAP domains, that of the E3 ligase was apparently independent of the rest of the molecule and was unaffected by addition of GTPγ*S* or GDP. Whether that is true also in the intracellular environment is not known. Although the ubiquitinated proteins failed to react with ARD1 antibodies, their increasing amounts and size during assays were paralleled by decreasing amounts of free ARD1 protein, consistent with auto-ubiquitination. Three bands of modified UbcH6 (E2) did react with UbcH6 antibodies as well as with those against ubiquitin (Vichi et al. 2005). Later studies of $ARD1^{-/-}$ mouse embryo fibroblasts stably expressing constructs for induced synthesis of ARD1 or a (C34A, H53A) mutant lacking E3 ligase activity supported the conclusion that auto-ubiquitination regulated ARD1 degradation and was responsible for maintaining its concentration at very low levels in all cells studied (Meza-Carmen et al. 2011). Mutant ARD1 without

E3 ligase activity accumulated in cells incubated with proteasomal inhibitors to levels sevenfold those usually seen.

Cytohesin-1 had been shown earlier to be a specific guanine nucleotide-exchange factor (GEF) activator for ARD1 (Vitale et al. 2000b). Since cytohesin-1 (and other cytohesins) had been reported to modify receptor tyrosine kinase action, effects of ARD1 or its mutants on EFGR were explored (Meza-Carmen et al. 2011). There was no evidence of E3 ligase involvement, but Arf domain function was clearly important. Actions in vesicular trafficking are probably the most extensively studied of all Arf functions. These require continuity of Arf cycling between inactive GDP-bound and active GTP-bound states. Overexpression of mutants with single amino acid replacements that abolish GTP binding (T418N), thereby blocking activation, or prevent GTP hydrolysis (K458I) both interrupt cyclic activation/inactivation, causing accumulation, respectively, of GDP or GDP-liganded proteins (thus referred to as ARD1-GDP or ARD1-GTP mutants). Amounts of EGFR were higher in ARD1-GDP and lower in ARD1-GTP cells than in those expressing wild-type (WT) ARD1 with a functional Arf domain, and without significant differences in RNA levels (Meza-Carmen et al. 2011). All findings were consistent with the conclusion that relatively rapid EGFR turnover enables more facile control of receptor protein levels and functions than would alterations in gene expression and necessary subsequent protein adjustments. Regardless of total levels, about 80% of the EGFR was on the cell surface. It was notable also that in ARD1-GDP and –GTP cells differences in total amounts of TGFβR III and insulin receptor (IR) were similar to those of EGFR, although the limited IR data did not reach statistical significance (Meza-Carmen et al. 2011). Potential functions of ARD1 in the regulation of these (and perhaps other) growth factor receptors, via internalization, signaling, and/or degradation, should be of considerable interest and importance.

ARD1: Action/Function as TRIM23

Description of TRIM 23 as a member of the TRIM protein family provided new perspective on ARD1 action (Reymond et al. 2001). Activation of transcription with induction of type I interferon production is critical for initiation of innate immune responses. Association of TRIM 23 (ARD1) with HCMV (human cytomegalovirus)-encoded protein UL144, which was known to activate NF-kB, was first recognized in a yeast two-hybrid screen undertaken to identify UL144-interacting proteins in human cells (Poole et al. 2009). Involvement of tumor necrosis factor receptor (TNFR)-associated factor 6 (▶ TRAF6) and/or TAK1 in HCMV-induced NF-kB activation was known, but the screen revealed no evidence of their direct interaction with the viral UL144 protein. Then, interaction of UL144 with TRIM23 was found and confirmed, showing direct interaction of the UL144 C-terminal Zn-finger with the TRIM23 N-terminal Zn-finger region. To verify those findings, tagged UL144, TRIM23, and TRAF6 or fragments thereof were overexpressed in human fibroblasts. All experimental data supported the conclusion that TRIM23 was required for NF-kB activation by UL144 (but not for its activation by TNF or dsRNA). TRIM23 was necessary for ubiquitination also of TRAF6, but it did not catalyze the modification (using ubiquitin lysine 63, not lysine 48 for both) that resulted evidently from TRAF6 auto-ubiquitination (Poole et al. 2009).

Rapid induction of the type I interferon response by viral infection is critical for an innate immune reaction. Involvement of several E3 ubiquitin ligases and their substrates provides complex feedback controls for the potentially harmful inflammatory reactions that result. To clarify some of these regulatory relationships, Arimoto et al. (2010) looked for effects of overexpression of one of the ligases, RNF-125, on gene expression and found a dramatic increase of >200% in TRIM23 mRNA. Exploration of TRIM 23 function revealed that it increased expression of an NF-kB-driven reporter gene in a NF-kB essential modulator (NEMO)-dependent manner. Direct interaction of the TRIM23 Arf domain with CC1 and LZ domains, which contain the sites of NEMO ubiquitination (via lysine 27), was demonstrated (Arimoto et al. 2010). Information regarding the role of UbcH5 as an E2 enzyme for TRIM23 ubiquitination of NEMO was notable, particularly perhaps the enhanced association of UbcH5 with NEMO after virus infection and the decreased NEMO ubiquitination by TRIM23 in UbcH5-depleted cells. Behavior of the alternatively spliced TRIM23 molecules (Venkateswarlu and Wilson 2011) in these interactions will surely be of interest, as an Arf domain contribution to intracellular trafficking might facilitate innate immune responses.

Summary

ARD1 was cloned because of its C-terminal Arf sequence (which lacks the first nine Arf amino acids). Initial investigation of the predicted 64-kDa protein was directed toward understanding how the Arf domain interacted with the non-Arf remainder of the molecule to accelerate hydrolysis of bound GTP using separately synthesized recombinant protein fragments. The region of the ARD1 molecule that serves as a GAP to terminate activation of the Arf moiety, including adjacent residues required for its functional interaction with Arf structure, appears to overlap B-box and C-C sequences of the TRIM23 molecule (Fig. 2). GDI action of 17 amino acids immediately N-terminal to the Arf domain mimics behavior of the N-terminal amphipathic α-helix of an Arf molecule that would precede the sequence designated Arf domain at ARD1 position 403 (Fig. 2). Both ARD1 and TRIM23 sequences assign E3 ubiquitin ligase actions to the same novel RING structure near the N-terminus. Generation of $ARD^{-/-}$ mouse embryo fibroblasts stably transfected with constructs for inducible expression of wild type or specifically mutated ARD1 molecules enabled demonstration that degradation of endogenous EGF receptor (EGFR) required continuity of the ARD1 GDP/GTP cycle. Cell content of TRIM23 was apparently maintained at low levels by auto-ubiquitination, with no TRIM23 contribution to EGFR ubiquitination. For auto-polyubiquitination of ARD1 in vitro with purified proteins, UbcH5a, 5b, 5c, and UbcH6 had been effective as E2 enzymes. UbcH5 was also involved in TRIM23 ubiquitination of NEMO. These and related findings may be a preview of the importance of only recently recognized antiviral actions of TRIM23.

References

Arimoto K, Funami K, Saeki Y, Tanaka K, Okawa K, Takeuchi O, Akira S, Murakami Y, Shimotohno K. Polyubiquitin conjugation to NEMO by tripartite motif protein 23 (TRIM23) is critical in antiviral defense. Proc Natl Acad Sci USA. 2010;107:36.

Ding M, Vitale N, Tsai S, Adamik R, Moss J, Vaughan M. Characterization of a GTPase-activating protein that stimulates GTP Hydrolysis by both ADP-ribosylation factor (ARF) and ARF-like proteins. J Biol Chem. 1996;271:2400–9.

Meza-Carmen V, Pacheco-Rodriguez G, Soo Kang G, Kato J, Donati C, Zhang C, Vichi A, Payne DM, El-Chemaly S, Stylianou M, Moss J, Vaughan M. Regulation of growth factor receptor degradation by ADP-ribosylation factor domain protein (ARD)1. Proc Natl Acad Sci USA. 2011;108:10454–9.

Mishima K, Tsuchiya M, Nightingale MS, Moss J, Vaughan M. ARD1, a 64-kDa guanine nucleotide-binding protein with a carboxyl-terminal ADP-ribosylation factor domain. J Biol Chem. 1993;268:8801–7.

Ozato K, Shin DM, Chang TH, Morse HCC III. TRIM family proteins and their emerging roles in innate immunity. Nat Rev Immunol. 2008;8:849–60.

Poole E, Groves I, Macdonald A, Pang Y, Alcami A, Sinclair J. Identification of TRIM23 as a cofactor involved in the regulation of NF-kB by human cytomegalovirus. J Virol. 2009;83:3581–90.

Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, Zanaria E, Messali S, Cainarca S, Guffanti A, Minucci S, Pelicci PG, Ballabio A. The tripartite motif family identifies cell compartments. EMBO J. 2001;20:2141–51.

Venkateswarlu K, Wilson LM. ADP-ribosylation factor domain protein 1. UCSD Molecule Pages. 2011. doi:10.1038/m.p.a003292.01.

Vichi A, Payne DM, Pacheco-Rodriguez G, Moss J, Vaughan M. E3 ubiquitin ligase activity of the trifunctional ARD1 (ADP-ribosylation factor domain protein 1). Proc Natl Acad Sci USA. 2005;102:1945–50.

Vitale N, Moss J, Vaughan M. ARD1, a 64-kDa bifunctional protein containing an 18-kDa GTP-binding ADP-ribosylation factor domain and a 46-kDa GTPase-activation domain. Proc Natl Acad Sci USA. 1996;93:1941–4.

Vitale N, Moss J, Vaughan M. Interaction of the GTP-binding and GTPase-activating domains of ARD1 involves the effector region of the ADP-ribosylation factor domain. J Biol Chem. 1997a;272:3897–904.

Vitale N, Moss J, Vaughan M. Characterization of a GDP dissociation inhibitory region of ADP-ribosylation factor domain protein ARD1. J Biol Chem. 1997b;272:25077–82.

Vitale N, Moss J, Vaughan M. Molecular characterization of the GTPase-activating domain of ADP-ribosylation factor domain protein 1 (ARD1). J Biol Chem. 1998a;273:2553–60.

Vitale N, Horiba K, Ferrans V, Moss J, Vaughan M. Localization of ADP-ribosylation factor domain protein 1 (ARD1) in lysosomes and Golgi apparatus. Proc Natl Acad Sci USA. 1998b;95:8613–18.

Vitale N, Ferrans V, Moss J, Vaughan M. Identification of lysosomal and Golgi localization signals in GAP and ARF domains of ARF domain protein 1. Mol Cell Biol. 2000a;20:7432–52.

Vitale N, Pacheco-Rodriguez G, Ferrans V, Riemenschneider W, Moss J, Vaughan M. Specific functional interaction of human cytohesin-1 and ADP-ribosylation factor domain protein (ARD1). J Biol Chem. 2000b;275:21331–9.

ARF GAP with Dual Pleckstrin Homology (PH) Domains

► ADAP1

Arf1-GAP

▶ ArfGAP1

Arf-Domain Protein

▶ ARD1/TRIM23

ARF-GAP, RHO-GAP, Ankyrin Repeat, and Plekstrin Homology Domains Containing Protein 3

▶ ARAP3

ArfGAP1

Moran Rawet-Slobodkin
Department of Biological Chemistry Ullman Building, Weizmann Institute of Science, Rehovot, Israel

Synonyms

ADP ribosylation factor GTPase-activating protein 1; Arf1-GAP

Historical Background

ArfGAP1 is a 415 aa soluble protein that reversibly interacts with the Golgi apparatus (Cukierman et al. 1995). Originally isolated from rat liver cytosol (Makler et al. 1995), it is the founding member of the ArfGAP family of proteins, all characterized by a conserved catalytic domain containing a zinc finger model whose structure was solved by Goldberg (1999).

The substrate of ArfGAP1, Arf1, is a key regulator of the COPI system that mediates vesicular transport in the ER–Golgi shuttle. Upon GDP to GDP exchange, Arf1 associates with the Golgi membrane and recruits the heptameric COPI coat (coatomer), which in turn sorts cargo proteins and polymerizes to form the coat cage surrounding the vesicle. The subsequent hydrolysis of Arf-bound GTP is required for the release of coatomer from the membrane, a prerequisite for vesicle fusion. This reaction requires the action of a GAP. ArfGAP1 was the first Arf-directed GTPase-activating protein purified and cloned. Thirty one ArfGAP proteins are currently known in human, three of which – ArfGAP1, 2, and 3 – are thought to regulate COPI-mediated transport though the Golgi. Silencing of ArfGAP1 or a combination of ArfGAP2 and ArfGAP3 in HeLa cells does not decrease cell viability; however, silencing all three ArfGAPs causes cell death. In yeast, two ArfGAPs –GCS1, the orthologue of ArfGAP1, and Glo3, the orthologue of mammalian ArfGAP2/3 – have been implicated in COPI-mediated transport and were shown to function as an essential pair (Poon et al. 1999).

Function of ArfGAP1

Most published data implicate ArfGAP1 as a regulator of the COPI system. However, various and sometimes conflicting reports exist on the role of ArfGAP1 in the biogenesis and consumption COPI vesicles.

First indication for a role of ArfGAP1 in deactivation of Arf1 at the Golgi was provided by the finding that overexpression of ArfGAP1 in cells results in redistribution of the Golgi and its fusion with the ER (Huber et al. 1998), an effect that was known before to result from the deactivation of Arf1 by the drug brefeldin-A.

Reconstitution of COPI vesicles from Golgi membranes revealed that blocking GTP hydrolysis by the use of GTPγS (Serafini et al. 1991) or an activating mutant of Arf1 (Tanigawa et al. 1993) lead to the production of vesicles that cannot uncoat. These findings lead to the prediction that ArfGAP activity should trigger coatomer release from membranes. Subsequent studies, however, suggested that GTP hydrolysis on Arf1 is required for efficient uptake of cargo into vesicles (Lanoix et al. 1999; Nickel et al. 1998; Pepperkok et al. 2000), implying that GAP activity may also promote cargo sorting that occurs during vesicle formation. Lee et al. (2005) presented evidences for a role of ArfGAP1 in regulating the binding of coatomer to cargo proteins, indicating a direct role of ArfGAP1 in regulating cargo sorting. This study further demonstrated a requirement of

ArfGAP1 catalytic activity for vesicle formation from Golgi membranes suggesting that ArfGAP1 plays a central role in coupling cargo sorting and vesicle formation. An additional function of ArfGAP1 in the vesicle fission was also proposed (Kartberg et al. 2010; Yang et al. 2006). Finally, ArfGAP1 has been shown to be involved in the regulation of asymmetric tethering between flat and curved membrane mediated by the Arf1 effector, GMAP210 (Drin et al. 2008).

Regulation of ArfGAP1 Activity

Experiments in vitro have suggested two mechanisms for the regulation of ArfGAP1 activity: stimulation by the COPI coat and by regulated recruitment to membranes.

The first mechanism concerning the role of coatomer was first described by Goldberg (Goldberg 1999). Using the catalytic domain of ArfGAP1 and a truncated Arf1 lacking the first 17 residues that can be loaded with GTP in the absence of lipids or detergents, Goldberg reported that the activity of the ArfGAP1 can be stimulated by up to 1,000-fold by coatomer. In contrast, investigating ArfGAP1 catalytic activity in liposomal system (Szafer et al. 2000) or on Golgi membranes (Szafer et al. 2001) using myristoylated membrane-bound Arf1 revealed only moderate or no stimulation by the addition of coatomer, respectively. Examination of the enzymology of ArfGAP1 suggested that coatomer allosterically regulates ArfGAP1 activity, affecting the affinity of ArfGAP1 for Arf-GTP but not the catalytic rate constant. These results further support the idea that coatomer has a regulatory role on the activity of ArfGAP1 (Luo and Randazzo 2008).

The second mechanism concerns the role of lipids. Although there is no evidence for direct interaction of ArfGAP1 with specific lipids, binding of ArfGAP1 to membranes results in increase in GAP activity by bringing it into proximity with its membrane-bound substrate, Arf1-GTP. The binding of ArfGAP1 to liposomes and its catalytic activity are both increased by chemical or physical conditions that create open spaces in the outer leaflet of the membrane bilayer such as the presence of diacylglycerols, phospholipids containing monounsaturated fatty acids (Antonny et al. 1997; Bigay et al. 2005), and high membrane curvature. Curvature-dependent activity of ArfGAP1 in vitro is of particular interest as it offers a mechanism that may be employed in vivo to ensure efficient targeting of ArfGAP1 to coated vesicles and/or to the highly curved rim of the Golgi cisternae where budding of COPI vesicles takes place. Bigay et al. (2005) have identified a motif in the center of ArfGAP1 that mediates the interaction with loosely packed lipids. This domain, termed ALPS (for ArfGAP1 lipid packing sensor) is unstructured in solution but in the presence of loosely packed lipids, hydrophobic residues in ALPS are inserted between lipid residues and ALPS folds into an amphipathic helix with serine/threonine residues forming the hydrophilic face. A function for ALPS–lipid interaction in vivo is suggested by the findings that the hydrophobic residues in ALPS are required for the interaction of ArfGAP1 with the Golgi apparatus (Parnis et al. 2006). In subsequent studies a second amphipathic motif – ALPS2 – with similar physicochemical characteristics was identified in ArfGAP1 (Levi et al. 2008; Mesmin et al. 2007). The two amphipathic motifs are separated by a short break and function cooperatively conferring liposome interaction and Golgi localization of ArfGAP1.

Summary

Mounting evidence implicates ArfGAP1 as critical regulator of the COPI system, yet its precise role has remained uncertain, with suggested functions ranging from an uncoating factor to an essential coat component required for cargo sorting and vesicle formation. While most studies have focused on the role of ArfGAP1 in the COPI system, ArfGAP1 also interacts with components of clathrin-coated carriers (including clathrin, AP-1, and AP-2), although the functional consequences of these interactions remains to be established.

References

Antonny B, Huber I, Paris S, Chabre M, Cassel D. Activation of ADP-ribosylation factor 1 GTPase-activating protein by phosphatidylcholine-derived diacylglycerols. J Biol Chem. 1997;272:30848–51.

Bigay J, Casella JF, Drin G, Mesmin B, Antonny B. ArfGAP1 responds to membrane curvature through the folding of a lipid packing sensor motif. EMBO J. 2005;24:2244–53.

Cukierman E, Huber I, Rotman M, Cassel D. The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization. Science. 1995;270:1999–2002.
Drin G, Morello V, Casella JF, Gounon P, Antonny B. Asymmetric tethering of flat and curved lipid membranes by a golgin. Science. 2008;320:670–3.
Goldberg J. Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatomer in GTP hydrolysis. Cell. 1999;96:893–902.
Huber I, Cukierman E, Rotman M, Aoe T, Hsu VW, Cassel D. Requirement for both the amino-terminal catalytic domain and a noncatalytic domain for in vivo activity of ADP-ribosylation factor GTPase-activating protein. J Biol Chem. 1998;273:24786–91.
Kartberg F, Asp L, Dejgaard SY, Smedh M, Fernandez-Rodriguez J, Nilsson T, Presley JF. ARFGAP2 and ARFGAP3 are essential for COPI coat assembly on the Golgi membrane of living cells. J Biol Chem. 2010;285:36709–20.
Lanoix J, Ouwendijk J, Lin CC, Stark A, Love HD, Ostermann J, Nilsson T. GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles. EMBO J. 1999;18:4935–48.
Lee SY, Yang JS, Hong W, Premont RT, Hsu VW. ARFGAP1 plays a central role in coupling COPI cargo sorting with vesicle formation. J Cell Biol. 2005;168:281–90.
Levi S, Rawet M, Kliouchnikov L, Parnis A, Cassel D. Topology of amphipathic motifs mediating Golgi localization in ArfGAP1 and its splice isoforms. J Biol Chem. 2008;283:8564–72.
Luo R, Randazzo PA. Kinetic analysis of Arf GAP1 indicates a regulatory role for coatomer. J Biol Chem. 2008;283:21965–77.
Makler V, Cukierman E, Rotman M, Admon A, Cassel D. ADP-ribosylation factor-directed GTPase-activating protein. Purification and partial characterization. J Biol Chem. 1995;270:5232–7.
Mesmin B, Drin G, Levi S, Rawet M, Cassel D, Bigay J, Antonny B. Two lipid-packing sensor motifs contribute to the sensitivity of ArfGAP1 to membrane curvature. Biochemistry. 2007;46:1779–90.
Nickel W, Malsam J, Gorgas K, Ravazzola M, Jenne N, Helms JB, Wieland FT. Uptake by COPI-coated vesicles of both anterograde and retrograde cargo is inhibited by GTPgammaS in vitro. J Cell Sci. 1998;111:3081–90.
Parnis A, Rawet M, Regev L, Barkan B, Rotman M, Gaitner M, Cassel D. Golgi localization determinants in ArfGAP1 and in new tissue-specific ArfGAP1 isoforms. J Biol Chem. 2006;281:3785–92.
Pepperkok R, Whitney JA, Gomez M, Kreis TE. COPI vesicles accumulating in the presence of a GTP restricted arf1 mutant are depleted of anterograde and retrograde cargo. J Cell Sci. 2000;113:135–44.
Poon PP, Cassel D, Spang A, Rotman M, Pick E, Singer RA, Johnston GC. Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping function. EMBO J. 1999;18:555–64.
Serafini T, Orci L, Amherdt M, Brunner M, Kahn RA, Rothman JE. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. Cell. 1991;67:239–53.
Szafer E, Pick E, Rotman M, Zuck S, Huber I, Cassel D. Role of coatomer and phospholipids in GTPase-activating protein-dependent hydrolysis of GTP by ADP-ribosylation factor-1. J Biol Chem. 2000;275:23615–19.
Szafer E, Rotman M, Cassel D. Regulation of GTP hydrolysis on ADP-ribosylation factor-1 at the Golgi membrane. J Biol Chem. 2001;276:47834–9.
Tanigawa G, Orci L, Amherdt M, Ravazzola M, Helms JB, Rothman JE. Hydrolysis of bound GTP by ARF protein triggers uncoating of Golgi-derived COP-coated vesicles. J Cell Biol. 1993;123:1365–71.
Yang JS, Zhang L, Lee SY, Gad H, Luini A, Hsu VW. Key components of the fission machinery are interchangeable. Nat Cell Biol. 2006;8:1376–82.

Arf-Like Protein 8B

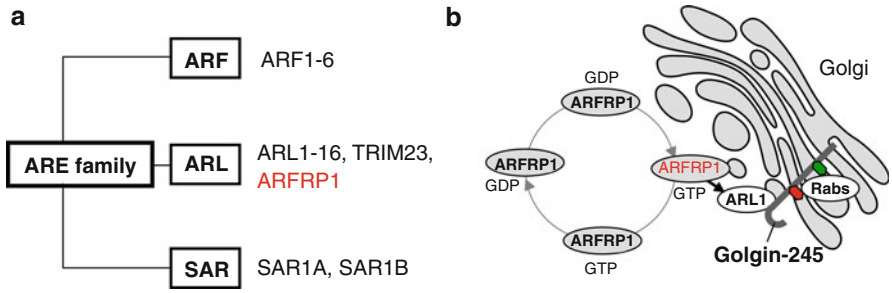
► Arl8b

ARFRP1 (ADP-Ribosylation Factor Related Protein 1)

Deike Hesse, Alexander Jaschke and Annette Schürmann
Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Historical Background

Due to the remote similarity of the ADP-ribosylation factor related protein 1 (ARFRP1) to other members of the ARF family, it was designated as a distant ARF family member (Fig. 1a). Discovered by the screening of adipocytes with degenerated primers for ARF proteins, ARFRP1 was shown to be highly conserved among species (97% identical amino acids between rat and human, 79% identical to *Saccharomyces cerevisiae*, and 74% identical to *Drosophila melanogaster*) and closest related to ARL1 (33% identical) and ARL3 (39% identical) (Schürmann et al. 1995, 1999). In comparison to the membrane association motif of other members of the ARF family (myristoylation), membrane association of ARFRP1 is mediated by acetylation of the initial methionine and interaction with an integral membrane protein Sys1 (Behnia et al. 2004;



ARFRP1 (ADP-Ribosylation Factor Related Protein 1), Fig. 1 ARFRP1, a member of the ARF family is recruited to the *trans*-Golgi upon its activation. (**a**) The ARF family of small GTPases consists of ARF, ARF-like (ARL), and SAR proteins (Kahn et al. 2006). (**b**) Inactive, GDP-bound ARFRP1 is located in the cytosol, active, GTP-bound ARFRP1 associates with membranes of the *trans*-Golgi. Active ARFRP1 initiates recruitment of ARL1 and its effector Golgin-245 to this compartment (Zahn et al. 2006)

Setty et al. 2003). The *Arfrp1* gene consists of eight exons and is located on distal mouse chromosome 2 (human chromosome 20) with the transcriptional start codon in exon 2 (Mueller et al. 2002a). The gene product, ARFRP1, is a 25 kDa protein with a ubiquitous expression pattern and an intrinsic GTPase activity (Schürmann et al. 1995). In contrast, guanine nucleotide exchange is relatively slow in an in vitro system suggesting the existence of a so-far unidentified GEF (guanine nucleotide exchange factor) for ARFRP1 to act as a fast GTP-dependent molecular switch (Schürmann et al. 1995).

Molecular Function of ARFRP1

In the active GTP-bound form ARFRP1 is located at the *trans*-Golgi (Fig. 1b) (Zahn et al. 2006). The yeast homologue of ARFRP1, Arl3p, acts sequentially to recruit golgin proteins to the Golgi membranes. In yeast, Arl3p brings Arl1p, the yeast homolog of ARL1, to the Golgi apparatus which is then responsible for the recruitment of the yeast golgin Imh1p (Panic et al. 2003; Setty et al. 2003). Golgins are conserved proteins found in different parts of the Golgi stack, and they are typically anchored to the membrane at their carboxyl termini by a transmembrane domain or by binding a small GTPase (Rab and ARL1). They appear to have roles in membrane traffic and Golgi structure, but their precise function is in most cases unclear (Munro 2011). In cell culture as well as in murine embryos, ARFRP1 controls the targeting of ARL1 and its effector Golgin-245 to the *trans*-Golgi (Fig. 1b) (Zahn et al. 2006, 2008). Upon inhibition of the expression of *Arfrp1* in cells or deletion of *Arfrp1* in mice, the *trans*-Golgi structure appeared altered as several *trans*-Golgi markers (TGN38, ARL1, Syntaxin6, Golgin-245) showed a different distribution pattern or a dissociation from the Golgi membranes (Hommel et al. 2010; Zahn et al. 2006, 2008). However, other Golgi proteins located in the *cis*- and *medial*-region of the Golgi apparatus (giantin, GM130, 58 k) seemed less affected by the lack of ARFRP1 (Hommel et al. 2010; Zahn et al. 2006, 2008).

In mammalian cells, ARFRP1 seems to inhibit ARF1-regulated pathways such as the activation of phospholipase D (PLD) (Schürmann et al. 1999). ARFRP1 binds the Sec7 domain of the ARF-specific nucleotide exchange factor cytohesin in a GTP-dependent manner. This interaction does not modify the activity of ARFRP1 but results in the inhibition of the ARF/Sec7-dependent activation of PLD in a system of isolated membranes and in HEK-293 cells transfected with a constitutively active mutant of ARFRP1 (Schürmann et al. 1999).

Knockout Models Explaining the Physiological Role of ARFRP1

In order to characterize the function of ARFRP1 in a mammalian organism, its gene was disrupted by gene-targeting approaches. Homozygosity for the conventional transgene causes embryonic lethality, whereas tissue-specific deletion of *Arfrp1* resulted in growth retardation according to lipid and glycogen storage defects.

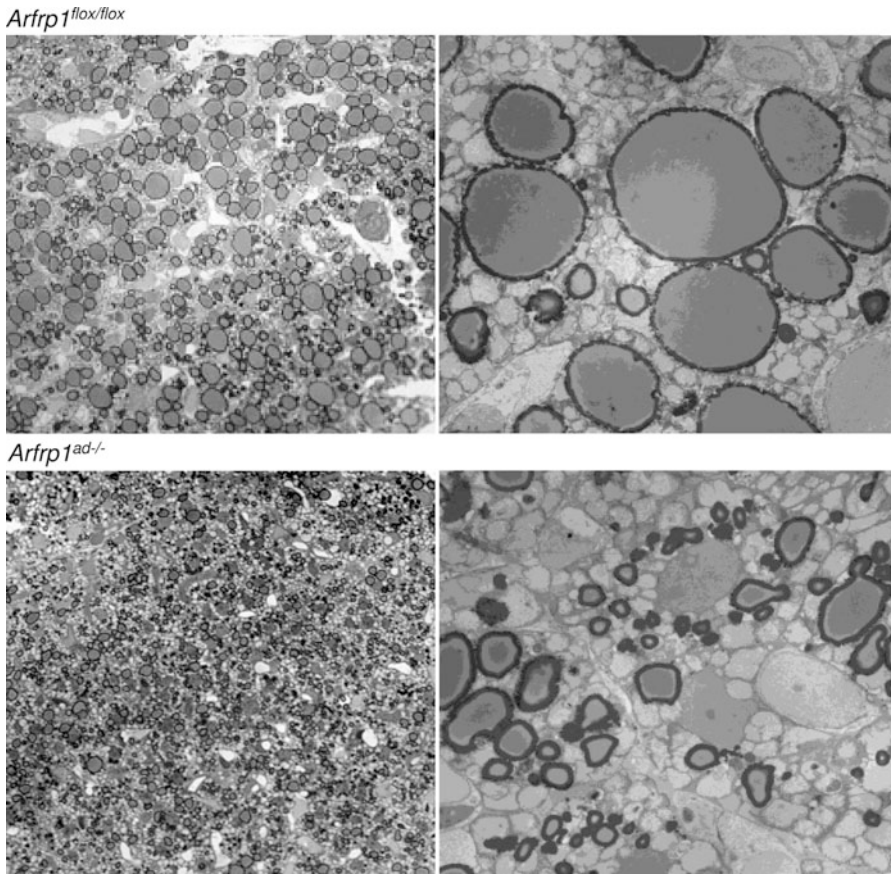
Adhesion Defects Responsible for Embryonic Lethality of Conventional *Arfrp1* Knockout Mice

Mueller et al. (2002b) showed that ARFRP1 is already important during early embryogenesis. The amount of *Arfrp1* mRNA was detectable from embryonic day 4.5 and increases during gastrulation and neurulation (Mueller et al. 2002b). The conventional deletion of *Arfrp1* in the mouse results in embryonic lethality during early gastrulation (Mueller et al. 2002b). *Arfrp1*-null mutant embryos seemed normal until embryonic day 5, but exhibited profound alterations of the distal part of the egg cylinder at day 6–6.5 due to a cell-adhesion defect (Mueller et al. 2002b; Zahn et al. 2008). Further investigations revealed that embryonic cells showed a mistargeting of E-cadherin to intracellular membranes which prevented epiblast cells to undergo an epithelial-to-mesenchymal transition, and resulted in a failure of mesoderm development (Mueller et al. 2002b; Zahn et al. 2008). This finding was confirmed in studies performed in intestinal epithelium of mice lacking *Arfrp1* specifically in the intestine (see below). Here retention of E-cadherin in intracellular membranes was observed, it was co-localized with a *cis*-Golgi marker (GM130) in epithelial intestinal cells. Moreover, a direct interaction of ARFRP1 with the E-cadherin/catenin complex was demonstrated by co-immunoprecipitation experiments (Zahn et al. 2008) indicating that ARFRP1 is essential for the correct trafficking of E-cadherin through the Golgi and finally for the correct cell surface localization of the E-cadherin complex.

Adipocyte-Specific Deletion of *Arfrp1* Resulting in Lipodystrophy and Reduced Survival

ARFRP1 is highly expressed in the adipose tissue of mice (Schürmann et al. 1995). Adipocyte-specific *Arfrp1*-deleted mice ($Arfrp1^{ad-/-}$) were generated with the Cre/loxP recombination system using the *Fatp4*/*aP2* promoter (He et al. 2003). Animals were born viable according to the expected Mendelian distribution but exhibited a markedly reduced survival with a mortality rate of 70% until weaning (Hommel et al. 2010). In addition, $Arfrp1^{ad-/-}$ mice showed a postnatal growth retardation accompanied by a reduced surface body temperature which presumably is responsible for the impaired survival. The most obvious phenotype of the $Arfrp1^{ad-/-}$ mice was a pronounced lipodystrophy indicated by the lack of subcutaneous and gonadal white adipose tissue depots as well as a significantly reduced amount of brown adipose tissue and an early hepatosteatosis. Oil-Red-O staining of brown adipose tissues of $Arfrp1^{ad-/-}$ and control littermates indicated an altered lipid storage associated with smaller lipid droplets in the brown fat cells (Hommel et al. 2010). One reason for the impaired lipid storage of $Arfrp1^{ad-/-}$ mice was shown to be a stimulation of lipolysis. The amount of phosphorylated hormone-sensitive lipase (HSL) was elevated, and the association of adipocyte triglyceride lipase (ATGL) with lipid droplets was enhanced in brown adipose tissue of $Arfrp1^{ad-/-}$ mice indicating that lipolysis was activated. In fact, siRNA-induced knockdown of *Arfrp1* in 3T3-L1 adipocytes increased basal lipolysis. A second cause for smaller lipid droplets in adipocytes lacking ARFRP1 was affiliated to a defective lipid droplet fusion. Electron microscopy showed that lipid droplets exhibited ultrastructural alterations such as a disturbed interaction of small lipid-loaded particles with larger lipid droplets (Fig. 2). The SNARE (soluble N-ethylmaleimide-sensitive-factor attachment receptor) protein SNAP23 (synaptosomal-associated protein) which is described to be involved in lipid droplet fusion (Boström et al. 2007) was predominantly located in the cytosol and plasma membrane in brown adipose tissue of $Arfrp1^{ad-/-}$ mice, whereas it was associated with small lipid droplets in controls. This suggested that ARFRP1 mediates lipid droplet growth via sorting of SNAP23. Thus, disruption of ARFRP1 in the adipose tissue led to a lipodystrophic phenotype by activating lipolysis and preventing the normal enlargement of lipid droplets via fusion events.

Since SNARE proteins (VAMP2, syntaxin-4, and SNAP23) have been implicated in the insulin-induced translocation of vesicles containing the glucose transporter GLUT4 to the plasma membrane of adipocytes (Hickson et al. 2000; Kawanishi et al. 2000; Bryant et al. 2002), subcellular distribution of GLUT4 in $Arfrp1^{ad-/-}$ adipocytes was studied. GLUT4 accumulated at the plasma membrane rather than being sequestered into an intracellular insulin-sensitive compartment as in control adipocytes (Fig. 3) (Hesse et al. 2010). A similar missorting of GLUT4 was produced



ARFRP1 (ADP-Ribosylation Factor Related Protein 1), Fig. 2 In the absence of *Arfrp1* in adipose tissues, the lipid droplets are much smaller as indicated in the ultrastructural analysis performed by electron microscopy (Hommel et al. 2010)

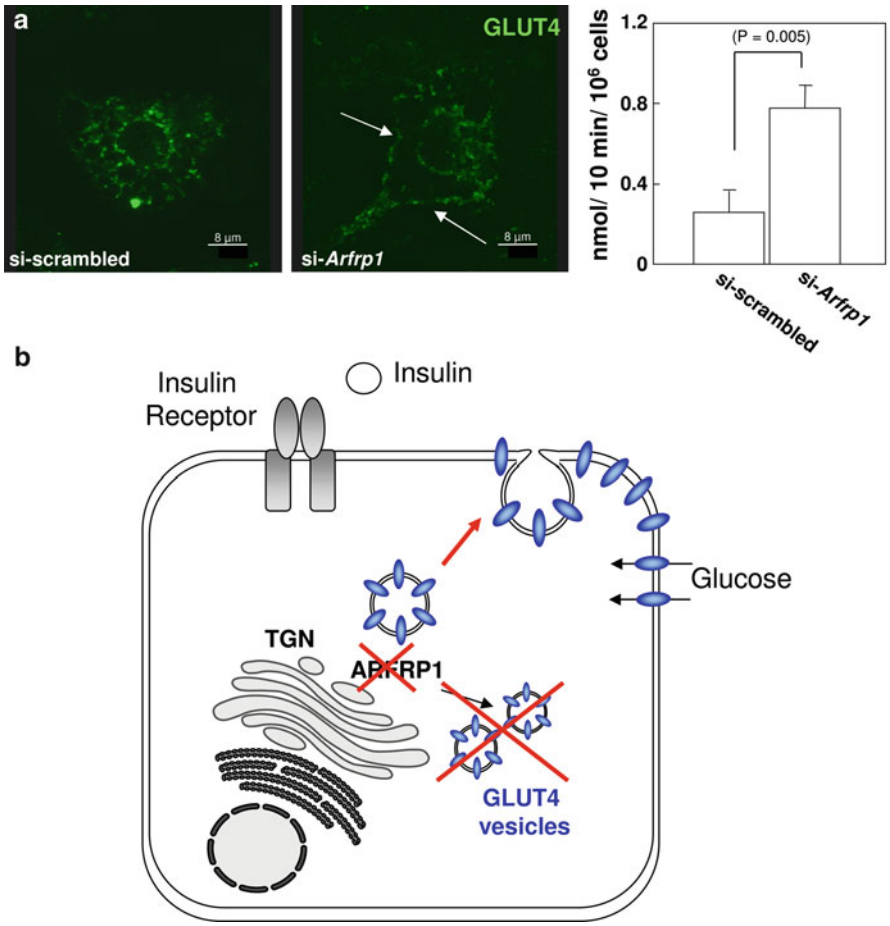
by siRNA-mediated knockdown of *Arfrp1* in 3T3-L1 adipocytes which led to a significantly elevated glucose transport. Thus, ARFRP1 appears to be involved in the sorting of GLUT4.

Deletion of *Arfrp1* in the Intestine Resulting in Fat Malabsorption

Conditional deletion of *Arfrp1* in the intestinal epithelium of mice ($Arfrp1^{vil-/-}$), as achieved by crossing $Arfrp1^{flox/flox}$ mice with transgenic mice expressing the Cre-recombinase under the villin promoter, resulted in an early postnatal growth retardation according to an impaired maturation and lipidation of chylomicrons (Jaschke et al., in revision).

$Arfrp^{vil-/-}$ mice revealed decreased levels of triglyceride and free fatty acid concentrations in the plasma, indicating that their growth retardation is the consequence of a malabsorption. Actually, lipid uptake elucidated by oral fat tolerance tests was impaired in $Arfrp1^{vil-/-}$ mice but fatty acids transport into the intestinal epithelium was normal and $Arfrp1^{vil-/-}$ mice accumulated lipid droplets in epithelial cells after an oil bolus. However, the release of resynthesized triglycerides was massively decreased, the apolipoprotein ApoA-I accumulated in the $Arfrp1^{vil-/-}$ epithelium, whereas its level in the plasma was reduced (Jaschke et al., in revision).

As stated above, ARFRP1 is required for recruitment of ARL1 and its effector, the golgin protein Golgin-245, to *trans*-Golgi membranes. Since several Rab proteins – involved in the regulation of vesicular trafficking – interact with Golgin-245, their subcellular distribution was studied in the $Arfrp1^{vil-/-}$ epithelium. Indeed Rab2 revealed a modified distribution in $Arfrp1^{vil-/-}$ epithelial cells as compared with $Arfrp1^{flox/flox}$ cells. Whereas Rab2 was predominantly located in the cytosol and only partially associated with membranes of the Golgi in control cells, it was mainly detected at large vesicular structures adjacent to the nuclei and co-localized with ApoA-I in



ARFRP1 (ADP-Ribosylation Factor Related Protein 1), Fig. 3 Downregulation of *Arfrp1* by siRNA results in a direct translocation of the glucose transporter GLUT4 to the cell surface without stimulation with insulin. (**a**) Immunocytochemical staining of GLUT4 in 3T3-L1 adipocytes that were transfected with scrambled or *Arfrp1*-specific siRNA (*left panel*). Glucose transport as detected by deoxyglucose uptake was significantly elevated in 3T3-L1 cells depleted for *Arfrp1* (*right panel*). (**b**) Predicted model of how GLUT4 vesicles are mistargeted to the plasma membrane in the basal unstimulated state when ARFRP1 is deleted (Hesse et al. 2010)

Arfrp1$^{vil-/-}$ cells (Fig. 4). These data indicated that an ARFRP1-ARL1-golgin-Rab2 cascade is required for intestinal chylomicron maturation in the Golgi.

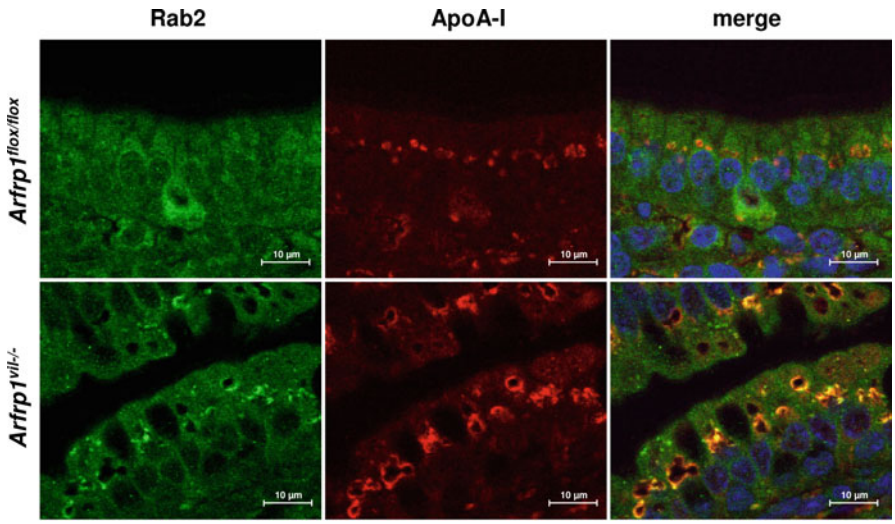
Deletion of *Arfrp1* in the Liver Impairing Glycogen Storage

The liver-specific deletion of *Arfrp1* resulted in a postnatal growth retardation accompanied by a significantly lower absolute and relative liver weight. The discrepancy between liver and body weight observed in *Arfrp1*$^{liv-/-}$ mice could at least partly be explained by the reduced glycogen storage which was reduced by 50% in knockout mice. This effect was referred to a reduced glucose uptake into the liver.

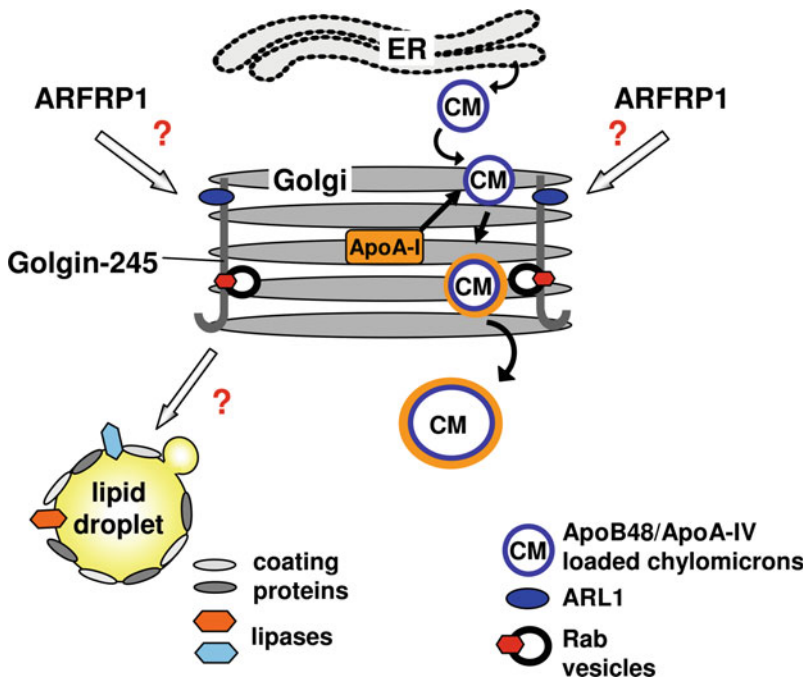
Immunohistochemical staining of the glucose transporter GLUT2 revealed a reduction of GLUT2 in the plasma membrane of *Arfrp1*$^{liv-/-}$ hepatocytes. In addition, total GLUT2 protein in lysates from livers of *Arfrp1*$^{liv-/-}$ mice was much lower compared to the controls. As the quantification of mRNA levels (*Slc2a2*) showed no alteration between the genotypes, it was speculated that a mistargeting of GLUT2 results in an advanced degradation of this transporter (Hesse et al., manuscript in preparation).

Suppression of ARFRP1 Expression in the Brain by Sleep Deprivation

ARFRP1 is not only expressed in peripheral tissues, it also shows a widespread distribution in the brain (Paratore et al. 2008). Highest expression levels of mRNA were determined by in situ hybridization and real-time PCR in the cerebral cortex, thalamic nuclei, colliculus, substantia nigra, and the granule cellular layer of the cerebellum. These brain areas show high



ARFRP1 (ADP-Ribosylation Factor Related Protein 1), Fig. 4 Co-localization of Rab2 with ApoA-I and its accumulation at Golgi membranes of intestinal *Arfrp1*$^{vil-/-}$ cells. Immunohistochemical detection of Rab2 (*left panels*), ApoA-I (*middle panels*), and the merged picture (*right panels*) in sections of the small intestine of 4-weeks old *Arfrp1*$^{flox/flox}$ and *Arfrp1*$^{vil-/-}$ mice that had free access to their diet



ARFRP1 (ADP-Ribosylation Factor Related Protein 1), Fig. 5 Proposed model of ARFRP1 action on lipid droplet formation and chylomicron maturation. ARFRP1 is necessary to recruit ARL1 to the Golgi, ARL1 binds to the scaffolding protein Golgin-245 which itself interacts with Rab proteins. *Left* part of the cartoon indicates that ARFRP1 is required for lipid droplet fusion and the regulation of lipases. *Right* part of the cartoon demonstrates the chylomicron formation in ER and Golgi. In the ER resynthesized triacylglycerol (TAG) is incorporated into ApoB48-containing pre-chylomicrons. Subsequently, ApoA-IV binds to the pre-chylomicrons which are then released to the *cis*-Golgi. ApoA-I is attached to the chylomicrons within the Golgi. ApoA-I loaded chylomicrons are then transported through the Golgi, released on the *trans*-site, and finally secreted into the lymph. Therefore, it is proposed that the ARFRP1-ARL1-golgin-Rab cascade is needed for an appropriate chylomicron assembly of ApoA-I and its transport through the Golgi

levels of neurotransmitter release, synaptic remodeling, and neuronal plasticity and therefore require extensive synaptic vesicle trafficking. Sleep deprivation alters the expression of genes involved in neuronal plasticity and synapse-related genes. In cerebral cortex the expression of *Arfrp1* was markedly reduced after sleep deprivation which could represent an adaptive response to the associated stress. Moderate levels of *Arfrp1* were detected in some amygdaloid nuclei, CA2 area, and dentate gyrus of the hippocampus, endopiriform nuclei, globus pallidus, striatum, molecular layer of cerebellum, and locus coeruleus. No expression of *Arfrp1* was observed in hypothalamic nuclei, CA1 and CA3 areas of the hippocampus and zona incerta.

Summary

ARFRP1 is a member of the family of ADP-ribosylation factors (ARFs) of GTPases which play a pivotal role in the regulation of membrane traffic. Activated, GTP-bound ARFRP1 associates with *trans*-Golgi membranes, and is required for the recruitment of ARF-like 1 (ARL1) and its effectors, specific golgin proteins to the *trans*-Golgi. ARFRP1 is essential for the correct targeting of several proteins (E-cadherin, GLUT4, GLUT2) but is also needed for the normal growth of lipid droplets (in adipose tissues) and the maturation of chylomicrons (in the small intestine) (Fig. 5). However, it is still not known how ARFRP1 is regulated, since no ARFRP1-specific GEF or GAP (GTPase-activating proteins) have been discovered so far. In addition, we did not solve the particular molecular action of ARFRP1 at the Golgi, how it initiates the ARL1-golgin-Rab cascade, and how this cascade finally modulates protein targeting, and lipid droplet and chylomicron maturation.

References

Behnia R, Panic B, Whyte JR, Munro S. Targeting of the Arf-like GTPase Arl3p to the Golgi requires N-terminal acetylation and the membrane protein Sys1p. Nat Cell Biol. 2004;6(5):405–13.

Boström P, Andersson L, Rutberg M, Perman J, Lidberg U, Johansson BR, Fernandez-Rodriguez J, Ericson J, Nilsson T, Borén J, Olofsson SO. SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. Nat Cell Biol. 2007;9(11):1286–93.

Bryant NJ, Govers R, James DE. Regulated transport of the glucose transporter GLUT4. Nat Rev Mol Cell Biol. 2002;3(4):267–77.

He W, Barak Y, Hevener A, Olson P, Liao D, Le J, Nelson M, Ong E, Olefsky JM, Evans RM. Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. Proc Natl Acad Sci USA. 2003;100(26):15712–7.

Hesse D, Hommel A, Jaschke A, Moser M, Bernhardt U, Zahn C, Kluge R, Wittschen P, Gruber AD, Al-Hasani H, Joost HG, Schürmann A. Altered GLUT4 trafficking in adipocytes in the absence of the GTPase Arfrp1. Biochem Biophys Res Commun. 2010;394(4):896–903.

Hesse D, Jaschke A, Augustin R, Hommel A, Püschel GP, Petzke KJ, Joost HG, Schürmann A. Altered glucose metabolism in mice lacking Arfrp1 in the liver.

Hickson GR, Chamberlain LH, Maier VH, Gould GW. Quantification of SNARE protein levels in 3 T3-L1 adipocytes: implications for insulin-stimulated glucose transport. Biochem Biophys Res Commun. 2000;270(3):841–5.

Hommel A, Hesse D, Völker W, Jaschke A, Moser M, Engel T, Blüher M, Zahn C, Chadt A, Ruschke K, Vogel H, Kluge R, Robenek H, Joost HG, Schürmann A. The ARF-like GTPase ARFRP1 is essential for lipid droplet growth and is involved in the regulation of lipolysis. Mol Cell Biol. 2010; 30(5):1231–42.

Jaschke A, Chung B, Hesse D, Kluge R, Zahn C, Moser M, Petzke KJ, Koepsell H, Heeren J, Joost HG, Schuermann A. The Golgi-associated GTPase ARFRP1 controls the final step of intestinal chylomicron maturation.

Kahn RA, Cherfils J, Elias M, Lovering RC, Munro S, Schurmann A. Nomenclature for the human *Arf* family of GTP-binding proteins: *ARF*, ARL, and SAR proteins. J Cell Biol. 2006;172(5):645–50.

Kawanishi M, Tamori Y, Okazawa H, Araki S, Shinoda H, Kasuga M. Role of SNAP23 in insulin-induced translocation of GLUT4 in 3 T3-L1 adipocytes. Mediation of complex formation between syntaxin4 and VAMP2. J Biol Chem. 2000;275(11):8240–7.

Mueller AG, Joost HG, Schürmann A. Mouse ARF-related protein 1: genomic organization and analysis of its promoter. Biochem Biophys Res Commun. 2002a;292(1):113–20.

Mueller AG, Moser M, Kluge R, Leder S, Blum M, Büttner R, Joost HG, Schürmann A. Embryonic lethality caused by apoptosis during gastrulation in mice lacking the gene of the ADP-ribosylation factor-related protein 1. Mol Cell Biol. 2002b;22(5):1488–94.

Munro S. The golgin coiled-coil proteins of the Golgi apparatus. Cold Spring Harb Perspect Biol. 2011;3(6):pii: a005256. doi:10.1101/cshperspect.a005256

Panic B, Perisic O, Veprintsev DB, Williams RL, Munro S. Structural basis for Arl1-dependent targeting of homodimeric GRIP domains to the Golgi apparatus. Mol Cell. 2003;12(4):863–74.

Paratore S, Parenti R, Cavallaro S. Distribution of ADP-ribosylation factor-related protein 1 in mouse brain. Arch Ital Biol. 2008;146(1):53–61.

Schürmann A, Massmann S, Joost HG. ARP is a plasma membrane-associated Ras-related GTPase with remote similarity to the family of ADP-ribosylation factors. J Biol Chem. 1995;270(51):30657–63.

Schürmann A, Schmidt M, Asmus M, Bayer S, Fliegert F, Koling S, Massmann S, Schilf C, Subauste MC, Voss M, Jakobs KH, Joost HG. The ADP-ribosylation factor (ARF)-related GTPase ARF-related protein binds to the ARF-specific guanine nucleotide exchange factor cytohesin and inhibits the ARF-dependent activation of phospholipase D. J Biol Chem. 1999;274(14):9744–51.

Setty SR, Shin ME, Yoshino A, Marks MS, Burd CG. Golgi recruitment of GRIP domain proteins by Arf-like GTPase 1 is regulated by Arf-like GTPase 3. Curr Biol. 2003;13(5):401–4.

Zahn C, Hommel A, Lu L, Hong W, Walther DJ, Florian S, Joost HG, Schürmann A. Knockout of Arfrp1 leads to disruption of ARF-like1 (ARL1) targeting to the trans-Golgi in mouse embryos and HeLa cells. Mol Membr Biol. 2006;23(6):475–85.

Zahn C, Jaschke A, Weiske J, Hommel A, Hesse D, Augustin R, Lu L, Hong W, Florian S, Scheepers A, Joost HG, Huber O, Schürmann A. ADP-ribosylation factor-like GTPase ARFRP1 is required for trans-Golgi to plasma membrane trafficking of E-cadherin. J Biol Chem. 2008;283(40):27179–88.

ARH9

▶ RhoC (RHOC)

ARHC

▶ RhoC (RHOC)

ARHGEF25

Katherine Figella[1], Brad Allen Bryan[1,2] and Mingyao Liu[3]
[1]Department of Biology, Ghosh Science and Technology Center, Worcester State University, Worcester, MA, USA
[2]Center of Excellence in Cancer Research, Department of Biomedical Sciences, Texas Tech University Health Sciences Center, El Paso, TX, USA
[3]Department of Molecular and Cellular Medicine, Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, TX, USA

Synonyms

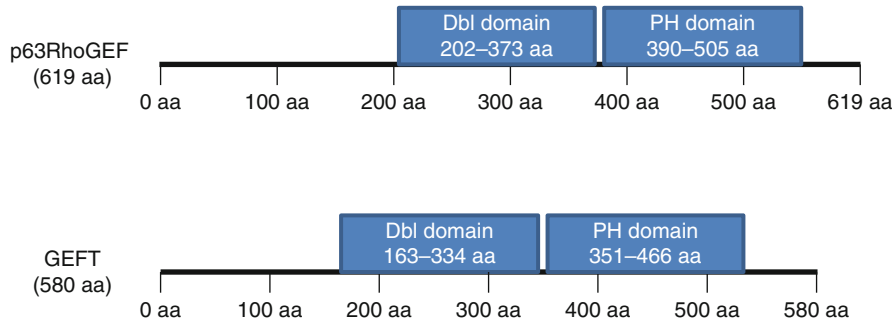
GEFT, p63RhoGEF; Rho Guanine Nucleotide Exchange Factor 25

Historical Background

The gene for ARHGEF25 is located on human chromosome 12q13.3, and its expression encodes a Rho-family guanine nucleotide exchange factor (GEF) protein composed of a Dbl homology domain and a pleckstrin homology domain flanked by short N- and C-termini (Souchet et al. 2002; Guo et al. 2003). ARHGEF25 belongs to a family composed of over 60 known human GEFs. This family of proteins serves as enzymes which catalyze the activation of the Rho family of small GTPases through stimulating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on Rho proteins, thus modulating a broad range of cellular processes in eukaryotic cells such as cell proliferation, gene expression, and actin cytoskeletal organization (Hall and Nobes 2000). Two known alternative splice variants of the ARHGEF25 gene have been identified (GEFT and p63RhoGEF) (Fig. 1), and these protein products share high homology with several other key Rho family GEFs including Trio, Kalirin, and MCF.2 cell line–derived transforming sequence (MCF2).

Regulation of Rho-Family GTPase Signaling by ARHGEF25 Proteins

Both GEFT and p63RhoGEF exhibit isoform specificity for the Rho-GTPases. p63RhoGEF appears to be ras homology gene family, member A (RhoA) specific, while GEFT has been shown to activate RhoA, ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division cycle 42 (Cdc42) in a cell type–dependent manner. For instance, overexpression of GEFT in fibroblasts drives activation of Rac1 and Cdc42 leading to membrane protrusions (Guo et al. 2003); however, GEFT regulates gene expression and cell differentiation in skeletal muscle, neuronal cells, and lens epithelial cells through activation of multiple GTPases (Bryan et al. 2004, 2005, 2006; Mitchell et al. 2011). Rho-GTPase specificity of this nature is not at all surprising as similar isoform-specific regulation of Rho family members has been observed with the ARHGEF25 paralog MCF2 as well as other GEFs (Komai et al. 2002). p63RhoGEF induces RhoA-dependent stress fiber formation in fibroblasts and in H9C2 cardiac myoblasts (Souchet et al. 2002), while GEFT stimulates the formation of lamellipodia, actin



ARHGEF25, Fig. 1 *ARHGEF25 protein domains*. The ARHGEF25 protein isoforms p63RhoGEF (619 amino acids) and GEFT (580 amino acids) both contain Dbl homology domains and pleckstrin homology domains. These isoforms differ only at their N-terminus, where p63RhoGEF contains extra 39 amino acids not present in GEFT. With the exception of this N-terminal region, both isoforms share 100% amino acid identity

microspikes, and filopodia, similar to overexpression of constitutively active Rac1 and Cdc42 mutants (Guo et al. 2003; Bryan et al. 2004, 2006). p63RhoGEF has been shown to promote serum response factor (SRF) activity through a RhoA-specific pathway, while GEFT reportedly modulates the activity and/or subcellular localization of a number of transcription factors including SRF, activating protein 1 (AP1), nuclear factor kappa beta (NFkappaβ), myogenic differentiation 1 (MyoD), peroxisome proliferator-activated receptor gamma (PPARγ), and activating transcription factor 2 (ATF2) through RhoA, Rac1, and Cdc42 signaling (Bryan et al. 2004, 2005). Moreover, overexpression of GEFT stimulates the activation of numerous downstream kinases including Rho-associated, coiled-coil containing protein kinase (ROCK), p21 protein–activated kinase 1 (▶ PAKI), p21 protein–activated kinase 5 (Pak5), mitogen-activated protein kinase 14 (MAPK14), mitogen-activated protein kinase 8 (MAPK8), and ▶ p42/44 MAPK (Bryan et al. 2004, 2005) and promotes crystallin gene expression in lens epithelial cells (Mitchell et al. 2011). p63RhoGEF is expressed most highly in the heart and brain (Souchet et al. 2002). The expression of GEFT is detected at the highest levels in the excitable tissues (brain, heart, and skeletal muscle), while lower levels of protein expression are observed in a number of diverse tissues (Guo et al. 2003). An extensive analysis of GEFT expression in the developing and adult eye was recently performed by Mitchell et al. (2011), detecting high levels of the protein in the neuroblastic layer and differentiating lens fibers of the late-stage mouse embryo, and in the postnatal corneal epithelium, lens epithelium, and throughout the retina.

ARHGEF25 Protein Regulation of Muscle Physiology

Both p63RhoGEF and GEFT appear to play important roles in skeletal, cardiac, and smooth muscle function. For instance, p63RhoGEF is expressed highly in cardiomyocytes, where it strongly colocalizes with ▶ myosin at the sarcomeric I-band (Souchet et al. 2002). These data suggest the p63RhoGEF may be connected directly or indirectly to actin thin filaments and may play an important role in cardiac muscle contraction. In vascular smooth muscle cells, knockdown of endogenous p63RhoGEF ablates angiotensin II-mediated proliferation, actin stress fiber formation, longitudinal focal adhesion arrangement, and peripheral distribution of vimentin (Wuertz et al. 2010). Moreover, Wuertz et al. demonstrated that depletion of endogenous p63RhoGEF ablates angiotensin II-induced smooth muscle contraction using an in vitro collagen matrix assay. GEFT is expressed highly in the embryonic mouse limb bud, during differentiation of skeletal muscle precursors, and in established adult skeletal muscle (Bryan et al. 2005). Moreover, GEFT expression in cardiotoxin-damaged skeletal muscle is detected at high levels during the late phases of tissue regeneration, suggesting a role in the differentiation phase rather than the proliferative phase of muscle regeneration. Subcellular fractionation reveals that GEFT is wholly cytoplasmic, and fluorescence staining demonstrates that GEFT strongly colocalizes to the actin cytoskeleton in both undifferentiated skeletal muscle precursors and in differentiated multinucleated myotubes. Exogenous overexpression of GEFT in skeletal muscle precursors leads to

activation of RhoA, Rac1, and Cdc42 and their downstream effector proteins, and promotes myogenesis, inhibition of adipogenesis, and enhances in vivo tissue regeneration following skeletal muscle injury (Bryan et al. 2005).

ARHGEF25 Protein Regulation of Neuronal Physiology

p63RhoGEF expression has been detected in the cell bodies of astrocytes and oligodendrocytes localized in the cerebellar cortex (Souchet et al. 2002), while GEFT levels are observed throughout the adult brain, with prominent expression in the hippocampus, Purkinje cells, and granular region of the cerebellum (Bryan et al. 2005). Subcellularly, GEFT colocalizes to actin-rich regions, particularly those found in axons and dendrites (Bryan et al. 2006). Exogenous expression of GEFT promotes dendritic outgrown in cultured hippocampal neurons, resulting in a higher abundance and increased size of mature dendritic spines compared to the control (Bryan et al. 2005). GEFT activates RhoA, Rac1, and Cdc42 signaling in neuronal cells, and strongly enhances neurite outgrowth in neuroblastoma cells in a Pak1/Pak5-dependent manner, as well promotes axon and dendrite formation during neuronal cell differentiation (Bryan et al. 2005, 2006). Additionally, GEFT expression is highly upregulated during retinoic acid- or dibutyric cyclic adenosine monophosphate (cAMP)-induced neuronal cell differentiation (Bryan et al. 2006). During neuronal development, neural precursor cells migrate, differentiate, and extend axons and dendrites to specific regions to form synapses with appropriate target cells. Multiple GEFs have been implicated in neuronal morphonesis, growth cone guidance, and neuronal dendritic spines, and these data suggest that GEFT could play a strong role in neuronal guidance and pathfinding.

ARHGEF25 Protein Regulation of Ocular Development

High levels of Rho GTPase expression in the developing and adult eye suggest their importance in the morphogenesis and maintenance of ocular components (Mitchell et al. 2007). GEFT expression has been detected in mice as early as 9 days p.c. in the neuroepithelial tissue adjacent to the lumen of the optic vessel, and is notably expressed in differentiating lens fibers at later developmental stages (Mitchell et al. 2011). In the adult mouse eye, GEFT is observed in corneal and lens epithelium, and in the axons and cell bodies of the retinal ganglion cells of the optic nerve layer, the nerve fibers of the inner and outer plexiform layers, and the photoreceptor layer containing the rods and cones. In both in vitro and ex vivo systems, GEFT promotes lens epithelium differentiation through regulation of the expression of multiple lens crystallin and filensin genes through modulating the activation and subcellular localization of Rac1.

ARHGEF25 and Oncogenic Properties

In addition to normal physiological properties, GEFT has been shown to promote the oncogenic properties of transformed cells. The gene encoding GEFT was initially identified using an enhanced retroviral mutagen strategy selecting for foci-forming complementary DNAs (cDNAs) expressed in a human brain library (Liu et al. 2000), and its overexpression strongly induces foci-formation, proliferation, and migration in transformed fibroblasts (Guo et al. 2003). Moreover, expression of the ARHGEF25 gene is significantly elevated in approximately 4% of adenomas and carcinomas utilizing a large scale microarray study on 950 human tumor lines (https://array.nci.nih.gov/caarray/project/woost-00041).

Known Protein–Protein Interactions with ARHGEF Proteins

p63RhoGEF has been shown to physically interact with the activated G-protein coupled receptors (GPCR) G-alpha(q) and G-alpha11, but not with G-alpha12 or 13 (Lutz et al. 2005). This interaction occurs independently and in competition with the activation of the canonical G-alpha(q/11) effector phospholipase C beta, and strongly enhances the activity of p63RhoGEF. As has been reported for many GEF proteins, in the unbound state the C-terminal pleckstrin homology domain of p63RhoGEF folds over and autoinhibits its Dbl catalytic domain (Rojas et al. 2007; Lutz et al. 2007; Shankaranarayanan et al. 2010). This autoinhibition is relieved upon interaction

of the GPCR with the highly conserved C-terminal extension of the p63RhoGEF PH domain, thus coupling GPCR stimulation to Rho-GTPase activation. Alternatively, p63RhoGEF can serve as an inhibitor of GPCR function by forming a stable complex with activated G-alpha16 to inhibit its activation of phospholipase beta2, Ras, and ▶ Stat3 activation via competitive inhibition (Yeung and Wong 2009).

Several inhibitors of p63RhoGEF activity have been recently identified. p63RhoGEF, G protein coupled receptor kinase 2 (GRK2), and RGS GTPase activating proteins form a ternary complex with G-alpha(q)-coupled receptors (Shankaranarayanan et al. 2008). RGS 2 and RGS4 serve as negative allosteric regulators of G-alpha(q) binding to p63RhoGEF, thus inhibiting RhoA activation and downstream signaling. Moreover, the mixed lineage kinase 3 (▶ MLK3), a MAPK3 protein that normally activates the JNK-dependent MAPK pathways, binds directly to p63RhoGEF and prevents its G-alpha(q)-mediated activation of Rho-signaling pathways (Swenson-Fields et al. 2008). GEFT was recently identified as a protein that colocalizes and directly interacts with the cytoplasmic region of the Popdc family integral membrane protein Bves (blood vessel epicardial substance) in cardiac, smooth, and skeletal muscle (Smith et al. 2008). This interaction inhibits GEFT activity and leads to a reduction in cell movement and an increase in cell roundness via blocking of Rac1 and Cdc42 activity.

Summary

The ARHGEF25 gene encodes for two key GEF isoforms, p63RhoGEF and GEFT. These proteins are responsible for modulation of the activity of Rho-GTPases and their downstream signaling pathways in muscle and neuronal cell lineages. Future studies will likely uncover more roles and signaling cross talk for these protein products in a variety of tissues.

References

Bryan B, Kumar V, Stafford LJ, Cai Y, Wu G, Liu M. GEFT, a Rho family guanine nucleotide exchange factor, regulates neurite outgrowth and dendritic spine formation. J Biol Chem. 2004;279(44):45824–32.

Bryan BA, Mitchell DC, Zhao L, Ma W, Stafford LJ, Teng BB, et al. Modulation of muscle regeneration, myogenesis, and adipogenesis by the Rho family guanine nucleotide exchange factor GEFT. Mol Cell Biol. 2005;25(24):11089–101.

Bryan BA, Cai Y, Liu M. The Rho-family guanine nucleotide exchange factor GEFT enhances retinoic acid- and cAMP-induced neurite outgrowth. J Neurosci Res. 2006;83(7):1151–9.

Guo X, Stafford LJ, Bryan B, Xia C, Ma W, Wu X, et al. A Rac/Cdc42-specific exchange factor, GEFT, induces cell proliferation, transformation, and migration. J Biol Chem. 2003;278(15):13207–15.

Hall A, Nobes CD. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci. 2000;355(1399):965–70.

Komai K, Okayama R, Kitagawa M, Yagi H, Chihara K, Shiozawa S. Alternative splicing variants of the human DBL (MCF-2) proto-oncogene. Biochem Biophys Res Commun. 2002;299(3):455–8.

Liu D, Yang X, Yang D, Songyang Z. Genetic screens in mammalian cells by enhanced retroviral mutagens. Oncogene. 2000;19(52):5964–72.

Lutz S, Freichel-Blomquist A, Yang Y, Rümenapp U, Jakobs KH, Schmidt M, et al. The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gq/11-coupled receptor signaling and RhoA. J Biol Chem. 2005;280(12):11134–9.

Lutz S, Shankaranarayanan A, Coco C, Ridilla M, Nance MR, Vettel C, et al. Structure of Galphaq-p63RhoGEF-RhoA complex reveals a pathway for the activation of RhoA by GPCRs. Science. 2007;318(5858):1923–7.

Mitchell DC, Bryan BA, Liu JP, Liu WB, Zhang L, Qu J, et al. Developmental expression of three small GTPases in the mouse eye. Mol Vis. 2007;13:1144–53.

Mitchell DC, Bryan BA, Liu L, Hu XH, Huang XQ, Ji WK, et al. GEFT, A Rho family guanine nucleotide exchange factor, regulates lens differentiation through a Rac1-mediated mechanism. Curr Mol Med. 2011;11(6):465–80.

Rojas RJ, Yohe ME, Gershburg S, Kawano T, Kozasa T, Sondek J. Galphaq directly activates p63RhoGEF and Trio via a conserved extension of the Dbl homology-associated pleckstrin homology domain. J Biol Chem 2007;282:29201–10.

Shankaranarayanan A, Thal DM, Tesmer VM, Roman DL, Neubig RR, Kozasa T, Tesmer JJ. Assembly of high order G alpha q-effector complexes with RGS proteins. J Biol Chem. 2008;283(50):34923–34.

Shankaranarayanan A, Boguth CA, Lutz S, Vettel C, Uhlemann F, Aittaleb M, et al. Galpha q allosterically activates and relieves autoinhibition of p63RhoGEF. Cell Signal. 2010;22(7):1114–23.

Smith TK, Hager HA, Francis R, Kilkenny DM, Lo CW, Bader DM. Bves directly interacts with GEFT, and controls cell shape and movement through regulation of Rac1/Cdc42 activity. Proc Natl Acad Sci USA. 2008;105(24):8298–303.

Souchet M, Portales-Casamar E, Mazurais D, Schmidt S, Léger I, Javré JL, et al. Human p63RhoGEF, a novel RhoA-specific guanine nucleotide exchange factor, is localized in cardiac sarcomere. J Cell Sci. 2002;115(Pt3):629–40.

Swenson-Fields KI, Sandquist JC, Rossol-Allison J, Blat IC, Wennerberg K, Burridge K, et al. MLK3 limits activated Galphaq signaling to Rho by binding to p63RhoGEF. Mol Cell. 2008;32(1):43–56.

Wuertz CM, Lorincz A, Vettel C, Thomas MA, Wieland T, Lutz S. p63RhoGEF–a key mediator of angiotensin II-dependent signaling and processes in vascular smooth muscle cells. FASEB J. 2010;24(12):4865–76.
Yeung WW, Wong YH. The RhoA-specific guanine nucleotide exchange factor p63RhoGEF binds to activated Galpha(16) and inhibits the canonical phospholipase Cbeta pathway. Cell Signal. 2009;21(8):1317–25.

ARHGEF8

▶ Net1 (Neuroepithelial Cell Transforming Gene 1 Protein)

Arl10c

▶ Arl8b

Arl8b

Aiysha Thompson and Venkateswarlu Kanamarlapudi
Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales, UK

Synonyms

Adp-ribosylation factor-like 10C; ADP-ribosylation factor-like 8B; Arf-like protein 8B; Arl10c; Gie1; Small G protein indispensable for equal chromosome segregation 1

Historical Background

Arf (ADP-ribosylation factor) proteins were first discovered as a membrane-bound cofactor in the cholera-toxin-dependent ADP-ribosylation of Gα (Kahn and Gilman 1984). They are a family of Ras-related small GTP-binding proteins, approximately 21 kDa in size, and are expressed abundantly in all eukaryotic cells and some bacteria (Quinn 1995; Dong et al. 2007). Arfs regulate membrane trafficking and actin cytoskeleton dynamics, which are important for cellular events such as endocytosis, cell secretion, cell adhesion, cell migration, and neurite outgrowth (D'Souza-Schorey and Chavrier 2006). They act as molecular switches for many cellular pathways by cycling between inactive GDP-binding and active GTP-binding forms. Arf proteins are activated by guanine-nucleotide exchange factors (GEFs) and inactivated by GTPase-activating proteins (GAPs) (Donaldson and Honda 2005; Quinn 1995). Numerous Arf GEFs and GAPs have been identified and characterized (Gillingham and Munro 2007; Kahn et al. 2005). The Arf family consists of six mammalian Arfs (Arf1–Arf6) (Kahn et al. 2006). Arf1–Arf5 localize to and act at the Golgi, whereas Arf6 localizes to and acts at the cell surface (Donaldson and Honda 2005; Gillingham and Munro 2007). The biological functions of Arfs occur through their specific interactions with several downstream effectors (Kahn et al. 2006).

In addition to Arfs, the Arf family contains a number of Arf-like (Arl) proteins. Arl proteins are related to Arf proteins structurally but not functionally (Kahn et al. 2006). Arl proteins share 40–60% sequence identity with Arfs. In contrast to Arf proteins, Arls cannot act as cofactors in cholera-toxin-catalyzed ADP-ribosylation of Gαs; cannot activate ▶ phospholipase D (PLD); and cannot functionally complement Arf1 or Arf2 in *Saccharomyces cerevisiae* (Hong et al. 1998; Kahn et al. 2006; Price et al. 1988; Sebald et al. 2003; Stearns et al. 1990; Tamkun et al. 1991). These Arls contain an amino-terminal amphipathic helix and the "interswitch" region (Kahn et al. 2006; Li et al. 2004; Pasqualato et al. 2002). In humans, there are 22 Arls (Arl1–Arl22), which have been well conserved in evolution (Hofmann and Munro 2006). Generally, membrane binding of Arl proteins occurs via an N-terminal amphipathic helix that is inserted into the lipid bilayer once activated. In addition, this N-terminal amphipathic helix contains a myristoyl or an acetyl group (Gillingham and Munro 2007).

The Arl8 subfamily contains two isoforms, Arl8a and Arl8b, which share 92% amino acid identity in human, and there is a single homolog (Arl8) in *Drosophila* and *Caenorhabditis elegans* but none in yeast. This suggests that Arl8 is highly conserved in multicellular eukaryotes (Li et al. 2004; Kahn et al. 2006). Arl8b was originally identified in macrophages as a mycobacterial phagosome harboring protein

(Garin et al. 2001) and also as a lysosomal membrane protein in rat liver (Bagshaw et al. 2005). Arl8b is 186 amino acids in length and has a molecular weight of approximately 22 kDa (Okai et al. 2004; Kahn et al. 2006). The protein contains an N-acetylated amphipathic helix, a putative effector domain, and highly conserved GTP-binding domains (G1-G5) (Okai et al. 2004; Hofmann and Munro 2006).

Protein Function and Regulation of Activity

The main functions of Arl8b discovered so far include a role in lysosome motility (Hofmann and Munro 2006), delivery of endocytosed macromolecules to lysosomes (Nakae et al. 2010), axonal transport of presynaptic cargo (Klassen et al. 2010), neurite formation (Haraguchi et al. 2006), and chromosome segregation (Okai et al. 2004). Arl8b has been found to localize to lysosomes, and its overexpression resulted in a microtubule-dependent accumulation of lysosomes at the cell periphery (Hofmann and Munro 2006; Bagshaw et al. 2006). Wild-type Arl8 or a constitutively active (GTP-bound) mutant (Arl8-Q75L), but not a constitutively inactive (GDP-bound) mutant (Arl8-T34N), localize to lysosomes when expressed exogenously in mammalian cells, indicating that the lysosomal localization of Arl8b depends on its guanine-nucleotide-bound status (Bagshaw et al. 2006). As previously mentioned, membrane binding of the Arf family proteins generally occurs via an N-terminal myristoyl group. However, Arl8b does not contain this motif but instead contains an N-terminally acetylated Met-Leu, which is essential for its lysosomal localization (Hofmann and Munro 2006). RNA-interference-mediated knockdown of human Nα-terminal acetyl transferase complex C (hMak3), which acetylates Met-Leu protein N termini, alters the subcellular localization of Arl8b, supporting the hypothesis that Arl8b is a hMak3 substrate in vivo (Starheim et al. 2009).

Okai et al. (2004) believed Arl8b to be involved in microtubule-related functions based on co-immunoprecipitation of Arl8 with β-tubulin and chromosomal segregation defects in *Drosophila* cell lines following short interfering (si)RNA-mediated knockdown of Arl8. Overexpression of Arl8 mutants that lacked the putative effector domains but still contained tubulin-binding capabilities induced micronucleus formation. The authors concluded that Arl8b may act as a molecular switch to regulate chromosomal segregation by associating with microtubules (Okai et al. 2004).

A recent paper by Nakae et al. (2010) has demonstrated Arl8 localization to lysosomes and its involvement in late endosome-lysosome fusion in *C. elegans*. A loss of Arl8 in *C. elegans* caused an increase in number of late endosome-lysosome vesicles, which are smaller than those in worms expressing wild-type Arl8. In an Arl8 knockout (*arl8*) mutant, late endosomal vesicles are unable to fuse with lysosomes and thus a loss of Arl8 critically reduces late endosome-lysosome hybrid formation, which in turn affects the delivery of endocytosed macromolecules to lysosomes. These results suggest that Arl8 may be important in the biogenesis and function of lysosome-related organelles. In addition, homozygous *arl8* worms from heterozygous *arl8* mothers develop to fertile adults, whereas hermaphrodites homozygous for *arl8* produce no viable embryos, suggesting that Arl8 is required maternally for embryonic development. Introduction of wild-type Arl8 or a constitutively active Arl8-Q75L mutant, but not a constitutively inactive Arl8-T34N mutant, efficiently rescued the embryonic lethality of the *arl8* mutant, suggesting the importance of Arl8 in its GTP-bound active form for normal embryogenesis. Furthermore, expression of human Arl8 proteins in *C. elegans arl8* mutants could also rescue the embryonic lethality, indicating that Arl8 has a conserved role in multicellular animals.

As mentioned above, Arl8b has been shown to localize with microtubules to the spindle mid-zone in late mitosis and to be involved in chromosomal segregation (Okai et al. 2004). However, two later studies found no apparent mitotic spindle localization of Arl8b in mammalian cell lines other than PC12 cells tested (Nakae et al. 2010; Hofmann and Munro 2006). The authors concluded that this role may be important in certain cell types under specific conditions.

It has also been suggested that Arl8b may have an important role in neurite formation because Arl8b, when overexpressed, accumulates at the growth cones in primary neurons and greatly affects the morphology of human embryonic kidney HEK293 cells by inducing the formation of long protrusions (Haraguchi et al. 2006). In the absence of Arl8, presynaptic cargo prematurely aggregates in the axons in *C. elegans*,

indicating that Arl8 acts as a regulator of presynaptic assembly (Klassen et al. 2010).

A study by Salilew-Wondim et al. (2010) showed higher levels of *Arl8b* gene expression in bovine embryos that resulted in calf delivery than in those that resulted in no pregnancy. This study highlighted the potential of *Arl8b* gene expression pattern in embryos as a predictor of pregnancy success in cattle. *Arl8b* has recently been identified as an endogenous reference gene (ERG) using multi-platform expression data and validated for quantitative gene expression analysis (Kwon et al. 2009). ERGs are useful in normalization of mRNA levels, which is essential for an accurate comparison of gene expression between different samples. Several studies have reported the expression variability of traditional ERGs (tERGs) such as GAPDH and ACTB in various tissues or disease status. It has been suggested that Arl8 is better reference gene than tERGs owing to its greater expression stability.

A recent study by Korolchuk et al. (2011) has shown that knockdown of Arl8b (along with Arla) resulted in disruption of the centrifugal movement of lysosomes along microtubules and reduction in the nutrient-dependent activation of ▶ mammalian target of rapamycin (mTOR)-C1. In contrast, overexpression of Arl8b increased ▶ mTOR-C1 activity by increasing the number of lysosomes at the tip of cell protrusions. These findings suggest that the positioning of lysosomes regulates the ▶ mTOR-C1 activity. Inhibiting ▶ mTOR-C1 activity by restricting lysosomes transport to the cell periphery through downregulation of Arl8b and Arl8a increases the number of autophagosomes formed even when cells are maintained in nutrient-rich conditions. Moreover, an increase in the transfer of autophagic cargo from autophagosomes to lysosomes, known as autophagic flux, and its subsequent degradation was also observed in cells with simultaneous knockdown of Arl8b and Arl8a.

Arl8b activity is regulated through exchange of bound GDP for GTP and hydrolysis of the bound GTP to GDP. Arl8b is active when it is bound to GTP and inactive when it is bound to GDP (Okai et al. 2004). However, it is unknown currently whether Arl8b requires GEFs for its activation and GAPs for its inactivation. No GEFs or GAPs that are specific for Arl8b have been identified so far. In addition, it is currently unknown whether Arf GEFs and GAPs are active on Arl8b.

Arl8b is N-acetylated, which is necessary for its localization, and thereby its activity in intact cells. However, it is currently unknown whether N-acetylation is required for the GTP-binding or GTPase activity of Arl8b. Several members of the Arf family require membranes or lipid vesicles and micromolar concentrations of Mg^{2+} for an increase in their in vitro GTP binding (Pasqualato et al. 2002). However, it is currently unknown whether Arl8b requires any of these cofactors to promote GTP binding.

Arl8b interacts with GDP and GTP (Okai et al. 2004). Bagshaw et al. (2006) concluded that the localization of Arl8b to lysosomes may occur only when the protein is present in its GTP-bound active state. The authors made a constitutively GTP-bound active (Arl8-Q75L) and a constitutively GDP-bound inactive (Arl8-T34N) mutants of Arl8b. When these mutants were expressed in mammalian cells, the GTP-bound mutant localized to lysosomes whereas the GDP-bound mutant did not, indicating that the lysosomal localization of Arl8b depends on its guanine-nucleotide-bound status (Bagshaw et al. 2006).

Arl8b has been shown to associate with β-tubulin independent of its guanine-nucleotide-bound status or the absence of its effector domain (Okai et al. 2004). Interaction between Arl8b and β-tubulin was shown by co-immunoprecipitation from the extracts of HeLa cells overexpressing Arl8b.

In addition, several interacting proteins for Arl8b have been identified in the Biogrid, IntAct, HPRD, Bind, UniProtKB, MINT, and STRING databases. However, these interactions are yet to be confirmed.

Haraguchi et al. (2006) demonstrated that short-term feeding of a high beef-tallow diet in mice results in the downregulation of Arl8b mRNA in the brain.

Major Sites of Expression and Subcellular Localization

Arl8b is ubiquitously expressed in various tissues and cell lines. Expression in human tissues includes the brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, and peripheral blood leukocytes. However, higher expression levels were observed in brain, heart, skeletal muscle, kidney, and placenta (Okai et al. 2004).

Arl8b has also been shown to be expressed in cell lines such as human HeLa and HEK293, rat PC12, mouse NIH3T3 and neuro2A, and mast cells (Okai et al. 2004).

Bagshaw et al. (2005) identified Arl8b as a lysosomal membrane protein by proteomic analysis. The lysosomal localization of Arl8b is supported by its colocalization with the lysosomal markers CD63, Lamp1, Lamp2, and NPC1 when expressed exogenously in mammalian cells (Hoffman and Munro 2006; Bagshaw et al. 2006; Nakae et al. 2010). In addition, Arl8b has been shown to change its localization from lysosomes to the mid-zone of spindle during mitosis (Okai et al. 2004). However, two later studies disproved this by showing Arl8b localization to lysosomes during mitosis (Hofmann and Munro 2006; Nakae et al. 2010). In neuronal cells, Arl8b localizes to the protruded core and the perinuclear regions (Haraguchi et al. 2006).

Arl8b is N-terminally acetylated, which is essential for its lysosome localization (Hofmann and Munro 2006). Arl8b becomes cytosolic when three hydrophobic amino acids in the N-terminal amphipathic helix are mutated (I5A, L8A, and F12A), which does not affect the N-acetylation of Arl8b (Hofmann and Munro 2006). This study indicates that the lysosomal localization of Arl8b may require not only the N-acetylation but also an intact N-terminal amphipathic helix region.

There are several anti-Arl8b antibodies that are commercially available, which could be used for studying Arl8b expression in tissues and cell lines. Two rabbit polyclonal antibodies raised against the C terminus (SAB1300305) and the center (SAB1300683) of Arl8 are available from Sigma and can be used for the detection of protein of human origin by Western blot, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry. Three rabbit polyclonal antibodies raised against synthetic peptide corresponding to internal regions of human ARL8b (LS-C102443, LS-C111980, and LS-C111981) are available from Lifespan Biosciences. These antibodies can be used for the detection of endogenous ARL8b by Western blot. Abnova supplies a mouse monoclonal antibody, clone 1A2 (H00055207-M01) raised against a full-length recombinant human ARL8, which can be used for the detection of recombinant protein by ELISA. An anti-Arl8b rabbit polyclonal antibody (13049-1-AP) available from ProteinTech group is suitable for recognition of Arl8b by Western blot and ELISA. Although no publications currently site any of these commercially available antibodies, the product data sheets supplied by companies contain evidence of their efficiency in the methods outlined above.

Phenotypes and Splice Variants

No studies on the phenotypic effects of Arl8b have been carried out in mammals because of the lack of *Arl8b* knockout mice. However, loss of Arl8 in *C. elegans* results in an increase in the number of late endosomal-lysosomal compartments that are small in size and inhibition of viable embryo production by hermaphrodites (Nakae et al. 2010). Knockdown of *Arl8b* in *Drosophila* S2 cells with siRNA resulted in chromosomal mis-segregation but no inhibition of mitotic progression (Okai et al. 2004). These abnormalities were also observed in HeLa cells following introduction of the dominant-negative mutants, Arl8b-T34N and Arl8b-N130I, into the cell. Therefore, the function of Arl8b in chromosome segregation must be conserved in animals. In addition, a recent study has shown that simultaneous knockdown of *Arl8b* and *Arl8a* additively enhanced autophagosome-lysosome fusion in HeLa cells (Korolchuk et al. 2011).

The human *Arl8b* gene is located on chromosome 3 and has eight exons. Nine splice variants have been predicted to be formed through alternative splicing of *Arl8b*, which differ from each other in length and amino acid sequence. However, it is not yet known whether the splice variants of *Arl8b* are functionally active or whether their expression levels differ.

Okai et al. (2004) raised a rabbit polyclonal antibody against the carboxy-terminal peptide (CLIQHSKSRRS) of human Arl8b and Arl8a and used in the recognition of Arl8 proteins by Western blot.

A rabbit antiserum raised against residues 18–186 of *Drosophila* Arl8 was used by Hoffman and Munro (2006) for the detection of endogenous *Drosophila* Arl8 by Western blot.

Nakae et al. (2010) generated an anti-Arl8 antibody by immunizing rabbits with a synthetic peptide (CDITLQWLIDHSKAQR) corresponding to the C terminus of *C. elegans* Arl8 and used it in the identification of Arl8 protein by Western blot.

Garin et al. (2001) have used a polyclonal antibody raised against the C-terminal region of Arl8b in Western blot analysis to indicate that the protein is highly enriched on phagosomes in macrophages. However, neither the immunogen sequence nor animal used for raising the antibody was reported in the publication.

Summary

ADP ribosylation factor (Arf)-like 8b (Arl8b) is a member of the Arf family of small GTP-binding proteins, which regulate membrane trafficking in eukaryotic cells. Arl8b is 186 amino acids in length and has a molecular weight of approximately 22 kDa. The protein contains an amino-terminally acetylated amphipathic helix, a putative effector domain, and GTP-binding domains. Like other small GTP-binding proteins, Arl8b acts as a molecular switch by cycling between the GDP-bound inactive and the GTP-bound active forms. Arl8b is primarily localized to lysosomes in its GTP-bound form. The N-terminal acetylation of Arl8b is also important for its localization to lysosomes. The main functions of Arl8b discovered so far include a role in microtubule-dependent accumulation of lysosomes at the cell periphery, delivery of endocytosed macromolecules to lysosomes, axonal transport of presynaptic cargo, neurite formation, and chromosome segregation. Recent studies also highlighted the potential of Arl8b as an endogenous reference gene for quantitative gene expression analysis and the use of its gene expression pattern in embryos as a predictor of pregnancy success in cattle. Arl8b has been shown to associate with β-tubulin independent of its guanine-nucleotide-bound status or the effector domain. It also interacts with several other proteins, but the interactions have not yet been confirmed. Arl8b is ubiquitously expressed in tissues and is especially high in brain, heart, skeletal muscle, kidney, liver, placenta, and lung and cell lines. Furthermore, *Arl8b* mRNA expression is downregulated in the brains of mice fed a high-fat diet. Studies on the phenotypic effects of loss of Arl8 in *Caenorhabditis elegans* revealed that Arl8 is required for embryonic development and late endosome-lysosome fusion. The human *Arl8b* gene is located on chromosome 3 and its alternative splicing is predicted to generate nine splice variants.

References

Bagshaw RD, Mahuran DJ, Callahan JW. A proteomic analysis of lysosomal integral membrane proteins reveals the diverse composition of the organelle. Mol Cell Proteomics. 2005;4:133–43.

Bagshaw RD, Callahan JW, Mahuran DJ. The Arf-family protein, Arl8b, is involved in the spatial distribution of lysosomes. Biochem Biophys Res Commun. 2006; 344:1186–91.

D'Souza-Schorey C, Chavrier P. ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol. 2006;7: 347–58.

Donaldson JG, Honda A. Localization and function of Arf family GTPases. Biochem Soc Trans. 2005;33:639–42.

Dong JH, Wen JF, Tian HF. Homologs of eukaryotic Ras superfamily proteins in prokaryotes and their novel phylogenetic correlation with their eukaryotic analogs. Gene. 2007;396:116–24.

Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, Sadoul R, Rondeau C, Desjardins M. The phagosome proteome: insight into phagosome functions. J Cell Biol. 2001;152:165–80.

Gillingham AK, Munro S. The small G proteins of the Arf family and their regulators. Annu Rev Cell Dev Biol. 2007;23: 579–611.

Haraguchi T, Yanaka N, Nogusa Y, Sumiyoshi N, Eguchi Y, Kato N. Expression of ADP-ribosylation factor-like protein 8B mRNA in the brain is down-regulated in mice fed a high-fat diet. Biosci Biotechnol Biochem. 2006;70:1798–802.

Hofmann I, Munro S. An N-terminally acetylated Arf-like GTPase is localised to lysosomes and affects their motility. J Cell Sci. 2006;119:1494–503.

Hong JX, Lee FJ, Patton WA, Lin CY, Moss J, Vaughan M. Phospholipid- and GTP-dependent activation of cholera toxin and phospholipase D by human ADP-ribosylation factor-like protein 1 (HARL1). J Biol Chem. 1998;273: 15872–6.

Kahn RA, Gilman AG. Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. J Biol Chem. 1984;259:6228–34.

Kahn RA, Volpicelli-Daley L, Bowzard B, Shrivastava-Ranjan P, Li Y, Zhou C, Cunningham L. Arf family GTPases: roles in membrane traffic and microtubule dynamics. Biochem Soc Trans. 2005;33:1269–72.

Kahn RA, Cherfils J, Elias M, Lovering RC, Munro S, Schurmann A. Nomenclature for the human Arf family of GTP-binding proteins: ARF, ARL, and SAR proteins. J Cell Biol. 2006;172:645–50.

Klassen MP, Wu YE, Maeder CI, Nakae I, Cueva JG, Lehrman EK, Tada M, Gengyo-Ando K, Wang GJ, Goodman M, Mitani S, Kontani K, Katada T, Shen K. An Arf-like small G protein, ARL-8, promotes the axonal transport of presynaptic cargoes by suppressing vesicle aggregation. Neuron. 2010;66:710–23.

Korolchuk VI, Saiki S, Lichtenberg M, Siddiqi FH, Roberts EA, Imarisio S, Jahreiss L, Sarkar S, Futter M, Menzies FM, O'Kane CJ, Deretic V, Rubinsztein DC. Lysosomal positioning coordinates cellular nutrient responses. Nat Cell Biol. 2011;13:453–60.

Kwon MJ, Oh E, Lee S, Roh MR, Kim SE, Lee Y, Choi YL, In YH, Park T, Koh SS, Shin YK. Identification of novel reference genes using multiplatform expression data and their validation for quantitative gene expression analysis. PLoS One. 2009;4:e6162.

Li Y, Kelly WG, Logsdon JM, Schurko AM, Harfe BD, Hill-Harfe KL, Kahn RA. Functional genomic analysis of the ADP-ribosylation factor family of GTPases: phylogeny among diverse eukaryotes and function in C. *elegans*. FASEB J. 2004;18:1834–50.

Nakae I, Fujino T, Kobayashi T, Sasaki A, Kikko Y, Fukuyama M, Gengyo-Ando K, Mitani S, Kontani K, Katada T. The arf-like GTPase Arl8 mediates delivery of endocytosed macromolecules to lysosomes in *Caenorhabditis elegans*. Mol Biol Cell. 2010;21:2434–42.

Okai T, Araki Y, Tada M, Tateno T, Kontani K, Katada T. Novel small GTPase subfamily capable of associating with tubulin is required for chromosome segregation. J Cell Sci. 2004;117:4705–15.

Pasqualato S, Renault L, Cherfils J. Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for "front-back" communication. EMBO Rep. 2002;3:1035–41.

Price SR, Nightingale M, Tsai SC, Williamson KC, Adamik R, Chen HC, Moss J, Vaughan M. Guanine nucleotide-binding proteins that enhance choleragen ADP-ribosyltransferase activity: nucleotide and deduced amino acid sequence of an ADP-ribosylation factor cDNA. Proc Natl Acad Sci USA. 1988;85:5488–91.

Quinn MT. Low-molecular-weight GTP-binding proteins and leukocyte signal transduction. J Leukoc Biol. 1995;58:263–76.

Salilew-Wondim D, Hölker M, Rings F, Ghanem N, Ulas-Cinar M, Peippo J, Tholen E, Looft C, Schellander K, Tesfaye D. Bovine pretransfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after embryo transfer. Physiol Genomics. 2010;42:201–18.

Sebald E, Krueger R, King LM, Cohn DH, Krakow D. Isolation of a new member of the ADP-ribosylation like factor gene family, ARL8, from a cartilage cDNA library. Gene. 2003;311:147–51.

Starheim KK, Gromyko D, Evjenth R, Ryningen A, Varhaug JE, Lillehaug JR, Arnesen T. Knockdown of human N alpha-terminal acetyltransferase complex C leads to p53-dependent apoptosis and aberrant human Arl8b localization. Mol Cell Biol. 2009;29:3569–81.

Stearns T, Kahn RA, Botstein D, Hoyt MA. ADP ribosylation factor is an essential protein in *Saccharomyces cerevisiae* and is encoded by two genes. Mol Cell Biol. 1990;10:6690–9.

Tamkun JW, Kahn RA, Kissinger M, Brizuela BJ, Rulka C, Scott MP, Kennison JA. The arflike gene encodes an essential GTP-binding protein in drosophila. Proc Natl Acad Sci USA. 1991;88:3120–4.

ARMD10

► TLR4, Toll-Like Receptor 4

ASK (Activator of S Phase Kinase)/ huDbf4 (Human)

► Dbf4

ASV

► c-Src Family of Tyrosine Kinases

ATF3 Activating Transcription Factor 3

Tsonwin Hai[1,3], Johnna Dominick[1,3] and Kun Huang[2]
[1]Department of Molecular and Cellular Biochemistry, Center for Molecular Neurobiology, Ohio State Biochemistry Program Ohio State University, Columbus, OH, USA
[2]Department of Biomedical Informatics, OSUCCC Biomedical Informatics Shared Resources, Ohio State University, Columbus, OH, USA
[3]Molecular & Cellular Biochemistry, Ohio State University, Columbus, OH, USA

Synonyms

CRG-5; LRF-1; LRG-21; TI-241

Historical Background

The term ATF was first used in 1987 to refer to a putative polypeptide with the activity to bind to the sites on the adenovirus E2, E3, and E4 promoters with sequences similar to the consensus TGACGT(C/A)(G/A) (Lee et al. 1987). However, it was later found that, instead of a single polypeptide, many polypeptides can bind to the sequence (Hai et al. 1988; Raychaudhuri et al. 1987). Cloning of the corresponding cDNAs using the consensus sequence to screen the expression library revealed seven different clones (Hai et al. 1989). These proteins were collectively called the ATF family of proteins. Over the years, identical or homologous cDNA clones have been isolated, expanding the size of this protein family (for previous reviews, see Hai and

Hartman 2001; Hai et al. 1999). Because the consensus ATF binding site is the same as the cAMP responsive element (CRE) (Montminy and Bilezsikjian 1987) and because ATF1 is 75% similar to the CRE-binding protein (▶ CREB) at the amino acid level (Hai et al. 1989), sometimes the ATF and ▶ CREB proteins are referred to as the ATF/▶ CREB or ▶ CREB/ATF family of proteins. ATF3 is a member of this family.

DNA Binding by ATF3

Both ATF and ▶ CREB proteins bind to DNA using their basic region/leucine zipper (bZip) motif and belong to a superfamily of bZip proteins that includes ATF, ▶ CREB, AP1 (Fos/Jun), C/EBP, and Maf families (see Hai 2006 for a dendrogram). These proteins can form cross-family heterodimers. In addition, many of these proteins can bind to each other's consensus sequences (which are similar to each other) or the composite sites (a previous review, Hai and Hartman 2001). Thus, their names reflect the history of discovery, rather than the differences (or similarities) between them. An unbiased way to view them is that they all belong to a superfamily of bZip transcription factors that form homodimers and selective heterodimers, with potentially overlapping DNA-binding sequences (for a few reviews on bZip proteins, see Amoutzias et al. 2007; Hurst 1995; Newman and Keating 2003; Vinson et al. 2002). Strictly speaking, there is no such site as the "ATF3 consensus binding sequence," since any sites bound by ATF3 are likely to be also recognized by other bZip proteins. When searching for potential ATF3 binding sites on a given promoter, one should scan the sequence not just for the consensus ATF sequence, but also the AP1 sequence (TGACTCA, one nucleotide deletion from the ATF site), and sequences with *several* deviations from either consensus. If potential sites are identified, it is necessary to test the binding experimentally. If no potential sites are identified, it does not necessarily mean that ATF3 does not regulate the promoters. It is possible that ATF3 can bind to the promoters at yet unidentified sites, or ATF3 can be recruited to these promoters via other proteins. Alternatively, ATF3 may regulate them indirectly via regulating other transcription factors that in turn regulate the promoters of interest. For more discussions on DNA binding by ATF3, see McConoughey et al. (2011).

Transcriptional Activity and Target Promoters of ATF3

Although the name "ATF" implies that the proteins are transcriptional activators, it is clear now that ATF3 can be an activator or repressor, depending on the promoter or cellular context (previous reviews, Hai 2006; Hai and Hartman 2001). Over the years, many potential ATF3 target genes (direct or indirect) have been identified. Table 1 lists some of the potential direct target genes that fulfill minimally two criteria: (a) ATF3 was shown to be recruited to their promoters in vivo by chromatin-immunoprecipitation (ChIP) assay; (b) their steady-state mRNA or protein levels are affected by ectopic expression or knockdown of ATF3. Note that changes in steady-state mRNA level could be due to change in transcription, or mRNA stability, or both, and the change in the steady-state protein level could be due to regulation at various steps. Thus, the second criterion by itself does not necessarily mean transcriptional regulation. In addition, it does not mean that the influence of ATF3 on the genes is direct or indirect. However, combined with the first criterion (recruitment of ATF3 to their promoters in vivo), it suggests that ATF3 may, at least in part, regulate their transcription. For some of the target genes listed in Table 1, ATF3 was shown to modulate their transcription in vivo by the indicated assay: pol II occupancy on the promoters/genes or the measurement of their pre-mRNA levels. Results based on transient transfection coupled with reporter assay were not included as evidence for in vivo transcription, because the assay does not address the issue of endogenous gene regulation. In addition, in vitro DNA binding (such as DNase footprint or electrophoretic mobility shift assay) were not included as evidence for ATF3 binding to the promoters. For potential ATF3 target genes identified using less stringent criteria, see McConoughey et al. (2011).

Biological Function of ATF3

ATF3 as a "Hub"

Overwhelming evidence indicates that the ATF3 mRNA level is low in many cell lines and tissues, but upregulated by a variety of signals, usually within 2 h of induction (see Hai (2006) for a short list, and see McConoughey et al. (2011) for more). One striking feature of ATF3 induction is that it is neither

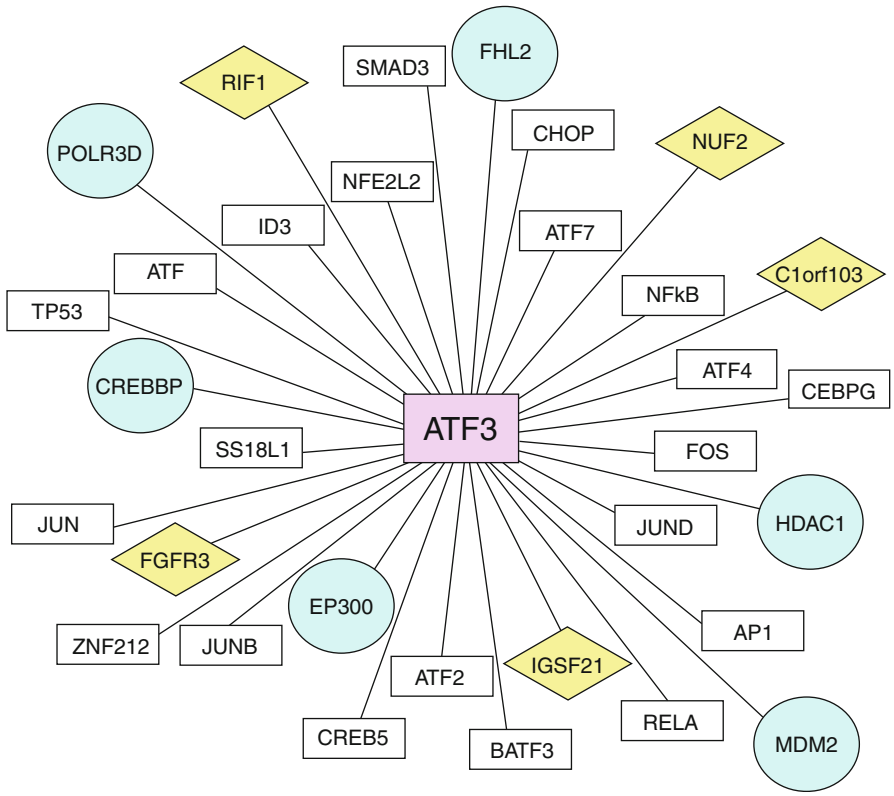
ATF3 Activating Transcription Factor 3, Table 1 Potential target genes of ATF3

Gene[a]	Cells[b] used in the indicated assays: ChIP	Western or RT-PCR	Transcription assays Pre-mRNA[a] or PolII[b]	Effect of ATF3	References
AdipoR1	HepG2	HepG2, MIN6N8, and C2C12	ND[4]	Repression	(Amoutzias et al. 2007)
AdipoR2	HepG2		ND	Repression	(Demidova et al. 2009)
bNIP3	INS-1	Mouse primary islets	ND	Activation	(Gilchrist et al. 2006)
Cdc25A	HCT116	HCT116	ND	Repression	(Hai 2006)
CCL2	INS-1	Mouse primary islets	ND	Activation	(Gilchrist et al. 2006)
CCL4	RAW264.7	Mouse peritoneal and bone marrow derived macrophages	ND	Repression	(Hai and Hartman 2001)
Cyclin D1	MEFs	Ras-transformed MEFs	MEFs[a]	Repression	(Hai et al. 1988)
FN-1	MCF10CA1a	MCF10CA1a	MCF10CA1a[b]	Activation	(Hai et al. 1989)
GLUT4	HEK293T	Mouse white adipose tissue	ND	Repression	(Hai et al. 1999)
HIF-2α	HeLa	MEFs	ND	Activation	(Hai et al. 2010)
IFN-γ	Mouse NK cells	Mouse NK cells	ND	Repression	(Ho et al. 2008)
IL-1β	INS-1	Mouse primary islets	ND	Activation	(Gilchrist et al. 2006)
IL-6	RAW264.7	RAW264.7	ND	Repression	(Hurst 1995)
	INS-1	Mouse primary islets	ND	Activation	(Gilchrist et al. 2006)
IL-12b	RAW264.7	RAW264.7	ND	Repression	(Hurst 1995)
IRS2	MIN6 and INS823/13	MIN6 and INS823/13	INS823/13[b]	Repression	(Kang et al. 2003)
MMP1	Primary mouse monocytes	THP-1	ND	Repression	(Khuu et al. 2007)
MMP13	MDA-MB231	MDA-MB231	ND	Activation	(Kim et al. 2009)
Noxa	INS-1	INS-1	ND	Activation	(Gilchrist et al. 2006)
$p15^{PAF}$	HaCaT	MEFs	ND	Activation	(Kim et al. 2010)
Slug	MCF10CA1a	MCF10CA1a	MCF10CA1a[b]	Activation	(Hai et al. 1989)
Snail	MCF10CA1a	MCF10CA1a	MCF10CA1a[b]	Activation	(Hai et al. 1989)
STAT1	MIN6N8	MIN6N8	ND	Activation	(Koh et al. 2010)
TNFα	RAW264.7	RAW264.7	ND	Repression	(Korb et al. 2008)
	INS-1	Mouse primary islets	ND	Activation	(Gilchrist et al. 2006)
TWIST1	MCF10CA1a	MCF10CA1a	MCF10CA1a [b]	Activation	(Hai et al. 1989)

[a]*Gene*: *AdipoR1* adiponectin receptor 1, *AdipoR2* adiponectin receptor 2, *bNIP3* Bcl-2/E1B-19 K-interacting protein 3, *Cdc25A* Cdc25 protein phosphastase type A, *CCL* Chemokine (CC motif) ligand, *FN-1* Fibronectin, *GLUT4* glucose transporter 4, *HIF-2α* hypoxia-inducible factor 2 alpha subunit, *IFN-γ* interferon-γ, *IL* interleukin, *IRS2* insulin receptor substrate 2, *MMP* matrix metalloproteinase, *Noxa* Latin for damage, $p15^{PAF}$ proliferating Cell Nuclear Antigen-associated factor KIAA0101, *STAT* signal transducer and activator of transcription, *TNF-α* tumor necrosis factor

[b]*Cells*: *C2C12* mouse myoblast cell line, *HaCaT* human skin keratinocyte cell line, *HCT116* human colon carcinoma cell line, *HEK293T* human embryonic kidney cell line, *HeLa* human cervical cancer cell line, *HepG2* human liver carcinoma cell line, *INS-1* rat insulinoma cell line, *INS823/13* rat insulinoma cell line derived from INS-1, *MCF10CA1a* human breast cancer cell line, *MDA-MB231* human breast cancer cell line, *MEFs* immortalized mouse embryonic fibroblasts, *MIN6* mouse pancreatic β cell line, *MIN6N8* mouse pancreatic β cell line, *RAW264.7* mouse leukemic monocyte macrophage cell line, *THP-1* human acute monocytic leukemia cell l

stimulus- nor cell type-specific. This lack of specificity raises an important question: What is the purpose of inducing ATF3? Previously, the idea that ATF3 is a hub of the cellular adaptive-response network to respond to signals perturbing homeostasis was put forth (Hai et al. 2010). This idea was based on the following observations (Amoutzias et al. 2007). A broad spectrum of stimuli can induce ATF3 (above) (Demidova et al. 2009). A variety of signaling pathways have been shown to induce ATF3, such as the JNK, Erk, p38, PKC, and NFκB pathways. This is consistent with the numerous binding sites on the



ATF3 Activating Transcription Factor 3, Fig. 1 Potential ATF3 interacting proteins

ATF3 promoters (ATF3 has at least two promoters, see a review, (Hai et al. 2010) that are recognized by transcription factors targeted by the above signaling pathways. (Gilchrist et al. 2006) Analysis of the amino acid sequence of ATF3 revealed many potential post-translational modification sites, again supporting the idea that ATF3 is a target for regulation by many signaling pathways. For detailed description and references, see Hai et al. (2010).

Additional observations to support the "hub" idea came from bioinformatics analysis. Using the Cytoscape program, Aderem and colleagues analyzed the known transcription factor protein–protein interaction network and found ATF3 to interact with a number of transcription factors, including AP1, CHOP, NFκB, and ▶ p53 (Gilchrist et al. 2006). As shown in Fig. 1, an expansion of this analysis to include all proteins – not just transcription factors – using the Ingenuity Pathway Analysis database revealed that ATF3 interacts with many proteins, further supporting the idea of ATF3 as a hub.

Figure 1 shows the potential ATF3-interacting proteins derived from the Ingenuity Pathway Analysis database. The level of evidence for their interaction varies, depending on the assays used in the studies. To address whether any interaction occurs in vivo in specific cell types, it is important to examine it using appropriate assays. White rectangle in the figure denotes classically defined sequence-specific transcription factors; blue circle denotes cofactors or regulators that modulate transcription factors; yellow diamond denotes other types of proteins.

Abbreviations used in Fig. 1 are the following. AP1: Activator protein 1; ATF: Activating Transcription Factor; BATF3: Basic leucine zipper transcription factor, ATF-like 3; C1orf103: Chromosome 1 open reading frame 103; CEBPG: CCAAT/enhancer binding protein gamma; CHOP: C/EBP homologous protein; CREB5:Cyclic AMP-responsive element-binding protein 5; CREBBP: CREB-binding protein; EP300: E1A binding protein p300; FGFR3: Fibroblast growth factor receptor 3; FHL2: Four and a half LIM domain protein 2; FOS: FBJ murine osteosarcoma viral oncogene homolog; HDAC1: Histone Deacetylase 1; ID3: Inhibitor of DNA-binding 3; IGSF21: Immunoglobin superfamily 21; JUN: Jun proto-oncogene; MDM2: Murine double minute

oncogene; NFE2L2: Nuclear factor (erythroid-derived 2)-like 2: NFkB: Nuclear Factor-KappaB; NUF2: NDC80 kinectochore complex component; POLR3D: Polymerase (RNA) III (DNA directed) polypeptide D; RELA: v-rel reticuloendotheliosis viral oncogene homolog A (avian); RIF1: RAP1 interacting factor 1; SMAD3: Mothers against decapentaplegic homolog 3; SS18L1: Synovial sarcoma translocation gene on chromosome 18 like protein 1; TP53: Tumor protein 53; ZNF212: Zinc finger protein 212

ATF3 as a Cell–Cell Communication Gene

By its nature as an immediate-early gene, ATF3 has far-reaching effects. Immediate-early genes encode transcription factors and are known to turn on/off genes encoding transcription factors, which in turn regulate downstream genes, leading to a cascade of changes in transcriptional programs. Thus, to understand ATF3 function, an important task is to elucidate its target genes – either direct or indirect targets. Table 1 lists some genes that are likely to be direct targets (see above for criteria for inclusion). However, ATF3 has been shown to affect the expression of many more genes (see previous reviews, Hai et al. 2010; McConoughey et al. 2011; Thompson et al. 2009). Due to the lack of evidence for ATF3 recruitment to their promoters in vivo, it is not clear whether they are direct or indirect target genes of ATF3. Among the diversity of ATF3 targets, some can be grouped into functional pathways or groups. (a) ATF3 modulates the expression of numerous inflammatory genes – not only in immune cells (such as macrophages, mast cells, and T cells) but also in non-immune cells (see database (Knob et al. 2008) and a previous review Hai et al. 2010). (b) ATF3 functions as a co-transcription factor for Smad3 to regulate many TGFβ target genes, such as those that affect cell motility and cell cycle (Kang et al. 2003; Yin et al. 2008; Yin et al. 2010). Furthermore, ATF3 forms a positive feedback loop on TGFβ: *ATF3* gene is induced by TGFβ (Kang et al. 2003) and its gene product upregulates the expression of *TGFβ* gene (Yin et al. 2010). Thus, ATF3 appears to play an important role in TGFβ signaling.

TGFβ and the inflammatory gene products (such as cytokines and chemokines) are all soluble factors. This, combined with the "hub" idea above, supports the following view of ATF3. Upon the disturbance of homeostasis by extra- or/and intra-cellular signals, one of the key genes that the cells turn on is ATF3. After induction, ATF3 initiates a cascade of changes in gene expression with a key consequence of releasing various soluble factors, which in turn disturb the homeostasis of the cells receiving the signals. Thus, ATF3 is a key molecule for cell–cell communication, both as a gene to respond to the signals and as a gene to send out signals for communication. This proposed view of ATF3 is supported by a recent cRNA microarray data that cell–cell communication is within the top ten functional groups of genes regulated by ATF3 (Wolford et al. in preparation).

Other Functions

The above two proposed functions of ATF3 are based on literature with a perspective of viewing ATF3 from a broad angle. They are not meant to be comprehensive and do not include the function of ATF3 in various cellular processes, such as apoptosis, cell cycle, cell motility, metabolism, and DNA repair. For those functions, see previous reviews (Hai et al. 2010; McConoughey et al. 2011; Thompson et al. 2009). In this context, two points relevant to the understanding of ATF3 functions are of interest.

(a) Although ATF3 is a transcription factor, its subcellular localization is not limited to the nucleus. MacLeod and colleagues reported low levels of cytoplasmic ATF3 (in addition to nuclear ATF3) in human breast tumor samples by immunohistochemistry (Wang et al. 2008). Similar cytoplasmic localization of ATF3 has also been observed by other investigators (not published). The subcellular localization of ATF3 is likely a regulated event, as suggested by the observation that ATF3 localizes in the cytoplasm of Stat1 knockdown hepatocytes but in the nucleus of the control knockdown cells (Kim et al. 2009). These are potentially interesting observations. As shown in the literature, some proteins have unexpected functions outside their originally identified subcellular location, such as the mitochondrial function of the transcription factor ▶ p53 (a review, Vaseva and Moll 2009) and the nuclear function of the cytoplasmic membrane protein epidermal growth factor receptor (EGFR, Liccardi et al. (2011) and references therein). Clearly, further analysis for the subcellular localization of ATF3 is required. It is intriguing that FGFR3 – a cytoplasmic membrane receptor – is a potential ATF3-interacting protein based on a yeast two-hybrid screen

(Stelzl et al. 2005). If this interaction can be validated in mammalian cells, it would be important to address the subcellular localization of their interaction (nuclear or non-nuclear) and the functional consequences.

(b) Several isoforms of ATF3 have been identified (a review, McConoughey et al. 2011). However, the functional importance of these isoforms and the regulation of their expression are not well understood. When investigating ATF3 functions, this is an area to consider.

Potential Roles of ATF3 in the Pathogenesis of Diseases

Inflammation: A Potential Unifying Component for the Roles of ATF3 in Various Diseases

As detailed in a previous review (Hai et al. 2010), ATF3 modulates the expression of many inflammatory genes. Work by several groups clearly identified ATF3 to play a role in modulating inflammatory responses in macrophages (Gilchrist et al. 2006; Khuu et al. 2007; Whitmore et al. 2007). Furthermore, using systems biology approach combined with genome-wide ChIP-on-chip analyses, Aderem and colleagues identified a large array of ATF3 target genes (not just inflammatory genes) in macrophages and generated an interactive database (Korb et al. 2008). In addition to macrophages, ATF3 also regulates inflammatory genes in other immune cells (such as $CD4^+$-T cells, natural killer cells, mast cells, and dendritic cells) and nonimmune cells (such as fibroblasts and epithelial cells). See Table 2 in Hai et al. (2010) for a list and references. Considering the importance of inflammation in the pathogenesis of various diseases, it is reasonable to speculate that the ability of ATF3 to modulate inflammatory response genes – either in the immune cells or nonimmune cells – plays a key role in its potential implication in various diseases [for more discussions, see Hai et al. (2010)]. Below is a brief review of the data for ATF3 in cancer.

ATF3 in Cancer

Various mouse models have been used to investigate the potential roles of ATF3 in the pathogenesis of diseases. These include transgenic mice ectopically expressing ATF3 in selective tissues, knockout mice deficient in ATF3, and orthotopic injection of cells with modulation of ATF3 levels. See Table 3 in a previous review (Hai et al. 2010) for a brief description of the mouse models and phenotypes. Taking this set of literature together with the in vitro data that ATF3 affects many cellular processes relevant to cancer development (such as apoptosis, cell cycle progression, angiogenesis; for a review, see McConoughey et al. (2011)), it is reasonable to conclude that ATF3 most likely plays a role in the pathogenesis of cancer. However, ATF3 does not simply inhibit or promote cancer; rather, its function varies depending on the cellular context. As shown in the literature, ATF3 can either inhibit or enhance processes such as apoptosis, cell cycle progression, and tumor formation. Since these reports were derived from vastly different cell lines or models with different contexts (see McConoughey et al. (2011) for some examples), one idea is that the role of ATF3 is affected by cellular context. To address what specific features of the cells may affect the roles of ATF3 in cancer development, one study utilized isogenic cell lines. These cells share the same genetic background except the genetic and/or epigenetic alterations that allow them to have varying degrees of malignancy. Interestingly, ATF3 enhances apoptosis in normal or untransformed epithelial cells but has an opposite effect on a malignant cell line derived from them (Yin et al. 2008). Thus, ATF3 plays a dichotomous role, depending on the degree of malignancy of the cells. This concept explains the phenotypes of many transgenic mice models ectopically expressing ATF3 in selective tissues (see Table 3 in Hai et al. (2010) for specifics). In general, ATF3 has deleterious effects on the corresponding tissues, since the cells are untransformed. An exception is the CK5-ATF3 transgenic mice (which express ATF3 in the basal epithelial cells by the bovine cytokeratin promoter 5); the mice developed mammary carcinoma in biparous mice (Wang et al. 2007; Wang et al. 2008). Presumably, after cycles of proliferation and apoptosis, the mammary epithelial cells in biparous mice develop cellular context allowing ATF3 to be "co-opted" and become pro-oncogenic.

All the above data are derived from cell lines in culture dish or mouse models; a critical question is whether the conclusion that ATF3 affects cancer development can be extrapolated to human. At present,

ATF3 has been detected in various tumors, such as breast, prostate, squamous cell carcinoma, and Hodgkin lymphoma (see Table 4 in Hai et al. (2010)). Due to its nature of induction by various stress signals, it is not surprising that ATF3 is expressed within the tumors. The question is whether this has any functional relevance. Does ATF3 play a causal or preventive role? Can its expression be used as a predictive marker (positive or negative) for clinical outcomes? Since ATF3 can be induced in different cell types by various signals, it is likely to be expressed in both cancer epithelial cells and stromal cells. Does its expression in stromal cells have any functional relevance? Considering the complexity of ATF3 biology, these are challenging questions and much work is required to address them.

Summary

ATF3 is a member of the bZip superfamily of transcription factors. This review puts forth two potential functions of ATF3 from a broad perspective. (a) ATF3 as a hub: Overwhelming evidence indicates that ATF3 is induced by a variety of extra- and intra-cellular signals. This, combined with other clues (such as the involvement of various signaling pathways in its induction and its interaction with many proteins), prompted the proposal that ATF3 functions as a "hub" of the cellular adaptive-response network to respond to signals perturbing homeostasis. (b) ATF3 in cell–cell communication: Since ATF3 is a transcription factor, it exerts its actions at least in part by regulating downstream target genes. Analyses of its target genes – either direct or indirect targets – revealed that a consequence of inducing ATF3 is to turn on a variety of genes encoding soluble factors, which in turn disturbs the homeostasis of the cells receiving the signals. Thus, ATF3 is a key molecule for cell–cell communication, both as a gene to respond to the signals and as a gene to send out signals for communication. Various mouse models have been used to investigate the potential roles of ATF3 in the pathogenesis of diseases. A previous review put forth the idea that the ability of ATF3 to modulate inflammatory response genes is a key component for the potential implication of ATF3 in various diseases. This review highlights the evidence and clues that ATF3 most likely plays a role in the pathogenesis of cancer – in a context-dependent manner, not simply anti- or pro-cancer. A critical question is whether the findings from cell culture and mouse models can be extrapolated to human. This is a challenging question; in addressing this issue, it is important to consider the complexity of ATF3 biology.

References

Amoutzias GD, Veron AS, Weiner 3rd J, Robinson-Rechavi M, Bornberg-Bauer E, Oliver SG, Robertson DL. One billion years of bZIP transcription factor evolution: conservation and change in dimerization and DNA-binding site specificity. Mol Biol Evol. 2007;24:827–35.

Demidova AR, Aau MY, Zhuang L, Yu Q. Dual regulation of Cdc25A by Chk1 and p53-ATF3 in DNA replication checkpoint control. J Biol Chem. 2009;284:4132–9.

Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Kennedy K, Hai T, Bolouri H, Aderem A. Systems biology approaches identify ATF3 as a negative regulator of toll-like receptor 4. Nature. 2006;441:173–8.

Hai T. The ATF transcription factors in cellular adaptive responses. In: Ma J, editor. Gene expression and regulation. Beijing/New York: Higher Education Press/Springer; 2006. p. 322–33.

Hai T, Hartman MG. The molecular biology and nomenclature of the ATF/CREB family of transcription factors: ATF proteins and homeostasis. Gene. 2001;273:1–11.

Hai T, Liu F, Allegretto EA, Karin M, Green MR. A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. Genes Dev. 1988;2:1216–26.

Hai T, Liu F, Coukos WJ, Green MR. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. 1989;3:2083–90.

Hai T, Wolfgang CD, Marsee DK, Allen AE, Sivaprasad U. ATF3 and stress responses. Gene Expr. 1999;7:321–35.

Hai T, Wolford CC, Chang YS. ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? Gene Expr. 2010;15:1–11.

Ho HH, Antoniv TT, Ji JD, Ivashkiv LB. Lipopolysaccharide-induced expression of matrix metalloproteinases in human monocytes is suppressed by IFN-gamma via superinduction of ATF-3 and suppression of AP-1. J Immunol. 2008;181:5089–97.

Hurst HC. Transcription factors 1: bZIP proteins. Protein Profile. 1995;2:101–68.

Kang Y, Chen CR, Massague J. A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol Cell. 2003;11:915–26.

Khuu CH, Barrozo RM, Hai T, Weinstein SL. Activating transcription factor 3 (ATF3) represses the expression of CCL4 in murine macrophages. Mol Immunol. 2007;44:1598–605.

Kim JY, Lee SH, Song EH, Park YM, Lim JY, Kim DJ, Choi KH, Park SI, Gao B, Kim WH. A critical role of STAT1 in streptozotocin-induced diabetic liver injury in mice: controlled by ATF3. Cell Signal. 2009;21:1758–67.
Kim JY, Song EH, Lee S, Lim JH, Choi JS, Koh IU, Song J, Kim WH. The induction of *STAT1* gene by activating transcription factor 3 contributes to pancreatic beta-cell apoptosis and its dysfunction in streptozotocin-treated mice. Cell Signal. 2010;22:1669–80.
Koh IU, Lim JH, Joe MK, Kim WH, Jung MH, Yoon JB, Song J. AdipoR2 is transcriptionally regulated by ER stress-inducible ATF3 in HepG2 human hepatocyte cells. FEBS J. 2010;277:2304–17.
Korb M, Rust AG, Thorsson V, Battail C, Li B, Hwang D, Kennedy KA, Roach JC, Rosenberger CM, Gilchrist M, Zak D, Johnson C, Marzolf B, Aderem A, Shmulevich I, Bolouri H. The innate immune database (IIDB). BMC Immunol. 2008;9:7.
Lee KAW, Hai TY, SivaRaman L, Thimmappaya B, Hurst HC, Jones NC, Green MR. A cellular protein, activating transcription factor, activates transcription of multiple E1a-inducible adenovirus early promoters. Proc Natl Acad Sci USA. 1987;84:8355–9.
Liccardi G, Hartley JA, Hochhauser D. EGFR nuclear translocation modulates DNA repair following cisplatin and ionizing radiation treatment. Cancer Res. 2011;71:1103–14.
McConoughey SJ, Wolford CC, Hai T. Activating Transcription Factor 3, UCSD-Signaling Molecule Pages. 2011. http://www.signaling-gateway.org/molecule/search?nm=ATF3.
Montminy MR, Bilezsikjian LM. Binding of a nuclear protein to the cyclic AMP response element of the somatostatin gene. Nature. 1987;328:175–8.
Newman JRS, Keating AE. Comprehensive identification of human bZIP interactions with coiled-coil arrays. Science. 2003;300:2097–101.
Raychaudhuri P, Rooney R, Nevins JR. Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. EMBO J. 1987;6:4073–81.
Stelzl U, Worm U, Lalowski M, Haenig C, Brembeck FH, Goehler H, Stroedicke M, Zenkner M, Schoenherr A, Koeppen S, Timm J, Mintzlaff S, Abraham C, Bock N, Kietzmann S, Goedde A, Toksoz E, Droege A, Krobitsch S, Korn B, Birchmeier W, Lehrach H, Wanker EE. A human protein-protein interaction network: a resource for annotating the proteome. Cell. 2005;122:957–68.
Thompson MR, Xu D, Williams BR. ATF3 transcription factor and its emerging roles in immunity and cancer. J Mol Med. 2009;87:1053–60.
Vaseva AV, Moll UM. The mitochondrial p53 pathway. Biochim Biophys Acta. 2009;1787:414–20.
Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M. Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol. 2002;22:6321–35.
Wang A, Arantes S, Conti C, McArthur M, Aldaz CM, MacLeod MC. Epidermal hyperplasia and oral carcinoma in mice overexpressing the transcription factor ATF3 in basal epithelial cells. Mol Carcinog. 2007;46:476–87.
Wang A, Arantes S, Yan L, Kiguchi K, McArthur MJ, Sahin A, Thames HD, Aldaz CM, Macleod MC. The transcription factor ATF3 acts as an oncogene in mouse mammary tumorigenesis. BMC Cancer. 2008;8:268.
Whitmore MM, Iparraguirre A, Kubelka L, Weninger W, Hai T, Williams BR. Negative regulation of TLR-signaling pathways by activating transcription factor-3. J Immunol. 2007;179:3622–30.
Yin X, DeWille J, Hai T. A potential dichotomous role of ATF3, an adaptive-response gene, in cancer development. Oncogene. 2008;27:2118–27.
Yin X, Wolford CC, McConoughey SJ, Ramsey SA, Aderem A, Hai T. ATF3, an adaptive-response gene, enhances TGFβ signaling and cancer initiating cell features in breast cancer cells. J Cell Sci. 2010;123:3558–65.

ATP Diphosphohydrolase

▶ E-NTPDase Family

ATP-Binding Cassette Transporter Subfamily A

▶ ABCA Transporters

ATPDase

▶ E-NTPDase Family

AtRabC1-C2b (*Arabidopsis thaliana*)

▶ RAB18

A-Type Endothelin Receptor

▶ Endothelin A Receptor (ETAR)

AUF1

▶ hnRNP D (AUF1)

Aurora Kinases

David Reboutier and Claude Prigent
CNRS, UMR 6061, Institut Génétique et Développement de Rennes, Rennes, France
Université Rennes 1, UEB, IFR, 140, Faculté de Médecine, Rennes, France

Aurora Kinases Family Members

Aurora-A: AIK, ARK1, AURA, AURORA2, BTAK, MGC34538, STK15, STK6, STK7
Aurora-B: AIK2, AIM-1, AIM1, ARK2, AurB, IPL1, STK12, STK5
Aurora-C: AIE2, AIK3, ARK3, AurC, STK13, Aurora-C

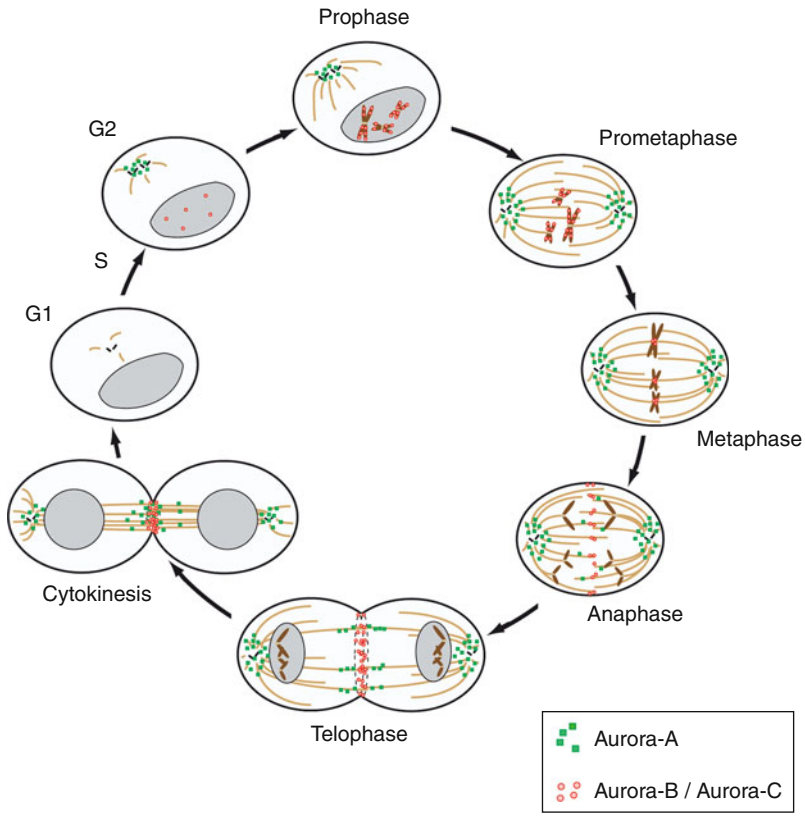
Historical Background

Every new cell is generated through division of an existing cell (one cell generates two cells) and survival of all organisms depends on reliable transmission of the genetic information from the mother cell to daughter cells. Consequently, all cellular components must be duplicated before cell divides, and these duplications must be achieved with extreme precision and reliability over generations. The eukaryotic cell (cell with a nucleus) has set up complex network of regulatory mechanisms to ensure a correct sequence of events that eventually leads to cell division. This network called the cell cycle control system consists mainly in a complex assembly of oscillating phosphorylation/dephosphorylation reactions. Several protein kinases and phosphatases are involved in these signaling cascades. Aurora kinases that belong to a family of serine/threonine kinases play such a role; they are critical for the establishment of mitotic spindle, centrosome duplication, and separation as well as maturation, chromosomal alignment, spindle assembly checkpoint, and cytokinesis. In the early 1990s, Chan and Botstein (1993) identified the first Aurora kinase in the budding yeast *Saccharomyces cerevisiae*. The kinase was originally named Ipl1 for Increase in Ploidy 1. Indeed, conditional temperature sensitive *ipl1*$^{ts-}$ mutant cells reveal abnormal ploidy induction, suggesting that the Ipl1 kinase is involved in controlling chromosomes segregation. Glover and colleagues (1995) then discovered a *Drosophila* homolog that they named Aurora. The kinase was identified during a search of mutants that affect centrosome cycle in *Drosophila*. The first human Aurora kinase was isolated simultaneously by two groups, one was searching homology with yeast Ipl1 and *Drosophila* Aurora in randomly sequenced cDNAs (They identified Aik "Aurora Ipl1–related Kinase") and the other one was mapping a chromosome region commonly amplified in breast cancer to search for potential oncogenes (they identified BTAK "breast tumor–activated kinase"). Rapidly, homology studies revealed several genes encoding Aurora kinases in the genome of multicellular organisms. They were first called AIRK for Aurora/Ipl1-related kinases. Thus, while yeast genome encodes only one Aurora kinase, a second gene was found in *D. melanogaster* through a genome sequencing program and simultaneously two new genes were found in *Homo sapiens*: STK12 and AIE2. Because all these kinases have been isolated simultaneously in different laboratories, they carried "esoteric" names strongly requiring a new nomenclature. This was achieved by dividing the Aurora family into three sub-members (Nigg 2001): Aurora-A, Aurora-B, and Aurora-C. Both Aurora-A and -B are expressed in proliferating cells, yet Aurora-A is associated predominantly with centrosomes and the spindle apparatus from prophase through telophase, whereas Aurora-B is prominent at the midzone during anaphase and in postmitotic bridges during telophase. Aurora-C seems to be restricted to germlines, is highly expressed in testis, but is also found overexpressed in tumors. Interestingly enough Aurora-C can fulfill Aurora-B function suggesting a close relationship. Chromosome localizations of Aurora kinase genes are as follows: Aurora-A (AURKA) on chromosome 20q13.2, Aurora-B (AURKB) on chromosome 17p13.1, and Aurora-C (AURKC) on chromosome 19q13.3.

Aurora Kinases Structure and Functions

Structure of Aurora Kinases

The catalytic domain of the Aurora kinases family is located in the carboxy terminus and is highly conserved among species. It contains 26 residues lining the ATP-binding active site that are characteristic of the Auroras. Interestingly, Aurora-A and Aurora-B

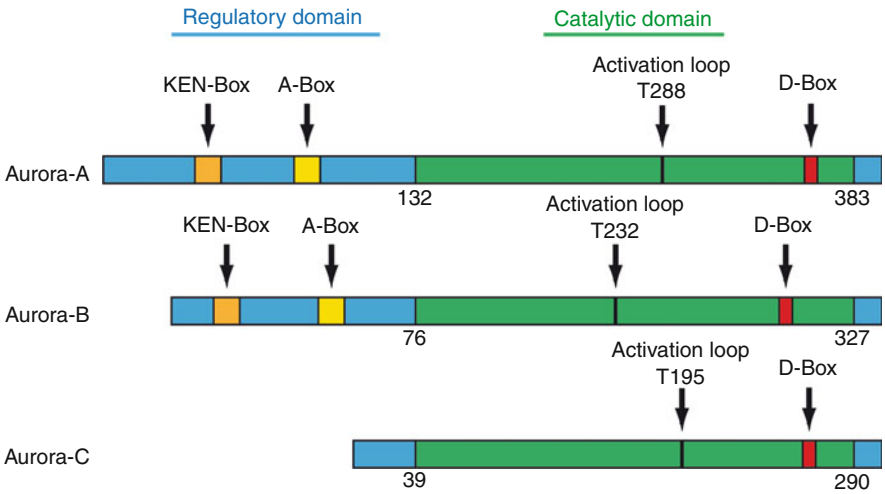


Aurora Kinases,
Fig. 1 Aurora kinases localization through cell cycle progression

sequences are so conserved that the mutation of a single residue in Aurora-A (G198N) is able to transform this kinase in a functional Aurora-B kinase (Hans et al. 2009). All Auroras possess a carboxyl terminal "destruction box" (D-box), but only Aurora-A has the amino terminal "D-box-activating domain box" required for the functional activation of D-box and subsequent degradation by the proteasome via the anaphase-promoting cyclosome complex (APC/c) pathway activated by CDH1. Aurora-B degradation also depends on proteasome and APC/c but needs a KEN-box and a A-box in the amino terminal part. Amino terminal extensions are of variable lengths and display little or no similarity (Fig. 1). Data suggest these domains may be involved in subcellular localization or substrate recognition for the proteins (Rannou et al. 2008).

Aurora-A Functions

Aurora-A is mainly localized at centrosome in interphase and at the poles of the spindle in mitosis (Fig. 2). Aurora-A first appears at centrosomes in G2, its targeting depends on several protein kinases also found at the centrosome, such as ▶ Pak1, Plk-1, and Cdk-11 (Vader and Lens 2008). At the centrosome Aurora-A participates in the recruitment of pericentrosomal materials (PCM), an event commonly called centrosome maturation. Aurora-A is for instance required for the centrosome localization and function of Centrosomin, NDEL1, LATS, TACC. Aurora-A depletion causes defects in centrosome maturation but also ch-TOG and MCAK mislocalization, two centrosomal proteins that are involved in microtubules polymerization and depolymerization, respectively, one of the major functions of the centrosome PCM. After maturation, the centrosomes migrate apart during late G2 to define the two poles of the bipolar mitotic spindle. This event requires Aurora-A function, since its invalidation leads to monopolar spindle formation. One hypothesis possibly explaining such phenotype could involve the Eg5 kinesin. Eg5, known to be involved in spindle bipolarity, is phosphorylated by Aurora-A. Yet the link between Eg5 phosphorylation and spindle bipolarity is still lacking.



Aurora Kinases, Fig. 2 Aurora kinases functions

Astral microtubules have also been implicated in centrosome's separation by connecting centrosomes to cellular cortex. One other hypothesis likely explaining involvement of Aurora-A in centrosome separation is that Aurora-A is implicated in astral microtubules formation by phosphorylating TACC on Ser863.

Aurora-A is involved in mitotic entry. On the first hand, Aurora-A phosphorylates and promotes the activation of the Cdc25B phosphatase at centrosomes. This event is dependent on the targeting of Aurora-A to the centrosome *via* the LIM protein Ajuba. Phosphorylation of CDC25B is required for initial centrosomal activation of Cyclin-B/Cdk-1. On the other hand, it was recently shown that Aurora-A promotes the G2 activation of Polo-like kinase-1 (Plk-1) through phosphorylation within the T-loop of Plk-1. Active Plk1 controls Cyclin-B/Cdk1 activity through phosphorylation of the Cdc25B phosphatase and degradation of the Cdk1 inhibitory kinase Wee1. Aurora-A might also be involved in both the DNA damage checkpoint and in the spindle assembly checkpoint. Marumoto et al. (2003) showed that loss of function of Aurora-A through microinjection of antibodies delays mitotic entry, while gain of function through overexpression leads to premature entry into mitosis in the presence of DNA damage. Anand et al. (2003) showed that overexpression of Aurora-A at levels equivalent to those observed in cancers leads to an override of the spindle assembly checkpoint (SAC). The function of this checkpoint is to prevent chromosome segregation until every one of them is bioriented through kinetochore/microtubule attachment. This is achieved by controlling the activity of the anaphase-promoting complex/cyclosome (APC/C). Once the checkpoint is satisfied, the inhibition is relieved on the APC/C that promotes anaphase and mitotic exit by targeting securin and cyclin-B for degradation by the proteasome.

In one word, in the presence of spindle defects induced by taxol treatment, cells should arrest in prometaphase because of the SAC. Cells overexpressing Aurora-A are resistant to taxol treatment, they exit mitosis despite an active SAC. This might explain centrosome amplification and polyploidy frequently observed after Aurora-A overexpression (Meraldi et al. 2002; Marumoto et al. 2003).

To fulfill its functions, Aurora-A needs to be activated. Several Aurora-A activators have been suggested, yet data are quite controversial. For example, Bora and Ajuba have been first described as Aurora-A activators. However, Bora turns out not to be a direct and general activator of Aurora-A but rather an intermediate stimulating PLK1 phosphorylation by Aurora-A. And Ajuba was demonstrated not to be an Aurora-A activator in *Drosophila*. TPX2, HEF1, and, more recently, CEP192 and Arpc1b were also described as Aurora-A activators. Activation mechanism through binding to TPX2 and CEP192 is well documented. TPX2 activates Aurora-A through conformational changes triggering protection of the T288 residue from dephosphorylation by PP1 phosphatase (Bayliss et al. 2003). CEP192 recruits Aurora-A to centrosomes and favors oligomerization leading to a strong activation.

Aurora-B

Aurora-B is a chromosome passenger protein, localized on chromosome and kinetochores in prophase, on kinetochores during prophase and metaphase, and at the midbody at the end of mitosis (Fig. 2). At the chromosome level, Aurora-B fulfills various functions. Aurora-B phosphorylates histone H3 at serine-10 during late G2/prophase thought to be required for chromosome condensation. Mutation of H3 serine-10 in *Tetrahymena thermophila* or *Schizosaccharomyces pombe* indeed caused chromosome condensation defects and subsequent segregation anomalies. Yet, the same mutation does not cause any similar defect in *Saccharomyces cerevisiae* and there is actually no evidence it could work in a similar way in human. Within condensin complexes, that play a major role in chromosomes condensation, the three non-SMC subunits are phosphorylated by Aurora-B. This phosphorylation is required for loading of condensin I complex on chromosomes.

Aurora-B is also involved in sister chromatid cohesion. This cohesion is maintained by a ring structure formed by the cohesin complex, that surrounds two sister chromatids. The ring is then broken at the metaphase-anaphase transition through a mechanism that involved both kinases Plk-1 and Aurora-B.

Aurora-B activity is required for the localization of the centromeric protein shugoshin-1 (Sgo1). In the absence of Aurora-B, instead of concentrating on centromeres, Sgo1 localizes diffusely along chromosome arms. The role of Sgo1 during mitosis is to protect centromeric cohesin from being degraded before the SAC has been satisfied. To protect cohesin, Sgo1 must bind to PP2A that locally counteracts PLK1 activity. Redistribution of Sgo1 from centromeres to chromosome arms in the absence of Aurora-B causes inappropriate cohesin protection along chromosome arms eventually inhibiting sister chromatids separation.

Aurora-B also participates in the SAC by regulating an enzyme involved in microtubule dynamics and that specifically controlled the biorientation of each chromosomes as well as the stability of the bipolar mitotic spindle. In a perfect spindle, each pair of every kinetochore is attached to microtubules emanating from different centrosomes building a bipolar spindle. Abnormal attachments can take various forms: (1) synthelic (in a kinetochore pair, both bind microtubules emanating from the same pole or centrosome) or (2) merothelic attachments (in a kinetochore pair, one binds to both centrosomes). Those two abnormal attachments are at the origin of a signal "lack of tension" in the spindle that will trigger a correction corresponding to an elimination of the abnormal attachment. The unattached kinetochores can then enter a new cycle of microtubule attachment until bipolar attachment is obtained. Two mechanisms involving Aurora-B in such corrections have been described. In the first one, Aurora-B phosphorylates the microtubule-depolymerizing enzyme MCAK and increases its recruitment to centromere. In the second one, Aurora-B phosphorylates kinetochore microtubule-capture factors such as Ncd80/Hec1 and Dam1. Phosphorylation reduces kinetochore affinity for microtubules whereas dephosphorylation stabilizes microtubule-kinetochore interactions. When biorientation is achieved, mitotic spindle comes under tension. This tension results in a movement of the kinetochores away from the kinase Aurora-B that is not able anymore to phosphorylate its target at the kinetochores. In summary, a single unattached kinetochore is sufficient to keep the SAC on in prometaphase. Aurora-B activity is required to arrest cell cycle progression in response to taxol that induces a lack of tension in the mitotic spindle. Attachments that do not generate tension are removed in an Aurora-B-dependent manner that triggers the recruitment of checkpoint proteins to kinetochores, resulting in inhibition of APC/C Cdc20.

After metaphase/anaphase transition, Aurora-B leaves kinetochores to concentrate at the spindle midzone and at the equatorial cortex to finally accumulates at the midbody. This protein localization from chromosome to midbody depends on microtubules and is characteristic of chromosome passenger protein essential for late mitotic events. Indeed, Aurora-B is the catalytic activity of the chromosomal passenger complex (CPC) consisting of Aurora-B itself, inner centromere protein (INCENP), borealin, and survivin. CPC plays an essential role in cytokinesis in a wide range of organisms. At the midbody Aurora-B phosphorylates the centralspindlin complex composed of the kinesin MKLP1/ZEN4 and the Rac GTPase–activating protein 1 (MgcRacGAP). This phosphorylation is required to signal the positioning of the cleavage furrow via phosphorylation. The centralspindlin complex then regulates events leading to RhoA activation positioning the actomyosin contracting ring. In budding yeast, Aurora-B (Ipl1) plays an additional role during cytokinesis by

controlling the NoCut pathway that prevents abscission (the final step of cytokinesis) when chromosomes have not been fully segregated and remain present at the site of abscission (Norden et al. 2006). Ipl1 controls the localization of the anillin-related proteins Boi1 and Boi2 to the ingression site, they both act as abscission inhibitors and prevent premature abscission and concomitant chromosome breakage.

Aurora-C

The third member of the Aurora family, Aurora-C, is a close relative to Aurora-B. Ectopically expressed Aurora-C shows the same mitotic localization pattern as Aurora-B. Aurora-C can also interact with INCENP and survivin, two CPC proteins. Overexpression of a kinase-dead mutant of Aurora-C interfered with Aurora-B function by displacing its binding partners. In normal physiological conditions, Aurora-C mRNA and protein were initially described to be expressed only in testis. Aurora-C is required for spermatogenesis and male fertility in mice. A recent study identified a homozygous mutation within the *Aurora-C* gene in a group of infertile men. The mutated *Aurora-C* gene yielded a truncated kinase activity-deficient Aurora-C protein. The spermatozoa in these men were polyploid, again indicating a role for Aurora-C in maintaining a stable karyotype during male meiosis. Aurora-C mRNA was detected in several human adult tissues, yet to significantly lower levels than in testis.

Aurora Kinases and Cancer

The chromosomal region in which *Aurora-A* gene is located is frequently amplified in human cancers. Many studies show a significant incidence of Aurora-A amplification and overexpression in human breast, bladder, ovarian, colon, and pancreatic cancers. Ectopic overexpression of Aurora-A transforms NIH3T3 cells and Rat 1 fibroblasts in vitro, and introduction of these transformed cells into nude mice results in tumor growth. Aurora-A mRNA and protein overexpression is not systematically correlated with the gene amplification (i.e., amplification of Aurora-A was detected in 3% of hepatocellular carcinoma (HCC), whereas more than 60% of HCCs overexpressed Aurora-A mRNA and protein). Apart from gene amplification, transcriptional activation and inhibition of protein degradation can also contribute to the elevated levels of Aurora-A expression. It is still unclear how Aurora-A triggers cellular transformation and tumorigenesis, and how important its kinase activity is during this process. For instance, two different studies showed contradictory results concerning the effect of overexpression of a kinase-dead version of Aurora-A (Meraldi et al. 2002; Anand et al. 2003). The oncogenic effect of Aurora-A overexpression is likely due to chromosome instability and directly due to its functions during mitosis. Aurora-A overexpression triggers centrosome amplification likely through a cytokinesis defect. Subsequent abnormal spindles (monopolar or multipolar) formation are precursors of aneuploidy that could contribute to genomic instability and to tumorigenesis. Also, abnormal spindle usually results in the maintenance of the SAC, which leads to abortive mitosis and cell death through apoptosis. Yet, in many tumor cells, there is a tight relation between Aurora-A overexpression and the tumor suppressor ▶ p53. Aurora-A directly phosphorylates p53 and controls its stability and transcriptional activity. Additionally, p53 directly inhibits Aurora-A function, potentially *via* its binding to the catalytic domain of Aurora-A. The effect of Aurora-A overexpression on tetraploidization and centrosome amplification thus depends on the p53 status. When Aurora-A is overexpressed in cells lacking p53, the newly generated tetraploid cells would continue their cell cycle progression, thus giving rise to polyploid cells with four centrosomes and finally leading to genomic instability. Aurora-A was also shown to physically bind to and phosphorylate BRCA1. BRCA1, like p53, is a tumor suppressor; its phosphorylation by Aurora-A causes a loss of function, making cells resistant to DNA damage and override checkpoint response.

Two recent studies in *Drosophila* and mouse showed that Aurora-A could yet function as a tumor suppressor. In *Drosophila*, Aurora-A is required for correct spindle orientation during asymmetric neuroblast division. Asymmetric stem cell division is required for the correct balance between stem cell self-renewal and differentiation. Disruption of asymmetric stem cell division through Aurora-A inactivation can give rise to stem cell overproduction and concomitant tumor growth. In mouse, Aurora-A heterozygosity results in a significant increased tumor incidence suggesting that Aurora-A may act as a haplo-insufficient tumor suppressor. Furthermore, Aurora-A heterozygous mouse embryonic

fibroblasts have higher rates of aneuploidy. These findings, together with the observation that Aurora-A levels are low in certain tumors, strongly suggest that a balanced Aurora-A level is critical for maintaining genomic stability. These data are important to keep in mind regarding the current efforts that are made to exploit Aurora-A inhibition by chemical inhibitors as an antitumor therapeutic.

The role of Aurora-B in tumorigenesis is not as clear as concerning Aurora-A. Only one study showed that overexpression of Aurora-B in CHO cells can promote aneuploidy and increase invasiveness in xenograft experiments. Yet, the chromosomal region containing Aurora-B has never been associated with amplification in tumors. Reports show that Aurora-B is overexpressed in certain tumor types, but it is not clear whether the observed overexpression of Aurora-B is due to the high proliferative index of cancerous cells or whether it is really related to tumorigenesis. Aurora-B overexpression was also shown to strongly enhance cellular transformation in cells expressing oncogenic Ras-V12. Thus, Aurora-B is probably not directly oncogenic but could participate in cell transformation in a particular cellular context.

Although the chromosomal region in which the Aurora-C gene is located is known to be deleted or translocated in certain human cancers cell lines, it is unclear whether Aurora-C deletion or overexpression plays a causative role in tumorigenesis even if a correlation was made between expression and aggressiveness of thyroid cancer.

Pharmacological Inhibitors of Aurora Kinases

The clearly established role for Aurora kinases in mitosis, accompanied by the evidence suggesting that deregulated Aurora-A and, to a lesser extent, Aurora-B expression is linked to tumorigenesis, raised the hypothesis that inhibiting these kinases might be a powerful antitumor strategy. The aim of this entry is not to give exhaustive data about Aurora kinase inhibitor (exhaustive details are given in these three excellent reviews: Boss et al. 2009; Katayama and Sen 2010; Karthigeyan et al. 2010) but to bring clear information important to know about pharmacological Aurora inhibition. Globally, and because the catalytic site is well conserved in Aurora kinases family, there does not exist any inhibitor to specifically target Aurora-A or Aurora-B. All inhibitors target the ATP-binding site to inhibit the catalytic activity of the kinase. Hesperadin and ZM447439 were the first proven small-molecule inhibitors of Aurora kinases. If Hesperadin is more effective on Aurora kinase B than on Aurora-A, ZM447439 inhibits both Aurora kinases A and B. Both compounds never entered clinical trials, probably due to the emergence of more potent and specific inhibitors of the Aurora kinases family. There are actually several new molecules that entered phase I and even phase II clinical trials. Some molecules are pan-Aurora inhibitors (i.e., VX-680, PHA-739358…), others are more selective for Aurora-B (i.e., AZD1152) or Aurora-A (i.e., MLN8054, MLN8237). Concerning pan-Aurora inhibitors, results suggest that effect is mainly directed against Aurora-B. Regarding these data, it is important to keep in mind that in vitro tests designed to assess inhibitor specificity do not take into account Aurora-interacting proteins (i.e., inhibitors or activators). Indeed, it is now known that binding of TPX2 to Aurora-A alters inhibitor interaction (Anderson et al. 2007). Specificity revealed in vitro thus does not reflect the real effect of the drug in vivo. Overall, the responses seen in the phase I studies reported to date in patients with solid tumors are rather disappointing. The majority of reports had only disease stabilization as best response, with very few exceptions. This highlights the fact that to increase the antitumor activity of the Aurora kinase inhibitors in the clinic, combination therapy with cytotoxic anticancer agents, radiotherapy, or other targeted agents might be used in the future. Regarding the possible role of Aurora-A as a tumor suppressor, one can also ask whether it is really judicious to specifically target the Aurora-A kinase or, in other words, whether there a risk to trigger tumorigenesis of noncancer cells in a patient treated with an Aurora kinase inhibitor. Further studies are needed to fully address these questions and there is a strong need to generate pertinent biomarkers to treat patients that really need these molecules.

Summary

Aurora proteins belong to a family of kinases involved in cell cycle regulation. In mammals, there exist three different Aurora kinases named Aurora-A, -B, and -C.

These proteins share a common structure but present different functions and subcellular localizations. Aurora-A is associated predominantly with centrosomes and the spindle apparatus from prophase through telophase and is mainly involved in mitotic spindle assembly. Aurora-B is prominent at the midzone during anaphase and in postmitotic bridges during telophase and participates in chromosomes segregation and cytokinesis. Aurora-C appears to possess functions similar to Aurora-B but its expression is restricted to testis. Due to their prominent roles in cell cycle regulation, Aurora kinases are frequently involved in tumorigenesis and are targets of pharmaceutical industry that aims at generating specific Aurora kinase inhibitors to treat cancer.

References

Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell. 2003; 3(1):51–62.

Anderson K, Yang J, Koretke K, Nurse K, Calamary A, Kirkpatrick RB, Patrick D, Silva D, Tummino PJ, Copeland RA, Lai Z. Biochemistry. 2007;46:10287–95.

Bayliss R, Sardon T, Vernos I, Conti E. Structural basis of aurora-A activation by TPX2 at the mitotic spindle. Mol Cell. 2003;12(4):851–62.

Boss DS, Beijnen JH, Schellens JH. Clinical experience with aurora kinase inhibitors: a review. Oncologist. 2009;14(8): 780–93.

Chan CS, Botstein D. Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. Genetics. 1993;135(3):677–91.

Glover DM, Leibowitz MH, McLean DA, Parry H. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. Cell. 1995;81(1):95–105.

Hans F, Skoufias DA, Dimitrov S, Margolis RL. Molecular distinctions between aurora A and B: a single residue change transforms aurora A into correctly localized and functional aurora B. Mol Biol Cell. 2009;20(15): 3491–502.

Karthigeyan D, Prasad SB, Shandilya J, Agrawal S, Kundu TK. Biology of aurora A kinase: implications in cancer manifestation and therapy. Med Res Rev. 2010;31:757–93.

Katayama H, Sen S. Aurora kinase inhibitors as anticancer molecules. Biochim Biophys Acta. 2010;1799(10–12): 829–39.

Marumoto T, Honda S, Hara T, Nitta M, Hirota T, Kohmura E, Saya H. Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. J Biol Chem. 2003;278 (51):61786–95.

Meraldi P, Honda R, Nigg EA. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53−/− cells. EMBO J. 2002;21(4):483–92.

Nigg EA. Nat Rev Mol Cell Biol. 2001;2(1):21–32.

Norden C, Mendoza M, Dobbelaere J, Kotwaliwale CV, Biggins S, Barral Y. The NoCut pathway links completion of cytokinesis to spindle midzone function to prevent chromosome breakage. Cell. 2006;125(1):85–98.

Rannou Y, Troadec MB, Petretti C, Hans F, Dutertre S, Dimitrov S, Prigent C. Localization of aurora A and aurora B kinases during interphase: role of the N-terminal domain. Cell Cycle. 2008;7(19):3012–20.

Vader G, Lens SM. The aurora kinase family in cell division and cancer. Biochim Biophys Acta. 2008;1786(1):60–72.

B

B cell Stimulatory Factor-2 (BSF-2)

► IL6

B1BKR

► Bradykinin Receptors

B_1R

► Bradykinin Receptors

B220

► CD45 (PTPRC)

B2BKR

► Bradykinin Receptors

B_2R

► Bradykinin Receptors

BAD (BCL-2-Associated Agonist of Cell Death), BBC6 (BCL2-Binding Component 6), BCL2L8 (BCL2-Like Protein 8)

► BCL-2 Family

BAF

► SWI/SNF Chromatin Remodeling Complex

BAK (BCL-2 Antagonist Killer), BCL2L7 (BCL-2 Like 7), CDN1

► BCL-2 Family

basic FGF

► FGF (Fibroblast Growth Factor)

BatK

► CSK-Homologous Kinase

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

BAX (BCl2-Associated X Protein), BCL2L4 (BCL-2 Like 4)

▶ BCL-2 Family

BC067047

▶ P-Rex

Bcl10-Interacting MAGUK Protein 3

▶ CARMA1

BCL-2 (B-Cell Lymphoma 2)

▶ BCL-2 Family

BCL-2 Family

Zuzana Saidak, Zakaria Ezzoukhry, Jean-Claude Maziere and Antoine Galmiche
Laboratoire de Biochimie, Inserm ERI12 – EA4292, Université de Picardie Jules Verne (UPJV), Amiens Cedex, France

Synonyms

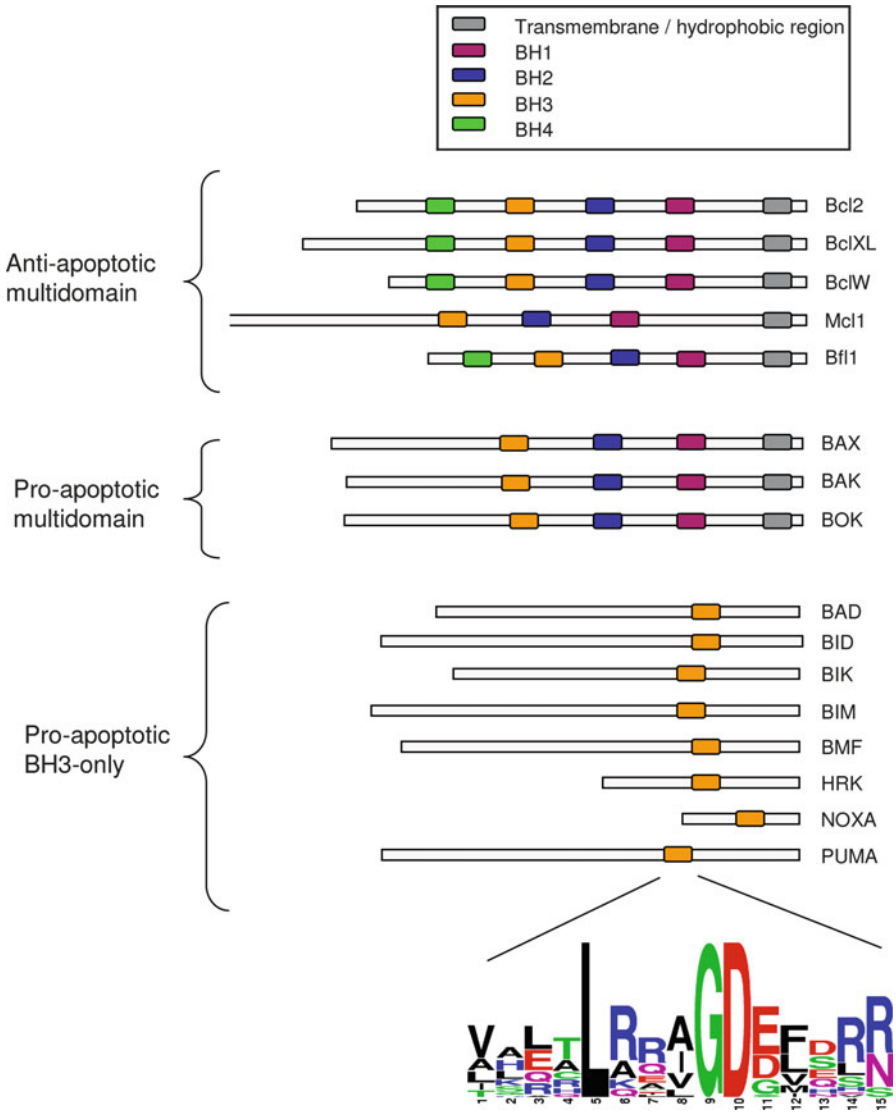
A1 (BCL2-related protein A1), BFL-1, BCL2L5 (BCL-2 like 5); BAD (BCL-2-Associated Agonist of cell Death), BBC6 (BCL2-Binding Component 6), BCL2L8 (BCL2-like protein 8); BAK (BCL-2 antagonist Killer), BCL2L7 (BCL-2 like 7), CDN1; BAX (BCl2-associated X protein), BCL2L4 (BCL-2 like 4); BCL-2 (B-cell lymphoma 2); BCL-W, BCL2L2 (BCL-2 like 2); BCL-XL, BCL2L, BCL2L1 (BCL-2 like 1); BID (BH3 Interacting domain Death agonist); BIK (BH3 interacting Killer), NBK; BIM (BCL-2 Interacting Mediator of cell death), BCL2L11 (BCL-2-like Protein 11), BOD; BMF (BCL-2-Modifying Factor); BOK (BCL-2 related Ovarian Killer), BCL2L9 (BCL-2 like 9); HRK (Harakiri BCL2 interacting protein), DP5 (Neuronal Death Protein-5); MCL-1 (Myeloid Cell Leukemia-1), BCL2L3 (BCL-2 like 3); NOXA, PMAIP (Phorbol-Myristate-Acetate-induced Protein), APR (Adult T cell leukemia-derived PMA-responsive); PUMA (p53 upregulated modulator of apoptosis), BBC3 (BCL2-Binding component 3)

Historical Background

The BCL-2 protein, the founding member of this family of proteins, was discovered in 1985. The gene *BCL2* was identified as the main protagonist in the chromosomal translocation t(14;18) in a subset of B-cell lymphomas, placing it under the control of the promoter of the immunoglobulin heavy chain genes (Cotter 2009). In contrast to previously identified oncogenes that mainly promote cell proliferation, BCL-2 was the first oncogene shown to inhibit cell death (Vaux et al. 1988). Since then other members of the BCL-2 family have been discovered, and the family of BCL-2 proteins now consists of approximately 20 members. In mammalian organisms, BCL-2 proteins play an essential role in the control of programmed cell death, and in particular apoptosis. Recent investigations have helped to unveil some facets of their regulation and their molecular mode of action on membrane organelles and mitochondria.

Structure and Classification of BCL-2 Proteins

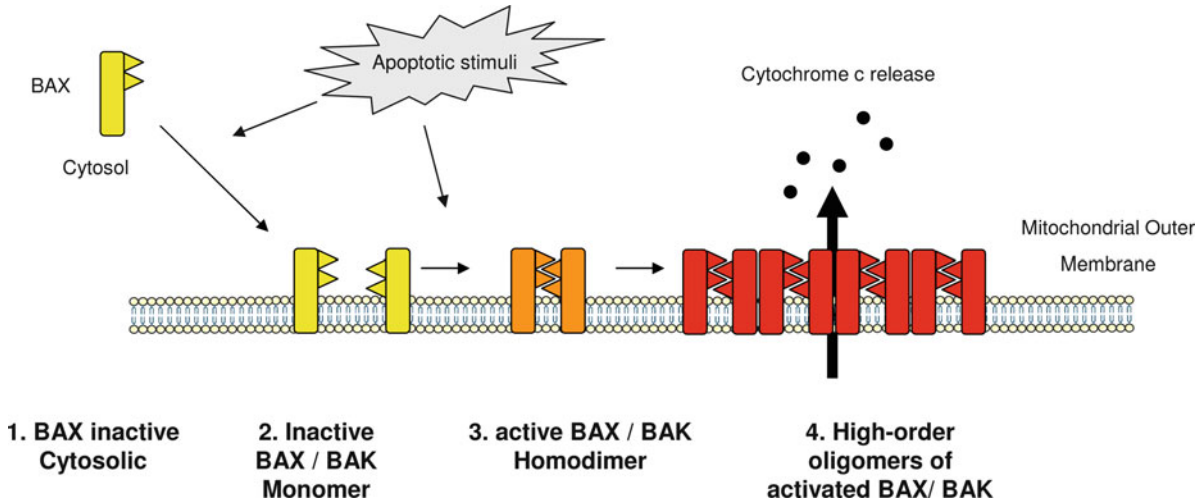
BCL-2 proteins are divided into three groups, based on functional as well as structural criteria (Chipuk et al. 2010): (1) functionally, depending on their effect on apoptosis – either pro- or anti-apoptotic; (2) structurally according to the presence of one or multiple homology domains – all members of the BCL-2 family share one or more regions of sequence homology, called the BCL-2 homology domains 1–4 (BH1 to BH4). The anti-apoptotic proteins of the BCL-2 family constitute the first group of proteins. They are multidomain proteins, usually containing BH1-BH4 domains. This group principally includes the proteins BCL-2, BCL-XL, MCL-1, and A1



BCL-2 Family,
Fig. 1 *Structural domains and organization of the BCL-2 proteins*. BCL-2 proteins can be classified according to their pro- or anti-apoptotic effects and the presence of one or multiple BCL-2-homology (BH) domains. The BH3 domain is the only domain of homology shared by all members of the family. It consists of 15 AA with a preference for the motif depicted in the sequence logo in the lower part of the figure (adapted from the server Prosite, http://expasy.org/cgi-bin/prosite/). While the membrane localization domains are indicated here for the multidomain BCL-2 proteins, several members of the BH3-only proteins also possess membrane targeting domains with an affinity for lipids

(Fig. 1). The corresponding proteins functionally counteract the pro-apoptotic proteins of the BCL-2 family. The pro-apoptotic proteins are divided into two groups: (1) the multidomain pro-apoptotic proteins (BAX, BAK, BOK) of the BCL-2 family, and (2) the BH3-only proteins, containing this sole homology domain (such as BID, BIM, PUMA, BAD, NOXA, BMF, HRK, BIK) (Fig. 1). The BH3 domain is, therefore, the only conserved region of homology among the proteins of the BCL-2 family. While this domain is an essential region for the activity of the BCL-2 proteins, it is short and consists of approximately 15 amino acids organized in an amphipathic helix (Fig. 1). The BH3 domain is also present in proteins that are only loosely connected to the BCL-2 family, such as the proteins MULE and Beclin-1.

Remarkably, despite important differences in their amino-acid sequences, all multidomain proteins of the BCL-2 family possess a similar secondary structure consisting mostly of α-helices and a similar overall fold (Chipuk et al. 2010). This similarity extends to the proteins that have opposing functions, either pro- or anti-apoptotic. In BCL-XL, the spatial juxtaposition of α-helices from the BH1-BH3 regions defines a globular structure with a hydrophobic groove on the surface of the molecule. This hydrophobic groove enables BCL-XL to interact with the BH3 domain of



BCL-2 Family, Fig. 2 *A model for the activation of BAX/BAK and the induction of MOMP*. The activation of the pro-apoptotic multidomain proteins BAX and BAK is an essential step that leads to mitochondrial membrane permeabilization and apoptosis. While BAK is constitutively present at the mitochondrial level, BAX is normally cytosolic. The first step in the activation of BAX consists of a cytosolic to membrane translocation, possibly occurring as a consequence of the release of the carboxy-terminal tail of BAX from an inhibitory internal interaction with the hydrophobic groove of this molecule. The second step consists of the activation of BAX/BAK per se and probably results in the shaping of the BH3 domains of BAX or BAK. Reciprocal interactions and homodimer formation are rendered possible once this shaping has allowed reciprocal interactions between their BH3 domains and hydrophobic grooves. Further interactions implicating other parts of BAX/BAK lead to higher order complex- and pore-formation, resulting in MOMP and apoptosis

pro-apoptotic proteins. In contrast to the multidomain proteins of the BCL-2 family, the BH3-only proteins are structurally diverse, with the exception of BID, which has an overall fold reminiscent of the multidomain proteins. Members of the BH3-only subset, such as BAD or BIM, tend to be intrinsically unfolded proteins and they probably acquire a stable fold only upon their interaction with other members of the BCL-2 family.

Mitochondrial Membrane Permeabilization by BCL-2 Proteins

In mammalian cells, mitochondrial outer membrane permeabilization (MOMP) is an early and crucial event during the induction of apoptosis (Tait and Green 2010). The MOMP leads to the release of pro-apoptotic factors, such as cytochrome c, into the cytosol. There, cytochrome c induces a cascade of biochemical events that lead to the activation of caspases, a family of proteases involved in the execution of the death sentence.

The proteins of the BCL-2 family are key players in the MOMP (Kuwana et al. 2002). The pro-apoptotic multidomain proteins of the BCL-2 family, i.e., BAX and BAK, play an essential role in the MOMP through their ability to form membrane-inserted oligomers (Chipuk et al. 2010; Westphal et al. 2010). How BAX and BAK insert and ultimately permeabilize mitochondrial membranes is a complex question and represents the focus of intense research. According to a commonly accepted model, several steps are required for BAX/BAX oligomerization and MOMP (Fig. 2). The first step consists of the mitochondrial recruitment of these proteins. While BAK is constitutively present at the mitochondrial level, BAX is cytosolic in healthy cells. In its cytosolic form, the C-terminal extremity of BAX is sequestered in its BH3-binding pocket and BAX is therefore locked in a monomeric, inactive form. The first step in BAX activation consists of the release of BAX from this intramolecular lock, and this step is a requisite for the insertion of BAX into the MOM. The next step is common to BAX and BAK, and consists of the direct activation of these proteins. Some proteins of the BH3-only subset, in particular BID, BIM, or PUMA, can directly activate BAX and BAK through direct contacts (Gavathiotis et al. 2008; Gallenne et al. 2009). The multimerization of BAX and BAK also requires the release of these molecules

from the inhibitory effect of anti-apoptotic proteins of the BCL-2 family, such as BCL-2, BCL-XL, or MCL-1. Anti-apoptotic proteins of the BCL-2 family negatively regulate the multimerization of BAX and BAK through two mechanisms: (1) the direct sequestration of BAX and/or BAK, and (2) indirectly, through the neutralization of the BH3-only proteins endowed with the ability to activate BAX/BAK, such as BID (Billen et al. 2008). Overall, the MOMP is a complex process that is intimately associated with the formation of complexes between proteins of the BCL-2 family. While the role of BCL-2 proteins in MOMP is well accepted, many questions about the precise mechanisms still remain, such as the exact nature of the pore formed by BAX and BAK and the contribution of accessory mitochondrial proteins to this process.

Regulation of the BCL-2 Network: Role of the BH3-Only Proteins

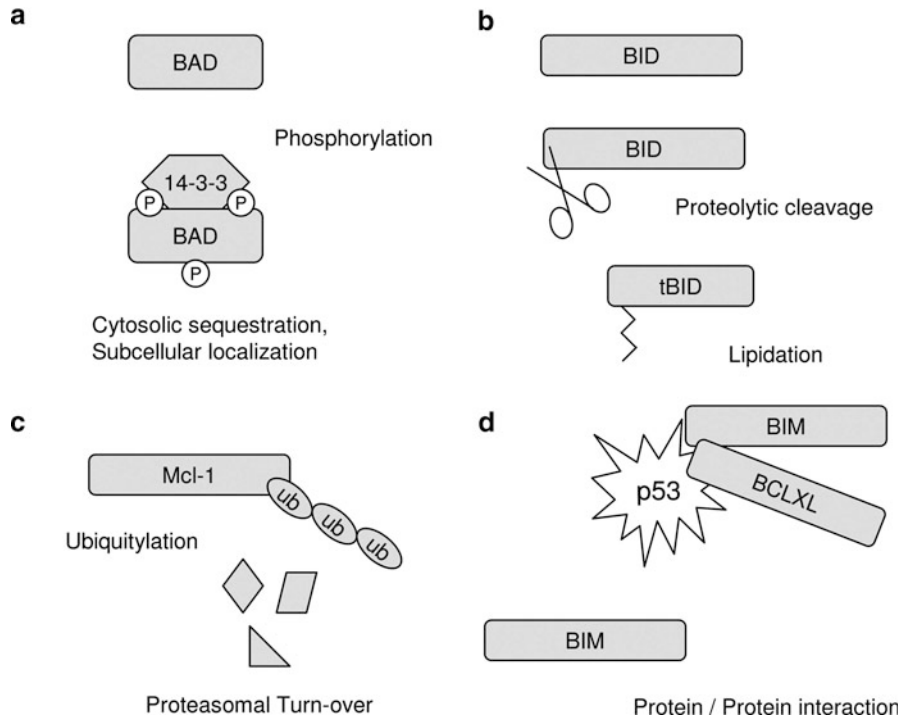
Cell survival is the result of a delicate balance between the activities of the pro- and anti-apoptotic proteins of the BCL-2 family. Apoptosis occurs when this balance is tipped over in favor of the pro-apoptotic signal. The BH3-only proteins play an upstream regulatory role in the BCL-2 network. While the proteins of this subset generally stimulate apoptosis, a complex picture of the mode of action of BH3-only proteins has recently emerged.

All BH3-only proteins are able to neutralize the anti-apoptotic proteins of the BCL-2 family, but the interaction of BH3-only proteins with anti-apoptotic BCL-2 proteins is characterized by its selectivity. There are large differences in the interaction spectra among BH3-only proteins (Chen et al. 2005; Certo et al. 2006). Some BH3-only proteins, such as BAD, neutralize selected anti-apoptotic proteins, such as BCL-2 and BCL-XL, while others, such as BIM and PUMA, bind all pro-survival proteins. Some BH3-only proteins, such as BIM, BID, and PUMA, do not only neutralize the anti-apoptotic proteins of the BCL-2 family, but can also activate the pro-apoptotic proteins BAX and BAK through labile interactions (Gavathiotis et al. 2008; Gallenne et al. 2009). These differences in terms of mode of action of the BH3-only proteins translate into differences in apoptotic potency, and proteins such as BAD behave more as sensitizers toward apoptosis rather than true inducers. The study of how the effector BCL-2 proteins are regulated in living cells has until now been a difficult task. New biochemical as well as functional approaches will certainly help to track the dynamic interactions between BCL-2 proteins, and to clarify the regulation of BCL-2 proteins during the life/death decision.

An important aspect of the regulation of BH3-only proteins is that they are kept under control by specific stimuli. Apoptosis-modulating stimuli operate on each BH3-only protein, via an array of regulations ranging from transcriptional to posttranslational (Fig. 3). For example, PUMA is induced transcriptionally following severe DNA damage, essentially through the activation of the transcription factor ▶ p53 (Yu and Zhang 2008). On the other hand, the BH3-only protein BAD is phosphorylated and thereby inactivated by pro-survival kinases, such as the kinase cascades RAF-MEK-ERK or PKB-mTOR. The survival of cells requires their constant exposure to trophic factors that activate these cascades, and BAD becomes activated by dephosphorylation in response to growth factor deprivation (Danial 2008). BID is another member of the BH3-only subset whose activity is under regulation through the engagement of a family of cell surface receptors known as death receptors. BID is activated by a proteolytic cleavage generating the truncated, active form of the molecule called tBID. Cellular studies have helped to establish the basics on the regulation of each BH3-only protein and the sentinel function of BH3-only proteins, but the regulation of BCL-2 proteins remains a complex topic, involving several protagonists with different tissue-specific expression patterns and partially redundant functions.

Various Physiological Functions

In addition to the regulation of programmed cell death, proteins of the BCL-2 family regulate several physiological processes. These processes are diverse, and range from the control of mitochondrial morphogenesis and Ca^{2+} fluxes in the endoplasmic reticulum to various aspects of cell metabolism. Cell proliferation and the integrity of the genome are also regulated by BCL-2 proteins, through interactions established with regulatory proteins of the cell cycle and DNA repair machinery. A detailed overview of these mechanisms is clearly beyond the scope of this chapter, but the



BCL-2 Family, Fig. 3 *Posttranslational regulation of proteins of the BCL-2 family*. The BCL-2 proteins are regulated through the direct modulation of their activation status, their subcellular localization, protein stability, or their functional sequestration. Posttranslational modifications, such as phosphorylations, proteolytic cleavage, ubiquitylation, lipidation, interaction with chaperones or with specific molecules are frequently encountered. For example, the protein BAD is regulated by phosphorylation and association with proteins of the 14-3-3 family (panel A). BID becomes active upon engagement of death receptors: A proteolytic cleavage by Caspase-8 creates a truncated version of this protein (tBID) and unmasks a site for N-myristoylation of this protein (panel B). The protein MCL-1 is regulated by ubiquitylation, a posttranslational modification that controls its turnover through proteasomal degradation (panel C). Finally, protein interactions can also regulate the activity of BCL-2 proteins. The protein p53 is able to functionally neutralize the anti-apoptotic proteins of the BCL-2 family, such as BCL-XL or MCL-1, despite the absence of a BH3 domain; by doing so, the cytosolic accumulation of p53 might release pro-apoptotic proteins of the BCL-2 family, such as BIM, from preexisting inhibitory interactions (panel D)

regulation of autophagy and inflammatory cytokine production by BCL-2 proteins provide two well-known examples. Autophagy is a process whereby cellular macromolecules or organelles become isolated inside cellular membrane and fuse with lysosomes to promote their elimination and recycling of their components. BCL-2 and BCL-XL have been shown to interact with Beclin-1, an essential regulator of autophagy. The interaction between Beclin-1 and BCL-2 is possible because Beclin-1 possesses a BH3 motif (Maiuri et al. 2007). BCL-2 and BCL-XL also play a role in the regulation of the metabolism of inflammatory cytokines, such as Interleukin-1, through molecular interactions established with the inflammasome, an intracellular protein complex involved in the regulation of Caspase-1, the enzyme responsible for the maturation processing of this cytokine (Bruey et al. 2007). The proteins of the BCL-2 family therefore exert pleiotropic effects that extend far beyond the regulation of programmed cell death.

BCL-2 Proteins and Cancer

Reduced sensitivity to apoptosis is one of the hallmarks of cancer cells. Deregulation of BCL-2 protein expression is frequently observed and it was shown to contribute to this disease (Yip and Reed 2008; Frenzel et al. 2009). Overexpression of the anti-apoptotic proteins of the BCL-2 family was the mechanism first reported to account for apoptosis resistance in cancer cells. While it is now well accepted that most

cancer cells present a reduced sensitivity to apoptosis due to modulation of the BCL-2 regulatory system, the mechanisms that lead to the altered regulation of BCL-2 proteins are complex. Alterations in the genome of cancer cells, epigenetic mechanisms, and posttranslational modifications often concur to shape the BCL-2 proteome in cancer cells (Yip and Reed 2008; Frenzel et al. 2009).

The regulation of the proteins of the BCL-2 family has attracted considerable attention as a possible approach for cancer treatment. Indeed, inducing tumor regression through the death of cancer cells is the main goal of cancer treatment, and most chemotherapeutic agents are apoptosis inducers in cancer cells (Fulda and Debatin 2006). In a growing number of situations, apoptosis of cancer cells induced by medical treatments was found to depend on the modulation of BCL-2 proteins: treatment-induced apoptosis could either be blocked by the overexpression of anti-apoptotic proteins, such as BCL-XL or MCL-1, or by the reduction of the expression of pro-apoptotic proteins of the BCL-2 family. For example, colorectal cancer cells with a BAX knockout were found to be insensitive to the commonly used chemotherapeutic agent 5-fluorouracil (Zhang et al. 2000). More recently, specific BH3-only proteins were found to account for cell death induced by specific targeted therapies. For example, the BH3-only protein BAD mediates the apoptotic response of liver cancer cells exposed to the kinase inhibitor sorafenib, currently the only medical treatment for this tumor (Galmiche et al. 2010).

The realization that BCL-2 proteins play a pivotal role in the response of cancers to medical treatments led to intense efforts aiming to identify compounds that would directly target these proteins and could be used as a new line of targeted therapies in oncology. In recent years great advancements have been made along this line, principally with the search for BH3-mimetic compounds that bind the hydrophobic groove formed by BH1-BH3 of the anti-apoptotic BCL-2 proteins, thus favoring apoptosis. To date, the compound with the best characterized BH3-mimetic activity is ABT-737 that was developed by the Abbott laboratories (Oltersdorf et al. 2005). ABT-737 binds with high affinity to the anti-apoptotic proteins BCL-2, BCL-XL, and BCL-W, but not to MCL-1, thus demonstrating a BAD-like reactivity. ABT-737 exerts a strong anticancer activity on Small Cell Lung Carcinoma cells, which frequently overexpress BCL-2 (Oltersdorf et al. 2005). An orally active derivative, ABT-263, has been developed. ABT-263 has shown promising effects in animal models with xenografted tumors, leading to sustained regression and demonstrating the safety of the inhibition of BCL-2 proteins in the entire organism. Studies aiming to test BCL-2 inhibitors in animal models that more closely mimic human tumors are now eagerly awaited. In parallel, the identification of compounds with reactivities that differ from those of ABT-737 as well as the understanding of cancer cell addiction to anti-apoptotic proteins of the BCL-2 family are the focus of future research. BCL-2 proteins have acquired the status of potential targets in oncology, and advances in this field are expected in the coming decade.

Summary

BCL-2 proteins are pivotal regulators of apoptosis. Over the past decade, intense research efforts have helped to better understand how these proteins mutually interact and regulate the mitochondrial membrane permeabilization, a critical step in apoptosis execution. In addition to their role as important effectors, BCL-2 proteins have also emerged as key integrators for the cell signaling pathways regulating programmed cell death. Extensive work still remains to fully understand the functionality of the intricate network of BCL-2 proteins, but recent advances have demonstrated the therapeutic potential of targeting BCL-2 proteins in cancer therapy. The introduction of drugs with a new mode of action, called BH3 mimetics, holds great promise in cancer research. It is also expected to facilitate the exploration of the physiological functions and the regulation of these important signaling molecules.

References

Billen LP, Kokoski CL, Lovell JF, et al. Bcl-XL inhibits membrane permeabilization by competing with Bax. PLoS Biol. 2008;6:e147.

Bruey JM, Bruey-Sedano N, Luciano F, et al. Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. Cell. 2007;129:45–56.

Certo M, Del Gaizo MV, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell. 2006;9:351–65.

Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell. 2005;17:393–403.
Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. Mol Cell. 2010;37:299–310.
Cotter TG. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer. 2009;9:501–7.
Danial NN. BAD: undertaker by night, candyman by day. Oncogene. 2008;27(Suppl 1):S53–70.
Frenzel A, Grespi F, Chmelewskij W, et al. Bcl2 family proteins in carcinogenesis and the treatment of cancer. Apoptosis. 2009;14:584–96.
Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene. 2006;25:4798–811.
Gallenne T, Gautier F, Oliver L, et al. Bax activation by the BH3-only protein Puma promotes cell dependence on antiapoptotic Bcl-2 family members. J Cell Biol. 2009;185:279–90.
Galmiche A, Ezzoukhry Z, François C, et al. BAD, a proapoptotic member of the BCL2 family, is a potential therapeutic target in hepatocellular carcinoma. Mol Cancer Res. 2010;8:1116–25.
Gavathiotis E, Suzuki M, Davis ML, et al. BAX activation is initiated at a novel interaction site. Nature. 2008;455:1076–81.
Kuwana T, Mackey MR, Perkins G, et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell. 2002;111:331–42.
Maiuri MC, Le Toumelin G, Criollo A, et al. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. EMBO J. 2007;26:2527–39.
Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature. 2005;435:677–81.
Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010;11:621–32.
Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. 1988;335:440–2.
Westphal D, Dewson G, Czabotar PE, Kluck RM. Molecular biology of Bax and Bak activation and action. Biochim Biophys Acta. 2010;1813:521–31.
Yip KW, Reed JC. Bcl-2 family proteins and cancer. Oncogene. 2008;27:6398–406.
Yu J, Zhang L. PUMA, a potent killer with or without p53. Oncogene. 2008;27(Suppl 1):S71–83.
Zhang L, Yu J, Park BH, et al. Role of BAX in the apoptotic response to anticancer agents. Science. 2000;290:989–92.

BCL-W, BCL2L2 (BCL-2 Like 2)

► BCL-2 Family

BCL-XL, BCL2L, BCL2L1 (BCL-2 Like 1)

► BCL-2 Family

BDKRB1

► Bradykinin Receptors

BDKRB2

► Bradykinin Receptors

BDR

► Hippocalcin

Beta-Catenin

Ken-Ichi Takemaru, Xingwang Chen and Feng-Qian Li
Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, USA

Synonyms

Catenin (cadherin-associated protein), beta 1 (88kD); Catenin beta; Catnb; Ctnnb; CTNNB1

Historical Background

Beta-catenin (β-catenin) (Armadillo in *Drosophila*) is a multifunctional protein involved in two essential cellular events: cell–cell adhesion and the canonical Wnt signaling pathway (Takemaru 2006). β-Catenin/armadillo (Arm) was initially identified as a segment polarity protein in *Drosophila* in the early 1980s, and later recognized as a key downstream effector of the Wnt pathway. Meanwhile, β-catenin was shown to be

an integral component of cadherin-mediated cell adhesion complexes. Over the past two decades, interdisciplinary research has tremendously advanced our knowledge of β-catenin function and its involvement in human disorders (Takemaru et al. 2008; Cadigan and Peifer 2009; MacDonald et al. 2009). At cell–cell adhesion junctions, β-catenin interacts with type-I cadherins and α-catenin, which in turn associates with the actin cytoskeleton. In canonical Wnt signaling, β-catenin acts as a transcriptional coactivator through its interaction with transcription factors and cofactors to stimulate expression of target genes. In recent years, aberrant activity of β-catenin signaling has been linked to various diseases, especially cancer.

Structural Features of β-Catenin

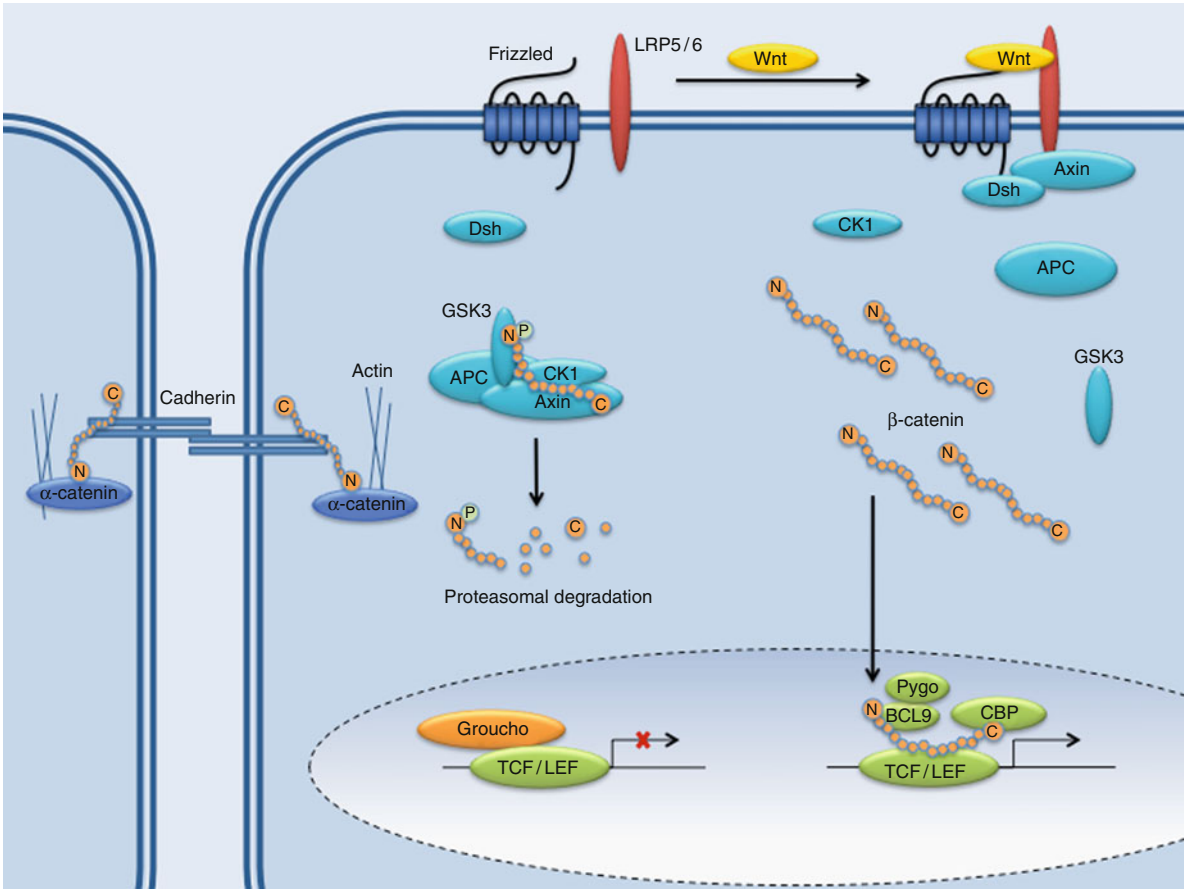
Human or mouse β-catenin consists of 781 amino acid residues, harboring a central structural core of 12 Arm repeats, flanked by unique N- and C-termini (Takemaru et al. 2008; Mosimann et al. 2009). The Arm repeat domain is highly conserved between vertebrates and other species but the terminal portions are diverged. The three-dimensional structure of the Arm repeat region has been determined, forming a twisted superhelical structure with a positively charged groove. Many β-catenin-binding partners bind to the Arm repeat domain. The precise structures of the N- and C-terminal tails remain unknown and may not form a rigid structure on their own. β-Catenin is subjected to posttranslational modifications such as ubiquitination, phosphorylation, and acetylation that control its protein stability, subcellular localization, and protein–protein interactions (Verheyen and Gottardi 2010). Plakoglobin (γ-catenin) is a close homologue of β-catenin in vertebrates and can fulfill some of the same functions (Zhurinsky et al. 2000).

β-Catenin as a Key Transcriptional Coactivator in the Canonical Wnt Pathway

β-Catenin is best known for its function as a transcriptional coactivator downstream of canonical Wnt signaling. Wnts are secreted extracellular proteins that play diverse roles in embryonic development and tissue homeostasis, including cell proliferation, cell fate decisions, and stem cell maintenance, as well as cell movement and polarity (Angers and Moon 2009; Cadigan and Peifer 2009; MacDonald et al. 2009). Core components of the Wnt/β-pathway are highly conserved in evolution from primitive cnidarians to humans.

Our current understanding of the Wnt/β-catenin signaling pathway is summarized in Fig. 1. In the absence of a Wnt ligand (Fig. 1, left), β-catenin is captured by the multi-protein "destruction complex," composed of the tumor suppressors Axin and adenomatous polyposis coli (APC), and the protein kinases casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). CK1 acts as a priming kinase and phosphorylates β-catenin at serine 45, allowing subsequent phosphorylation at threonine 41, serine 37, and serine 33 by GSK3. Phosphorylated β-catenin is then recognized by the E3 ubiquitin ligase receptor β-TrCP and targeted for ubiquitin-mediated proteasomal degradation. Therefore, under unstimulated conditions, cytosolic β-catenin is maintained at low levels. In the nucleus, the DNA-binding HMG-box T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins keep Wnt target genes off by recruiting transcriptional corepressors such as Groucho (TLE). Extracellularly, the activity of Wnts is regulated by several secreted antagonists including Dickkopfs (DKKs), secreted frizzled-related proteins (sFRPs), and Wnt inhibitory factors (WIFs). Upon engagement with the seven transmembrane frizzled (Fz) receptors and the low-density lipoprotein receptor-related protein (LRP) coreceptors LRP5/6 (Fig. 1, right), Wnts trigger activation of the cytoplasmic protein disheveled (Dsh) and phosphorylation of the cytoplasmic tail of LRP5/6. This promotes recruitment of Dsh and Axin to the receptor complex at the plasma membrane, resulting in inhibition of β-catenin phosphorylation and degradation. Consequently, β-catenin accumulates in the cytoplasm and then translocates into the nucleus where it displaces Groucho and forms a complex with TCF/LEF transcription factors, leading to activation of Wnt target genes. Thus, activation of the Wnt pathway at the cell surface is ultimately translated into changes in gene expression through the TCF/β-catenin complex in the nucleus.

It is noteworthy that several negative regulators of β-catenin signaling, including APC, Axin, and Chibby (Cby), have been shown to contain both nuclear localization and nuclear export signals that enable them to shuttle between the nucleus and cytoplasm, and facilitate nuclear export of β-catenin (Willert and Jones



Beta-Catenin, Fig. 1 A simplified current model of the Wnt/β-catenin signaling pathway. β-Catenin has a dual function, acting in both cell adhesion and canonical Wnt signaling. See text for details

2006; Cadigan and Peifer 2009; MacDonald et al. 2009). In contrast, nuclear β-catenin interactors, such as TCF and BCL9/Pygopus (Pygo), appear to retain β-catenin in the nucleus. Detailed information on Wnt signaling can be found on the Wnt Homepage (http://www.stanford.edu/~rnusse/wntwindow.html).

Mechanisms of Target Gene Activation by β-Catenin

β-Catenin exerts its activation potential through assembly of coactivator and chromatin-remodeling complexes (Willert and Jones 2006; Takemaru et al. 2008; Mosimann et al. 2009). The C-terminal activation domain of β-catenin interacts with various positive cofactors such as the histone acetyltransferases CBP/p300, SWI/SNF ATPase subunit BRG1, and Parafibromin (Hyrax; a component of the RNA polymerase II-associated PAF1 complex). On the other hand, the N-terminal portion of β-catenin directly binds to the bridging molecule BCL9 (Legless), which in turn recruits the PHD-finger protein Pygo. Other β-catenin coactivators include TIP49 (Pontin), MED12, TRRAP, MLL1/2, and TBL1/TBLR1. The signaling activity of β-catenin is negatively regulated by its antagonists such as ICAT and Cby. There is also evidence that the TCF/β-catenin complex can function as a transcriptional repressor (Cadigan and Peifer 2009; MacDonald et al. 2009).

A considerable number of direct target genes of the TCF/β-catenin complex have been identified in various model systems including c-Myc, cyclinD1, Axin2, and TCF/LEF (for a comprehensive list of Wnt target genes, see the Wnt homepage). In general, cellular responses to Wnt signals vary significantly among

different cell types, and many Wnt/β-catenin target genes are regulated in a cell-type specific manner. There are a number of reagents/tools available to monitor β-catenin signaling activity including cell-based reporters, transgenic reporter animals, and direct β-catenin target genes (Moon et al. 2004; Barker and Clevers 2006; Chien et al. 2009).

β-Catenin at the Crosstalk with Other Signaling Pathways

Besides the canonical Wnt pathway, β-catenin signaling activity is positively or negatively regulated by a variety of other signaling pathways including Akt (protein kinase B), Src, PTEN, p53, NF-κB, epidermal growth factor (EGF), integrin-linked kinase (ILK), insulin-like growth factor (IGF), and prostaglandin E2 (PGE2) (Moon et al. 2004; MacDonald et al. 2009).

In addition to TCF/LEF factors, β-catenin has been shown to serve as a coactivator or, in some cases, a corepressor for many DNA-binding transcription factors including members of the nuclear hormone receptor family and HMG-box-containing Sox proteins (Takemaru et al. 2008; MacDonald et al. 2009). For instance, the vitamin A, vitamin D, and androgen receptors physically interact with β-catenin in a ligand-dependent fashion to potentiate activation of their target genes, while suppressing expression of TCF/β-catenin-dependent genes. Thus, it is apparent that β-catenin, via these transcription factors, could impact a broader range of gene expression programs.

β-Catenin in Development and Disease

The Wnt/β-catenin pathway has been studied extensively in a wide spectrum of model organisms including *C. elegans*, *Drosophila*, zebrafish, *Xenopus*, and mouse, and proven to be essential for numerous aspects of embryonic development such as segmentation, axis formation, and brain patterning (Cadigan and Nusse 1997; Chien et al. 2009). In mice, β-catenin deficiency results in embryonic lethality at the gastrulation stage (Grigoryan et al. 2008). Over the last decade, through the use of conditional mouse models, β-catenin has been activated and inactivated in various tissues in a temporal and tissue-specific manner (Grigoryan et al. 2008). These studies revealed important roles of Wnt/β-catenin signaling in development and homeostatic maintenance of many organs. In adults, Wnt/β-catenin signaling is crucial for maintaining self-renewal of pluripotent stem cells in skin, blood, intestine, and brain, and for tissue regeneration and repair following injury (Reya and Clevers 2005; Clevers 2006; Stoick-Cooper et al. 2007). Remarkably, recent studies identified the Wnt/β-catenin target and orphan receptor Lgr5 (GPR49) as a marker for stem cells in the adult intestinal epithelium and hair follicle (Barker and Clevers 2010).

More recently, dysregulation of Wnt/β-catenin signaling activity has been linked to the pathogenesis of a wide range of human diseases such as bone density defects and cancer (Logan and Nusse 2004; Clevers 2006; MacDonald et al. 2009).

Loss-of-function mutations in the Wnt coreceptor LRP5 are associated with osteoporosis-pseudoglioma syndrome (OPPG) characterized by low bone mass and loss of vision. Conversely, activating mutations in LRP5 cause increased bone density. These findings clearly demonstrate that Wnt/β-catenin signaling positively regulates bone formation.

Constitutively activated β-catenin signaling, due to loss-of-function mutations in APC or Axin or gain-of-function mutations in β-catenin itself, is associated with a variety of human malignancies including melanoma and colon and hepatocellular carcinomas (Polakis 2000; Takemaru et al. 2008). Remarkably, greater than 70% of colon cancers show aberrant Wnt/β-catenin signaling activity. Mutations in APC or Axin compromise their function within the β-catenin destruction complex, while oncogenic mutations in the N-terminal regulatory domain of β-catenin block its degradation via the ubiquitin-proteasome pathway. In addition, some tumor types show loss of expression of the secreted Wnt antagonists sFRPs and WIF1 due to epigenetic silencing by hypermethylation (Barker and Clevers 2006; Takemaru et al. 2008). All of these alterations ultimately lead to stabilization and nuclear translocation of β-catenin, followed by activation of target gene expression. Hence, β-catenin is an attractive molecular target for cancer therapeutics as well as other Wnt-related diseases. To date, small molecules that disrupt TCF/β-catenin or CBP/β-catenin interaction or stabilize Axin protein and therefore inhibit β-catenin-dependent transcription have been reported (Moon et al. 2004; Barker and Clevers 2006; Takemaru et al. 2008).

Summary

β-Catenin plays crucial roles in diverse biological processes as a pivotal component of cell–cell adhesion and Wnt signaling. It serves as a protein network hub by mediating numerous protein–protein interactions to ensure proper development and homeostasis of multiple tissues. Recent advances in genome-wide RNAi screens and proteomics approaches greatly facilitate the identification of novel β-catenin regulators (Angers and Moon 2009). The realization that β-catenin signaling is perturbed in various human diseases continues to fuel worldwide research efforts in the future. Certainly, a better understanding of β-catenin functions has broad impact on human diseases, stem cell biology, and regenerative medicine.

References

Angers S, Moon RT. Proximal events in Wnt signal transduction. Nat Rev Mol Cell Biol. 2009;10:468–77.

Barker N, Clevers H. Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov. 2006;5:997–1014.

Barker N, Clevers H. Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology. 2010;138:1681–96.

Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes Dev. 1997;11:3286–305.

Cadigan KM, Peifer M. Wnt signaling from development to disease: insights from model systems. Cold Spring Harb Perspect Biol. 2009;1:a002881.

Chien AJ, Conrad WH, Moon RT. A Wnt survival guide: from flies to human disease. J Invest Dermatol. 2009;129:1614–27.

Clevers H. Wnt/beta-catenin signaling in development and disease. Cell. 2006;127:469–80.

Grigoryan T, Wend P, Klaus A, Birchmeier W. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. Genes Dev. 2008;22:2308–41.

http://www.stanford.edu/~rnusse/wntwindow.html

Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20:781–810.

MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 2009;17:9–26.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet. 2004;5:691–701.

Mosimann C, Hausmann G, Basler K. Beta-catenin hits chromatin: regulation of Wnt target gene activation. Nat Rev Mol Cell Biol. 2009;10:276–86.

Polakis P. Wnt signaling and cancer. Genes Dev. 2000;14:1837–51.

Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature. 2005;434:843–50.

Stoick-Cooper CL, Moon RT, Weidinger G. Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. Genes Dev. 2007;21:1292–315.

Takemaru K-I, Catenin beta. UCSD-nature molecule pages. 2006; doi:10.1038/mp.a000506.01

Takemaru K-I, Ohmitsu M, Li F-Q. An oncogenic hub: beta-catenin as a molecular target for cancer therapeutics. Handb Exp Pharmacol. 2008;186:261–84.

Verheyen EM, Gottardi CJ. Regulation of Wnt/beta-catenin signaling by protein kinases. Dev Dyn. 2010;239:34–44.

Willert K, Jones KA. Wnt signaling: is the party in the nucleus? Genes Dev. 2006;20:1394–404.

Zhurinsky J, Shtutman M, Ben-Ze'ev A. Plakoglobin and beta-catenin: protein interactions, regulation and biological roles. J Cell Sci. 2000;113:3127–39.

bFGF

▶ FGF (Fibroblast Growth Factor)

Bgp

▶ CEACAMs

BGT-1

▶ GAT (GABA Transporters)

bHLHb27

▶ Inhibitor of DNA Binding 4 (ID4)

BID (BH3 Interacting Domain Death Agonist)

▶ BCL-2 Family

BIK (BH3 Interacting Killer), NBK

▶ BCL-2 Family

BIM (BCL-2 Interacting Mediator of Cell Death), BCL2L11 (BCL-2-Like Protein 11), BOD

▶ BCL-2 Family

Bimp3

▶ CARMA1

BKB1R

▶ Bradykinin Receptors

BKB2R

▶ Bradykinin Receptors

BKR1

▶ Bradykinin Receptors

BKR2

▶ Bradykinin Receptors

BM-90

▶ Fibulins

BMF (BCL-2-Modifying Factor)

▶ BCL-2 Family

BOK (BCL-2 Related Ovarian Killer), BCL2L9 (BCL-2 like 9)

▶ BCL-2 Family

Bp50

▶ CD40

Bradykinin Receptors

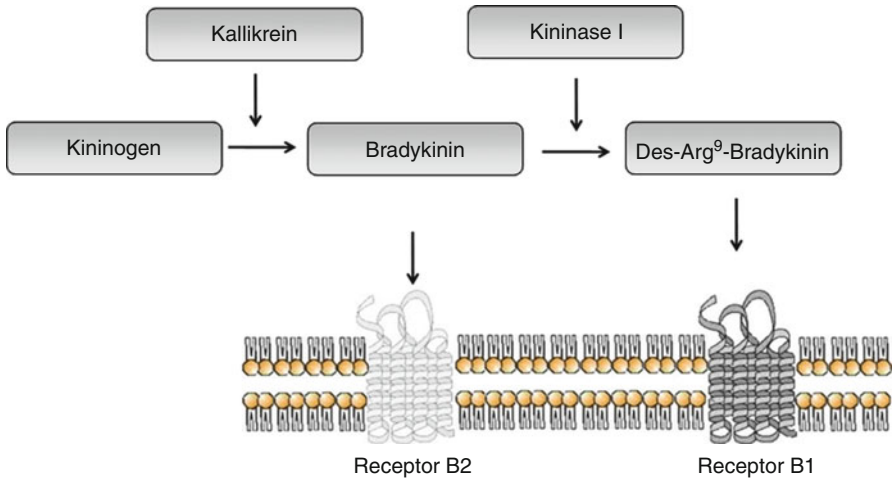
Vicência Sales and João Bosco Pesquero
Biophysics Department, Universidade Federal de São Paulo, São Paulo, Brazil

Synonyms

B1BKR; B_1R; B2BKR; B_2R; BDKRB1; BDKRB2; BKB1R; BKB2R; BKR1; BKR2; Kinin B_1; Kinin B_2

Historical Background

A primary mediator of inflammation, the nonapeptide bradykinin (BK) is a pharmacologically active peptide of the kinin group released in tissues and circulation as a consequence of coagulation cascade activation, more specifically by the kininogen cleavage by kallikrein. The enzyme kallikrein was described in 1930 by Werle and Frey. It was the first component of the kallikrein-kinin system (KKS) discovered, followed by the identification of bradykinin (BK) by Rocha e Silva and colleagues in 1949. In 1970s Regoli and coworkers characterized molecularly the two subtypes of kinin receptors B_1 and B_2, based in their pharmacological and expression profiles differences (Fig. 1). These findings enabled the subsequent development of different agonists and antagonists for these receptors (Leeb-Lundberg et al. 2005). The genes encoding these receptors were cloned in 1990s and after that, animal models for the study of this system were generated: the B_2 knockout



Bradykinin Receptors, Fig 1 Kallikrein-kinin system

mice (Borskowki et al. 1995), the B_1 knockout mice (Pesquero et al. 2000), and the knockout mice for both kinin receptors (Cayla et al. 2002).

Structural Aspects

The BK receptors are typical G protein coupled receptor (GPCR), consisting of a single polypeptide chain that spans the membrane seven times, with the N-terminal domain being extracellular and the C-terminal domain being intracellular. These receptors are present in different species of mammals like human, monkey, rats, mice, rabbit, and others.

In humans both receptors, B_1 and B_2, are homologues preserving 36% of identity at the amino acid level (Leeb-lundbeerg et al. 2005). These receptors are encoded by three-exon genes. B_1 receptor gene is in tandem with the B_2 receptor gene, located sequentially (5′ direction) separated by only 12 kb at cromossome 14q32 in humans. This composition can vary between species like the deletion of exon 2 in mice (Cayla et al. 2002).

Knockout animal models of each kinin receptor gene by homologous recombination have been done. The B_2 receptor knockout mice are fertile, apparently healthy, and when smooth muscle or neurons of these mice are stimulated with bradykinin they failed to produce response (Borkowski et al. 1995). The B_1 receptor knockout mice are healthy, fertile, normotensive, and they are analgesic in behavioral tests of chemical and thermal nociception (Pesquero et al. 2000). The generation of a knockout mouse of both receptors ($B_1B_2^{-/-}$) was also done. Due to the fact that both genes are in close chromosomal position, $B_1B_2^{-/-}$ mice could not be obtained by simple breeding of the single knockout lines. The B_1 receptor gene was inactivated in embryonic stem cells derived from B_2-deficient animals. These animals are normotensive and protected from endotoxin-induced hypotension (Cayla et al. 2007). Recently, another model of double-knockout of kinin receptors was generated by complete deletion of the gene locus (Kakoki et al. 2010).

Pharmacological Aspects

Kinins are locally released from their origin molecules, the kininogens, as a result of limited proteolysis by a class of serine proteases called kallikreins. The metabolite generated is the nonapeptide bradykinin or a decapeptide, kallidin (Lys-BK). Kinins cleavage by the kininase II also named angiotensin converting enzyme (ACE) generates inactive metabolites terminating bradykinin activity. The action of carboxipeptidases on kinins generates des-Arg^9-BK (DBK) or Lys-des-Arg^9-BK (Lys-DBK). The B_2 receptor has high affinity for the intact kinins, those generated by either plasma or tissue kallikreins, BK and Lys-BK, in all mammalian species. B_1 receptor responds to different kinin metabolites, either DBK or Lys-DBK, generated by arginine carboxypeptidases, such as carboxypeptidase N and M. In humans, plasma

kallikrein forms BK, whereas tissue kallikreins form kallidin. In rodents, both plasma and tissue kallikrein generate BK. Receptor affinity for agonist ligands: B_2 receptor, BK ≈ Lys-BK >> des-Arg^9-BK and Lys-des-Arg^9-BK; B_1 receptor, Lys-des-Arg^9-BK > Lys-BK ≈ des-Arg^9-BK >>BK (Leeb-Lundberg et al. 2005).

Peptide antagonists for the kinin B_1 receptor were the first antagonists generated based on modifications of the agonist structure, such as [Leu8]des-Arg^9-BK and Lys-[Leu8]des-Arg^9-BK. The search for antagonists showed that the spatial orientation of the C-terminal region of the peptide molecule is critical for antagonism. Many antagonists the for B_2 receptor have been generated. The most known peptide antagonist is the icatibant or HOE-140. Non-peptide ligands for the kinin receptors have been designed and are yet a great field of study, since peptides are generally poor drugs for oral bioavailability and brain penetration (Leeb-Lundberg et al. 2005).

Signaling Pathways

In different species both kinin receptors are identified as seven transmembrane G protein coupled receptor. Various signal transduction mechanisms have been described for kinins depending on the cellular type. BK or DBK stimulates B_2 or B_1 receptors, respectively. Through the phospholipase C pathway (by Gq activation), kinin signaling leads to inositol 3-phosphate (IP_3) generation and intracellular calcium mobilization, whereas through the ▶ phospholipase A_2 pathway (activated through Gi or calcium-dependent mechanisms) it leads to arachidonic acid release, also by activating the endothelium nitric oxide synthase (eNOS) and producing nitric oxide (NO). B_2 receptor has also been found to directly interact with other eNOS in a G protein-independent manner (Leeb-Lundberg et al. 2005).

BK also transiently promotes tyrosine phosphorylation of ▶ MAP Kinases and activates a Janus-activated kinase/STAT (JAK-STAT) pathaway. This involves tyrosine phosphorylation of both the Janus-activated kinase family tyrosine kinase Tyk2 and STAT3 followed by STAT3 nuclear translocation. B_2 activates multiple transcription factors that regulate the induction of several cytokines involved in tissue injury and inflammation as well as B_1 receptor induction. Besides these classical pathways, IL-1β and ▶ TNF-α can stimulate the expression of B_1 and B_2 receptors by pathways involving activation of ▶ NF-κB and MAPKs. Although the B_1 and B_2 receptors seem to couple to similar cellular signal transduction pathways, the patterns of signaling are different (Leeb-Lundberg et al. 2005; Brechter et al. 2008).

B_1 and B_2 receptor form homodimers and these receptors were found to spontaneously heterodimerize. Heterodimerization was associated with a specific proteolytic degradation of the participating B_2 receptor and an increase in both agonist-dependent and -independent signaling of the heterologous receptor complex. The existence of a B_2 receptor and angiotensin receptor 1 (B_2/AT1) heterodimeric complex may have implications for blood pressure. The B_2/ACE interaction modulates ACE activity (Sabatini et al. 2008).

B_2 receptor function is controlled by short-term mechanisms involving fast ligand dissociation, receptor desensitization and internalization, and, after long-term stimulation, downregulation of the receptor occurs. In contrast, B_1 receptors elicit persistent responses and signaling that are subjected to very limited desensitization and receptor internalization with very slow ligand dissociation (Couture et al. 2001).

Kinins and Disease

The kallikrein-kinin system (KKS) is present in numerous pathologies and the role it plays may vary. It can maintain the danous state of disease or play a protective role, as summarized below in Table 1.

Generated during inflammation and tissue injury, bradykinin contributes to the initiation and maintenance of inflammation, to exciting and sensitizing sensory nerve fibers, thus producing pain as reviewed by Couture and coalleagues in 2001. Thus the B_2 receptor is involved in acute inflammation, including increased vascular permeability, venoconstriction, arterial dilatation, and pain through the activation of sensory nerve terminals. This receptor has a limited role in the cellular component of the inflammatory response involving leukocyte recruitment within the microcirculation. The activation of B_2 receptors in sensory neurons promotes hyperalgesia. Bradykinin can sensitize nociceptors following the release of prostaglandins, cytokines, and

Bradykinin Receptors, Table 1 Kinin receptors' presence in various diseases

Disease	Effect
Inflammation	Pro-inflammatory[a]
Pain	Hyperalgesia[a]
Infection	BK increase:
	Vascular leakage and vasodilation[a]
	Arterial vasodilatation[a]
Imune system (autoimmune diseases)	Imune cells stimulation and regulation[b]
Bone (arthritis and periodontitis)	Stimulate bone resorption[c]
Respiratory system (asthma and rhinitis)	Increase in the expression of kinins[d]
Neurological disease	
Alzheimer	Improvement of cognitive deficits[e]
Epilepsy	Deleterious and protective effects[e]
Sclerosis	B_1R increases blood–brain barrier permeability[f]
Kidney nephrophaty	Chemokine production[g]
	Macrophage accumulation[g]
Metabolism	
Diabetes	Prevention of progression of insulin-dependent diabetes[h]
Obesity	B_2 absence enhance senescence in mice[i]
	$B_1^{-/-}$ mice are protect from diet-induced obesity[j]
Cardiovascular	Hypertrophy[k]
	Cardiopathy[k]
	Hypertension[k,l]
	Atherosclerosis[m]
Liver	Attenuates fibrosis/hepatocellular damage[n]
Cancer	Tumor growth[l]
	Angiogenesis stimulation[l]

[a]Couture et al. (2001)
[b]Schulze-Topphoff et al. (2009)
[c]Brechter et al. (2008)
[d]Proud (1998)
[e]Lemos et al. (2010)
[f]Schulze-Topphoff et al. (2009)
[g]Klein et al. (2010)
[h]Kakoki et al. (2010)
[i]Kakoki et al. (2006)
[j]Mori et al. (2008)
[k]Sharma (2003)
[l]Leeb-Lundberg et al. (2005)
[m]Merino et al. (2009)
[n]Kouyoumdjian et al. (2005)

nitric oxide either from sensory neurones, endothelial and immune cells or fibroblasts in addition to its interaction with mast cell mediators. The blockade of B_2 receptors located on sensory neurons may be responsible for the analgesic property of B_2 receptor antagonists. The pro-inflammatory effects of B_1 receptors include promotion of blood-borne leukocyte trafficking, edema and pain. B_1 receptors are primarily involved in persistent inflammatory pain and are expressed in macrophages, fibroblasts, or endothelial cells, where they may be responsible for inflammation mediators releasing (prostaglandins, cytokines, and nitric oxide) that sensitize or activate the nociceptors.

Because of its multicellular location and the mode of persistent signaling mechanism, the B_1 receptor is likely to exert a strategic role in inflammatory diseases, particularly those with an immune etiology (asthma, rheumatoid arthritis, multiple sclerosis, and diabetes). In addition to the pro-inflammatory effects of kinin receptors, B_1 receptors may exert a protective effect in brain inflammatory diseases such as multiple sclerosis by reducing T-lymphocyte infiltration into the brain (Schulze-Topphoff et al. 2009).

Kinins exert influence on multiple players of the immune system (i.e., macrophages, dendritic cells, T and B lymphocytes). BK is capable of modulating the activation, proliferation, migration, and effector functions of immune cells. Kinin receptors seem to be important in autoimmune conditions, such as rheumatoid arthritis, lupus, and myasthenia gravis (Schulze-Topphoff et al. 2008).

Kinin receptors are present in osteoblasts, osteoclasts, and fibroblasts, linking the kallikrein-kinin system with rheumatoid arthritis, periodontitis, and bone resorption. They can stimulate bone resorption through prostaglandins. Kinin B_1 and B_2 receptors synergistically potentiate IL-1β and TNF-α-induced prostaglandin biosynthesis in osteoblasts by a mechanism involving increased levels of cycloxygenase-2 (Brechter et al. 2008).

Many studies have demonstrated increased kinin generation associated with asthma, allergic rhinitis, and during viral rhinitis (Proud 1998). The first studies began with the analysis of the presence of kinins after allergen stimulation in allergic subjects and absence of them in non-allergic subjects. The inflammatory infiltration and relation between kinins and the chronic phase of the disease were then observed. Kinins are also associated with the release of the mast cell granule constituents, histamine, and tryptase, major mediators of acute phase. The kinin concentration increase during asthma is associated with the augment in histamine

and other inflammatory markers, including eicosanoids. The administration of bradykinin by nasal spray to the upper airways of normal, nonatopic subjects, or of asymptomatic atopic individuals has been shown to result in the dose-dependent induction of symptoms of nasal obstruction, modest rhinorrhea, nasal irritation, and sore throat, but not sneezing (Proud 1998).

Kinin receptors are involved with brain damage in different forms. They act in multiplus sclerosis, epilepsy, and Alzheimer's disease. Kinin B_2 receptor promotes survival and protects against brain injury by suppression of apoptosis and inflammation induced by ischemic stroke. In epilepsy, the kinin B_2 receptor also plays a neuroprotector effect and the kinin B_1 receptor plays a deleterious, pro-epileptogenic action in animal models (Leeb-Lundberg et al. 2005). Kinin receptors are involved in neurodegeneration and increase of amyloid-b concentration, associated with Alzheimer's disease (Lemos et al. 2010). More recently it was shown that during the aging process, the B_1 receptor could be involved in neurodegeneration and memory loss. Nevertheless, the B_2 receptor is apparently acting as a neuroprotective factor (Lemos et al. 2010). In inflammatory brain disease, like sclerosis, kinin B_1 receptors are important in limitating migration of lymphocytes through the central barrier and inflammation in the brain (Schulze-Topphoff et al. 2009).

Kinins receptors are present in the kidney and are involved with kidney disease, such as renal failure and nephropathy. Since kinin receptors are present in patients in end stage of renal failure, treatment with a B_1 receptor antagonist reduces both glomerular and tubular lesions and improve renal function trough the reduction of renal chemokine expression and macrophage accumulation in glomerulonephritis (Klein et al. 2010). Genetic association between B_1 receptor polymorphisms and end-stage renal failure have been reported, as the B_2 receptor polymorphism is associated with diabetic nephropathy (Leeb-Lundberg et al. 2005).

Lack of B_1 and B_2 receptors exacerbates diabetic complications, enhances the nephropathy (glomerulonephritis), neuropathy (decrease the time of nervous impulse), and bone mineral loss caused by insulin-dependent diabetes in mice (Kakoki et al. 2010). The development of diabetic retinopathy increases vascular permeability, neovascularization, inflammation and B_2 activation contributes to vascular permeability and edema, which suggests the correlations between the KKS and microvascular complications of diabetes. Studies performed in diabetic mice demonstrated that the absence of B_2 receptor in these animals increases indicators of senescence like alopecia, skin atrophy, kyphosis, osteoporosis, testicular atrophy, lipofuscin accumulation in renal proximal tubule and testicular Leydig cells, and apoptosis in the testis and intestine (Kakoki et al. 2006).

The kinin B_2 receptor agonist BK may participate in the regulation of substrate utilization by several tissues by improving blood flow and substrate delivery to the tissues and also by promoting translocation of glucose transporters. It appears to improve the release of insulin and improve insulin sensitivitiy. Furthermore, insulin may activate the kallikrein-kinin system, which consequently may increase its metabolic effects. However, in experimental diabetes mellitus, BK may participate in the inflammatory reaction leading to Langerhans islets destruction (Damas et al. 2004). Kinin B_1 receptor is involved in obesity, as shown by Mori et al. The absence of B_1 receptor in mice decreases plasma leptin levels, increases leptin sensibility, protects mice from diet-induced obesity (diet with 45% of fat), and augments energy expenditure.

The KKS has importante role in various pathological processes of the cardiovascular system, such as hypertension, cardiac failure, ischemia, left ventricular hypertrophy, and endotoxemia. There is activation of BK activity in endotoxemia. On the other hand, it seems that there is deficient activity of the KKS in hypertension, cardiac ischemia, and development of left ventricular hypertrophy. These pathological states might be due to a genetic abnormality of the KKS or downregulation of the BK receptors (Sharma 2003). Several studies have detected a significant association between the B_2 receptor 58 C/T polymorphism and hypertension (Leeb-Lundberg et al. 2005). Kinin B_1 receptor deficiency aggravates atherosclerosis and aortic aneurysms in mice under cholesterolemic conditions, supporting an antiatherogenic role for the kinin B_1 receptor (Merino et al. 2009).

The KKS is also present in the liver and is related to liver disease. BK can induce portal hypertensive response when injected in the liver. This hepatic hypertensive response to BK is mediated by the B_2 receptor and modulated by the L-Arg/nitric oxide pathway. There is also evidence of the participation of BK in

the pathogenesis of vasodilatation and ascites formation in cirrhotic patients (Kouyoumdjian et al. 2005).

Finally, the ability of BK to stimulate vessel growth and increase vascular permeability may contribute to the biological behavior of tumors. Evidence for increased generation of kinins and kinin receptors detection in different types of cancer has been reported (Leeb-Lundberg et al. 2005).

Summary

Considering the knowledge gathered since the classical pharmacological models were established and the more recently gene target animal models, much has been changed concerning the kinin receptors function. In the beginning, the kinin receptors were first implicated with pain and inflammation. Nowadays they are still important in this area of study; however, they have been implicated with different diseases like asthma, arthritis, sepsis, kidney disease, hypertension, cardiopathy, diabetes, and cancer among others. In the last years, new implications of kinin receptors in obesity and immunology are described, as well as interaction of kinin receptors and other proteins like ACE and AT1 receptor. These implications will bring new possibilities for therapies involving kinin ligands (agonists and antagonists). Moreover, ongoing tests with new drugs affecting the KKS are on the way. The main goal is to develop more potent and tissue specific ligands, with increased disposability, central permeability, and reduced collateral effects. The field of study of these receptors is wide and promising.

References

Borkowski JA, Ransom RW, Seabrook GR, Trumbauer M, Chen H, Hill RG, Strader CD, Hess JF. Targeted disruption of a B_2 bradykinin receptor gene in mice eliminates bradykinin action in smooth muscle and neurons. J Biol Chem. 1995;270(23):13706–10.

Brechter AB, Persson E, Lundgren I, Lerner UH. Kinin B_1 and B_2 receptor expression in osteoblasts and fibroblasts is enhanced by interleukin-1 and tumour necrosis factor-alpha. Effects dependent on activation of NF-kappaB and MAP kinases. Bone. 2008;43(1):72–83.

Cayla C, Merino VF, Cabrini DA, Silva Jr JA, Pesquero JB, Bader M. Structure of the mammalian kinin receptor gene locus. Int Immunopharmacol. 2002;2(13–14):1721–7 (Review).

Cayla C, Todiras M, Iliescu R et al. Mice deficient for both kinin receptors are normotensive and protected from endotoxin-induced hypotension. FASEB J. 2007;21(8):1689–98.

Couture R, Harrisson M, Vianna RM, Cloutier F. Kinin receptors in pain and inflammation. Eur J Pharmacol. 2001;429(1–3):161–76 (Review).

Damas J, Garbacki N, Lefèbvre PJ. The kallikrein-kinin system, angiotensin converting enzyme inhibitors and insulin sensitivity. Diabetes Metab Res Rev. 2004;20(4):288–97 (Review).

Kakoki M, Kizer CM, Yi X, Takahashi N, Kim HS, Bagnell CR, Edgell CJ, Maeda N, Jennette JC, Smithies O. Senescence-associated phenotypes in Akita diabetic mice are enhanced by absence of bradykinin B_2 receptors. J Clin Invest. 2006;116(5):1302–9.

Kakoki M, Sullivan KA, Backus C, Hayes JM, Oh SS, Hua K, Gasim AM, Tomita H, Grant R, Nossov SB, Kim HS, Jennette JC, Feldman EL, Smithies O. Lack of both bradykinin B_1 and B_2 receptors enhances nephropathy, neuropathy, and bone mineral loss in Akita diabetic mice. Proc Natl Acad Sci USA. 2010;107(22):10190–5.

Klein J, Gonzalez J, Decramer S, Bandin F, Neau E, Salant DJ, Heeringa P, Pesquero JB, Schanstra JP, Bascands JL. Blockade of the kinin B_1 receptor ameloriates glomerulonephritis. J Am Soc Nephrol. 2010;21(7):1157–64.

Kouyoumdjian M, Nagaoka MR, Borges DR. Kallikrein-kinin system in hepatic experimental models. Peptides. 2005;26(8):1301–7 (Epub 26 Apr 2005. Review).

Leeb-Lundberg LM, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol Rev. 2005;57(1):27–77 (Review).

Lemos MT, Amaral FA, Dong KE, Bittencourt MF, Caetano AL, Pesquero JB, Viel TA, Buck HS. Role of kinin B_1 and B_2 receptors in memory consolidation during the aging process of mice. Neuropeptides. 2010;44(2):163–8.

Merino VF, Todiras M, Mori MA, Sales VM, Fonseca RG, Saul V, Tenner K, Bader M, Pesquero JB. Predisposition to atherosclerosis and aortic aneurysms in mice deficient in kinin B_1 receptor and apolipoprotein E. J Mol Med. 2009;87(10):953–63.

Mori MA, Araújo RC, Reis FC, Sgai DG, Fonseca RG, Barros CC, Merino VF, Passadore M, Barbosa AM, Ferrari B, Carayon P, Castro CH, Shimuta SI, Luz J, Bascands JL, Schanstra JP, Even PC, Oliveira SM, Bader M, Pesquero JB. Kinin B_1 receptor deficiency leads to leptin hypersensitivity and resistance to obesity. Diabetes. 2008;57(6):1491–500.

Pesquero JB, Araujo RC, Heppenstall PA, Stucky CL, Silva Jr JA, Walther T, Oliveira SM, Pesquero JL, Paiva AC, Calixto JB, Lewin GR, Bader M. Hypoalgesia and altered inflammatory responses in mice lacking kinin B_1 receptors. Proc Natl Acad Sci USA. 2000;97(14):8140–5.

Proud D. The kinin system in rhinitis and asthma. Clin Rev Allergy Immunol. 1998;16(4):351–64 (Review).

Sabatini RA, Guimarães PB, Fernandes L, Reis FC, Bersanetti PA, Mori MA, Navarro A, Hilzendeger AM, Santos EL, Andrade MC, Chagas JR, Pesquero JL, Casarini DE, Bader M, Carmona AK, Pesquero JB. ACE activity is modulated by kinin B_2 receptor. Hypertension. 2008;51(3):689–95.

Schulze-Topphoff U, Prat A, Bader M, Zipp F, Aktas O. Roles of the kallikrein/kinin system in the adaptive immune system. Int Immunopharmacol. 2008;8(2):155–60 (Epub 22 Aug 2007. Review).
Schulze-Topphoff U, Prat A, Prozorovski T, Siffrin V, Paterka M, Herz J, Bendix I, Ifergan I, Schadock I, Mori MA, Van Horssen J, Schröter F, Smorodchenko A, Han MH, Bader M, Steinman L, Aktas O, Zipp F. Activation of kinin receptor B_1 limits encephalitogenic T lymphocyte recruitment to the central nervous system. Nat Med. 2009;15(7):788–93.
Sharma JN. Does the kinin system mediate in cardiovascular abnormalities? Na overview. J Clin Pharmacol. 2003;43(11):1187–95 (Review).

Breast Cancer Antiestrogen Resistance Protein 1 (BCAR1)

▶ p130Cas

*Br*east Tumor *K*inase (Brk)

▶ Protein Tyrosine Kinase-6 (PTK6)

BRG1

▶ SWI/SNF Chromatin Remodeling Complex

BRL-Ras

▶ Rab7a in Endocytosis and Signaling

BRM

▶ SWI/SNF Chromatin Remodeling Complex

BRPK

▶ PTEN-Induced Kinase 1 (PINK1)

BTG/TOB

G. Sebastiaan Winkler and Rachel Doidge
School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK

Synonyms

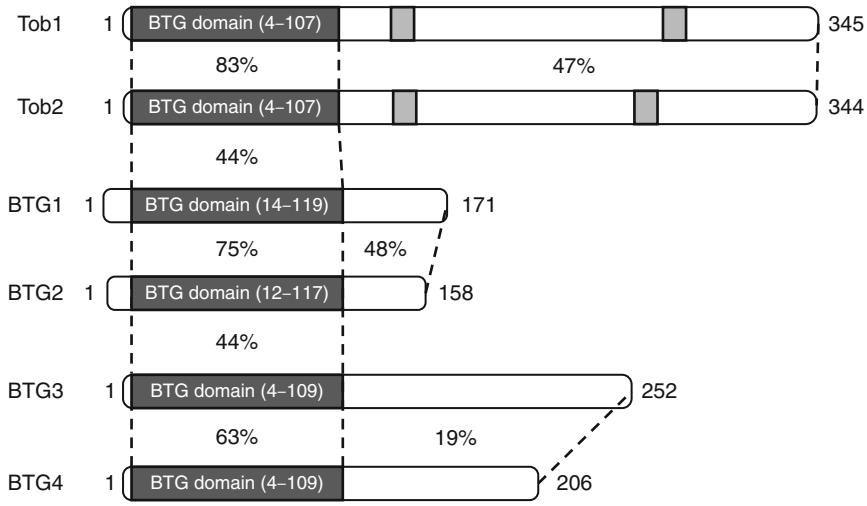
BTG1; BTG2 (TIS21, PC3); BTG3 (ANA, TOB5); BTG4 (PC3B); TOB1 (TOB, transducer of ERBB2); TOB2 (transducer of ERBB2 2)

Historical Background

The human BTG/TOB proteins form a small family of six proteins, which share a conserved N-terminal domain and anti-proliferative activity (Matsuda et al. 2001; Tirone 2001; Winkler 2010). BTG2 was discovered first by two laboratories: as the immediate/early response gene PC3 in rat PC12 cells stimulated with nerve growth factor and as TIS21 in mouse 3T3 fibroblasts in response to treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). The discovery of BTG1 (B-cell translocation gene 1), as a gene involved in a chromosomal translocation associated with chronic lymphocytic leukemia, suggested the presence of a new family of anti-proliferative genes. These findings were extended by the discovery of TOB1, which was found as an interacting protein of the ErbB2 tyrosine kinase receptor (HER2). The remaining three members, BTG3 (ANA), BTG4 (PC3B), and TOB2, were identified based on sequence homology of the conserved N-terminal domain. The preferred gene names by the Human Genome Nomenclature Committee are BTG1, BTG2, BTG3, BTG4, TOB1, and TOB2.

Regulation of Gene Expression: mRNA Deadenylation

The conserved N-terminus is known as the BTG domain (Pfam number PF07742) and comprises 104–106 amino acids. The C-terminal regions are less conserved and confer additional functions to the family members. Sequence analysis of both the BTG



BTG/TOB, Fig. 1 Schematic overview of the human BTG/TOB protein family. The approved gene names used by the human genome nomenclature committee are used. Indicated are the N-terminal BTG/TOB domains (*dark gray*) and PAM2 motifs (*gray*). The pair-wise percentage identities were determined using the Clustalw2 multiple sequence alignment program. The length of the proteins (amino acids) and the position of the BTG domain are also indicated

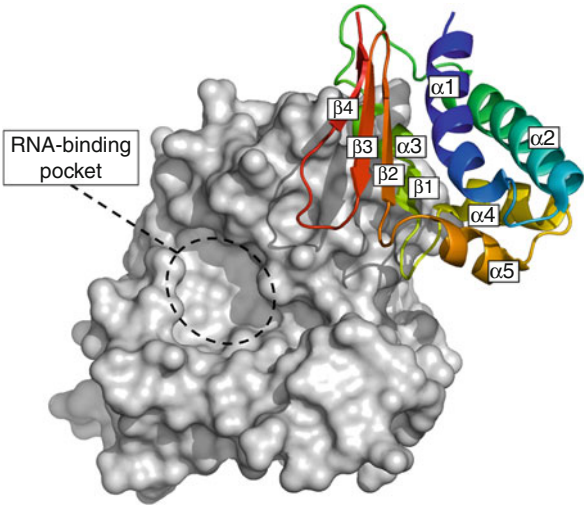
domain and the C-terminal regions suggests that TOB1 and TOB2, as well as BTG1 and BTG2, are highly similar, whereas BTG3 and BTG4 are more distantly related (Fig. 1). The BTG/TOB proteins are implicated in the regulation of gene expression by at least two distinct mechanisms.

The best characterized role of the BTG/TOB proteins in gene expression is mediated via the BTG homology domain, which interacts with the CNOT7 (Caf1a) and CNOT8 (Caf1b) subunits of the Ccr4-Not complex (Mauxion et al. 2009; Bartlam and Yamamoto 2010; Wiederhold and Passmore 2010; Winkler 2010). The highly similar CNOT7 and CNOT8 proteins are deadenylase enzymes, which shorten and remove the poly(A) tails of cytoplasmic mRNA resulting in translational repression and mRNA degradation (Bartlam and Yamamoto 2010; Wiederhold and Passmore 2010; Winkler 2010). The interaction of all BTG/TOB proteins (except BTG4) with either CNOT7 and/or CNOT8 is experimentally confirmed, and a specific role in the regulation of deadenylation and mRNA degradation was demonstrated for BTG2, TOB1, and TOB2. Both TOB1 and TOB2 contain PAM2 motifs in their C-terminal regions, which allow them to interact with poly(A)-binding protein 1 (PABPC1). During termination of translation, several proteins containing a PAM2 motif are consecutively recruited to the mRNA by PABPC1: following binding of the translation termination complex eRF1-eRF3 and the PAN2-PAN3 deadenylase, TOB1 recruits the Ccr4-Not deadenylase via interactions between the conserved BTG domain and the CNOT7 and CNOT8 deadenylase subunits. This sequence of events implicates TOB1 – as well as the related TOB2 protein – in mRNA deadenylation coupled to termination of translation. Alternatively, TOB1 can be recruited to specific mRNAs by sequence-specific RNA-binding proteins. For example, cytoplasmic polyadenylation element-binding protein 3 (CPEB3) binds TOB1 resulting in mRNA destabilization (Hosoda et al. 2011).

Several protein structures illuminate the molecular details of the interaction between BTG/TOB proteins and the CNOT7/CNOT8 deadenylase enzymes. The BTG domain is characterized by two long anti-parallel α-helices in the N-terminus of the domain that are part of a four-helix bundle and three β-sheets at the C-terminus of the domain (Fig. 2). Comparison of the structure of the free BTG domain of TOB1 with the domain in complex with the CNOT7 deadenylase indicates that the BTG domain does not undergo significant rearrangements upon binding. The RNA-binding catalytic site of the CNOT7 deadenylase appears to be separated from the residues important for binding to the BTG domain. In agreement with this, binding of the BTG domain of TOB1 does not influence the catalytic activity of the CNOT7 deadenylase.

Regulation of Gene Expression: Transcription

In addition to their role in mRNA deadenylation, BTG/TOB proteins can regulate gene expression at the level



BTG/TOB, Fig. 2 Structure of the BTG domain of TOB1 in complex with the CNOT7 (Caf1a) deadenylase enzyme. The representation was generated using structure 2d5r deposited in the Protein Data Bank (PDB) using Pymol (www.pymol.org). The BTG domain of TOB1 is represented by a multicolored cartoon. Indicated are the five α-helices and four β-sheets. The surface of the CNOT7 (Caf1a) deadenylase enzyme is represented in gray. Circled is a deep pocket that binds poly(A) RNA and corresponds to the catalytic center

of transcription (Matsuda et al. 2001; Tirone 2001; Winkler 2010). Several reports point to the ability of the BTG/TOB proteins to interact with DNA-binding transcription factors and modulate their ability to bind their cognate DNA sequence elements. Both BTG1 and BTG2 can interact with Hoxb9, a homeobox DNA-binding transcription factor, through their extreme N-terminus (residues 1–14). This interaction enhances the ability of Hoxb9 to bind to its consensus DNA sequence. Thus, this may increase the transcription rates of Hoxb9 target genes, which may contribute to the anti-proliferative function of BTG1 and BTG2. TOB1 and TOB2 have the most extensive C-terminal regions within the protein family. This region of TOB1 mediates interactions with a number of Smad transcription factors, altering their ability to bind to DNA. As a consequence, TOB1 regulates the expression of Smad target genes, such as the cytokine IL-2 promoter in quiescent T cells. BTG3 presents a third example of this mode of action: BTG3 can interact with E2F1, a transcription factor important for S-phase entry and cell-cycle progression. BTG3 binds E2F1 through its N-terminal region, which, in this case, inhibits DNA binding of the E2F1, thereby reducing the overall transcription rate of E2F1-responsive promoters and cell proliferation.

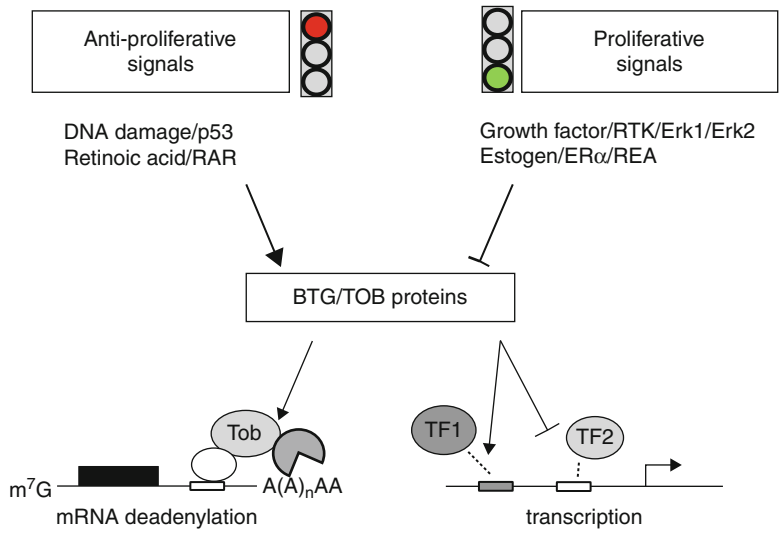
Finally, BTG1 and BTG2 can interact with protein arginine methyltransferase 1 (PRMT1) through a short β-sheet region (also known as Box C) just outside the BTG domain, which is not conserved in other BTG/TOB proteins. PRMT1 specifically methylates the arginine 3 residue of histone H4 in vitro and in vivo, which facilitates subsequent acetylation of histone H4 tails by p300 and gene activation. Thus, this raises the possibility that BTG1 and BTG2 could be involved in the regulation of chromatin modifications.

Effectors of Signaling Pathways

There are a variety of different signaling pathways that exploit the anti-proliferative properties of BTG/TOB proteins either positively or negatively by regulating the cellular levels of these proteins by transcriptional and post-translational mechanisms (Fig. 3). Both TOB1 and BTG2 are phosphorylated upon stimulation with growth factors by the Erk1/Erk2 kinases at serine residues in the C-terminus. This results in subsequent deactivation, which – in the case of TOB1 – leads to increased cyclin D1 expression and enhanced activation of CDK4, driving cell proliferation forward.

In MCF7 cells (an estrogen receptor–expressing breast cancer cell line), BTG2 mRNA can be regulated both positively and negatively by signaling through nuclear receptor transcription factors. BTG2 expression is activated when MCF7 cells are treated with retinoic acid through direct binding of the ▶ retinoic acid receptor (RAR)/RXR heterodimers to three retinoic acid response elements (RARE) in the BTG2 promoter region. Conversely, BTG2 expression is reduced when MCF7 cells are treated with estrogen through ▶ estrogen receptor ERα and its co-repressor REA.

BTG2 and BTG3 are both downstream targets of the ▶ p53 signaling pathway. Both proteins are direct transcriptional targets for p53 and play a role in the p53-mediated response to DNA damage (Rouault et al. 1996; Ou et al. 2007). In embryonic mouse fibroblasts, BTG2 plays critical role in suppressing transformation through oncogenic Ras by acting as a downstream effector of p53 (Boiko et al. 2006). BTG2 expression downregulates cyclin D1, cyclin E1, and the phosphorylation of retinoblastoma (Rb), slowing the cell cycle rate and preventing cellular transformation.



BTG/TOB, Fig. 3 Signaling pathways impinge on BTG/TOB proteins. Both antiproliferative and proliferative signals impinge on BTG/TOB family members by upregulation/activation or inhibition, respectively. In turn, BTG/TOB proteins can participate in the regulation of gene expression by deadenylation (left) or transcriptional mechanisms (right). See text for further details

Finally, TOB1 and BTG2 are implicated in signaling of TGF family members through Smad transcription factors. This was demonstrated in both quiescent T cells activated by CD28, which impinges on TGF-β signaling, and in bone-forming osteoblast cells upon stimulation by bone morphogenic protein (BMP) 2, a TGF family member.

Bone Formation: TOB1 and TOB2

The generation of mice containing null alleles of TOB1, TOB2, BTG2, and BTG3 uncovered a role for these proteins in bone formation and resorption (Yoshida et al. 2003; Park et al. 2004; Ajima et al. 2008; Miyai et al. 2009). The contrasting phenotypes observed in TOB1 and TOB2 knockout mice are of particular interest. Mice lacking TOB1 are apparently normal, but display increased bone volume and bone density. Interestingly, in a mouse model for estrogen-deficiency-induced osteoporosis, the increased bone mineralization in TOB1 null mice can compensate for bone loss associated with induced osteoporosis since ovariectomized TOB1 knockout mice have a bone mineral density and volume comparable to (sham operated) control mice (Usui et al. 2004). The increased bone density in TOB1$^{-/-}$ mice is due to enhanced bone formation, and osteoclast parameters are unchanged as compared to control mice. A similar increase in bone density is observed in mice lacking the CNOT7 deadenylase (Washio-Oikawa et al. 2007). As observed in TOB1 null mice, CNOT7 knockout mice do not display altered osteoclast parameters, suggesting that the role of TOB1 in bone formation is mediated via its interactions with the CNOT7 deadenylase subunits of the Ccr4-Not complex.

By contrast, mice lacking TOB2 display decreased bone mass due to an increased number of differentiated osteoclast cells. TOB2 interacts with the ▶ vitamin D receptor and reduces expression of RANKL, a vitamin D–induced gene. In agreement with this notion and the observation that osteoclast parameters are unaltered in CNOT7 knockout mice, TOB2 is a repressor of vitamin D–induced osteoclast formation (Ajima et al. 2008).

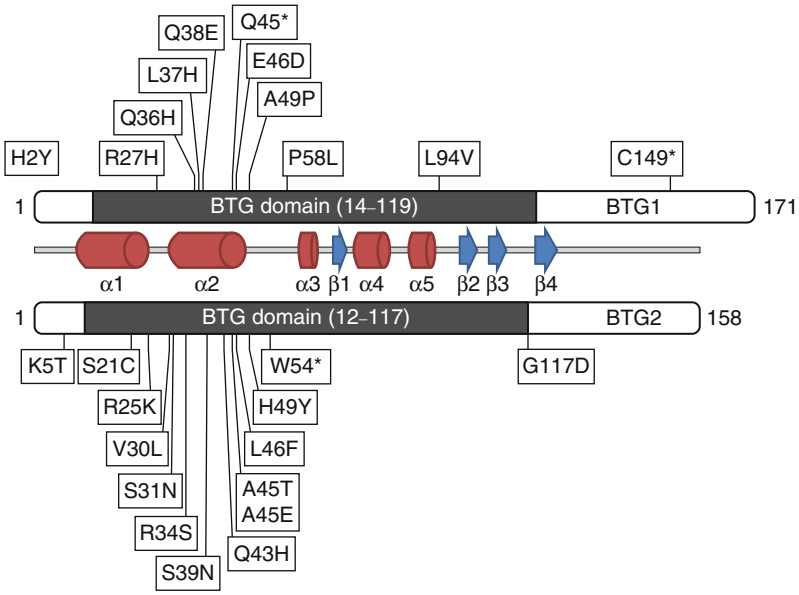
Cancer and Tumorigenesis

The discovery of BTG2 as an effector of the tumor suppressor function of p53, as well as the critical role of TOB1 in Ras-mediated transformation, strongly implicates these BTG/TOB proteins as important cellular components that contribute to the prevention of tumorigenesis (Rouault et al. 1996; Suzuki et al. 2002; Boiko et al. 2006). In agreement with this notion, expression of BTG/TOB genes is reduced or undetectable in a variety of clinical cancer samples (Table 1). In particular, the presence of increased levels of phosphorylated, inactive TOB1, and the absence of TOB1 protein levels correlate with tumor

BTG/TOB, Table 1 Relationship of BTG/TOB expression and cancer. To date, no changes in TOB2 expression are reported in human cancer samples

Gene	Cancer		References
TOB1	Lung	Spontaneous tumor formation[a]	Yoshida et al. (2003)
	Lung	Decreased expression/increased phosphorylation[b]	Iwanaga et al. (2003)
	Lymph node	Spontaneous tumor formation[a]	Yoshida et al. (2003)
	Liver	Spontaneous tumor formation[a]	Yoshida et al. (2003)
	Breast	Reduced expression in human breast cancer cell lines	Helms et al. (2009)
	Thyroid	Decreased mRNA expression[b]	Ito et al. (2005)
	Pancreatic	Induced expression inhibits tumorigenesis in nude mice	Yanagie et al. (2009)
BTG1	Leukemia	Reduced/undetectable expression[b]	Cho et al. (2004)
	Lymphoma	Somatic mutations[b]	Morin et al. (2011)
BTG2	Breast	Reduced expression and re-localization (nuclear to cytoplasm)[b]	Kawakubo et al. (2006)
	Renal	Reduced mRNA levels[b]	Stuckman et al. (2004)
	Prostate	Low/undetectable mRNA levels[b]	Ficazzola et al. (2001)
	Brain	Induced expression inhibits medulloblastomas (transgenic mice)	Farioli-Vecchiolo et al. (2007)
	Lymphoma	Somatic mutations[b]	Morin et al. (2011)
BTG3	Lung	Increased lung tumor formation[a]	Yoneda et al. (2009)
	Lung	Reduced expression in adenocarcinoma samples[b]	Yoneda et al. (2009)
	Renal	Reduced mRNA expression[b]	Majid et al. (2009)
BTG4	Colon	Reduced mRNA expression[b]	Toyota et al. (2008)

[a]Observations made using mouse knock-out models
[b]Observation made using human clinical cancer samples and biopsies



BTG/TOB, Fig. 4 Mutations identified in BTG1 and BTG2 in non-Hodgkin lymphoma. Mutations in BTG1 and BTG2 identified by RNA-sequencing data from over 100 non-Hodgkin lymphomas (Morin et al. 2011. Nature; doi: 10.1038/nature10351). In some cases, both alleles contained mutations. Indicated are schematic representations of BTG1 and BTG2, the location of the BTG domain, and the presence of secondary structure elements based on the crystal structure of BTG2 (PDB structures 3dju, 3djn and 3e9v)

grade in a panel of lung cancer samples. Similarly, expression of BTG3 is reduced in the majority of lung cancer cell lines and clinical samples derived from lung cancer patients. Furthermore, BTG1 and BTG2 are frequently found to be mutated in non-Hodgkin lymphomas (Fig. 4). Such mutations are seemingly present in a mutually exclusive manner as compared to p53 mutations, suggesting a causative role as a component of the p53 pathway in this type of cancer. It is yet unknown how the identified mutations in BTG1 and BTG2 interfere with the function of the encoded gene products.

An important role for BTG/TOB proteins in the suppression of tumorigenesis is further evident from mouse knockout models. Disruption of TOB1 in mice results in susceptibility to a variety of cancers, including lung tumors, which is also observed in mice lacking BTG3 (Table 1). Thus, a direct role of several BTG/TOB proteins in the suppression of tumorigenesis and cancer development is now clearly established.

Summary

The understanding of the function and mechanisms through which the BTG/TOB proteins act have rapidly advanced in the past few years. The best characterized role of the BTG/TOB proteins is mediated by the interaction of the BTG homology domain with the CNOT7 and CNOT8 deadenylase subunits of the Ccr4-Not complex, which impacts on mRNA deadenylation. In addition, BTG/TOB proteins are also involved in the regulation of transcription and, possibly, the establishment of histone H4 modifications through the interactions of BTG1 and BTG2 with the PRMT1 methyltransferase. Mouse models have uncovered the importance of these proteins in the biology of bone and cancer. Reduced expression of BTG/TOB proteins is observed in a variety of clinical samples, and mutations in BTG1 and BTG2 are found in non-Hodgkin lymphoma. It remains to be determined whether BTG/TOB proteins regulate cell proliferation through mRNA degradation or transcriptional mechanisms, or both. Furthermore, there are still many questions with respect to unique and/or redundant roles of the individual BTG/TOB proteins.

References

Ajima R, Akiyama T, Usui M, Yoneda M, Yoshida Y, Nakamura T, Minowa O, Noda M, Tanaka S, Noda T, Yamamoto T. Osteoporotic bone formation in mice lacking tob2; involvement of Tob2 in RANK ligand expression and osteoclasts differentiation. FEBS Lett. 2008;582:1313–8.

Bartlam M, Yamamoto T. The structural basis for deadenylation by the CCR4-NOT complex. Protein Cell. 2010;1:443–52.

Boiko AD, Porteous S, Razorenova OV, Krivokrysenko VI, Williams BR, Gudkov AV. A systematic search for downstream mediators of tumor suppressor function of p53 reveals a major role of BTG2 in suppression of Ras-induced transformation. Genes Dev. 2006;20:236–52.

Cho JW, et al. Identification of B-cell translocation gene 1 as a biomaker for monitoring the remission of acute myeloid leukemia. Proteomics. 2004;4:3456–63.

Farioli-Vecchiolo S, et al. Inhibition of medulloblastoma tumorigenesis by the antiproliferative and pro-differentiative gene PC3. FASEB J. 2007;21:2215–25.

Ficazzola MA, et al. Antiproliferative B cell translocation gene 2 protein is downregulated post-transcriptionally as an early event in prostate carcinogenesis. Carcinogenesis. 2001;22:1271–9.

Helms MW, et al. TOB1 Is Regulated by EGF-Dependent HER2 and EGFR Signaling, Is Highly Phosphorylated, and Indicates Poor Prognosis in Node-Negative Breast Cancer. Cancer Res. 2009;69:5049–56.

Hosoda N, Funakoshi Y, Hirasawa M, Yamagishi R, Asano Y, Miyagawa R, Ogami K, Tsujimoto M, Hoshino S. Antiproliferative protein Tob negatively regulates CPEB3 target by recruiting Caf1 deadenylase. EMBO J. 2011;30:1311–23.

Ito Y, et al. Phosphorylation and inactivation of Tob contributes to the progression of papillary carcinoma of the thyroid. Cancer Lett. 2005;220:237–42.

Iwanaga K, et al. Alteration of expression or phosphorylation status of tob, a novel tumor suppressor gene product, is an early event in lung cancer. Cancer Lett. 2003;202:71–9.

Kawakubo H, et al. Loss of B-cell translocation gene-2 in estrogen receptor-positive breast carcinoma is associated with tumor grade and overexpression of cyclin D1 protein. Cancer Res. 2006;66:7075–82.

Majid S, et al. BTG3 tumor suppressor gene promoter demethylation, histone modification and cell cycle arrest by genistein in renal cancer. Carcinogenesis. 2009;30:662–70.

Matsuda S, Rouault J, Magaud J, Berthet C. In search of a function for the TIS21/PC3/BTG1/TOB family. FEBS Lett. 2001;497:67–72.

Mauxion F, Chen CY, Seraphin B, Shyu AB. BTG/TOB factors impact deadenylases. Trends Biochem Sci. 2009;34:640–7.

Miyai K, Yoneda M, Hasegawa U, Toita S, Izu Y, Hemmi H, Hayata T, Ezura Y, Mizutani S, Miyazono K, Akiyoshi K, Yamamoto T, Noda M. ANA deficiency enhances bone morphogenetic protein-induced ectopic bone formation via transcriptional events. J Biol Chem. 2009;284:10593–600.

Morin et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature. 2011;476:298–303.

Ou YH, Chung PH, Hsu FF, Sun TP, Chang WY, Shieh SY. The candidate tumor suppressor BTG3 is a transcriptional target of p53 that inhibits E2F1. EMBO J. 2007;26:3968–80.

Park S, Lee YJ, Lee HJ, Seki T, Hong KH, Park J, Beppu H, Lim IK, Yoon JW, Li E, Kim SJ, Oh SP. B-cell translocation gene 2 (Btg2) regulates vertebral patterning by modulating bone morphogenetic protein/smad signaling. Mol Cell Biol. 2004;24:10256–62.

Rouault JP, Falette N, Guehenneux F, Guillot C, Rimokh R, Wang Q, Berthet C, Moyret-Lalle C, Savatier P, Pain B, Shaw P, Berger R, Samarut J, Magaud JP, Ozturk M, Samarut C, Puisieux A. Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nat Genet. 1996;14:482–6.

Stuckman, et al. Impaired expression of the cell cycle regulator BTG2 is common in clear cell renal cell carcinoma. Cancer Res. 2004;64:1632–8.

Suzuki T, Junko KT, Ajima R, Nakamura T, Yoshida Y, Yamamoto T. Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. Genes Dev. 2002; 16:1356–70.

Tirone F. The gene PC3(TIS21/BTG2), prototype member of the PC3/BTG/TOB family: regulator in control of cell growth, differentiation, and DNA repair? J Cell Physiol. 2001;187:155–65.

Toyota M, et al. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res. 2008;68:4123–32.

Usui M, Yoshida Y, Tsuji K, Oikawa K, Miyazono K, Ishikawa I, Yamamoto T, Nifuji A, Noda M. Tob deficiency superenhances osteoblastic activity after ovariectomy to block estrogen deficiency-induced osteoporosis. Proc Natl Acad Sci USA. 2004;101:6653–8.

Washio-Oikawa K, Nakamura T, Usui M, Yoneda M, Ezura Y, Ishikawa I, Nakashima K, Noda T, Yamamoto T, Noda M. Cnot7-null mice exhibit high bone mass phenotype and modulation of BMP actions. J Bone Miner Res. 2007;22:1217–23.

Wiederhold K, Passmore LA. Cytoplasmic deadenylation: regulation of mRNA fate. Biochem Soc Trans. 2010;38:1531–6.

Winkler GS. The mammalian anti-proliferative BTG/Tob protein family. J Cell Physiol. 2010;222:66–72.

Yanagie H, et al. Tumor growth suppression by adenovirus-mediated introduction of a cell-growth-suppressing gene tob in a pancreatic cancer model. Biomed Pharmacother. 2009;3:275–86.

Yoneda M, et al. Deficiency of antiproliferative family protein Ana correlates with development of lung adenocarcinoma. Cancer Sci. 2009;100:225–32.

Yoshida Y, Nakamura T, Komoda M, Satoh H, Suzuki T, Tsuzuku JK, Miyasaka T, Yoshida EH, Umemori H, Kunisaki RK, Tani K, Ishii S, Mori S, Suganuma M, Noda T, Yamamoto T. Mice lacking a transcriptional corepressor Tob are predisposed to cancer. Genes Dev. 2003;17:1201–6.

BTG1

► BTG/TOB

BTG2 (TIS21, PC3)

► BTG/TOB

BTG3 (ANA, TOB5)

► BTG/TOB

BTG4 (PC3B)

► BTG/TOB

BY55

► CD160

C

C7

► CXCL10

CAAX Farnesyltransferase

► Protein Farnesyltransferase

Cadherins

Frans van Roy
Molecular Cell Biology Unit, Department for Molecular Biomedical Research, VIB and Ghent University, Ghent, Belgium

Synonyms

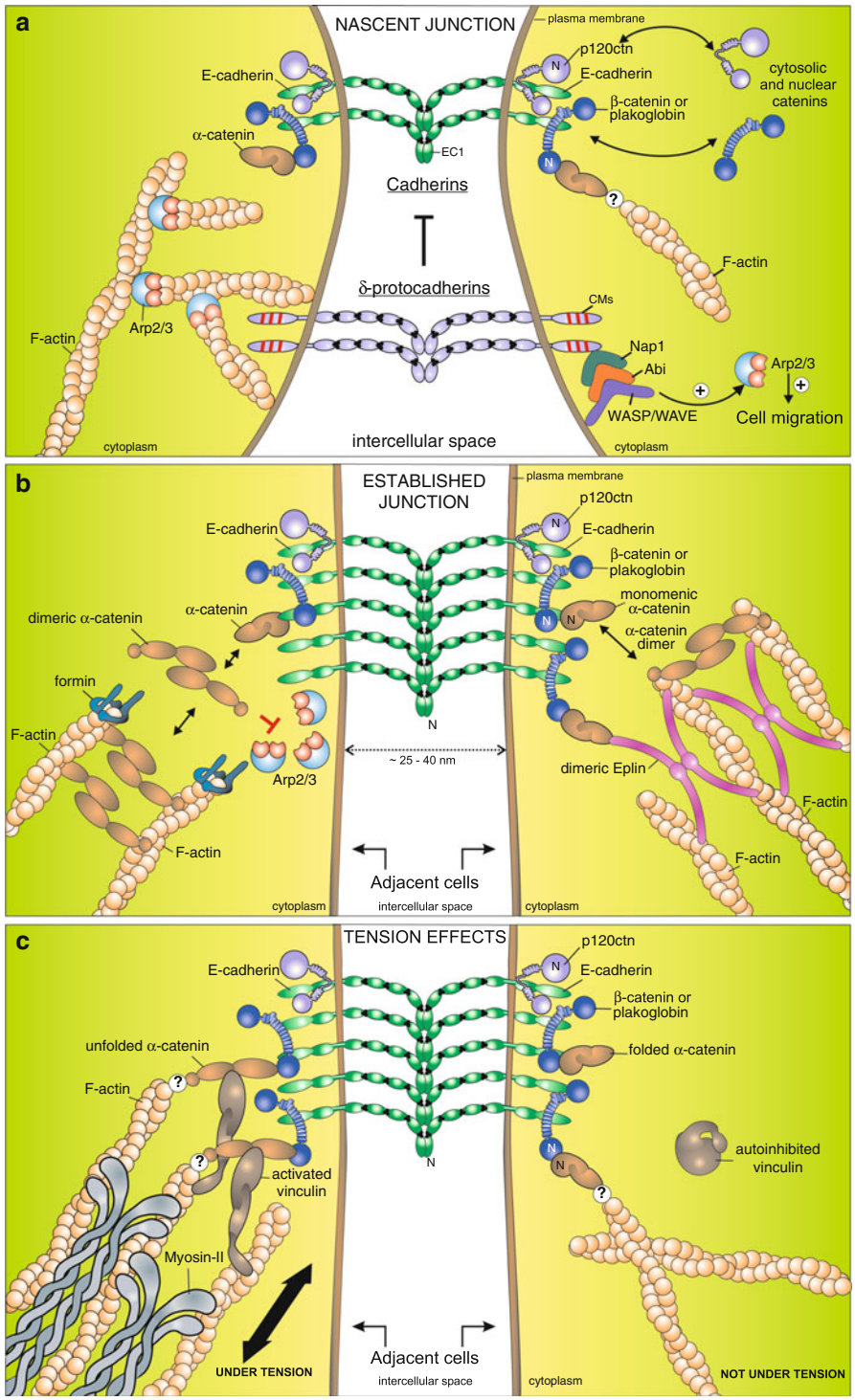
CELSR; Classic cadherins; Dachsous; Fat; Flamingo cadherins; Protocadherins; Type-I cadherins

Historical Background: The Larger Cadherin Superfamily

Cadherins comprise a large protein superfamily of calcium-dependent proteins associated with the plasma membrane. They are hallmarked by at least two consecutive extracellular cadherin repeats (ECs) in their extracellular domain. Epithelial cadherin (E-cadherin or cadherin-1) is the founding family member and represents the classic or type-I cadherins. These cadherins mediate specific and strong cell-cell adhesion and play key roles in local organization of cytoskeletal structures and in signaling cascades, as documented below. The mature E-cadherin contains an ectodomain of about 550 amino acids (AA) comprising five ECs, a single transmembrane (TM) domain, and a cytoplasmic domain (CD) of about 150 AA. The CD has two highly conserved domains for binding p120ctn and related protein family members, as well as β-catenin and the homologous plakoglobin (γ-catenin), all of which belong to the armadillo protein family (reviewed by van Roy and Berx 2008) (Fig. 1). Association of p120ctn with the juxtamembrane domain (JMD) of cadherins protects them from premature endocytosis and hence stabilizes the junctions. On the other hand, in a classical cadherin-catenin complex (CCC), β-catenin forms a molecular bridge between the cadherins and α-catenins, the latter of which are vinculin-related proteins that bind F-actin. However, recent data reported by the groups of Nelson and Weis have cast doubt on this model (reviewed in Nelson and Fuchs 2010). These authors showed that monomeric α-catenin can integrate into the CCC but does not associate with F-actin, whereas homodimeric α-catenin dissociates from the CCC but binds F-actin. In contrast, other actin-binding linker molecules, such as α-actinin and Eplin, might be involved in anchoring cadherin-dependent junctions to the cytoskeleton (Fig. 1).

Cadherins were originally discovered as mediators of specific cell-cell adhesive structures, often called adherens junctions (AJ) (Gumbiner 2005; Nelson and Fuchs 2010). The basic and well-documented model for this function involves two modes of interaction

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



Cadherins, Fig. 1 Alternative states of the classic cadherin-catenin complex (CCC) at the junction between two neighboring epithelial cells (see text for details and references). (**a**) Nascent junction: Transmembrane classic cadherins form zipper-like intercellular junctions based on homophilic molecular interactions between their EC1 and EC2 domains. Their cytoplasmic domains contain conserved binding sites for the armadillo proteins p120ctn and β-catenin (although not drawn that way, p120ctn and β-catenin can interact simultaneously with a single cadherin molecule). p120ctn and β-catenin also have

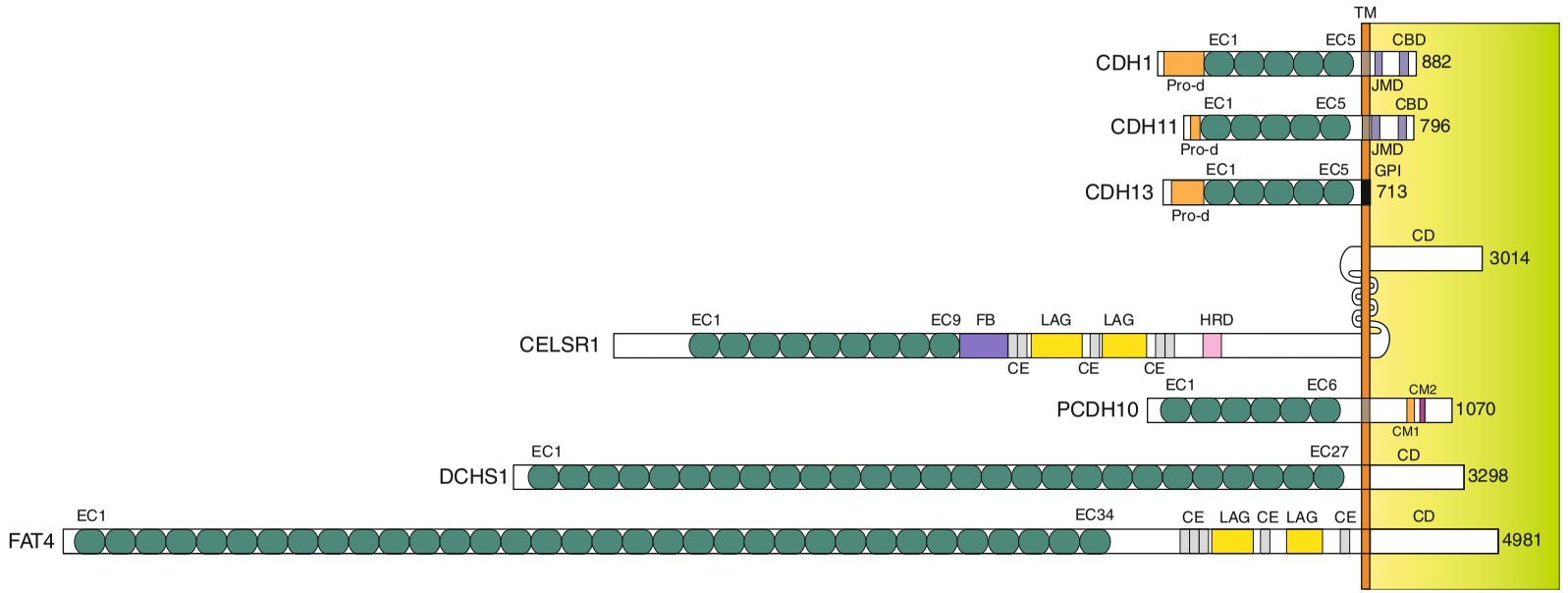
between the most amino-terminal ECs (called EC1): homophilic (between identical cadherin species) and homotypic (between identical cell types). Both *cis* (between neigboring cadherin molecules in the same plasma membrane) and *trans* interactions (between apposing cadherin molecules) have been described in considerable detail (van Roy and Berx 2008). Cell-cell adhesion mediated by classic cadherins is considered the basis of cell sorting out during both embryonic development and the morphogenesis of most organs (Gumbiner 2005; Halbleib and Nelson 2006; Nelson and Fuchs 2010). Although the cadherin activity during these important processes might be explained by cadherins serving as cell-type specific molecular glues, it is increasingly clear that signaling by and to cadherins plays major roles. Indeed, cadherins are required not only for rigid, static cell-cell contacts, but also for regulation of dynamic morphogenetic cell movements by active remodeling of intercellular junctions and the associated cytoskeletal structures (Nishimura and Takeichi 2009; Harris and Tepass 2010).

Presently, mammalian genomes are known to contain over 100 genes encoding cadherins or cadherin-related proteins (Hulpiau and van Roy 2011). Some of them are more recent evolvements, such as desmogleins and desmocollins, which form the backbone of desmosomes (strong cell junctions in tissues under high physical stress, like epidermis and heart). The so-called clustered protocadherins form another recently evolved cadherin subtype. Protocadherins seem to be less involved in tight cell-cell adhesion and may play greater roles in cell recognition and signaling. On the other hand, recent phylogenetic analyses have shown the existence of six ancient types of cadherins and cadherin-related proteins (Hulpiau and van Roy 2011) (Fig. 2). These are the classical cadherins (CDH), the FAT cadherins, the FAT-like cadherins, the Dachsous cadherins (DCHS), the flamingo (Fmi) or CELSR cadherins, and several cadherin-related proteins, such as cadherin-related 23 (CDHR23). Several of these cadherin family members were originally discovered in the fruit fly, and their genetic analyses have revealed important functions in signaling. The protocadherins emerged slightly later in evolution. They were originally thought to be specific for deuterostomes, but recent studies revealed their presence in cnidarians but not in the fruit fly or the nematode, which probably lost them during evolution.

From these studies, several hallmarks became clear: ancient family members always have long ectodomains with numerous ECs, which during evolution became progressively shorter (Hulpiau and van Roy 2011). Current classic and desmosomal cadherins have five ECs whereas protocadherins have six to seven ECs (Fig. 2). This, together with the finding of many long cadherin-like proteins in unicellular choanoflagellates, indicates that the cadherin family members originally served in extracellular sensing and cell-cell recognition rather than in specific cell-cell adhesion.

Cadherins characteristically have an ectodomain that is largely or exclusively composed of ECs, but their TM domains and in particular their CDs are much more diverse (Fig. 2). Quite often, cadherins have their own subfamily-specific CD, often with one to several motifs. These CDs are conserved within the same subfamily, but apparently they are not related to each

Cadherins, Fig. 1 (continued) roles in the cytoplasm and nucleus (not detailed here). Further, the CCC contains monomeric α-catenin, which via partly unknown intermediate proteins (?) links the CCC to the actin cytoskeleton. In a nascent junction, F-actin grows and branches by the action of the Arp2/3 protein complex. Certain δ-protocadherins (including protocadherin-10/OL-protocadherin) interfere with formation of tight CCC-dependent junctions and induce cell migration by (strong?) activation of Arp2/3, leading to formation of lamellipodia. This is because the cytoplasmic domain of δ-protocadherins, which has conserved motifs (CMs), recruits a Nap1-Abi-WASP/WAVE complex. (**b**) Molecular interactions in an established CCC-dependent junction. Monomeric α-catenin in the CCC can associate with the actin-bundling protein Eplin. On the other hand, it can dissociate from the CCC, dimerize, and locally bundle F-actin. Dimeric α-catenin is thought to inhibit the Arp2/3 complex, so that F-actin grows merely linearly by the action of formins. (**c**) Recently, evidence was obtained for tighter cadherin-dependent junctions in cells under mechanical tension, for example, during morphogenetic movements and wound repair. While in cells not under tension (depicted on the *right*) both vinculin and α-catenin are in a less active, folded configuration, they become unfolded when tension is applied. Local tension is generated by the action of myosin-II on F-actin (depicted on the *left*). This stretches α-catenin to a configuration that recruits and activates vinculin. Vinculin in turn binds the actomyosin complex. In this way, the reinforced mechanical coupling between the CCC and the cytoskeleton protects the junction against the tension while transmitting the force over the tissue. Modified after (van Roy and Berx 2008), and after (Yonemura et al. 2010)



Cadherins, Fig. 2 Domain structure of representative members of the cadherin superfamily in man (*Homo sapiens*). All proteins are drawn to scale and aligned at their transmembrane domains (TM). They are depicted as precursor proteins with their extracellular N-terminal ends on the left. Sizes (number of AA) are indicated on the right and range between about 800 and 5,000 AA. The cadherin families are exemplified as follows (from top to bottom). The CDH family is represented by E-cadherin (CDH1), cadherin-11, and cadherin-13. The latter has an atypical glycosyl phosphatidyl inositol anchor (GPI). The CELSR/Flamingo family is represented by CELSR1. These proteins have a seven-TM domain. The protocadherin (PCDH) family is represented by protocadherin-10. The Dachsous family is represented by DCHS1. Finally, the FAT family is represented by FAT4. The following protein domains are annotated: *CBD* (conserved cadherin-specific) β-catenin-binding domain; *CD* unique cytoplasmic domain; *CE* Cysteine-rich EGF repeat-like domain; *CM1* and *CM2* conserved motifs in the CDs of particular protocadherins; *EC* extracellular cadherin repeat; *FB* Flamingo box; *HRD* Hormone receptor domain (in CELSR/Flamingo proteins); *JMD* (conserved cadherin-specific) juxtamembrane domain, binding p120ctn; *LAG* laminin A globular domain; *Pro-d* prodomain. Modified after Berx and van Roy (in Nelson and Fuchs 2010)

other and often not at all to the "standard" CD of classic cadherins. Cadherin-13, also called T-cadherin (truncated), stands out because it is the only known cadherin lacking a TM domain. Instead, it is linked to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (reviewed by Berx and van Roy in Nelson and Fuchs 2010). Moreover, the recently resolved structure of its EC1 domain is unusual. The flamingo/CELSR cadherins have, besides an ectodomain of nine ECs, a 7-TM domain, which is exceptional for cadherins.

Signaling by Classic Cadherin-Catenin Complexes

Many major mechanisms are known to influence the roles of classic cadherins in cell structure and function. (1) Specific interactions between cells are influenced by the generation and stabilization versus destabilization and internalization of junctions composed of cadherins. This also has consequences for other types of junctions, such as tight junctions and gap junctions, which are not composed of cadherins. (2) By forming either static or dynamic cell-cell junctions, cadherins have a flexible influence on cytoskeletal structures, such as cortical F-actin/myosin-II (actomyosin) networks as well as microtubules, which in turn influence the form, polarity, and behavior of cells. (3) Either by physically sequestering β-catenin and p120ctn in stable junctions at the cell surface or by functional control of these armadillo proteins, cadherins can modulate the signaling roles of these key proteins in the cytoplasm and/or nucleus. Reciprocally, stable exposition of classic cadherins at the cell surface is due to p120-mediated inhibition of endocytosis, and stable association of β-catenin is needed for a functional CCC. (4) Cadherin ligation by itself is an excellent inhibitor of cell proliferation and a stimulator of cell polarity and differentiation, because cadherins engaged in adhesion serve as a hub in various signaling pathways, particularly in those involving small GTPases. A selection of published studies illustrates and consolidates these various signaling possibilities.

1. During both normal embryogenesis and pathological processes, such as invasive cancer, cadherin-type switching is a frequent occurence that forms the basis for the migration of particular cells and for generation of new structures with new functions (reviewed by Berx and van Roy in Nelson and Fuchs 2010). A notable example is the dynamic but highly controlled behavioral change of cells derived from the neural crest.
2. On the one hand, junctional p120ctn stabilizes the cadherins, but on the other hand, cadherins counteract cytoplasmic p120ctn, which can modulate members of the Rho GTPase family (reviewed by Pieters et al. 2012). Also, it has recently become clear that junctional αE-catenin can serve as a tension transducer for the CCC (Yonemura et al. 2010) (Fig. 1c). When an external force is applied to epithelial cells, for instance during wound healing, actomyosin-driven tension induces an unfolded conformation in αE-catenin so that it can recruit vinculin to the CCC. This serves as a positive feedback because it reinforces mechanical coupling between actomyosin networks and the CCC. At the same time, the force-dependent αE-catenin/vinculin interaction strengthens the intercellular junctions and contributes to tissue integrity despite the external force.
   On the other hand, αE-catenin dimers were shown to preferentially dissociate from the CCC and to locally block the binding of the actin nucleating and branching factor ARP2/3 complex (reviewed in Nelson and Fuchs 2010) (Fig. 1b). At the same time, binding of αE-catenin to formin is promoted in a way that facilitates the formation of elongated F-actin fibers that can associate effectively with myosin-II (Fig. 1b and c). More recently, it was demonstrated that AJs interact also with microtubules, either the plus ends of microtubules via β-catenin or the minus ends via p120ctn (reviewed by Harris and Tepass 2010). Association of the AJ with microtubules facilitates junctional assembly and also allows the AJ to influence intracellular structuring. For instance, the orientation of both symmetric and asymmetric cell divisions are influenced by AJs (reviewed by Harris and Tepass 2010).
3. By sequestering β-catenin in the junctions, this important signaling molecule is prevented from entering the nucleus and activating LEF/TCF-dependent gene activation (▶ Beta-Catenin). Similarly, p120ctn in complex with classic cadherins cannot shuttle into the nucleus, where it would

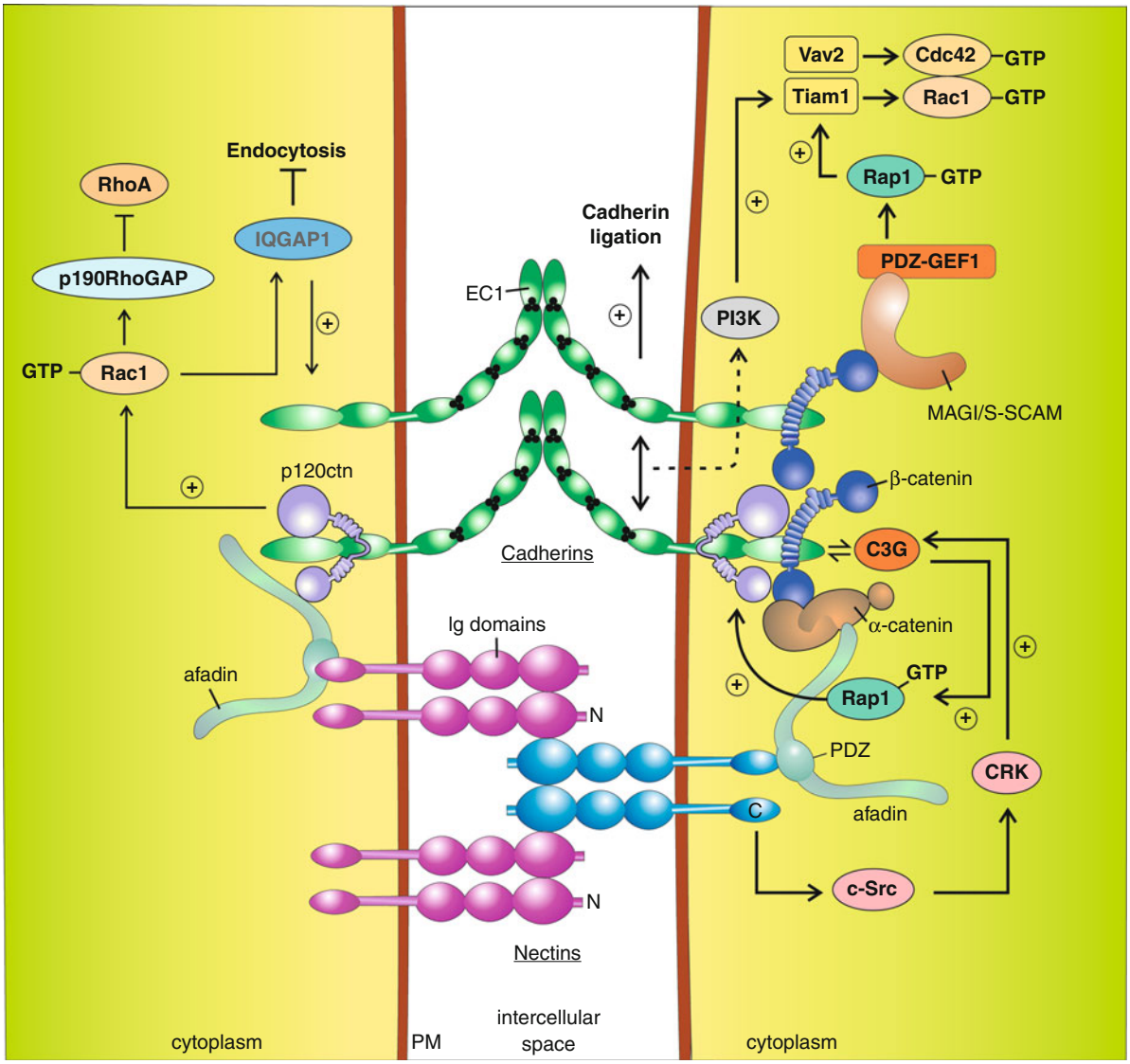
contribute to Wnt signaling by retrieving the transcriptional inhibitor Kaiso to the cytoplasm. In the case of E-cadherin, p120ctn binding to the JMD of classic cadherins shields both a di-leucine motif required in mammalian cells for clathrin-mediated endocytosis and a Tyr residue that, when phosphorylated, is recognized by Hakai, an E3 ubiquitin ligase (reviewed by Niessen et al. 2011) (see next section).

4. Native cadherin ligation, that is, the interaction in *trans* between cadherins across cell-cell boundaries, can result in two types of signaling: increased juxtacrine signaling by neighboring non-cadherin molecules, and a direct effect on cadherin-mediated signaling. To dissociate the first type of adhesion-dependent processes from the second type, researchers trigger cell-associated cadherins with, for instance, beads coated with recombinant cadherin ectodomains. Studies of this type have revealed a major role for small GTPases of the Rho family (Kooistra et al. 2007; Watanabe et al. in Nelson and Fuchs 2010; Niessen et al. 2011; Pieters et al. 2012). Although the effects seen are complex and much dependent on the cadherin type involved, as well as on cell type and cell activation status, several useful models have emerged. For instance, E-cadherin ligation induces local and transient activation of Rac1 and Cdc42 GTPases (Fig. 3). This might occur by activation of appropriate GEFs (guanine nucleotide exchange factors) such as Tiam1 and Vav2, and it appears to be dependent on association of p120ctn with cadherin. Two potential mediators of cadherin-induced GTPase activation are PI3K, which is stimulated upon E-cadherin ligation and leads to Tiam1 activation, and Rap1, a Ras-like small GTPase that has numerous effectors, including Vav2 and Tiam1 (Kooistra et al. 2007).

   Rap1 by itself can be activated upon cadherin ligation in at least four possible ways (Fig. 3). (a) The E-cadherin cytoplasmic domain transiently recruits the Rap-GEF C3G in nascent junctions. (b) Junctional β-catenin recruits MAGI scaffold proteins and PDZ-GEF1, and this macromolecular complex activates Rap1. (c) Nectins, which are immunoglobulin-like adhesion molecules, promote cadherin-dependent junction formation (reviewed by van Roy and Berx 2008) and also induce Rap1 activation by a process involving c-Src, Crk, and C3G (Hoshino et al. 2005). (d) Various RTKs can associate with classic cadherins (see below) and trigger signaling pathways leading to Rap1 activation. Interestingly, afadin (AF6), an actin-binding adaptor protein, couples the cytoplasmic domains of nectins to junctional p120ctn and α-catenin (van Roy and Berx 2008) (Fig. 3). Afadin has a Rap1-binding domain and is indeed recruited to nascent junctions through the action of Rap1. There, it strengthens the binding of p120ctn to E-cadherin, what counteracts the endocytosis of unligated E-cadherin and hence contributes to maturation of cadherin-based and nectin-based junctions (Hoshino et al. 2005).

   When and where are such cadherin-modulated signals important? Nascent epithelial junctions show a restricted membrane zone with active GTP-Rac1, recruitment of Arp2/3, and increased lamellipodial activity at the site of the expanding intercellular contacts (reviewed by Nelson and Fuchs 2010; Niessen et al. 2011). Subsequent expansion and completion of cell-cell adhesion is dependent on RhoA activation at the distal edges of the intercellular contacts, which locally induces actomyosin contractility. The cadherin-induced activation of Rac1 (and also Cdc42) can have different consequences, including actin nucleation through activation of WASP-WAVE family proteins and the Arp2/3 complex, local inhibition of RhoA by activation of p190RhoGAP, and inhibition of E-cadherin endocytosis through IQGAP1, which is an F-actin cross-linking protein and a downstream target of Rac and Cdc42 (Izumi et al. 2004) (Fig. 3).

   Interestingly, similar signaling molecules are at play also in neural synapses (reviewed by Brigidi and Bamji 2011). Postsynaptic spine morphology is indeed dependent on modulation of small GTPases by p120ctn, the related neural δ-catenin, and α-catenin. So, clustering of postsynaptic N-cadherin with associated p120ctn and α-catenin leads to recruitment of afadin, which in turn recruits the Rac-GEF kalirin-7, promoting Rac1-dependent widening of the spine head. Moreover, p120ctn and δ-catenin can inhibit RhoA, which regulates spine length and density. These findings collectively show that functional interactions of catenins, be it β-catenin, p120ctn, or α-catenin, with small GTPases and actin remodeling proteins is a recurrent theme that has evident implications for correct tissue morphogenesis (reviewed by Harris and Tepass 2010).



Cadherins, Fig. 3 Interactions between ligation of nectins, ligation of classic cadherins and activation of small GTPases. Nectins are members of the immunoglobulin(Ig)-like family of adhesion proteins. They homopolymerize in *cis* and heteropolymerize in *trans*. Their C-terminal ends bind to the PDZ domain of afadin. Afadin binds to a variety of proteins, including α-catenin, p120ctn, and Rap1, a Ras-like small GTPase. Rap1 is converted into its GTP-bound active form by the action of either Rap-GEF C3G or PDZ-GEF1. This occurs initially in nascent E-cadherin-containing junctions as C3G binds transiently to the cytoplasmic domain of E-cadherin (where it competes with β-catenin). Nectins promote C3G activation and junctional maturation via a c-Src/CRK signaling pathway. In this macromolecular complex, strengthening of the binding of p120ctn to E-cadherin stimulates activation of Rac1, which leads to inactivation of RhoA and activation of IQGAP1. The latter is an F-actin cross-linking protein counteracting E-cadherin endocytosis and in that way stimulating junctional maturation. Cadherin ligation by itself stimulates PI3K, leading to activation of the GEF proteins Vav2 and Tiam1, which in turn activate Rac1 and Cdc42. Upon junctional maturation, C3G on the cadherin tail is displaced by β-catenin, which interacts with scaffold proteins of the MAGI/S-SCAM family. Interaction of these scaffold proteins with PDZ-GEF1 further increases the local concentration of active Rap1-GTP, which in turn further activates Vav2 and Tiam1. Modified after (van Roy and Berx 2008), and after (Pieters et al. 2012)

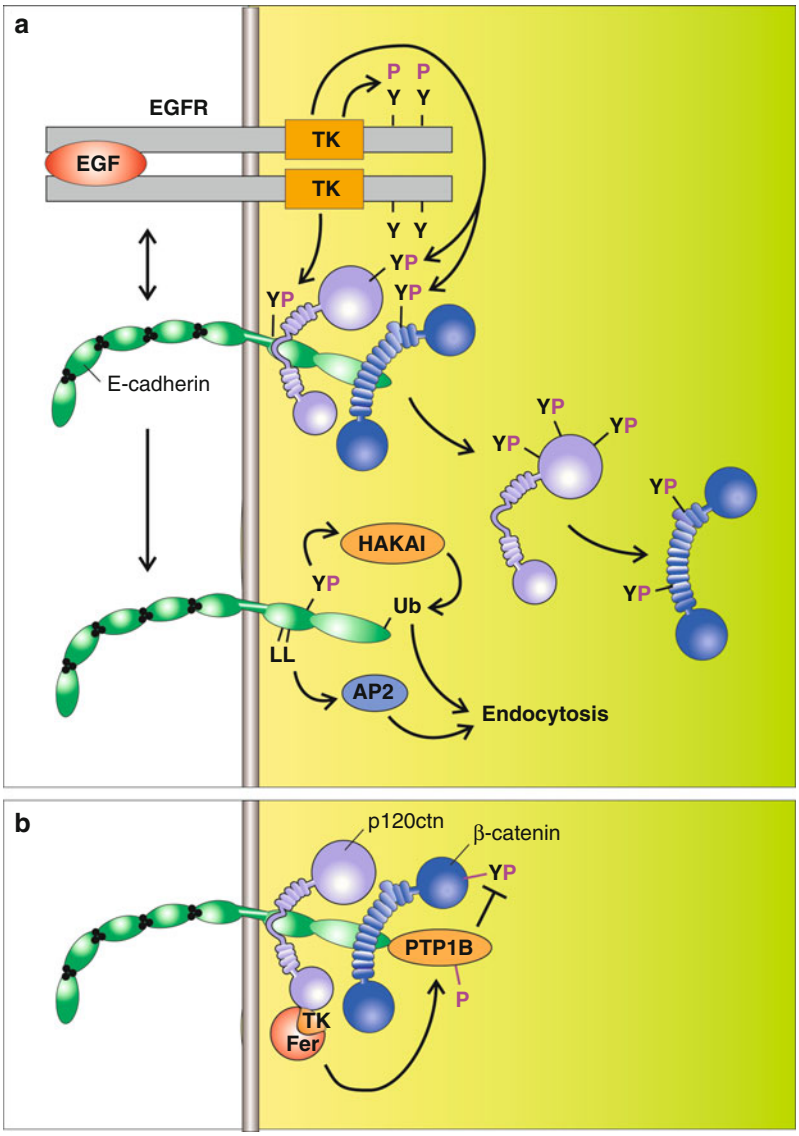
Signaling by Posttranslational Modification of Classic Cadherins

Of the wide variety of possible posttranslational modifications that might affect and modulate classic cadherins and associated proteins, (de)phosphorylation and proteolytic processing have been studied in most detail. For instance, the cytoplasmic domains of classic cadherins as well as the associated armadillo proteins p120ctn and β-catenin are efficient substrates for various tyrosine kinases, both receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases such as Src, Fyn, and Fer (reviewed by Niessen et al. 2011; Pieters et al. 2012). Tyrosine phosphorylation of cadherin and catenin is generally thought to perturb their functions, but there is ample evidence that weak or transient phosphorylation under physiological conditions stimulates cadherin-mediated adhesion. Ligation of E-cadherin stimulates the c-Src signaling pathway in a delicate biphasic way: weak signals are supportive of E-cadherin-based cell-cell contacts in a positive feedback, whereas strong signals inhibit E-cadherin functionality in a negative feedback (reviewed by Niessen et al. 2011).

Interestingly, cadherins can interact, in a cadherin-type specific way, with transmembrane signaling proteins like RTKs and phosphotyrosine phosphatases, and this influences both transcellular and intracelluar signaling. For instance, the E-cadherin ectodomain interacts physically with the ectodomains of EGFR and the HGF-receptor c-Met (Fig. 4a). These interactions either stimulate or inhibit ligand-induced receptor activities, through co-recruitment of RTKs by E-cadherin to the cell surface or by co-endocytosis of E-cadherin with the receptor (reviewed by van Roy and Berx 2008). On the other hand, the associated RTKs can negatively affect E-cadherin functionality by tyrosine phosphorylation of E-cadherin and the associated catenins. In the case of N-cadherin, the ectodomain has been demonstrated to interact molecularly and functionally with the FGF-receptor ectodomain to prevent FGFR internalization and allow sustained receptor activation and downstream signaling (reviewed by Berx and van Roy in Nelson and Fuchs 2010). In endothelial cells, VEGFR2 associates with the CD instead of the ectodomain of VE-cadherin (cadherin-5). This interaction also prevents receptor internalization and signaling, and this ultimately inhibits cell growth (reviewed by Niessen et al. 2011). VE-cadherin is tyrosine phosphorylated in response to a wide spectrum of signals, including triggering by VEGF and various inflammatory mediators.

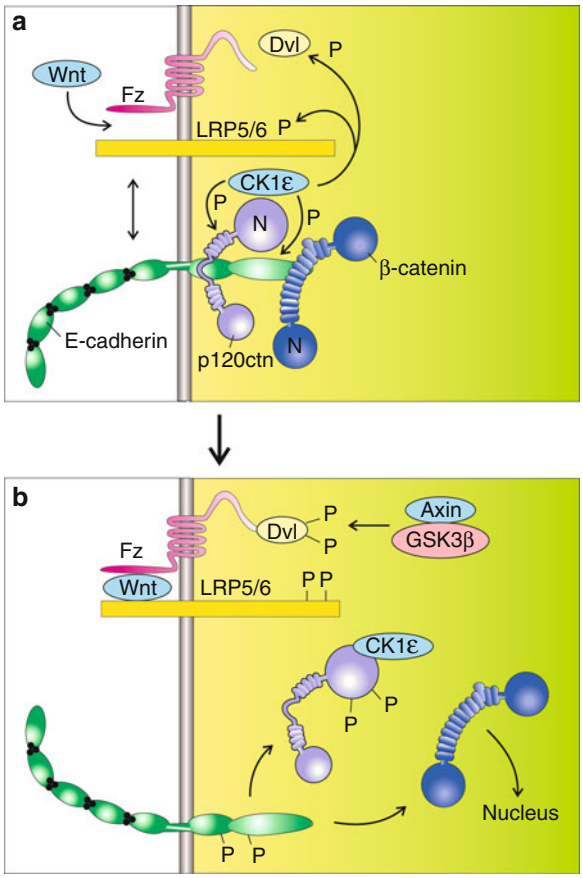
Generally, the cellular response to cadherin/catenin phoshorylation appears to be much influenced by the cadherin type, the cell type, and the microenvironment. Therefore, the following interactions serve as representative examples rather than as rules of thumb. In adherent cells expressing N-cadherin, the cadherin-associated p120ctn recruits the non-receptor tyrosine kinase Fer, which activates the protein Tyr phosphatase PTP1B (PTPN1) when this is complexed to junctional β-catenin (Fig. 4b). This promotes junctional integrity (reviewed by Pieters et al. 2012). Interestingly, a similar mechanism of junctional stabilization occurs in excitatory synapses, where junctional p120ctn at the presynaptic membrane recruits Fer, leading to activation of the Tyr phosphatase SHP2 (PTPN11), which in turn dephosphorylates β-catenin and promotes junctional integrity at synapses (Lee et al. 2008) (Fig. 4b). Further, synaptic functionality is promoted by the following specific molecular interactions with junctional β-catenin (reviewed by Brigidi and Bamji 2011). The PDZ-binding motif at the C-terminus of β-catenin acts as a scaffold for recruitment of various proteins to the cadherin/catenin complex, including the presynaptic scaffolding protein Scribble and the postsynaptic scaffolding protein S-SCAM (Magi2). Synaptic vesicles (SVs) are recruited via Scribble to the presynaptic membrane, whereas at the postsynaptic side, junctional S-SCAM recruits the cell adhesion protein neuroligin-1 (NL1) to the synapse. NL-1 engages in trans-synaptic binding with neurexin and this enhances the clustering of SVs in the presynaptic compartment (reviewed by Brigidi and Bamji 2011). Likewise, δ-catenin binds several PDZ-domain-containing proteins, including postsynaptic receptor scaffolding proteins. δ-catenin is a p120ctn homolog exclusively expressed in the brain. Moreover, the ectodomain of N-cadherin also associates with the AMPA receptor GluR2, and this interaction appears to be essential for GluR2-mediated spine maturation.

When RTKs or oncogenic tyrosine kinases are active, phosphotyrosine sites on p120ctn and cadherin cytoplasmic domains recruit SH2-containing kinases and phosphatases. These enzymes act in a complex and concerted manner on junctional, cytoskeletal, and signaling protein substrates. A major consequence can be

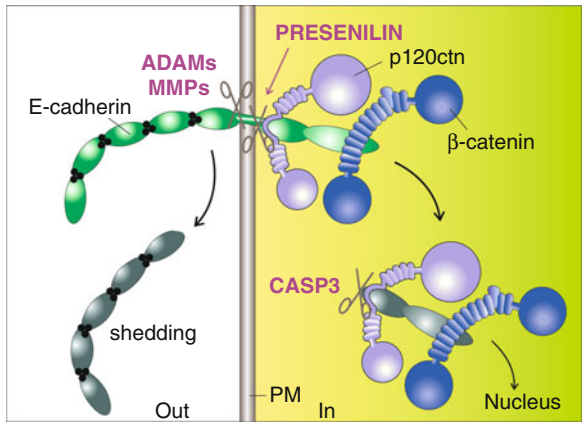


Cadherins, Fig. 4 Tyrosine phosphorylation of the cadherin/catenin complex (CCC) influences its stability. (**a**) The ectodomain of E-cadherin forms a complex (*bidirectional arrows*) with the epidermal growth factor receptor (EGFR). Triggering of dimeric EGFR by its ligand EGF activates its tyrosine kinase (TK) activity, leading to cross-phosphorylation of EGFR and phosphorylation of several components of the CCC on Tyr (Y) residues (arrows and -YP annotations). This leads to release of catenins from the CCC, which exposes a di-Leu motif on the cytoplasmic domain of E-cadherin. Recognition of this motif by the clathrin adaptor AP2 induces endoctysosis of E-cadherin. Moreover, the P-Tyr on the cytoplasmic domain of E-cadherin is recognized by the E3 ubiquitin ligase Hakai. Ubiquitinated E-cadherin is quickly endocytosed. (**b**) On the other hand, junctional p120ctn can associate with the non-receptor tyrosine kinase Fer. This leads to activation of the protein Tyr phosphatase PTP1B in complex with junctional β-catenin, which counteracts the action of other tyrosine kinases, including EGFR, on β-catenin. Not shown here is that increased Fer activity leads to Tyr phosphorylation also of β-catenin, stimulating its release from the CCC. Modified after (Pieters et al. 2012)

phosphorylation of critical Tyr residues on β-catenin, leading to disassembly of β-catenin from cadherin tails on the one hand and from α-catenin on the other hand (Fig. 4a). Moreover, junctional p120ctn can also recruit the Ser/Thr kinase CK1ε, which upon activation (for instance, by Wnt signaling) phosphorylates and activates several Wnt receptor components, which form a macromolecular complex with the



Cadherins, Fig. 5 Action of casein kinase-1ε (CK1ε) on a macromolecular complex of E-cadherin with the Wnt receptor modulates the Wnt signaling pathway. (**a**) In the absence of the ligand Wnt, the Ser/Thr kinase CK1ε associates with junctional p120ctn, whereas E-cadherin associates with the Wnt co-receptor LRP5/6 (*bidirectional arrows*). Upon binding of Wnt, the complex grows by association between LRP5/6 and the Wnt receptor Frizzled (Fz), followed by recruitment of Dishevelled (Dvl). By then, CK1ε becomes activated and phosphorylates LRP5/6, Dvl, p120ctn, and the cytoplasmic tail of E-cadherin (*arrows*). (**b**) On the one hand, these phosphorylation events trigger Wnt signaling by recruiting (and thus inhibiting) Axin and GSK-3β, while β-catenin is released from the CCC and can shuttle to the nucleus, where it stimulates transcription. On the other hand, phosphorylated E-cadherin dissociates from both LRP and p120ctn, which increases its turnover. Modified after (Casagolda et al. 2010)



Cadherins, Fig. 6 Junctional E-cadherin is sensitive to proteolytic processing. Action of metalloproteases (MMPs) and ADAMs (A Disintegrin and Metalloprotease) can lead to shedding of the ectodomain of cadherins. This reduces intercellular adhesion directly, as well as indirectly by interfering elsewhere in the tissue or organism with the clustering/signaling by full-length cadherins. The remaining cell-associated cadherin fragment is released from the plasma membrane by the actions of presenilin (γ-secretase) and then caspase-3. The final C-terminal fragment, which is still bound to the armadillo catenins, might shuttle as a complex to the nucleus, or it might first release these catenins, which then might translocate separately to the nucleus. In either case, transcription of specific target genes would be activated. Modified after (van Roy and Berx 2008)

E-cadherin/catenin complex (Casagolda et al. 2010) (Fig. 5). However, CK1ε also phosphorylates the cadherin tail and p120ctn. This leads to dissociation of both β-catenin and p120ctn/CK1ε from E-cadherin. Wnt signaling is then terminated for two reasons: CK1ε is released from the signalosome, and the release of p120ctn from the JMD of the E-cadherin tail removes Fer and PTP1B from the complex and exposes Tyr residues in the cadherin tail. When these residues become phosphorylated, they serve as docking sites for the E3 ubiquitin ligase Hakai; this results in ubiquitination and internalization of E-cadherin (Fig. 4a) (Pieters et al. 2012). Whether this scenario is valid for all cell types that are responsive to Wnt signals is an open question.

Posttranslational processing is another means for modulating the adhesive and signaling functions of cadherin-catenin complexes. Briefly, furin-mediated removal of a prodomain is essential for initiating adhesion by classic cadherins (reviewed by van Roy and Berx 2008). Moreover, the ectodomain of classic cadherins is shed upon cleavage by various metalloproteases, including transmembrane ADAMs. This phenomenon is probably relevant to cancer progression (reviewed by Berx and van Roy in Nelson and Fuchs 2010). In turn, the remaining membrane-associated cadherin fragments can be further cleaved by presenilin-1/γ-secretase or by caspase-3 (Fig. 6).

The final C-terminal fragments can retain biological activity by binding various proteins, including armadillo-family catenins, and by translocation into the nucleus (reviewed by Berx and van Roy in Nelson and Fuchs 2010; Niessen et al. 2011).

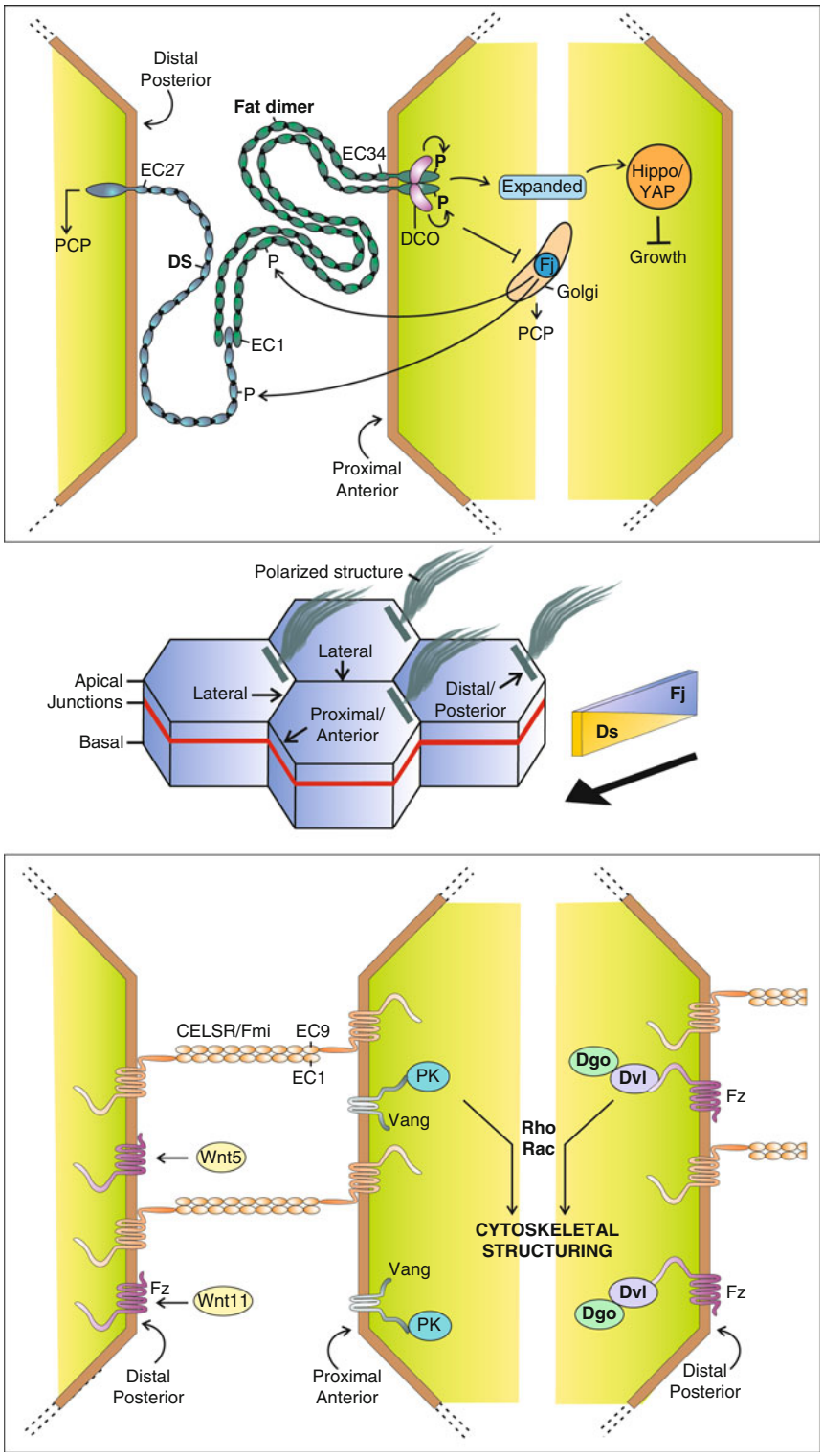
Signaling by Nonclassic Cadherins

Compared to classic cadherins, knowledge on nonclassic and cadherin-related atypical cadherins is still sparse. Nevertheles, it points to multiple signaling functions.

Cadherins with atypical membrane association are the GPI-anchored cadherin-13 and flamingo/CELSR with 7-TM domains. Upon homophilic ligation to endothelial cell surfaces, cadherin-13 becomes linked to Grp78/BiP. The latter is normally ER-retained but can be secreted under particular conditions. Formation of this complex with Grp78 triggers the anti-apoptotic Akt kinase pathway. This has important consequences for tumor-associated angiogenesis (reviewed by Berx and van Roy in Nelson and Fuchs 2010). The flamingo (Fmi)/CELSR proteins are particularly well known for their roles in planar cell polarity (PCP), both in fruit fly (where flamingo is also called starry night or stan) and in vertebrates (Gray et al. 2011). PCP (Fig. 7, middle) has turned out to be central for a variety of important biological processes: convergence and extension movements during embryonic development, inner ear development, hair follicle and cilia polarization, tangential neuronal migration, and development of heart, kidney, and lung (Gray et al. 2011). At the distal side of polarized cells, Fmi/CELSR forms a molecular complex with Frizzled receptors preferentially, while at the proximal side a complex of Fmi/CELSR with Van gogh (Vang, also called strabismus or stan), is preferred (Fig. 7, bottom). In addition to these heterophilic interactions, Fmi/CELSR molecules interact homophilically in *trans* across distal-proximal membrane boundaries, but not at lateral cell boundaries. Unfortunately, the structures of these various molecular interactions have not been reported. Anyhow, cellular asymmetry is assumed to be generated on the basis of the heterophilic interaction partners of Fmi/CELSR. Outside these asymmetric junctional complexes, Fmi/CELSR is unstable due to a high rate of endocytosis.

While Fmi/CELSR is considered a core protein that mediates PCP at the cellular level, two other atypical cadherin proteins, Fat and Dachsous (Ds), are considered either upstream or parallel signaling proteins for PCP, as they mediate PCP at a more global embryonic or organ level (Gray et al. 2011). In mammals, Fat4 is the true ortholog of fly Fat, and DCHS1 is the ortholog of Ds (Hulpiau and van Roy 2011). It is noteworthy that the cytoplasmic domain of FAT4 deviates completely from those of FAT1 to FAT3. Both Fat and Ds have very long ectodomains with numerous EC repeats, and they interact heterophilically with each other at cell junctions. Unfortunately, the structure of these ectodomains is not resolved. Polarity across embryos/organs is mediated both by increased Ds expression at the proximal side and by increased four-jointed (Fj) at the distal side (Sopko and McNeill 2009) (Fig. 7, middle). Fj is a kinase that phosphorylates the ectodomains of Fat and Ds. In fly, strong triggering of Fat by Ds leads to *cis*-dimerization of Fat and to phosphorylation by the associated Dco (Disc Overgrown, the fly homolog of CK1ε) (Fig. 7, top). Importantly, actively signaling Fat not only mediates PCP but, at least in *Drosophila*, also activates the Hippo signaling pathway by a complex, multistep and still incompletely understood mechanism (Badouel and McNeill 2011). The Hippo or Yap pathway is an emerging signaling network with critical importance for controlling organ size, and its derangement is being reported in an increasing number of human cancer types (Badouel and McNeill 2011).

Genuine protocadherins comprise more than half of the cadherin species in mammals and have typical cytoplasmic domains completely different from those of classic cadherins. Reportedly, they have much weaker or even undetectable adhesion potential compared to classic cadherins (for instance, Biswas et al. 2010). Consequently, they are believed to have a signaling function rather than an adhesive function. Unfortunately, analyses of protocadherins are fairly few, and functional studies have been performed only on a few protocadherins and mostly in *Xenopus* (Nishimura and Takeichi 2009). The cytoplasmic domain of arcadlin, which corresponds to the rat protocadherin-8, interacts in synaptic junctions with N-cadherin. Excitation of hippocampal neurons upregulates arcadlin, and this promotes N-cadherin internalization, which is accelerated by the homophilic



Cadherins, Fig. 7 Role of various members of the cadherin superfamily in planar cell polarity (PCP). *Central panel*: Scheme of a typical tissue with established PCP. Cells are arranged in a sheet with a polarized structure (for instance, sensory bristles on a fly wing). Besides having lateral membranes, cells thus have a proximal (anterior) membrane and a distal (posterior)

interaction of arcadlin ectodomains and involves an associated MAPKKK named TAO2β (reviewed by Nishimura and Takeichi 2009). A similar observation of cross-inhibition involves the induction by activin of PAPC expression in *Xenopus*, which is linked to decreased adhesion activity of C-cadherin, a classic cadherin in frog. For protocadherin-10 (OL-protocadherin), a specific interaction between its CD and the actin-organizing complex Nap1/WAVE has been reported (Nishimura and Takeichi 2009). Protocadherin-10 is enriched at sites of cell-cell contact and it recruits Nap1/WAVE, which in turn leads to weakening of the classic cadherin-catenin complex and upregulation of the cell motility machinery (Fig. 1a). These molecular interactions might explain the aberrant migration of particular neurons in mice in which protocadherin-10 is knocked out. One could therefore conclude that a general function of protocadherins is to negatively regulate the activity of classic cadherins. However, protocadherin-19 in zebrafish acts synergistically with N-cadherin to control cell migration during anterior neurulation (Biswas et al. 2010). This protocadherin is also thought to be linked to WAVE regulation. It will thus be challenging to unravel in detail the underlying mechanisms of protocadherin functions.

Summary

This entry deals with selected examples of intra- and cross-cellular signaling by members of the large cadherin superfamily. Much attention is paid to E-cadherin, which as founder of the superfamily, is an extensively studied "classic" cadherin. As a mediator of specific intercellular junctions, both static and dynamic ones, it has a pivotal role in epithelial cell behavior, tissue formation, and suppression of cancer. Compelling evidence has been gathered that E-cadherin is much more than "molecular glue," because it serves as a hub for several signaling pathways. On the one hand, association of the armadillo protein p120ctn with the juxtamembrane domain of classic cadherins, as well as activation of various small GTPases, contribute to junctional stability. On the other hand, cadherin ligation generates signals that contribute to cell differentiation and inhibit growth stimulatory signaling, in part by binding via its C-terminal domain the proto-oncogenic β-catenin. Important modulators of cadherin-associated signaling are cytoskeleton components, such as actomyosin, and several enzymes, in particular receptor tyrosine kinases and counteracting phosphatases. E-cadherin is also processed by several proteases to generate various fragments with signaling activity. The many other members of the superfamily include protocadherins, which may act negatively on classic cadherins in various ways. The flamingo/CELSR cadherins and the very extended Dachsous and Fat cadherins play important roles in the signaling toward planar cell polarity. In conclusion, signaling by cadherins is a rapidly growing research field with multiple proven or proposed links to mammalian pathologies. Recent investigations on cadherin-linked signaling raised many interesting questions that remain to be addressed by multidisciplinary approaches.

Cadherins, Fig. 7 (continued) membrane. A spatial gradient of Dachsous (Ds) and an inverse gradient of four-jointed (Fj) contribute to long-range polarity (indicated by the *arrow*). This is further detailed in the *top panel*, showing a speculative model of *Drosophila* cells with PCP. The very long Ds protein in the apical region of a distal membrane interacts with Fat in a proximal membrane of a juxtaposed cell (the interaction via the EC1 domains of these two cadherin family members is speculative). This induces *cis*-dimerization of Fat, followed by phosphorylation of their Fat cytoplasmic domains by the recruited kinase Discs Overgrown (Dco). This leads to inhibition of Fj, which is a Golgi-localized kinase that can phosphorylate the ectodomains of both Ds and Fat. Inverse spatial gradients of Ds and Fj over the tissue (*middle panel*) are believed to direct PCP. Moreover, the activated Fat dimer triggers, via complex signaling (not shown here), the growth inhibitory Hippo/YAP signaling pathway. One of the mechanisms involved is recruitment of Expanded, a FERM-domain-containing protein, to the apical side of the membrane. *Bottom panel*: Polarized expression pattern of the core polarity proteins Frizzled (Fz) and Van Gogh (Vang, also named Strabismus/Stbm) in juxtaposed plasma membranes. At the extracellular side, Fz becomes activated by Wnt5 or Wnt 11. At the cytoplasmic side, Fz is associated with Dishevelled (Dvl) and Diego (Dgo), whereas Vang is associated with Prickle (Pk). The assymetric distribution of these proteins leads to a polarized cytoskeleton structure via modulation of Rho and Rac signaling. Fz and Vang interact heterophilically across the intercellular space in a poorly understood way to which homophilic interaction of Flamingo (CELSR/Fmi) cadherins contribute (the interaction between EC1 and EC9 domains of overlapping Flamingo molecules is speculative). Modified after Berx and van Roy in (Nelson and Fuchs 2010), and after (Badouel and McNeill 2011)

References

Badouel C, McNeill H. SnapShot: the hippo signaling pathway. Cell. 2011;145:484–e1.

Biswas S, Emond MR, Jontes JD. Protocadherin-19 and N-cadherin interact to control cell movements during anterior neurulation. J Cell Biol. 2010;191:1029–41.

Brigidi GS, Bamji SX. Cadherin-catenin adhesion complexes at the synapse. Curr Opin Neurobiol. 2011;21:208–14.

Casagolda D, Del Valle-Perez B, Valls G, Lugilde E, Vinyoles M, Casado-Vela J, et al. A p120-catenin-CK1epsilon complex regulates Wnt signaling. J Cell Sci. 2010;123:2621–31.

Gray RS, Roszko I, Solnica-Krezel L. Planar cell polarity: coordinating morphogenetic cell behaviors with embryonic polarity. Dev Cell. 2011;21:120–33.

Gumbiner BM. Regulation of cadherin-mediated adhesion in morphogenesis. Nat Rev Mol Cell Biol. 2005;6:622–34.

Halbleib JM, Nelson WJ. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. Genes Dev. 2006;20:3199–214.

Harris TJ, Tepass U. Adherens junctions: from molecules to morphogenesis. Nat Rev Mol Cell Biol. 2010;11:502–14.

Hoshino T, Sakisaka T, Baba T, Yamada T, Kimura T, Takai Y. Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. J Biol Chem. 2005;280:24095–103.

Hulpiau P, van Roy F. New insights into the evolution of metazoan cadherins. Mol Biol Evol. 2011;28:647–57.

Izumi G, Sakisaka T, Baba T, Tanaka S, Morimoto K, Takai Y. Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments. J Cell Biol. 2004;166:237–48.

Kooistra MRH, Dube N, Bos JL. Rap1: a key regulator in cell-cell junction formation. J Cell Sci. 2007;120:17–22.

Lee SH, Peng IF, Ng YG, Yanagisawa M, Bamji SX, Elia LP, et al. Synapses are regulated by the cytoplasmic tyrosine kinase Fer in a pathway mediated by p120catenin, Fer, SHP-2, and beta-catenin. J Cell Biol. 2008;183:893–908.

Nelson WJ, Fuchs E, editors. Cell-cell junctions. Cold Spring Harbor perspectives in biology. Woodbury: Cold Spring Harbor Laboratory Press; 2010.

Niessen CM, Leckband D, Yap AS. Tissue organization by cadherin adhesion molecules: dynamic molecular and cellular mechanisms of morphogenetic regulation. Physiol Rev. 2011;91:691–731.

Nishimura T, Takeichi M. Remodeling of the adherens junctions during morphogenesis. Curr Top Dev Biol. 2009;89:33–54.

Pieters T, van Roy F, van Hengel J. Functions of p120ctn isoforms in cell-cell adhesion and intracellular signaling. Front Biosci. 2012;17:1669–94.

Sopko R, McNeill H. The skinny on fat: an enormous cadherin that regulates cell adhesion, tissue growth, and planar cell polarity. Curr Opin Cell Biol. 2009;21:717–23.

van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci. 2008;65:3756–88.

Yonemura S, Wada Y, Watanabe T, Nagafuchi A, Shibata M. α-Catenin as a tension transducer that induces adherens junction development. Nat Cell Biol. 2010;12:533–42.

cADPr Hydrolase

► CD38

Calcipressin1

► Regulator of Calcineurin 1 (RCAN1)

Calcitriol Receptor

► VDR, The Vitamin D Receptor

Calcium and DAG-Regulated Guanine Nucleotide Exchange Factor II

► RasGRP1

Calcium Release Channel

► Ryanodine Receptor (RyR)

Calcium-Activated Neutral Protease

► Calpain

CalDAG-GEFII

► RasGRP1

Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase

► Phosphodiesterase 1

Calpain

Volker Nimmrich, Anton Bespalov and Achim Möller
Neuroscience Research, GPRD, Abbott,
Ludwigshafen, Germany

Synonyms

Calcium-activated neutral protease; EC 3.4.22.52; EC 3.4.22.53; Non-lysosomal cysteine protease

Historical Background

Calpains are a family of non-lysosomal cysteine proteases that are activated by calcium. Calcium-dependent protease activity has already been detected in 1964 in brain tissue from rats, and this activity was later related to calpain. Yet, the identification of the protein started off with structural analysis of muscle tissue and its alteration by *post mortem* degradation (history broadly reviewed by Goll et al. 1990, 2003): In the late 1960s, Wayne Busch and Darrel Goll studied the physiological effects of Ca^{2+} in tissue specimen of rabbit muscle. When muscle strips were left overnight in a 1 mM Ca^{2+} buffer solution, it became apparent that the Z-line (a structural element separating sarcomers in skeletal muscle) had completely disappeared. Busch and Goll subsequently incubated the stripes in EGTA, thereby preventing degradation of the Z-line. With the help of Marvin Stromer, they performed electron microscopy studies showing that the degradation process was completely restricted to the Z-line, leaving other structural elements unaltered. In 1972, Busch and colleagues isolated a protein fraction from muscle fibers which caused removal of the Z-disk in a preparation of rabbit skeletal muscle at Ca^{2+} concentrations higher than 0.1 mM. The Z-line remained intact when Ca^{2+} was substituted by EGTA, strengthening the view that this activity required calcium. In 1976, Bill Dayton joined this group to isolate the protein (at that time called "Ca^{2+}-activated factor"). There was need for larger tissue quantities which required a switch of species from rabbit to pigs. From this preparation, Dayton and colleagues could isolate a sufficient amount of protein with Ca^{2+}-dependent proteolytic activity, which showed Z-disk removing characteristics. They also revealed that this protease contained two subunits, an 80-kDa subunit and a 30-kDa subunit. The protein was later named m-calpain, and is now mainly referred to as calpain 2. The name calpain was given to indicate that this is a *cal*cium-dependent protease with pa*pain*-like activity.

Ron Mellgren identified a second Ca^{2+}-dependent protease eluting at a different salt concentration than m-calpain. The Ca^{2+} concentration necessary for activation was in the μM range, and this protein was later named μ-calpain (now called calpain 1). This protein is also a heterodimer consisting of a catalytic 80-kDa subunit and a small 30-kDa subunit. The cDNAs for the large subunit of the two calpains have been cloned in the 1990s, and described as gene products of CAPN1 (coding for calpain 1) and CAPN2 (coding for calpain 2). The gene encoding the 30-kDa subunit has also been cloned and referred to as CAPN4. In 1989, further calpains beyond calpain 1 and calpain 2 were discovered, and over the last two decades 15 calpain-like genes have been identified in various tissues, and implicated with a variety of diseases. Only nine of them have the typical calmodulin-like EF-hand sequence and are commonly called "typical calpains," as opposed to the "atypical calpains," which do not contain an EF-hand domain (Saez et al. 2006).

Since the mid-1970s it was suspected that there must be a natural inhibitor of calpain in purified fractions of the enzyme and, in 1982, the first detailed report on an intrinsic inhibitor of calpain from human erythrocytes, called calpastatin, was published. Calpastatin is specific for calpain 1 and calpain 2, and does not appear to inhibit any other protease (Rachel et al. 2008). A number of synthetic calpain inhibitors, mostly of peptidic nature, are now commercially available. However, none of them is specific to calpain (Pietsch et al. 2010).

In the late 1980s, it was discovered that calpain dysfunction underlies muscular dystrophies. Since then, calpains have been suggested to be involved in a number of different pathologies, and the information on the pathophysiology of the calpain system has been increasing tremendously in the last two decades (Zatz and Starling 2005). Yet, the physiological role of calpains remains to a large extent unclear.

Biological Properties and Regulation

The "classical" calpains (1 and 2) are heterodimers comprising an 80-kDa subunit (catalytic subunit) and a common 30-kDa subunit (regulatory subunit). The catalytic domains of both calpains share about 60% sequence homology, and are comprised of four domains (I–IV). Domain II is further divided into two subdomains, IIa and IIb. In the absence of calcium, the catalytic triad consisting of Cys105 in domain I and His262 and Asn286 in domain II is separated, indicating that a conformational change is required for activation. Domain IV shares some sequence identity with calmodulin and contains five EF-hand Ca^{2+}-binding sites. The regulatory subunit constitutes two domains (V and VI), with EF-hand binding sites in domain VI (Strobl et al. 2000).

The cellular regulation of calpain and its inhibition by calpastatin is rather complex (Khorchid and Ikura 2002). Structural analysis revealed that the Ca^{2+}-free enzyme is catalytically inactive. A rise in the Ca^{2+} concentration above 1 mM induces a conformational change in the calpain molecule, inducing its proteolytic properties. Calcium appears to bind to more sites of the protein than just the EF-hand motifs, and it is not yet resolved which binding sites are required for activation of proteolytic activity. Interestingly, the concentration of 1 mM for the activation of calpain is not reached under physiological conditions, as the total intracellular Ca^{2+} concentration normally does not exceed 500 nM. It is likely that calpain can only be activated in cellular subdomains with increased Ca^{2+} concentration (e.g., in proximity to Ca^{2+} channels, synaptic terminals, etc.). Phospholipids appear to modulate the Ca^{2+} requirements of calpain, and it is possible that localization of calpain to the plasma membrane would enhance catalytic activity. Recent studies in fibroblasts have revealed that calpain 2 can be activated independently of calcium. Mitogen-activated protein kinase (▶ MAP Kinases), for example, activates calpain 2 by phosphorylation. As a result, calpain 2 is rapidly activated in dendrites and dendritic spines, thereby participating in cytoskeletal reorganization. Calpain 2 is also phosphorylated by the serine/threonine protein kinase ERK, leading to an activation of calpain and subsequent changes in tau cleavage, cell motility, and adhesion.

The contribution of the small "regulatory" subunit to calpain regulation is not fully understood. It has initially been speculated that the catalytic subunit is activated by dissociation of the small subunit from the core molecule. However, it appears that proteolytic activity is also present when the subunits are associated. The 30-kDa subunit may also be involved in folding of the 80-kDa subunit (as a molecular chaperone), thereby regulating function.

Physiological Function

The last decades have revealed the involvement of calpain in several pathological conditions. In contrast, less is known about the exact physiological function of calpain. It likely fulfills multiple roles, which is indicated by its great number of cellular substrates. It is therefore surprising that inhibition of calpain in animals by synthetic inhibitors is largely harmless.

Probably the best described function of calpain is its contribution to the (re)organization of the cytoskeletal system during a number of cellular events. The involvement of calpains in cell movement is well documented (Glading et al. 2002). Some cytoskeletal proteins including those assembling into intermediate filaments (vimentin, desmin, neurofilament protein) are cleaved by calpain and may allow adaptation of cellular architecture. Spectrin, a building block of the sub-membraneous cytoskeleton as well as cross-linkers of cytoskeletal filaments (e.g., talin) are also substrates for calpain, further underlining its involvement in structural modulation. Notably, a number of muscular elements including myosin and titin (which anchors actin and myosin to the Z-disk) are calpain substrates and possibly mediate the involvement of calpain in muscle diseases. Calpain also degrades dystrophin, a protein anchoring actin to the plasma membrane of muscle cells. Proteins of the microtubular system (MAP1 and MAP2) are also cleaved, as well as cell adhesion molecules (e.g., N-cadherin). In addition, calpain appears to be involved in cytoskeletal protein cleavage in platelet aggregation. Finally, calpain modulates cytoskeletal organization during myoblast fusion.

It is generally thought that calpain is central to a number of signaling pathways (Vosler et al. 2008). For example, RhoA activity is directly affected by calpain, thereby inhibiting cell spreading. Calpain also appears to be involved in apoptosis. Calpain inhibitors reportedly block proliferation at the G1 phase

suggesting that calpain contributes to cell cycle. In some tissues calpains may have highly specialized functions. In neurons, for example, calpains appear to play a central role in regulating synaptic function and plasticity (Wu and Lynch 2006), and may mediate long-term potentiation (LTP), a cellular form of learning and memory.

Calpains and Disease

Calpains have been suggested to be involved in a number of CNS diseases, especially those accompanied by chronic neurodegenerative processes (Liu et al. 2008). Excessive stimulation of NMDA receptors leads to calpain-mediated neurodegenerative cascades, which can be prevented by inhibition of calpain in several in vivo models. Calpain is activated in a number of CNS disorders, including Alzheimer's disease, Parkinson's disease, and Huntington's disease. It was recently shown that amyloid-β-induced nucleus basalis degeneration (a brain region affected during Alzheimer's disease) can be prevented by calpain inhibition in rats. At the cellular level, calpain mediates amyloid-β-induced cleavage of the presynaptic protein dynamin, thereby causing deficits in synaptic function. Calpain exacerbates tau pathology in Alzheimer's disease by cleaving p35, thereby activating cdk5, one of the kinases hyperphosphorylating tau protein. Calpain-mediated cdk5 activation also contributes to dopaminergic cell death in Parkinson's disease. A dysfunctional calpain system is also present in Huntington's disease, where active calpain directly cleavages the protein huntingtin, leading to a toxic accumulation of a protein fragment.

Beside chronic neurodegenerative diseases, calpain contributes to cell death in traumatic brain injury (Saatman et al. 2010) and cerebral ischemic processes (Bevers and Neumar 2008). Both pathologies involve excitotoxic cascades, which are prevented by calpain inhibition. For example, knockdown of calpain 1 increases long-term survival and protects hippocampal function in transient forebrain ischemia. Calpain is activated in the penumbra of the ischemic injury site and some calpain inhibitors have been shown to be neuroprotective. Similarly, calpain inhibition was effective in rat models of traumatic brain injury. Interesting, brain damage in both ischemia as well as traumatic brain injury can be prevented, when calpain inhibitors are given after the insult, indicating that calpain is involved in part of the downstream cascade of the excitotoxic process.

Calpain activation also facilitates degeneration of cardiac tissue after myocardial ischemia/reperfusion injury (Inserte et al. 2009). Both calpain 1 and calpain 2 are increased in ventricular muscle after coronary artery ligation in rats, and inhibition of calpain protects from ischemic cardiac muscle degeneration in animal models of myocardial infarction or in isolated rabbit heart. In an ischemia/reperfusion model in pigs, calpain inhibition decreased infarct size and improved ventricular contractility, and improved overall hemodynamic function.

Finally, there is strong evidence for the involvement of calpains in myopathies of skeletal muscle (Zatz and Starling 2005). Mutations in the gene encoding calpain 3 are responsible for the autosomal recessive disorder limb-girdle muscular dystrophy type 2A. Recent data suggest that mitochondrial abnormalities in calpain 3 deficient muscles may be the underlying mechanism.

Summary

Ubiquitously expressed and with numerous known substrates, calpain plays a significant role in cellular physiology. When activated, calpain cleaves various membrane components, cytosolic enzymes, and regulatory and structural proteins. Interaction with its substrates may lead to rapid changes in cellular functioning. While calpain activation is critically involved in numerous diseases including degenerative changes in the brain, muscles, retina, kidney, and other tissues, the physiological role is less understood. For an activity-regulated system like the calpain system, parameters like duration, intensity, and localization set the course for either a physiological or a pathological process. For example, activation of NMDA receptors activates calpain, leading to a cleavage of synaptic NMDA receptor subunits thereby preventing overexcitation. In contrast, excessive stimulation (possibly involving extrasynaptic NMDA receptors) induces calpain overactivation leading to downstream excitotoxic cascades and neurodegeneration. Analysis of the conditions which turn calpain activation from physiological function into pathophysiological offense is important not only for a full appreciation of the

signaling role of this molecule, but also for the development of novel therapeutics exploiting the biological significance of the calpain system.

References

Bevers MB, Neumar RW. Mechanistic role of calpains in postischemic neurodegeneration. J Cereb Blood Flow Metab. 2008;28:655–73.

Glading A, Lauffenburger DA, Wells A. Cutting to the chase: calpain proteases in cell motility. Trends Cell Biol. 2002;12:46–54.

Goll DE, Kleese WC, Okitani A, Kumamoto T, Cong J, Kapprell H-P. Historical background and current status of the Ca-dependent proteinase system. In: Intracellular calcium-dependent proteolysis. Boca Raton: CRC Press; 1990.

Goll DE, Thomson VF, Li H, Wei W, Cong J. The calpain system. Physiol Rev. 2003;83:731–801.

Inserte J, Barrabes JA, Hernando V, Garcia-Dorado D. Orphan targets for reperfusion injury. Cardiovas Res. 2009;83:169–78.

Khorchid A, Ikura M. How calpain is activated by calcium. Nat Struc Biol. 2002;9:239–41.

Liu J, Liu MC, Wang KKW. Calpain in the CNS: from synaptic function to neurotoxicity. Sci Signal 1re1. 2008;29:152–8.

Pietsch M, Chua K, Abell AD. Calpains: attractive targets for the development of synthetic inhibitors. Curr Top Med Chem. 2010;10:270–93.

Rachel AH, Campbell RL, Davies PL. Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. Nature. 2008;456:409–12.

Saatman KE, Creed J, Raghupathi R. Calpain as a therapeutic target in traumatic brain injury. Neurotherapeutics. 2010;7:31–42.

Saez ME, Ramirez-Lorca R, Moron FJ, Ruiz A. The therapeutic potential of the calpain family: new aspects. Drug Discov Today. 2006;11:917–23.

Strobl S, Fernandez-Catalan C, Braun M, Huber R, Masumoto H, Nakagawa K, Irie A, Sorimachi H, Bourenkowi G, Bartuniki H, Suzuki K, Bode W. The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. PNAS. 2000;97:588–92.

Vosler PS, Brennan CS, Chen J. Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. Mol Neurobiol. 2008;38:78–100.

Wu H-Y, Lynch DR. Calpain synaptic function. 2006;33:215–36.

Zatz M, Starling A. Calpains and disease. New Engl J Med. 2005;352:2413–23.

cAMP Phosphodiesterase-4

► PDE4

Cancer Osaka Thyroid Oncogene

► TPL2

Cancer-Associated Retinopathy Antigen

► Recoverin

CAR-Antigen

► Recoverin

CARD11

► CARMA1

CARD-Containing MAGUK Protein 1

► CARMA1

CARMA1

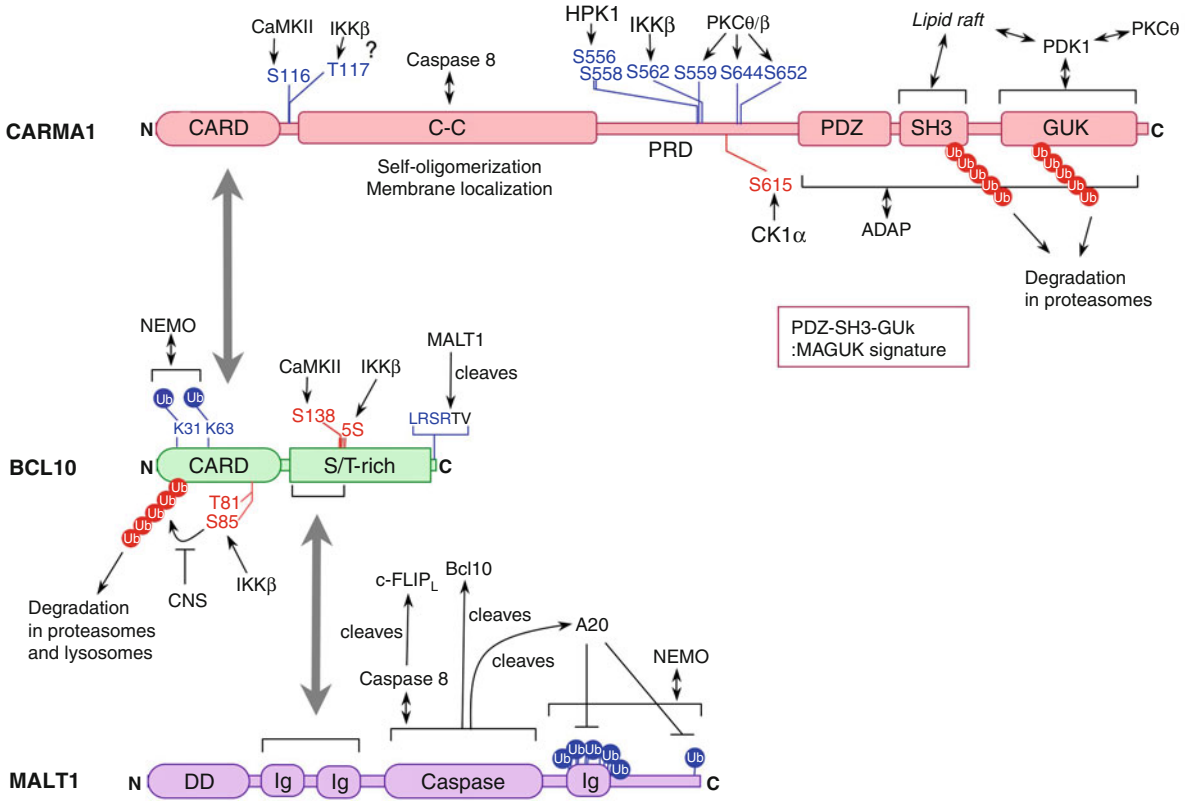
Hiromitsu Hara
Division of Molecular and Cellular Immunoscience, Department of Biomolecular Sciences, Saga University, Saga, Japan

Synonyms

Bcl10-interacting MAGUK protein 3; Bimp3; CARD11; CARD-containing MAGUK protein 1; Caspase recruitment domain family, member 11

Historical Background

The mucosa associated lymphoid tissue lymphoma (MALTL)-associated molecules, B-cell lymphoma

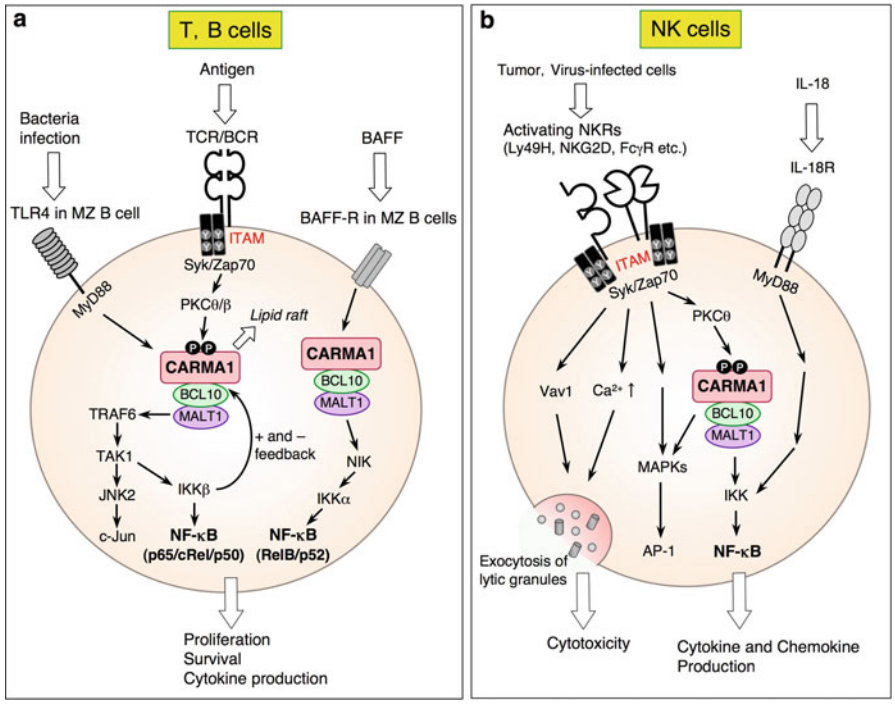


CARMA1, Fig. 1 Domain structure, regulatory phosphorylation sites, mono- or polyubiquitination sites, and proteolytic cleavage sites of CBM molecules. CBM, CARMA1-BCL10-MALT1; CARD, caspase-recruitment domain; C-C, coiled-coil domain; PDZ, PSD95, DLGA, and ZO1 homology domain; SH3, Src-homology 3 domain; GUK, guanylate kinase domain; S/T-rich, serine/threonine-rich domain; DD, death domain; Ig, immunoglobulin-like domain; Ub, ubiquitin; S, serine; T, threonine; K, lysine;5S, five serines; CNS, COP9 signalosome; CaMKII, calmodulin-dependent kinase II; HPK1, hematopoietic progenitor kinase 1; CK1α, casein kinase 1α; PDK1, 3′-phosphoinositide-dependent kinase 1; ADAP, degranulation-promoting adaptor protein. The figure refers to the human CARMA1, BCL10, and MALT1 protein. Blue text denotes positive regulatory sites and red text denotes negative regulation sites

10 (BCL10) and MALTL translocation gene 1 (MALT1), form a complex and play an essential role in nuclear factor κB (NF-κB) activation through immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors and some G-protein-coupled receptors (Hara and Saito 2009). CARMA1 is a member of CARD-containing membrane-associated guanylate kinase (MAGUK) family and was found to be a binding partner of BCL10 via CARD-CARD interaction (Bertin et al. 2001; Gaide et al. 2001), similar to CARD9 and the other member of CARD-MAGUKs, CARMA2 and CARMA3. Among the CARD-MAGUK families, only CARMA1 is highly and selectively expressed in lymphoid organs such as thymus, lymph node, and spleen. It is thought that CARMA1 binds to BCL10-►MALT1 complex following triggering through lymphocyte antigen receptors to form a signaling complex referred to as CARMA1-BCL10-MALT1 (CBM) complex (Fig. 1).

CARMA1 in the Development and Function in T and B Lymphocytes

Immunoreceptors such as T-cell receptors (TCRs), B-cell receptors (BCRs), some members of Fc receptors (FcRs), and activating ►NK receptors (NKRs) transduce activation signals by associating with signaling chains (e.g., CD3s, Igα, Igβ, FcRγ, and DAP12) containing ITAM (consensus; YxxL-x6-8-YxxL) in their cytoplasmic domains (Fig. 2). Upon engagement of ITAM-coupled



CARMA1,
Fig. 2 (**a**) Schematic signaling pathway mediated by CARMA1 in T and B lymphocytes. Syk, spleen tyrosine kinase; Zap70, zeta-chain-associated kinase; TRAF6, TNF receptor associated factor 6; TAK1, TGF-beta activated kinase 1; NIK, NF-κB inducing kinase; ▶ MyD88, myeloid differentiation factor 88. (**b**) Schematic signaling pathway mediated by CARMA1 in NK cells. Vav1, vav 1 guanine nucleotide exchange factor

receptors, an activation signal cascade is initiated with phosphorylation of specific tyrosines in ITAMs, culminating in the activation of transcription factors including NF-κB, nuclear factor-activated T cells (NFAT), and activating protein-1 (AP-1).

Genetic studies using knockout mice of CARMA1, BCL10, and MALT1 as well as a CARMA1-deficient Jurkat cell line have demonstrated an essential role of CBM complex in antigen receptor signaling (Fig. 2a). The T-cell phenotype of CARMA1-deficient (*CARD11*$^{-/-}$) mice closely resembles that of BCL10-deficient (*BCL10*$^{-/-}$) and of MALT1-deficient (*MALT1*$^{-/-}$) mice (Hara et al. 2003; Ruefli-Brasse et al. 2003; Ruland et al. 2001, 2003). Peripheral mature T cells from these knockout mice show almost complete abrogation of proliferation and cytokine production upon stimulation through TCR or with a direct protein kinase C (PKC) activator, PMA plus Ca^{2+}-ionophore (P/I). Accordingly, these mice exhibit severely impaired T-cell immunity. The loss of CBM molecules abrogates TCR-or P/I-induced NF-κB activation owing to defective activation of I-κB kinase (IKK), whereas calcium mobilization and proximal tyrosine phosphorylation are unaffected. In addition, CARMA1 and MALT1 deficiency affect the activation of the MAPK JNK, particularly JNK2, but not the other MAPKs, Erk, and p38.

The CBM deficiency does not affect overall development of thymocytes, with normal numbers of the $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ cells, but increased $CD4^-CD8^-$ cells for unclear reasons (Hara et al. 2003; Ruland et al. 2001). No overt developmental defects in conventional CD4 and CD8 T cells are observed in the peripheral lymphoid organs of these knockout mice; however, the number of natural-occurring regulatory T cells (nTregs) is markedly reduced both in the thymus and periphery (Molinero et al. 2009; Schmidt-Supprian et al. 2004). This phenotype is likely attributed to the reduced expression of the transcription factor Foxp3, the master regulator of Treg development, in thymic nTreg precursor cells in *CARD11*$^{-/-}$ mice because the TCR-induced NF-κB activation directly promotes the transcription of Foxp3 (Long et al. 2009).

Deficiency of CBM molecules in B cells, similar to that in T cells, results in abrogated BCR-induced NF-κB activation and thereby defects in B-cell proliferation and survival, although one line of *MALT1*$^{-/-}$ mice has exhibited only mild defects in B-cell activation (Fig. 2a) (Hara et al. 2003; Ruefli-Brasse et al. 2003; Ruland et al. 2001, 2003). Reduced follicular (FO) and marginal zone (MZ) B cells in spleen and an almost complete absence of peritoneal B-1 B cells have been

consistently observed in *CARD11*$^{-/-}$, *BCL10*$^{-/-}$, and *MALT1*$^{-/-}$ mice. CARMA1 and probably BCL10 control JNK activation through BCRs, whereas MALT1 might be dispensable for it. ▶ CD40-induced proliferation is also defective in splenic B cells with CBM deficiency, possibly owing to the defective development of MZ B cells, which are the major cells responding to CD40 stimulation.

The involvement of CBM complex in toll-like receptor (TLR) signaling in B cells has been suggested, although it remains a controversial issue. *CARD11*$^{-/-}$ whole splenic B cells show impaired proliferation in response to LPS (Hara et al. 2003). A study that compared FO and MZ splenic B cells revealed that BCL10 deficiency affected only MZ B cells responding to LPS due to impaired NF-κB activation (Fig. 2a) (Xue et al. 2003).

In addition to the essential role of CBM complex in canonical NF-κB signaling downstream of TCRs and BCRs, it also acts in the noncanonical NF-κB pathway through B-cell activation of the TNF family (BAFF) receptor, which regulates the survival of MZ B cells (Fig. 2a). Lack of MALT1 impairs BAFFR-induced phosphorylation and degradation of NF-κB2 precursor p100 (Tusche et al. 2009). The *MALT1*$^{-/-}$ MZ but not FO B cells exhibit reduced survival and anti-apoptotic gene induction in response to BAFF in vitro, likely owing to the elevated expression and defective BAFFR-induced downregulation of TRAF3, a negative modulator of the BAFFR-induced survival signal particularly in MZ B cells. The phenotypes of BAFF-Tg mice, including increased basal serum Ig, MZ B cells and B1 B cells, spontaneous germinal center formation, and Ig deposition in the kidney, all disappear in the absence of MALT1 or BCL10.

CARMA1 in NK-Cell Development and Function

Upon triggering of activating NKRs, NK cells attack targets through two defined effector functions: the cytotoxicity and the production of pro-inflammatory cytokines and chemokines. Studies have revealed that CARMA1, BCL10, and MALT1 are essential for production of cytokines and chemokines induced by multiple activating NKRs, including FcγRIII, NK1.1, Ly49H, Ly49D, and NKG2D; in contrast, the cytotoxicity of NK cells induced by these activating NKRs is not affected by CBM deficiency (Fig. 2b) (Gross et al. 2008; Hara et al. 2008). CBM deficiency does not influence either maturation or the repertoire formation of peripheral NK cells. The loss of CBM results in impaired NF-κB activation following activation of NKRs, whereas ▶ Vav1 phosphorylation and Ca^{2+} mobilization, both of which regulate exocytosis of lytic granules, are unaffected. Contribution of CBM to MAPK activation remains controversial. Similar to T cells, PKCθ activity is required for NF-κB activation through activating-NKR. TNF- or IL-18R-mediated NF-κB activation does not require CBM in NK cells.

Signaling Regulation of CBM

Multiple regulation mechanisms, involving phosphorylation, ubiquitylation, oligomerization, caspase activation, and recruitment to plasma membrane, have been proposed to control CBM-signaling (Fig. 1) (Hara et al. 2010).

Upon activation of antigen receptors, CARMA1 and BCL10 are phosphorylated by several kinases. Phosphorylation of CARMA1 by PKCθ (mainly in T cells) and PKCβ (mainly in B cells) in the PKC-regulated domain (PRD) likely transform CARMA1 to an active one that is accessible to BCL10 and other downstream molecules. Phosphorylation of CARMA1 by CaMKII facilitates the interaction between CARMA1 and BCL10. CaMKII also phosphorylates BCL10 but this phosphorylation is involved in the attenuation of the signaling. ▶ HPK1 phosphorylates CARMA1 within the PRD and is involved in both JNK and NF-κB activation although the precise mechanism is unclear. Phosphorylation of CARMA1 by IKKβ promotes signaling activation by enhancing the assembly of CBM complex. IKKβ also phosphorylates BCL10 within the MALT1-interacting S/T-rich domain and within CARD upon TCR stimulation. The former interferes with IKK ubiquitination by causing disengagement of BCL10 from MALT1, and the latter induces BCL10 degradation in the proteasome, thus negatively regulating the signaling. CK1α associates with the PRD of CARMA1after TCR stimulation and contributes to initial NF-κB activation; however, subsequent phosphorylation of the PRD by CK1α contributes to the negative feedback of the signaling.

Upon receptor stimulation, CARMA1 recruits downstream molecules and triggers oligomerization and ubiquitination cascades. BCL10-dependent MALT1

oligomerization induces activation of the E3 ubiquitin ligase ▶ TRAF6, which in turn activates the IKK complex through lysine (K) 63-linked ubiquitylation of the regulatory subunit of IKK NEMO. BCL10 and MALT1 also undergo K63-linked ubiquitination in the CARD domain and the C-terminal region, respectively, upon T-cell activation, which provide docking surfaces for the recruitment of NEMO. MALT1 itself has an E3-ligase activity and targets MALT and NEMO for ubiquitination. In contrast, ubiquitination also acts for signaling inhibition. CARMA1 is K48-linked-polyubiqutinated after receptor stimulation, leading to degradation of CARMA1 in the proteasome, which is dependent on the phosphorylation by PKCs on PRD. cIAP likely targets this ubiquitination of CARMA1. The E3 Cbl-b promotes mono-ubiquitination of CARMA1, which is involved in the anergy induction in NK T cells. BCL10 undergoes degradation following ubiquitination of CARD after receptor stimulation, which contributes to the termination of signaling. NEDD, cIAP, β-TrCP, and Itch have been suggested as E3 ubiquitin ligases of BCL10. The CNS5 and CNS2 of the COP9 signalosome fine-tune IKK activation by interfering with the polyubiquitination and degradation of BCL10. The deubiquitinating enzyme A20 catalyzes the removal of the K63-linked ubiquitin chains on MALT1 and therefore regulates the duration and strength of signals.

MALT1 and CARMA1 interact with Caspase-8 and thereby regulate the Caspase-8-c-FLIPL-mediated NF-κB activation pathway. Paracaspase activity of MALT1 fine-tunes CBM-signaling by cleaving BCL10 and A20. The BCL10 cleavage is required for TCR-induced cell adhesion to the extracellular matrix protein fibronectin. The cleavage of A20 by MALT1 disrupts the inhibitory effect of A20.

Membrane recruitment of signaling components is a crucial event in CBM-mediated NF-κB activation. BCL10, PKCθ, PKCβ, MALT1, pro-caspase-8, c-FLIPL, and the IKK complex are recruited into lipid rafts after antigen receptor stimulation. CARMA1 resides in both the cytoplasm and lipid rafts in resting cells, but the amount in lipid rafts increases after activation. CARMA1 controls the recruitment of PKCθ, BCL10-MALT1, and IKK complexes to lipid rafts. The adapter protein ▶ ADAP acts as a linker between the TCR-ZAP-70-SLP-76 signaling complex and CBM by binding to CARMA1. PDK1 recruits PKCθ and CARMA1 to lipid rafts upon TCR stimulation.

CBM in Lymphomas

The chromosomal translocations, t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21), have been well characterized in MALTL. MALT1 gene was originally identified in the break point of t(11;18)(q21;q21) (Du 2007). This translocation generates API2-MALT1 fusion products comprising the N-terminus of API2 and the C-terminus of MALT1. The fusion product, but neither API2 nor MALT1 alone, is capable of activating NF-κB. The translocations, t(1;14)(p22;q32) and t(14;18)(q32;q21), bring the BCL10 and MALT1 genes under the regulatory control of the Ig heavy chain (IgH) enhancer, respectively, leading to dysregulated expression of these genes and aberrant NF-κB activation. In addition, BCL10 gene amplification has been reported in pancreatic cancer and nodal diffuse large B-cell lymphoma (DLBCL). Similarly, MALT1 gene amplification was found in cell lines of MZ B-cell lymphoma and DLBCL. While normal B cells express BCL10 in the cytoplasm, MALTL cells bearing t(11;18)(q21;q21) and t(1;14)(p22;q32) express the protein predominantly in the nucleus, indicating a possible relationship between aberrant BCL10 nuclear localization and tumorigenesis.

Among the subtypes of DLBCL, the least curable activated-B-cell-like (ABC) subtype DLBCLs, but not the germinal center B-cell-like (GCB) subtype, rely on constitutive NF-κB signaling for survival (Hara et al. 2010). A loss-of-function RNA interference screen for genes required for survival of ABC DLBCLs revealed that CARMA1 is a key upstream signaling component responsible for the constitutive IKK activation in ABC DLBCLs but not GCB DLBCLs (Ngo et al. 2006). In line with this, oncogenic missense mutations of CARMA1 gene, all within exons encoding the C-C, have been found in ABC DLBCLs (Lenz et al. 2008). These mutations constitutively activate the NF-κB pathway and enhance antigen receptor signaling to NF-κB, possibly owing to aggregate the formation of the mutant proteins. The oncogenic forms of CARMA1 promote proteolytic activity of MALT1. Inhibition of MALT1 activity with the inhibitor z-VRPR-fmk specifically affects the growth and survival of ABC DLBCLs.

The CBM-regulated BAFFR signaling also contributes to the development of B lymphomas. BCL10-transgenic mice elevate BAFF expression

and specifically promote survival of MZ B cells, and some mice develop splenic MZ lymphomas (MZL) (Li et al. 2009). BAFF overexpression, with concomitant nuclear expression of BCL10 and NF-κB activation, is associated with *Helicobacter pylori*-independent growth of gastric DLBCL with histological evidence of MALTL (Kuo et al. 2008).

Summary

NF-κB plays a central role in the activation and survival of lymphocytes. CARMA1 is a CARD-MAGUK family adaptor protein originally found as a binding partner of BCL10 via CARD-CARD interaction. CARMA1 and the MALTL-related proteins BCL10 and MALT1 form so-called CBM complex following receptor stimulation. CBM complex is essential for the canonical NF-κB activation signaling through TCRs, BCRs, and activating NKRs, as well as for the noncanonical NF-κB signaling through BAFF, thereby regulating proliferation, survival, and effector functions of T, B, and NK cells. Multiple regulation mechanisms involving phosphorylation, ubiquitylation, oligomerization, caspase activation, and recruitment to plasma membrane have been proposed to control NF-κB activation signaling through CARMA1. The chromosomal translocations and the amplification of MALT1 and BCL10 genes are associated with the development of MALTL and DLBCL. ABC DLBCLs, but not GCB DLBCLs, rely on constitutive NF-κB activation via CBM-signaling for its survival. Oncogenic missense mutations of CARMA1 gene within the coiled-coil domain, leading to constitutive activation of the NF-κB pathway and enhancing antigen receptor signaling to NF-κB, have been found in ABC DLBCL. The CBM-regulated BAFF signaling also contributes to the development of MALTL, MZL, and DLBCL. Thus, CBM-signaling may be a promising therapeutic target of specific B lymphomas.

References

Bertin J, Wang L, Guo Y, Jacobson MD, Poyet JL, Srinivasula SM, Merriam S, DiStefano PS, Alnemri ES. CARD11 and CARD14 are novel caspase recruitment domain (CARD)/membrane-associated guanylate kinase (MAGUK) family members that interact with BCL10 and activate NF-kappa B. J Biol Chem. 2001;276:11877–82.

Du MQ. MALT lymphoma: recent advances in aetiology and molecular genetics. J Clin Exp Hematop. 2007;47:31–42.

Gaide O, Martinon F, Micheau O, Bonnet D, Thome M, Tschopp J. Carma1, a CARD-containing binding partner of Bcl10, induces Bcl10 phosphorylation and NF-kappaB activation. FEBS Lett. 2001;496:121–7.

Gross O, Grupp C, Steinberg C, Zimmermann S, Strasser D, Hannesschlager N, Reindl W, Jonsson H, Huo H, Littman DR, et al. Multiple ITAM-coupled NK-cell receptors engage the Bcl10/Malt1 complex via CARMA1 for NF-kappaB and MAPK activation to selectively control cytokine production. Blood. 2008;112:2421–8.

Hara H, IIzasa E, Nakaya M, Yoshida H. L-CBM signaling in lymphocyte development and function. J Blood Med. 2010;1:93–104.

Hara H, Ishihara C, Takeuchi A, Xue L, Morris SW, Penninger JM, Yoshida H, Saito T. Cell type-specific regulation of ITAM-mediated NF-kappaB activation by the adaptors, CARMA1 and CARD9. J Immunol. 2008;181:918–30.

Hara H, Saito T. CARD9 versus CARMA1 in innate and adaptive immunity. Trends Immunol. 2009;30:234–42.

Hara H, Wada T, Bakal C, Kozieradzki I, Suzuki S, Suzuki N, Nghiem M, Griffiths EK, Krawczyk C, Bauer B, et al. The MAGUK family protein CARD11 is essential for lymphocyte activation. Immunity. 2003;18:763–75.

Kuo SH, Yeh PY, Chen LT, Wu MS, Lin CW, Yeh KH, Tzeng YS, Chen JY, Hsu PN, Lin JT, Cheng AL. Overexpression of B cell-activating factor of TNF family (BAFF) is associated with Helicobacter pylori-independent growth of gastric diffuse large B-cell lymphoma with histologic evidence of MALT lymphoma. Blood. 2008;112:2927–34.

Lenz G, Davis RE, Ngo VN, Lam L, George TC, Wright GW, Dave SS, Zhao H, Xu W, Rosenwald A, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science. 2008;319:1676–9.

Li Z, Wang H, Xue L, Shin DM, Roopenian D, Xu W, Qi CF, Sangster MY, Orihuela CJ, Tuomanen E, et al. Emu-BCL10 mice exhibit constitutive activation of both canonical and noncanonical NF-kappaB pathways generating marginal zone (MZ) B-cell expansion as a precursor to splenic MZ lymphoma. Blood. 2009;114:4158–68.

Long M, Park SG, Strickland I, Hayden MS, Ghosh S. Nuclear factor-kappaB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. Immunity. 2009;31:921–31.

Molinero LL, Yang J, Gajewski T, Abraham C, Farrar MA, Alegre ML. CARMA1 controls an early checkpoint in the thymic development of FoxP3+ regulatory T cells. J Immunol. 2009;182:6736–43.

Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G, Lam LT, Dave S, Yang L, Powell J, Staudt LM. A loss-of-function RNA interference screen for molecular targets in cancer. Nature. 2006;441:106–10.

Ruefli-Brasse AA, French DM, Dixit VM. Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. Science. 2003;302:1581–4.

Ruland J, Duncan GS, Elia A, del Barco BI, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS, Mak TW. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell. 2001;104:33–42.

Ruland J, Duncan GS, Wakeham A, Mak TW. Differential requirement for Malt1 in T and B cell antigen receptor signaling. Immunity. 2003;19:749–58.
Schmidt-Supprian M, Tian J, Grant EP, Pasparakis M, Maehr R, Ovaa H, Ploegh HL, Coyle AJ, Rajewsky K. Differential dependence of CD4 + CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. Proc Natl Acad Sci USA. 2004;101:4566–71.
Tusche MW, Ward LA, Vu F, McCarthy D, Quintela-Fandino M, Ruland J, Gommerman JL, Mak TW. Differential requirement of MALT1 for BAFF-induced outcomes in B cell subsets. J Exp Med. 2009;206:2671–83.
Xue L, Morris SW, Orihuela C, Tuomanen E, Cui X, Wen R, Wang D. Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells. Nat Immunol. 2003;4:857–65.

Cas Scaffolding Protein Family Member 1 (CASS1)

▶ p130Cas

Casein Kinase 2

▶ Casein Kinase II

Casein Kinase II

Jacob P. Turowec[1], Nicole A. St. Denis[2] and David W. Litchfield[1,3]
[1]Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada
[2]Centre for Systems Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada
[3]Department of Oncology, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

Synonyms

Casein kinase 2; CK2; CKII; Csnk2; Protein kinase CK2

Historical Background

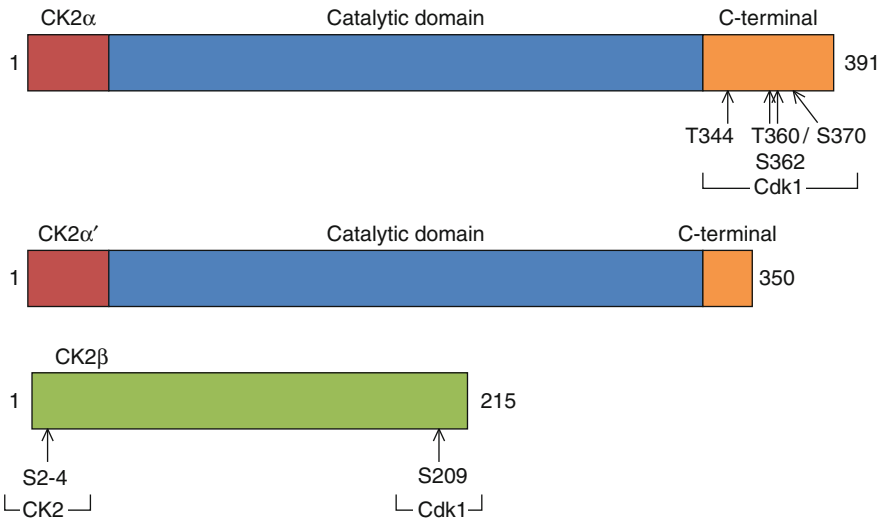
Protein kinase CK2 was isolated almost 60 years ago on the basis of its ability to phosphorylate the milk protein Casein (Allende and Allende 1995). As a result, it was originally designated "Casein kinase II," though this misnomer is less frequently used because of the lack of physiological significance for the phosphorylation of Casein by CK2. Ironically, the enzyme/substrate relationship between CK2 and Casein illustrates somewhat of a recurring theme. In this respect, over 50 years of research on CK2 has resulted in a compilation of a very large number of putative CK2 substrates while the functional analysis of these phosphorylation events has lagged comparatively behind. In large part, the identification of many CK2 substrates has been promoted by its relatively simple and somewhat unique preference for acidic specificity determinants (S/T – X – X- E/D/pS/pY) (Meggio and Pinna 2003). Even before the onset of the large-scale identification of phosphorylation sites, there were more than 300 candidate substrates identified with thousands of potential CK2 substrates now predicted on the basis of global phosphoproteomics and computational analyses (Pinna and Allende 2009). The promiscuity of CK2 in both its number of substrates and the cellular functions in which it has been implicated comes as little surprise when considering its lack of strict regulation (Litchfield 2003; Olsten and Litchfield 2004). Work to date has not yielded a unifying mechanism to explain the regulation of CK2 in cells. In this respect, it has been suggested that CK2 is constitutively active, although there is evidence that there are changes in the phosphorylation of some of its substrates in cells suggesting that its activity could in fact be regulated. Despite the uncertainties about its cellular regulation, evidence for pathogenic misregulation of CK2 in various human malignancies was observed over 20 years ago; CK2 activity and expression are elevated in a number of human cancers, while inhibition of CK2 results in cellular death (Duncan and Litchfield 2008; Trembley et al. 2009). This entry will outline structural and enzymatic features of CK2 and will highlight some of the mechanisms that may contribute to its physiological regulation. Also outlined is an overview of its cellular functions and its emergence as a candidate for molecular-targeted therapy.

Structural and Enzymatic Features of CK2

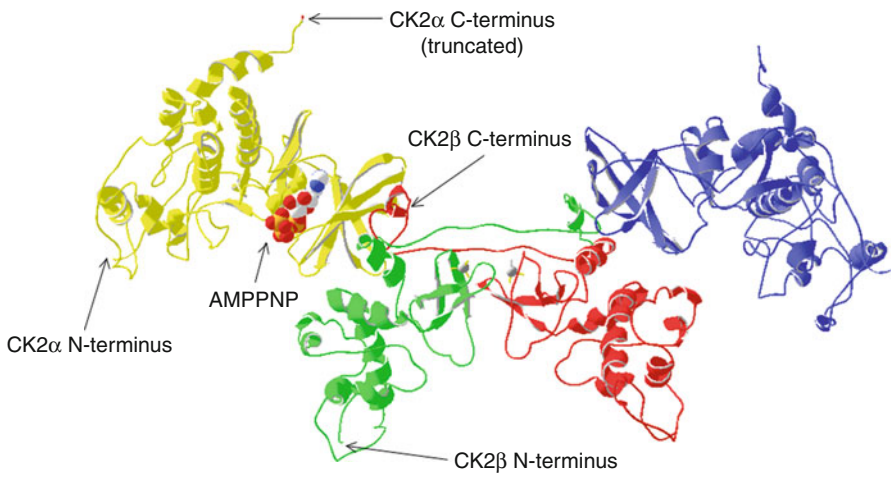
Protein kinase CK2 is ubiquitously expressed in eukaryotes and has generally been considered to be composed of two regulatory CK2β subunits and two catalytic subunits (CK2α and/or CK2α′) (Litchfield 2003). CK2α and CK2α′ are 90% similar within their catalytic domains, but differ substantially in their C-terminal domains (Fig. 1). The close similarity of CK2α′ and CK2α results in an inability to distinguish them on the basis of their catalytic properties as they appear to share the same enzymatic features. For example, the substrate specificity of CK2α and CK2α′ appears to be indistinguishable, with both proteins exhibiting a minimal consensus motif of S/T – X – X – D/E/pS/pY. The regulatory CK2β subunit is a 25 kDa protein in humans that displays little sequence homology with any other protein, but with exceptional conservation between species. While CK2 was traditionally considered to be a tetrameric enzyme, there are indications that its catalytic and regulatory subunits may also exist in cells independent of tetrameric complexes (reviewed in Olsten and Litchfield 2004; Bibby and Litchfield 2005). Although some of the enzymatic characteristics of tetrameric CK2 can be distinguished from that of the free catalytic subunits, a striking feature of CK2 is that both of these forms of CK2 are catalytically competent. In this respect, CK2 is distinct from Cyclin-dependent kinases where catalytic activity is strictly dependent on the presence of an activating Cyclin or from second messenger-dependent kinases such as PKA or PKC where catalytic activity is suppressed by an auto-inhibitory subunit or domain.

Although CK2β is not strictly required to turn on or turn off the catalytic activity of CK2α or CK2α′, it is apparent that it can affect the activity of the catalytic subunits and modulate substrate selectivity (Meggio and Pinna 2003; Olsten and Litchfield 2004; Bibby and Litchfield 2005). While the majority of CK2 substrates can be effectively phosphorylated by either tetrameric CK2 or free catalytic CK2 subunits, the regulatory CK2β subunit generally enhances thermal stability of the catalytic subunits and confers a modest increase (typically 3–5 fold) in catalytic activity toward most substrates. By comparison, there are other proteins such as eIF2β, where CK2β is required to enable efficient phosphorylation, or Calmodulin, where the presence of CK2β leads to complete inhibition of phosphorylation. The demonstration that Calmodulin is phosphorylated at its CK2 sites in cells was amongst the first evidence provided to suggest that free catalytic subunits exist, and are functionally active, in cells. In a similar vein, CK2 independent functions and regulatory mechanisms of CK2β have been explored (reviewed by Bibby and Litchfield 2005). In this respect, it is interesting to note that CK2β is synthesized in excess of the catalytic subunits. Though it is believed that CK2β is quickly degraded when it is not incorporated into tetrameric CK2 complexes, a number of reports highlight potential CK2-independent interactions of CK2β including interactions with a number of other protein kinases (e.g., c-Mos, Chk1, and A-Raf). Collectively, these findings illustrate not only the ability of CK2β to control the substrate specificity of CK2, but also raise the specter that individual CK2 subunits exist outside the holoenzyme and are governed by unique modes of regulation. At this point however, the precise mechanisms that drive tetramer formation and dissociation in cells remain unclear.

Structural studies have yielded many insights into the unique features of protein kinase CK2 (Niefind et al. 2009). First, its constitutive activity is apparent in virtually all of its more than 30 solved structures. In many instances, protein kinases contain an activation loop that can exist in either an open or closed confirmation that governs kinase activity. In the case of MAPKs, for example, phosphorylation of this loop by upstream protein kinases results in the open confirmation, thus activating the MAPK and promoting downstream phosphorylation of pathway constituents. By comparison, structures of CK2 provide evidence for its permanently open conformation and constitutive activation. Second, the structure of the CK2 holoenzyme, consisting of both catalytic and regulatory subunits, revealed the tetrameric configuration of CK2 (Fig. 2). CK2β dimerizes to form the core of the tetramer, and individual catalytic subunits bind to the regulatory subunit dimer. Interestingly, the interaction surface between CK2β and the catalytic subunits is considered strikingly small given the extremely tight interactions that have been measured between the two subunits. Lastly, structures of CK2 in complex with purine analogues revealed unique features of its ATP binding domain in relation to the ATP binding domain of other protein kinases. In CK2, the



Casein Kinase II, Fig. 1 Schematic representation of CK2 subunits. Linear representation of the catalytic isoforms CK2α and CK2α′ and the regulatory subunit CK2β. In vivo phosphorylation sites are indicated, including the four mitotic phosphorylation sites on the extended C-terminal of CK2α. Also noted are the mitotic and autophosphorylation sites located on CK2β



Casein Kinase II, Fig. 2 Crystal structure of tetrameric CK2. Catalytic subunits are shown in *blue* and *yellow* and regulatory subunits are shown in *green* and *red*. Also indicated is a non-hydrolyzable ATP analog, AMPPNP, in the active site of one of the catalytic subunits. Note that the C-terminal tail of CK2α is truncated in this structure (missing amino acids 337–391). The structure of the C-terminus is unknown. This representation of the CK2 tetramer was generated using Swiss PDB Viewer; PDB File 1JWH (Niefind et al. 2001)

ATP binding domain is collapsed and relatively thin in one plane, while being wider in the second plane. The widening allows for utilization of GTP as a phosphate donor, a relatively unique characteristic amongst protein kinases, while the thinning in the other plane provides the opportunity for the rational design of specific, small molecule ATP competitive inhibitors that exploit van der Waals interactions in CK2. A more detailed discussion of strategies for the development of CK2 inhibitors will be presented later.

Physiological Regulation of CK2

As mentioned previously, the activity of CK2 in cells is not subject to absolute "on/off" regulation by individual events such as posttranslational modifications, protein/protein interactions, or small molecule secondary messengers that are observed with other major protein kinase families such as MAPKs, CDKs, and PKCs, respectively. Rather, on the basis of structural characteristics (discussed above), the ability to isolate active, recombinant enzyme from bacterial sources and, most

importantly, the observation that activity is neither turned on nor turned off in response to a variety cellular stimuli that modulate a large number of other protein kinases, CK2 is considered to be constitutively active. While it is apparent that CK2 lacks strict on-off regulation, it is conceivable that a number of more subtle mechanisms may contribute to its regulation in cells, including protein interactions and temporal or stimulus specific changes in function that are discussed below.

The observation that CK2 phosphorylates proteins in multiple cellular compartments supports the hypothesis that interacting proteins regulate CK2 function by directing it to specific locations within the cell (Olsten and Litchfield 2004; Filhol and Cochet 2009). In this way, specific subpopulations of CK2 could be regulated by interacting proteins that permit spatial access to substrates, much the same way that AKAPs modulate the localization and function of PKA. One protein that may regulate CK2 in this manner is ▶ CKIP-1, a PH-domain containing protein that localizes CK2α, but not CK2α′, to the plasma membrane (Canton and Litchfield 2006). Changes in the expression of CKIP-1 induce alterations in cell morphology and the actin cytoskeleton. Since CKIP-1 interacts with CPα, a subunit of the heterodimeric actin capping protein that can be phosphorylated by CK2, these observations are consistent with a working model suggesting that CKIP-1 could participate in the regulation of the actin cytoskeleton by modulating the CK2 catalyzed phosphorylation of CPα. In addition to CKIP-1, CK2 has a large number of other potential interaction partners. Again, given the large number of CK2 substrates that are located in numerous cellular locations, further elucidation of its interaction partners and investigation of their spatial distribution could reveal important new insights into the regulation and physiological functions of CK2.

Though not necessarily responsible for global changes in CK2 activity, it has been demonstrated that discrete populations of CK2 are modulated in response to specific stimuli (Filhol and Cochet 2009). For example, in response to UV radiation, CK2 interacts with the FACT complex, which consists of SSRP1 and hSPT16. Binding of CK2 to the FACT complex results in increased phosphorylation of ▶ p53 by CK2 at Ser 392, an event believed to increase the transcription of apoptotic and cell cycle regulatory genes. Likewise, the regulation of specific CK2 protein interactors and substrates has been observed in nocodazole-arrested cells where CK2α has been shown to be phosphorylated at four proline-directed phosphorylation sites that can be phosphorylated by CDK1 in vitro (Fig. 1) (St-Denis and Litchfield 2009). Phosphorylation of these sites within its unique C-terminal domain promotes interactions between CK2α and the peptidyl-prolyl isomerase ▶ Pin1. Interactions with Pin1 modulate the ability of CK2 to phosphorylate Topoisomerase IIα in vitro at sites that are known to be phosphorylated in mitotic cells. Collectively, the examples of FACT and Pin1 illustrate mechanisms by which the ability of CK2 to phosphorylate specific proteins can be modulated by interactions with other cellular proteins. Given the large number of protein interactions that have been reported for CK2, it is likely that other proteins will exert similar effects to modulate discrete populations of CK2 in cells.

The isoform-specific interactions of proteins such as CKIP-1 and Pin1 also reveal independent forms of regulation for CK2α as compared to CK2α′, an observation consistent with the demonstration of distinct phenotypes for CK2α and CK2α′ knockout mice (Dominguez et al. 2009). CK2α′ knockout mice are viable but exhibit defects in spermatogenesis in males which results in infertility. By comparison, CK2α knockout mice are embryonic lethal with embryos exhibiting significant defects in heart development. Taken together, these contrasting phenotypes suggest unique functions for both catalytic subunits, though CK2α appears more able to compensate for a loss of CK2α′. Furthermore, it is conceivable that unique regulatory mechanisms of CK2α are responsible for its greater importance to murine viability.

Though some progress has been made regarding the regulation of CK2 via posttranslational modifications and protein/protein interactions, relatively little is known about the transcriptional regulation of CK2. As previously noted, the expression of CK2 is elevated in rapidly dividing cells and a number of human cancers. The precise mechanism by which the expression of CK2 isoforms, apparent housekeeping genes, are upregulated in cancers has not been thoroughly characterized, though a number of putative binding sites for various transcription factors have been identified (Pyerin and Ackermann 2003). For example, it is intriguing that all of the human CK2 genes contain response elements for the Ets1 transcription factor that is regulated by mitogen-activated pathways.

Other factors that may contribute to the regulation of CK2 levels include ubiquitination and degradation by the proteasome (Zhang et al. 2002).

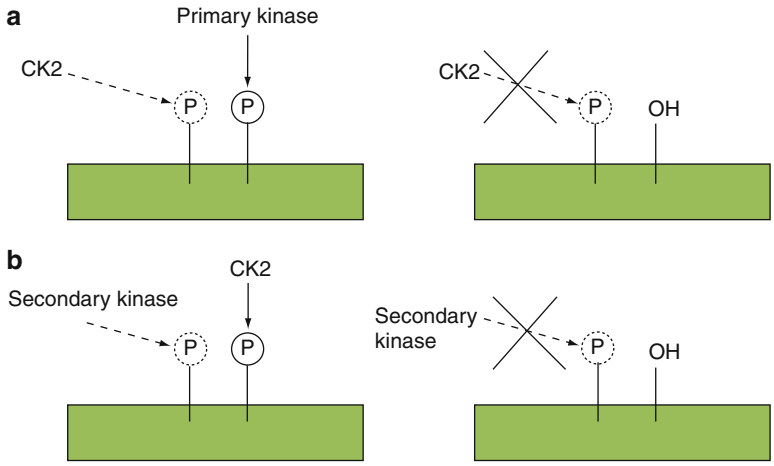
Cellular Functions of CK2

In consideration of its large number of documented substrates (and the ever-expanding list of prospective substrates that are appearing in rapidly populating phosphoproteomic databases), it is not surprising that CK2 has been implicated in a broad range of cellular processes including cell proliferation and survival, apoptosis, circadian rhythms, viral infection, and transcriptional control (Duncan and Litchfield 2008; St-Denis and Litchfield 2009; Mizoguchi et al. 2006). Of particular interest to human disease are the observations that CK2 is over-expressed in a number of human cancers, including kidney, mammary gland, lung, head and neck and prostate, and that targeted over-expression of CK2 in mice T-cells and mammary glands results in lymphoma and mammary adenocarcinomas (Duncan and Litchfield 2008). Furthermore, synergistic effects in tumor formation are observed in mice when over-expressing CK2 in concert with the ▶ Myc or Tal-1 oncogenes or in p53 (−/−) backgrounds (Xu et al. 1999). Conversely, short hairpin RNA (shRNA) knockdown and pharmacological inhibition studies in a variety of model systems results in cell death, reinforcing the notion that targeted inhibition of CK2 represents a promising therapeutic strategy for the treatment of human cancers (Trembley et al. 2009; Ruzzene and Pinna 2010). Though it is clear that global changes in CK2 activity regulate processes pertaining to cellular proliferation and survival, the precise molecular mechanisms by which CK2 exerts these functions are still incompletely understood. In the following discussion, some of the recent advances made in dissecting the specific role of CK2 in these cellular processes will be highlighted.

CK2 has been implicated in many signaling pathways directly involved in controlling the rate of cellular proliferation (Duncan and Litchfield 2008). As noted above, coordinated overexpression of CK2 and Myc in murine T-cells results in lymphoma development. Supporting the pathogenic synergism between CK2 and Myc is the observation that CK2 phosphorylates Myc and prevents its proteasome-dependent degradation, allowing increased transcriptional activity of proliferative and survival genes. Similarly, CK2 is believed to function at many levels within the Wnt signaling pathway, ultimately promoting ▶ Beta-Catenin stability, dissociation from APC, and the transcription of pro-survival and proliferative genes.

In a related vein, evidence for the direct involvement of CK2 in the control of cell cycle progression is continually mounting (St-Denis and Litchfield 2009). Knockout studies in genetically tractable organisms such as yeast highlight a requirement for CK2 in G1/S and G2/M transitions. Similarly, knockdown and pharmacological inhibition of CK2 in mammalian cells results in attenuation of cell cycle progression. A specific role for CK2 in mitosis has also been revealed in mammalian cells, where disruption of mitotic phosphorylation of CK2α leads to multiple mitotic defects, including chromosome missegregation and induction of mitotic catastrophe, a form of cell death. CK2 substrates involved in mitotic progression have also been identified, including the aforementioned Topoisomerase IIα, whose phosphorylation is regulated by mitosis-specific interaction of Pin1 with CK2α, and the cell cycle regulatory protein kinase Wee1. The phosphorylation of Wee1 reveals the intricate relationships between protein kinases since prior phosphorylation of Wee1 by CDK1 generates a consensus phosphorylation motif for CK2, which leads to the degradation of Wee1 and entry into mitosis. Wee1 is a prime example of the participation of CK2 in hierarchical phosphorylation (Fig. 3a). Given its preference for acidic determinants, including phosphoserine and phosphotyrosine, there will undoubtedly be other substrates that are phosphorylated by CK2 only after prior phosphorylation by other protein kinases. It is also noteworthy that CK2 can participate in hierarchical phosphorylation by enabling subsequent phosphorylation by another protein kinase (Fig. 3b). For example, the CK2-catalyzed phosphorylation of glycogen synthase is a prerequisite for its subsequent phosphorylation by ▶ GSK-3. Overall, the participation of CK2 in hierarchical phosphorylation, both as a primary and as a secondary protein kinase, reveals its capacity to participate in complex regulatory events with other protein kinases.

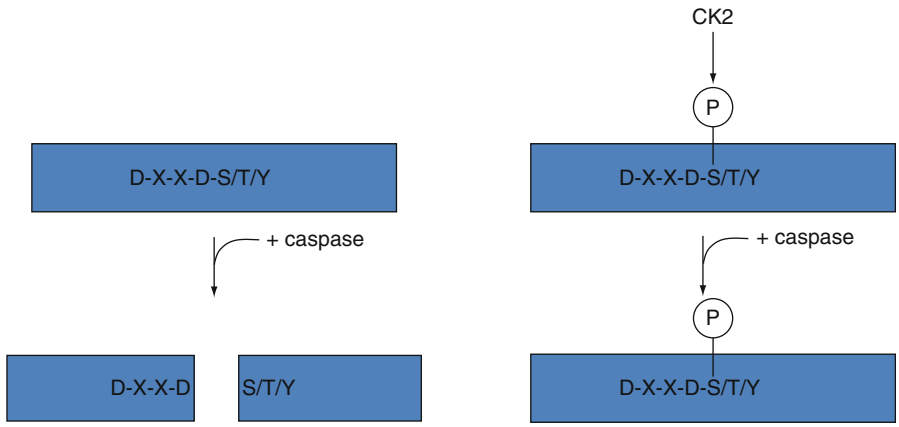
The observation that shRNA-mediated knockdown and pharmacological inhibition of CK2 results in cell death has accelerated the identification of CK2 functions within apoptotic pathways (Duncan and Litchfield 2008). One provocative example involves



Casein Kinase II, Fig. 3 Hierarchical phosphorylation events involving CK2. (**a**) A hypothetical example of CK2 acting as a secondary kinase in a hierarchical phosphorylation event whereby previous phosphorylation of a protein renders it a substrate of CK2. One example of this hierarchal phosphorylation, as discussed in the text, is observed when CDK1 primes Wee1 for phosphorylation by CK2. (**b**) A depiction of CK2 phosphorylating and priming a protein for subsequent phosphorylation by a secondary kinase is shown. This form of regulation is observed, for example, on glycogen synthase, where phosphorylation by CK2 creates a phosphorylation site for GSK-3

the CK2-dependent regulation of PML – a tumor suppressor protein responsible for the formation of PML bodies in the nucleus, which acts to promote senescence and apoptosis (Scaglioni et al. 2006). Interestingly, phosphorylation of PML by CK2 results in its proteasome-mediated degradation. Moreover, CK2 activity and PML protein levels are inversely correlated in non-small cell lung cancers. The fact that PML overexpression induces senescence and apoptosis underscores the potential importance of CK2 in PML regulation and, ultimately, cell survival. A role for the direct regulation of apoptotic machinery by CK2 has also recently been observed (reviewed in Duncan et al. 2010). Bid, a member of the Bcl-2 family of apoptotic signaling molecules, functions to permeabilize the mitochondrial membrane upon cleavage by Caspase-8 and, therefore, promote the activation of downstream caspases. However, phosphorylation of Bid at CK2 phosphorylation sites proximal to the caspase-8 cleavage site blocks processing, mitochondrial permeabilization, and the progression of apoptosis. Notably, the strict requirement for acidic residues in both the caspase recognition site and the CK2 minimal consensus motif may illustrate a more widespread apoptotic regulatory mechanism for CK2 than is currently appreciated (Fig. 4). Along these lines, other substrates with overlapping CK2 and caspase regulation motifs have been identified (including Connexin 45.6, murine Caspase-9, Max, HS-1, ▶ PTEN, and Presenilin-2). In all of these instances, phosphorylation by CK2 confers protection from caspase cleavage. These observations may provide at least a partial explanation for the enhanced survival that is observed in cancer cells with elevated levels of CK2. In this respect, given its constitutive activity, elevated levels of CK2 could result in increased phosphorylation of caspase substrates to attenuate caspase-mediated death pathways.

It is important to reiterate the vast repertoire of cellular functions involving CK2 and the diversity of its substrates. Even before the inception of predictive analyses that probe phospho-proteomic databases for putative CK2 substrates, CK2 was believed to have over 300 substrates, including over 60 transcription factors, 80 signaling molecules, and 40 viral proteins (Meggio and Pinna 2003). However, as a result of the constitutive activity of CK2 and its relatively simple consensus motif determinants, some analyses of phospho-proteomic databases have predicted the number of CK2 substrates to number in the thousands (Pinna and Allende 2009). While the systematic identification of all CK2 substrates remains a daunting task, a more complete evaluation of substrates will undoubtedly uncouple the pathogenic and physiological functions of CK2. Along these lines, an important avenue of future research will involve validating putative substrates. One appealing approach will



Casein Kinase II,
Fig. 4 Modulation of caspase cleavage by phosphorylation. Shown here is an example where phosphorylation within a caspase degradation motif renders a substrate refractory to caspase cleavage

certainly employ bioinformatic consensus motif analyses of phosphoproteomic databases to identify likely physiological targets of CK2, followed by in vitro phosphorylation assays that test the ability of CK2 to directly phosphorylate these sites (Turowec et al. 2010). In this sense, demonstration of direct phosphorylation of putative substrates by CK2 as well as the existence of these phosphorylation sites in cells should be adopted as the gold standard for classifying substrates as bona fide. Furthermore, sorting of biologically relevant CK2 substrates by GO designations will not only act to further clarify the physiological function of CK2, but may streamline the identification of substrates with pathological significance. Likewise, the demonstration that CK2 can participate in complex hierarchical regulatory mechanisms, such as protection of caspase substrates from degradation or acting as a primary or secondary kinase in hierarchical protein phosphorylation, may also be exploited by bioinformatics and/or high-throughput analyses that attempt to expand upon the role of CK2 in signaling pathways pertaining to cellular proliferation (Figs. 3 and 4) (St-Denis and Litchfield 2009; Duncan et al. 2010).

Emergence of CK2 as a Candidate for Molecular-Targeted Therapy

The overexpression of CK2 in a number of human malignancies and its participation in multiple pro-survival signaling pathways has driven the development of pharmacological inhibitors of CK2 for use as candidate lead compounds for molecular-targeted therapy (Sarno and Pinna 2008). Rational development of specific ATP competitive inhibitors using structural information, as well as virtual screening of chemical libraries has led to the identification of a number of CK2 inhibitors with inhibitory constants (Ki) in the micromolar range (Sarno et al. 2005). Preclinical studies of a number of these inhibitors, including DRB, Emodin, TBB, TBBz, DMAT, IQA, and TBCA, resulted in the induction of apoptosis in a number of cancer cells (Duncan and Litchfield 2008; Ruzzene and Pinna 2010). Despite these advances, the challenge of developing ATP competitive specific protein kinase inhibitors is exacerbated by the high degree of conservation exhibited amongst all human protein kinases, and to a lesser extent even other ATP binding proteins (Prudent and Cochet 2009; Prudent et al. 2010). Indeed, off-target effects of CK2 inhibitors are apparent and remain relatively uncharacterized.

To address concerns of off-target effects that result from ATP binding domain conservation, the development of CK2 inhibitors that bind at allosteric sites has recently been pursued (Prudent and Cochet 2009; Prudent et al. 2010). Using high-throughput screening of chemical libraries, polyoxometalates have been identified as a class of compounds that bind outside of both the ATP binding domain and the CK2α/β interface, and may function by docking in the vicinity of the activation loop and locking it in the closed confirmation. Another intriguing mode of modulating CK2 function is to regulate tetramer formation by blocking the ability of CK2β to bind the catalytic subunits. Preventing holoenzyme formation is predicted to decrease the cellular half-life of CK2 due to the relative instability of

individual subunits. Furthermore, it should also reduce catalytic activity by three- to fivefold, as CK2β has a generally stimulatory effect toward catalytic activity, but may also stimulate phosphorylation of substrates that are negatively regulated by CK2β. While the physiological consequences of inhibiting tetramer formation remain unknown, the prospect that it could function to block CK2 functions related to cellular proliferation and apoptosis remain plausible.

One limiting aspect pertaining to the characterization of CK2 inhibitors as being effective cellular pharmacological agents is the lack of bona fide biomarkers. As noted earlier, the preclinical utility of CK2 inhibitors to date has generally been measured by gross phenotypic changes, such as apoptosis, as opposed to a decrease in phosphorylation of a particular biomarker (Ruzzene and Pinna 2010). By developing tools that directly monitor the phosphorylation status of specific cellular substrates, the distinction between off-target effects and specific CK2 inhibition would be more easily clarified as biomarker status could be correlated to gross phenotypic changes. Furthermore, the use of phosphospecific antibodies, for example, as markers for CK2 activity would also be useful in identifying pathological samples that exhibit hyperactive CK2 activity and thus, are most likely to respond to treatment with CK2 inhibitors.

Summary

Since its discovery in 1954, much has been learned regarding the enzymatic characteristics, physiological regulation, and cellular functions of protein kinase CK2. Structural studies have yielded many insights into the unique features of CK2, such as its constitutive activity and ability to use GTP as a phosphate donor. While CK2 appears to be constitutively active, it is likely that a number of distinct mechanisms such as spatial regulation through protein interactions and possibly the regulated assembly or disassembly of tetrameric CK2 will govern how the phosphorylation of many of its substrates is controlled in cells. However, on the whole, many details regarding the precise manner by which CK2 is regulated in cells remain to be defined. Though the expanding list of CK2 substrates has illuminated many cellular functions, including roles pertaining to cell survival, proliferation, and involvement in diseases such as cancer, it is clear that CK2 has many undefined substrates whose phosphorylation status dictates unknown roles. In this sense, future endeavors should involve the use of phosphoproteomic databases, which contain a tremendous resource of physiological protein phosphorylation sites, to guide the identification of bona fide CK2 substrates. In expanding our knowledge of CK2 substrates, it is more likely that the physiological and pathogenic functions of CK2 will be uncoupled and that the generation of convenient biomarkers capable of monitoring CK2 activity in cells will be established. Tools such as these will greatly accelerate the development and benchmarking of current and future generations of inhibitors, which in turn will feedback and drive the discovery of CK2 functions and benefit the treatment of cancers where CK2 hyperactivity may be an oncogenic driver.

References

Allende JE, Allende CC. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. FASEB J. 1995;9:313–23.

Bibby AC, Litchfield DW. The multiple personalities of the regulatory subunit of protein kinase CK2: CK2 dependent and CK2 independent roles reveal a secret identity for CK2beta. Int J Biol Sci. 2005;1:67–79.

Canton DA, Litchfield DW. The shape of things to come: an emerging role for protein kinase CK2 in the regulation of cell morphology and the cytoskeleton. Cell Signal. 2006; 18:267–75.

Dominguez I, Sonenshein GE, Seldin DC. Protein kinase CK2 in health and disease: CK2 and its role in Wnt and NF-kappaB signaling: linking development and cancer. Cell Mol Life Sci. 2009;66:1850–7.

Duncan JS, Litchfield DW. Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. Biochim Biophys Acta. 2008;1784:33–47.

Duncan JS, Turowec JP, Vilk G, Li SS, Gloor GB, Litchfield DW. Regulation of cell proliferation and survival: convergence of protein kinases and caspases. Biochim Biophys Acta. 2010;1804:505–10.

Filhol O, Cochet C. Protein kinase CK2 in health and disease: cellular functions of protein kinase CK2: a dynamic affair. Cell Mol Life Sci. 2009;66:1830–9.

Litchfield DW. Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. Biochem J. 2003;369:1–15.

Meggio F, Pinna LA. One-thousand-and-one substrates of protein kinase CK2? FASEB J. 2003;17:349–68.

Mizoguchi T, Putterill J, Ohkoshi Y. Kinase and phosphatase: the cog and spring of the circadian clock. Int Rev Cytol. 2006;250:47–72.

Niefind K, Guerra B, Ermakowa I, Issinger O-G. Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. EMBO J. 2001;20:5320–31.
Niefind K, Raaf J, Issinger OG. Protein kinase CK2 in health and disease: protein kinase CK2: from structures to insights. Cell Mol Life Sci. 2009;66:1800–16.
Olsten ME, Litchfield DW. Order or chaos? An evaluation of the regulation of protein kinase CK2. Biochem Cell Biol. 2004;82:681–93.
Pinna LA, Allende JE. Protein kinase CK2 in health and disease: protein kinase CK2: an ugly duckling in the kinome pond. Cell Mol Life Sci. 2009;66:1795–9.
Prudent R, Cochet C. New protein kinase CK2 inhibitors: jumping out of the catalytic box. Chem Biol. 2009;16:112–20.
Prudent R, Sautel CF, Cochet C. Structure-based discovery of small molecules targeting different surfaces of protein-kinase CK2. Biochim Biophys Acta. 2010;1804:493–8.
Pyerin W, Ackermann K. The genes encoding human protein kinase CK2 and their functional links. Prog Nucleic Acid Res Mol Biol. 2003;74:239–73.
Ruzzene M, Pinna LA. Addiction to protein kinase CK2: a common denominator of diverse cancer cells? Biochim Biophys Acta. 2010;1804:499–504.
Sarno S, Pinna LA. Protein kinase CK2 as a druggable target. Mol Biosyst. 2008;4:889–94.
Sarno S, Salvi M, Battistutta R, Zanotti G, Pinna LA. Features and potentials of ATP-site directed CK2 inhibitors. Biochim Biophys Acta. 2005;1754:263–70.
Scaglioni PP, Yung TM, Cai LF, Erdjument-Bromage H, Kaufman AJ, Singh B, et al. A CK2-dependent mechanism for degradation of the PML tumor suppressor. Cell. 2006;126:269–83.
St-Denis NA, Litchfield DW. Protein kinase CK2 in health and disease: from birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. Cell Mol Life Sci. 2009;66:1817–29.
Trembley JH, Wang G, Unger G, Slaton J, Ahmed K. Protein kinase CK2 in health and disease: CK2: a key player in cancer biology. Cell Mol Life Sci. 2009;66:1858–67.
Turowec JP, Duncan JS, French AC, Gyenis L, St-Denis NA, Vilk G, Litchfield DW. Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in cells. Method Enzymol. 2010;484:471–93.
Xu X, Landesman-Bollag E, Channavajhala PL, Seldin DC. Murine protein kinase CK2: gene and oncogene. Mol Cell Biochem. 1999;191:65–74.
Zhang C, Vilk G, Canton DA, Litchfield DW. Phosphorylation regulates the stability of the CK2beta subunit. Oncogene. 2002;23:3754–64.

Casein Kinase-I

▶ Glycogen Synthase Kinase-3

Caspase Family

Dave Boucher and Jean-Bernard Denault
Department of pharmacology, Université de Sherbrooke, Sherbrooke, QC, Canada

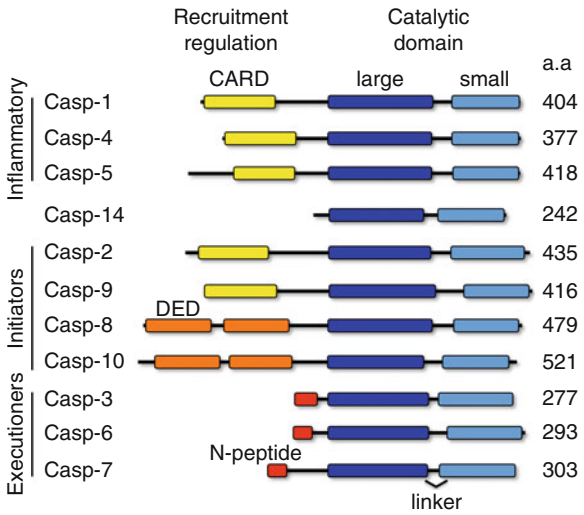
Historical Background

The importance of proteases in cell signaling is well established. This chapter presents the protease family of caspases, which performs limited proteolysis on a wide range of substrates, with molecular consequences ranging from inactivation to gain-of-function to accelerated degradation. It is important to emphasize that caspases are signaling proteases and not degrading enzymes, akin to lysosomal cathepsins or digestive enzymes. The first family member, caspase 1, was identified in 1989 (Black et al. 1989). Caspases are principally implicated in inflammation and apoptosis.

The term "programmed cell death" (PCD) was coined in 1964 by Lockshin and coworkers and originally designated the breakdown of tissues during development, especially in insects. Later, in 1972, Ker and colleagues described apoptosis for the first time. However, both concepts (PCD and apoptosis) are now considered synonymous. The first genetic evidence of "programming" in apoptosis came with works by H. Robert Horvitz's group in 1986. They reported the identification of genes required for the execution of cell death in the roundworm *Caenorhabditis elegans*. Finally, it took the work of Black and coworkers, who identified the mammalian cysteine protease caspase 1 as responsible for the maturation of pro-IL (interleukin)-1β, to realize that one of the genes found by Horvitz's group, *CED-3* (*C. elegans* cell death), encoded a cysteine protease homologous to caspase 1. These groundbreaking works paved the way for the identification of caspases responsible for apoptosis in mammals and other family members with roles in inflammation and keratinization of epithelia.

Caspases

Caspase stands for *c*ysteine *asp*artyl-specific prote*ase*. They are members of clan CD peptidases, i.e., with



Caspase Family, Fig. 1 *Schematic representation of the human caspase family*. Human caspases are grouped according to their main in vivo role: cytokine activators (inflammatory), initiator, and executioner apoptotic caspases. The five known domains of caspases are color coded: CARD (*yellow*) and DED (*orange*) are involved in recruitment, regulation, and activation; the N-peptide of executioner caspases (*red*) that is removed during apoptosis; the large subunit (*blue*), and small subunit (*light blue*) forming the catalytic domain are separated by a linker of variable length. Caspases are drawn to scale and the number of amino acids of the longest known isoform of each protein is shown on the right

a cysteine nucleophile and catalytic residue in the order His-Cys in the protein sequence. They form the C14 family of cytosolic cysteine peptidases, which is defined by strict specificity for the hydrolysis of aspartyl bonds. Their structure is highly conserved and composed of two domains: the N-terminal domain and the catalytic domain (Fig. 1). The catalytic domain, or unit, is defined as having one large and one small subunit separated by a flexible interdomain connector of variable lengths (hereafter referred to as the linker). This segment is sensitive to proteolysis and is always cleaved during activation. Cleavage of the catalytic domain gives rise to fragments often referred to as p20/p10. The catalytic unit is structured by 6 β-strands and 5 α-helices assembled in an open α/β barrel fold (Fig. 2a). The large subunit contains the catalytic dyad His-Cys that controls the hydrolysis of the peptide bond. The small subunit possesses most residues that form the substrate binding pocket, including a critical arginine, a residue involved in aspartate recognition by the substrate binding pocket. Based on the available literature, catalysis follows the same steps as serine and other cysteine peptidases. Fuentes-Prior and Salvesen have described in great depth the topology of the substrate binding site and catalytic cycle of caspases (Fuentes-Prior and Salvesen 2004a). Due to space limitations, we will not discuss it further. Importantly, active caspases are obligate dimers, and this feature is at the root of the activation mechanism.

Caspases are classified in three main categories based on their functions (Fig. 1): apoptotic caspases (caspases 2, 3, 6, 7, 8, 9, and 10), inflammatory caspases (caspases 1, 4, and 5), and caspase 14 which is involved in epithelial differentiation. However, accumulating evidence has clearly demonstrated roles of some caspases outside their traditional realm (Yi and Yuan 2009).

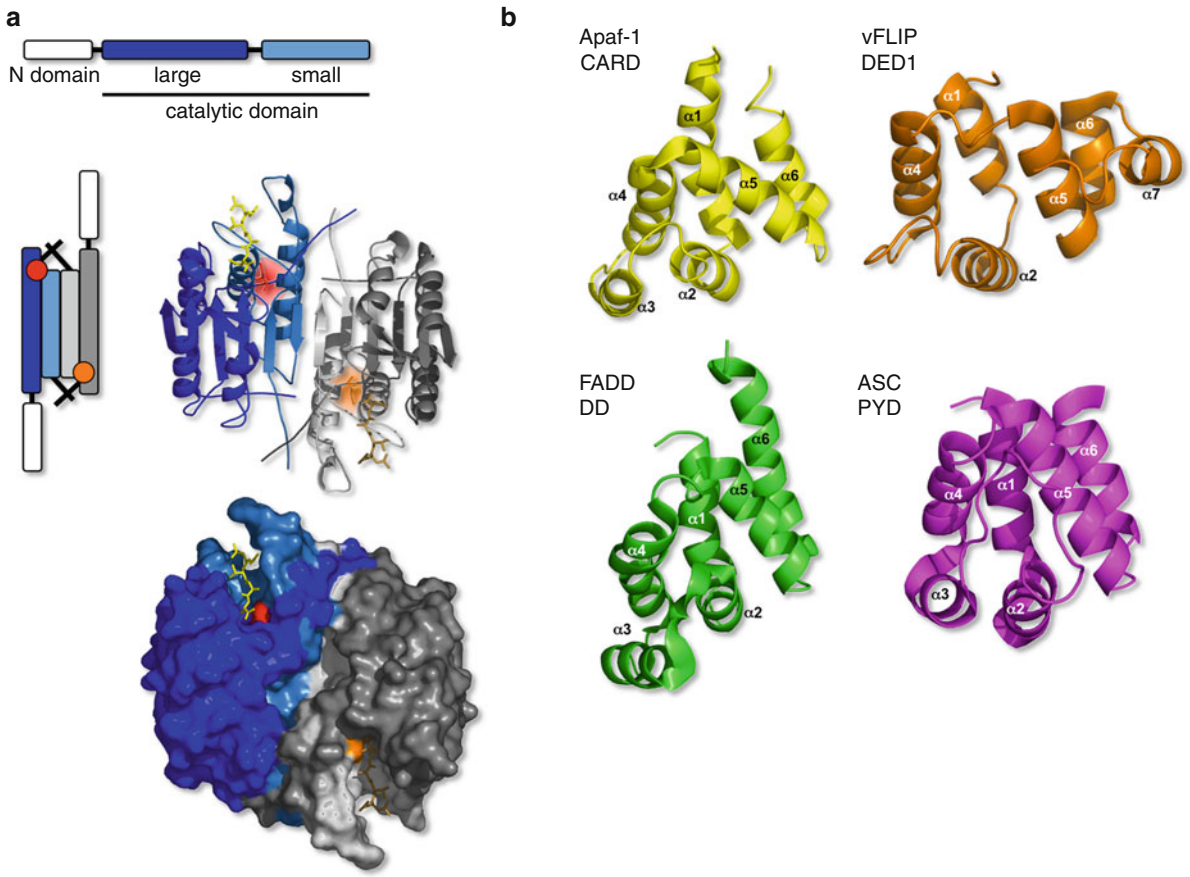
Inflammatory Caspases

In humans, three inflammatory caspases (caspases 1, 4, and 5) coexist, caspase 1 being the prototypical member of this group. The similar domain organization and the fact that their genes are all located on chromosome 11 suggest a rather recent evolution. These caspases are expressed in most, if not all, tissues, but mRNA levels are higher in inflammatory cells, such as monocytes (Martinon and Tschopp 2007).

Inflammasomes

In healthy cells, inflammatory caspases rest as cytosolic monomeric inactive enzymes. It is through recruitment by their N-terminal CARD (caspase-recruitment domain) to multimeric platforms named inflammasomes that they gain activity. Similar to other homotypic interaction domains, DD (death domain) and DED (death effector domain), CARD is a member of the death domain superfamily, and all of its members share a similar fold (Fig. 2b) (Park et al. 2007). The function of an inflammasome is to bring enough caspase molecules in close proximity to provoke their dimerization and activation [the so-called induced-proximity model (Salvesen and Dixit 1999)]. Subsequently, cleavage of the linker occurs, which further stabilizes the dimer.

Inflammasomes are multimeric complexes composed of several different proteins (Schroder and Tschopp 2010). At their core, they contain a NLR (NOD [nucleotide-binding oligomerization domain]-like receptor): a NOD protein, a (NOD)-like



Caspase Family, Fig. 2 *Caspase domain structures.* (**a**) General structure of caspases. Caspase structure is highly conserved and composed of two domains: the N-terminal domain and the catalytic domain. The catalytic domain (*red*/*orange*), or unit, is defined as having one large (*blue*/*gray*) and one small subunit (*light blue*/*gray*) separated by a flexible linker of variable lengths. This segment is sensitive to proteolysis and is always cleaved during activation. Cleavage of the catalytic domain gives rise to fragments often referred to as p20/p10. The catalytic unit is structured by six β-strands and five α-helices assembled in an open α/β barrel fold. The large subunit contains the catalytic dyad His-Cys that controls the hydrolysis of the peptide bond. The cleaved linker interacts with the other end of the other catalytic unit and stabilizes the active site. Active caspase 3: PDB 1CP3. (**b**) CARD, DED, DD, and PYD share similar fold of the death domain family. *CARD* caspase-recruitment domain, *DED* death effector domain, *DD* death domain, *PYD* pyrin domain are members of the DD superfamily, all members share a globular helicoidal structure made of six antiparallel α-helices. Homophylic interactions between each domain are dictated by specific residues. Apaf-1 CARD: PDB 1C15; vFLIP DED1: PDB 2F1S; FADD DD: PDB 1E3Y; ASC PYD: PDB 1UCP

receptor protein (NLRP) family member or an Ipaf (ICE [IL-1β converting enzyme]-protease activating factor) protein. The complex also includes ASC (apoptosis-associated speck-like protein containing a CARD) and caspase 1 (Bryant and Fitzgerald 2009). Caspase 5, but not caspase 4, has also been reported as part of inflammasomes (Martinon et al. 2002). In humans, 22 NLRs have been identified; thus, at least as many different inflammasomes may exist (Schroder and Tschopp 2010). With the exception of NLRP10, all NLRs are composed of an LRR (leucine rich-repeat), a central NOD, and one to three variable homotypic protein-protein interaction domains involved in protein recruitment. LRRs recognize a wide range of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), muramyl dipeptide, lethal toxin, crystalline factors, and endogenous damaged-associated patterns (DAMPs). By a mechanism that is still poorly understood, a specific LRR recognizes a particular PAMP or DAMP, a macromolecular platform assembled via the NOD, and ASC is recruited. Subsequently, caspase 1

monomers join the complex and dimerize (Bryant and Fitzgerald 2009). Despite little information, the current view is that the many NLRs exist to detect various bacterial, viral, or other chemical entities, and most, if not all, lead to caspase 1 activation. To add to the complexity of multiple NLRs, several decoy CARD-only proteins (COPs) have been described that can compete with caspases at the activation platforms (Stehlik and Dorfleutner 2007).

Like caspase 1, caspase 4 activation requires its dimerization and cleavage of its linker domain. By analogy, caspase 5 activation likely resembles that of caspases 1 and 4, but further studies are needed to clarify this point.

Inflammatory Caspases Functions

Active caspase 1 cleaves cytosolic cytokine precursors to their mature form. Based on small peptidic substrate libraries, caspase 1 prefers (W/Y)XXD↓ motifs (Thornberry et al. 1997) with a small residue in P1', a motif that is found in caspases 1, 4, and 5 and in pro-IL-1β. Pro-IL-1β and pro-IL-18 are the only caspase 1 inflammatory mediators identified to date. Recent proteomic data suggest that caspase 1 is responsible for the bulk of cleavage events versus other inflammatory caspases (Agard et al. 2010). Caspase 4 cleaves caspase 3, IL-1β, and IL-1F7B (Luthi and Martin 2007). The transcription factor Max is a substrate of caspase 5, and cleavage occurs at an unconventional glutamate residue instead of aspartate (Luthi and Martin 2007).

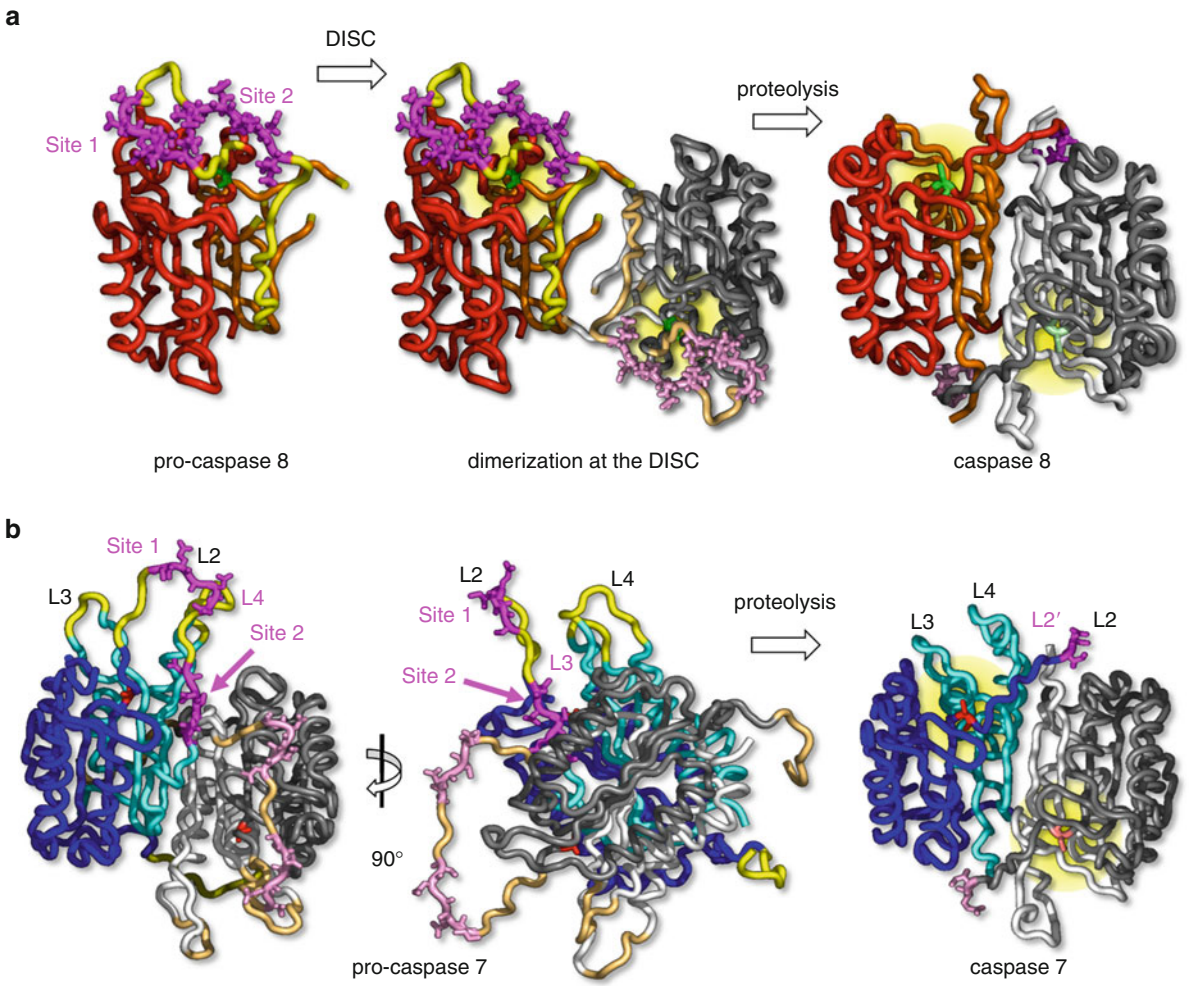
Other substrates of caspase 1 include caspase 7 and PARP (poly[ADP-ribose] polymerase), which are cleaved during pyroptosis (Lamkanfi 2011). This process is a peculiar form of cell death related to inflammation and macrophage death, thus providing a link between inflammation and part of the apoptotic machinery (Lamkanfi 2011).

Apoptotic Caspases: The Initiators

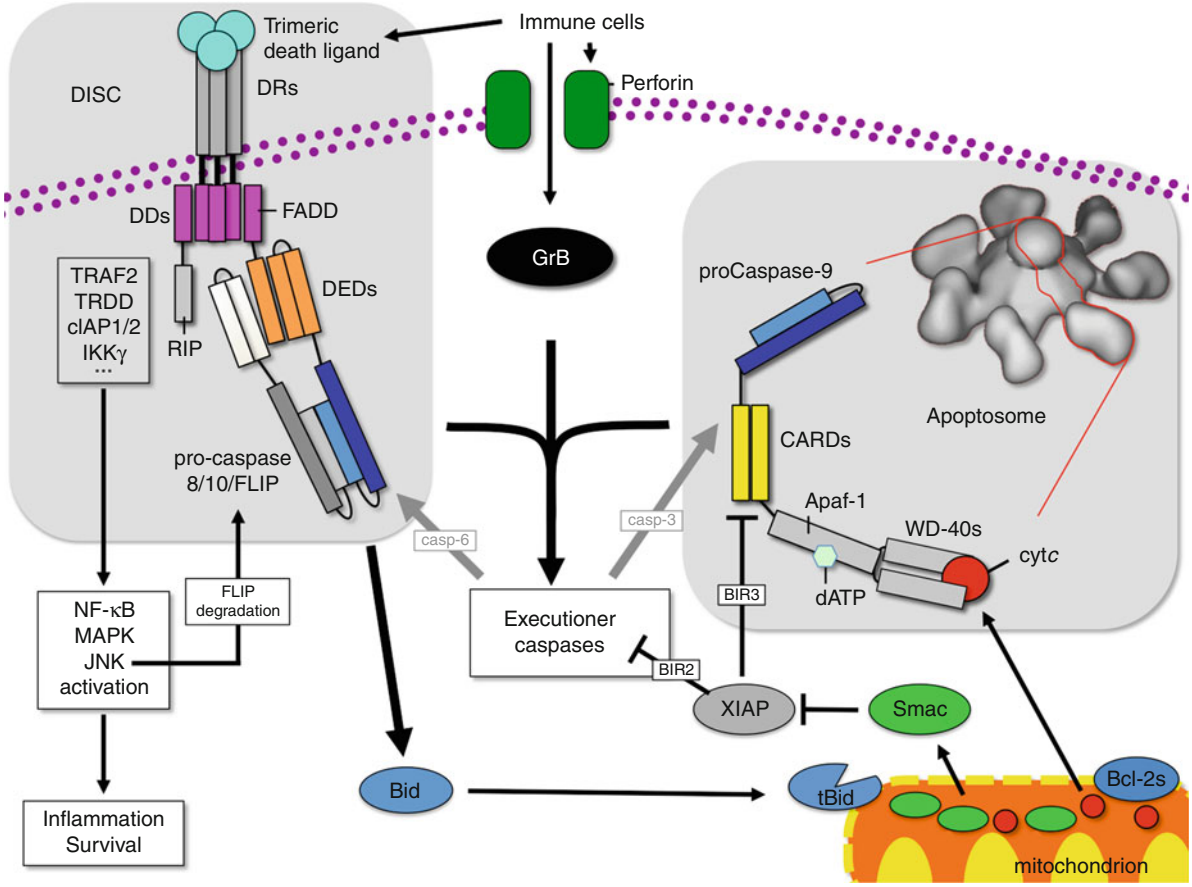
Apoptotic caspases are expressed in all tissues and cell types. They are integrators of apoptotic stimuli, and their activity is required for the activation of executioner caspases, their main substrates (Fig. 4). Like inflammatory caspases, initiators possess a protein-protein interaction domain at the N-terminus (Fig. 1), and they are activated by dimerization on macromolecular platforms (Mace and Riedl 2010a) (Fig. 3a). For a long time, cleavage of initiators has been viewed as synonymous to activation. However, the picture is far more complex than this model. Cleavage of the linker, which gives rise to the characteristic large and small subunits, always occurs during apoptosis. However, in a reconstituted system, uncleavable caspase 9 sustains caspase activation to the same extent as the fully cleaved protease. In vitro proteolysis is not necessary for initiator activation (Boatright and Salvesen 2003). Consequently, cleavage of an initiator caspase does not equate to activation. However, recent work has shown that cleavage is necessary for caspase 8-driven apoptosis *in cellulo* (Oberst et al. 2010). Therefore, cleavage seems to play a role not in activation per se, but rather in modulating the activity of the active caspase. Interestingly, executioner caspase 6 can cleave caspase 8, and caspase 3 cleaves caspase 9. The role of cleavage of caspase 9 by caspase 3 is to relieve it from the inhibition set by the endogenous XIAP (X-linked IAP [inhibitor of apoptosis protein]) caspase inhibitor. In contrast, cleavage of caspase 8 produces a more stable caspase dimer (Pop et al. 2007). Because mice do not have caspase 10, little attention has been paid to this caspase in humans. Nevertheless, it is known that, similar to other initiators, this caspase is activated by dimerization (Wachmann et al. 2010). Importantly, unprocessed caspase 10 possesses high activity on Bid (BH3 interacting domain death agonist), but efficient cleavage of its other targets requires linker proteolysis (Wachmann et al. 2010). Initiator caspases activate two major intracellular pathways to drive apoptosis: the extrinsic and the intrinsic pathways.

The Extrinsic Pathway

The extrinsic pathway (Fig. 4) drives the activation of caspases 8 and 10. It is initiated by ligation of preassembled trimeric death receptors by their cognate ligand (Guicciardi and Gores 2009). These ligands are membrane bound in their more potent form and are normally produced by cytotoxic lymphocytes and natural killer cells, which play a crucial role in immunosurveillance against viral infection and cancer. Based on X-ray crystallography, ligation of death receptors would enable the opening of the cytoplasmic region of the receptor and exposed DD. This would signal the recruitment of the adaptor FADD (Fas [fibroblast associated]-associated with death domain)



Caspase Family, Fig. 3 *Conformational changes during caspase activation.* (**a**) Initiator caspase activation: caspase 8. The NMR structure of pro-caspase 8 catalytic domain shows a monomeric (*left*) entity and has the general fold of an active catalytic unit (*right*). However, the dimer interface provided by the small subunit (*orange*) is disorganized. Interestingly, and contrary to executioner pro-caspase 7, the rather long linker is structured and sits over the unformed catalytic domain where it contacts several residues from loop L1. Recruitment at the DISC brings molecules in close proximity and caspase 8 dimerizes. The activity gained by this process allows cleavage of caspase 8, which further stabilizes the active form. However, it is not clear whether proteolysis occurs in *cis* (self-cleavage) or *trans* (cleavage by a neighboring molecule). Zymogen: PDB 2K7Z; active caspase 8: PDB 1QDU. (**b**) Executioner caspase activation: caspase 7. The zymogen (*left* and *middle*) and the active form (*right*) of caspase 7 adopt a similar fold, a globular arrangement of two catalytic units (*one blue*, *one gray*), each of which is composed of two subunits, the large and small (*dark* and *light colors*). The main differences between them involve three flexible loops (L2–L4, *yellow*) surrounding the unformed catalytic site and substrate binding pocket of the zymogen. The transition to the active form is driven by proteolysis at one of the two cleavage sites (*magenta*) found in the L2 loop. It is noteworthy that in the zymogen dimer, L2 loops adopt different conformation. The L3 loop, which is implicated in the formation of substrate binding pocket, is reoriented following cleavage of the caspase linker, allowing the formation of the substrate binding pocket in the mature form. The L4 loop turns 60° toward the opposite direction and flattens the catalytic groove. Together with loop L2, loop L4 forms a loop bundle, a structure implicated in the stabilization of the active site in the active form of caspase 7. The L2 loop changes drastically upon its cleavage and interacts with the same loop (L2') of the other catalytic unit and stabilizes the active site by crucial interactions. The cleavage of L2 loops leads to the reorientation of the catalytic cysteine in a solvent accessible conformation and enables the catalytic activity of caspases. Zymogen: PDB 1GQF; active caspase 7: PDB 1F1J



Caspase Family, Fig. 4 *Apoptotic pathways*. There are two fundamental pathways leading to caspase activation. The intrinsic pathway depends on the cell's internal control mechanisms, which are integrated at the mitochondrion by Bcl-2 family members (*blue ovals*). If proapoptotic members dominate, the mitochondrion then releases, among others, cyt*c* (*red*) and Smac. With dATP (*lime*), cyt*c* causes Apaf-1 protein oligomerization into an heptameric structure, the apoptosome, that regroups caspase-recruitment domains (CARD; *yellow*) at its apex. This allows the gathering of initiator caspase 9 by virtue of its CARD and its activation by dimerization. Smac (*light green*), which is co-released along with cyt*c*, relieves the inhibition of caspases by XIAP (BIR2 domain inhibits caspase 3 and 7 whereas BIR3 inhibits dimerization of caspase 9) and amplifies the signal initiated by cyt*c*. The second pathway, called extrinsic, originates from outside the cell and requires the ligation of death receptors (DRs) such as Trail-Rs, CD95/Fas, or TNF-Rs. Their ligands are produced by natural killer cells and cytotoxic T lymphocytes. Their activation provokes the assembly of a death-inducing signaling complex (DISC) that will recruit the adaptor protein FADD and initiator caspase 8 and 10 (and also FLIP) by virtue of DEDs (*orange*) and DDs (*magenta*) homotypic interactions. The high concentration of initiator caspases recruited at the DISC allows their activation through dimerization, which can be prevented by excess FLIP. Once active, the initiators cleave executioner caspases 3 and 7 (Fig. 5) to activate them. Depending of the DR, the DISC has the possibility to activate inflammatory and survival pathways through the recruitment of various proteins. The Bcl-2 family member Bid is proteolyzed by initiator caspase 8 and 10 to give tBid (truncated Bid), which provides a way for the extrinsic pathway to activate the intrinsic pathway. Some immune cells can inject in the cytosol of doomed cells using perforin pores the serine protease granzyme B (GrB) complementing the extrinsic pathway. GrB directly activate executioner caspases. Finally, some regulating steps are provided by executioner caspases through their ability to cleave the initiator caspases (*gray arrows*), although these events do not activate the initiators

by homophilic interactions between the newly exposed DD and FADD's DD. On the contrary, NMR (nuclear magnetic resonance) studies have proposed a model that does not involve structural opening, but instead the sandwiching of Fas and FADD's DDs into a pentameric organization of Fas/FADD heterodimer. Interestingly, this latter arrangement is similar to the one observed for the proposed caspase 2 activation platform (Hymowitz and Dixit 2010). No matter which model prevails, both highlight the importance

of receptor clustering for death-inducing signaling complex (DISC) formation. The other domain found on FADD is a DED that is also present in initiator caspases 8 and 10. This four-component complex, ligand-receptor-adaptor-caspase, constitutes the basis of the extrinsic pathway. DISC composition and signaling varies greatly depending on the initiating ligand-receptor pair (reviewed in Guicciardi and Gores 2009a). All assemblies use FADD adaptor protein to link the death receptor to caspase 8, but the tumor necrosis factor (TNF) receptor type 1-associated DD (TRADD) is also used as an adaptor by TNFα receptor.

Bound receptors cluster in lipid rafts and are internalized. Subsequently, the cytosolic side of the DISC becomes a micro-domain of DED and recruits caspases 8 and 10. The locally high concentration of initiator caspase molecules causes its dimerization through induced proximity (Salvesen and Dixit 1999), activation and cleavage of the caspase. A layer of regulation is provided by cFLIP (cellular FLICE [full-length ICE] inhibitory protein) (Thome et al. 1997). The long form of FLIP ($FLIP_L$) is a catalytically disabled caspase 8-like protein and competes with initiator caspases at the DISC at high level of expression, thus damping the death signal. Alternatively, low levels of $cFLIP_L$ can serve as a caspase 8 dimer partner and support caspase activation. In general, death signaling is strong during or after internalization, but weak when receptors are at the plasma membrane, where likely all of them generate pro-survival signals through the NFκB (nuclear factor κB) or mitogen-activated protein (MAP) pathways (Varfolomeev et al. 2005). A key mediator of the pro-survival effect is the kinase RIP1 (receptor-interacting serine/threonine-protein 1) that binds to all death receptors and FADD using its own DD. Post-translational modifications, such as phosphorylation, proteolytic cleavage by caspase 8, and ubiquitination, dictate the various effects of this kinase (Cho et al. 2009). Some particularities of each complex are briefly summarized below (reviewed in (Guicciardi and Gores 2009a)).

The Fas/CD95 DISC

Fas/CD95 (fibroblast associated) was discovered as the antigen to an antibody recognizing T lymphocytes and B cells (Oehm et al. 1992) and is considered a tumor suppressor. Compared to TNF-R1 (TNF receptor 1) and to a lesser extent Trail-R1/2 (TNF-related apoptosis-inducing ligand receptor 1/2; see ▶ APO2L/TRAIL), Fas does not display strong pro-survival effects unless its proapoptotic signaling is masked. For example, tumor cells with disabled Fas-mediated apoptosis will activate the pro-survival NFκB and MAP kinase pathways (Shikama et al. 2003; Barnhart et al. 2004). In non-pathological conditions, Fas signaling via extracellular signal-regulated kinase (ERK) is critical for neuronal regeneration, stellate cell proliferation after hepatic injury, and is required for activated T-cell and thymocyte proliferation. However, despite these few examples, Fas is essentially a death receptor.

The mechanism by which Fas is activated is the best understood among death receptors. After ligation by FasL (Fas ligand), the adaptor FADD is recruited, and the receptor gets palmitoylated, which constitutes the signal for receptor clustering in the lipid raft. Subsequently, Fas is internalized in endosomes in a clathrin-dependent manner, where it continues to recruit and promote caspase 8 and 10 activation. In some cells (so-called type II cells, e.g., hepatocytes, pancreatic β-cells), direct Fas-driven caspase activation is insufficient to cause apoptosis. Consequently, the intrinsic pathway also contributes via the cleavage of Bid by caspases 8 and 10 (Wachmann et al. 2010), which will engage the mitochondrion to release cytochrome *c* (cyt*c*). The discriminating difference between type I and II cells is the level of XIAP, which needs to be overcome for apoptosis to proceed (Jost et al. 2009). Although this dichotomy was initially described for Fas, it also applies to the other death receptors.

The TNFα DISC

The TNF receptor system is comprised of two receptors, TNF-R1 and TNF-R2, but only TNF-R1 is considered a true death receptor because TNF-R2 does not have a DD. Three ligands bind TNF-R1: the membrane bound and soluble form of TNFα and TNFβ, also known as the lymphocyte-derived cytokine α (LTα). However, the focus will be on TNFα signaling because it is the most studied. TNFα is produced by many immune cell types, hepatocytes, and fibroblasts, and plays a crucial role in inflammation, proliferation, and differentiation. The complex formed after TNFα ligation to TNF-R1 is peculiar in that its primary signaling is pro-survival (complex I) rather than apoptotic. It involves the recruitment of RIP1, TRAF2

(TNF receptor-associated factor 2), cIAP1/2 (cellular inhibitors of apoptosis protein 1/2), and maybe TRADD, although the presence of the latter is controversial. This complex also includes IKKγ (inhibitors of κB kinase)/NEMO (NFκB essential modulator). The association of RIP1 and TRAF2 activates two signaling routes. First, it promotes differentiation, proliferation, and inflammation via NFκB activation (Wajant and Scheurich 2001), and second, it stimulates JNK (c-Jun N-terminal kinase) activity. Together, both routes counteract each other because, despite the fact that at a low level of activity the JNK pro-death signal is counteracted by NFκB activation, sustained JNK activity leads to the activation of caspases 8 and 10, and apoptosis.

The pro-death DISC efficiently assembles only after internalization of the receptor, following which FADD and both initiators are recruited to promote cell death. Until now, there is no specific event that switches signaling by TNF-R1 from pro-survival to apoptosis. Instead, the build-up of pro-survival signaling before pro-death signaling strikes the balance between life and death by TNFα signaling.

The Trail-R1/2 DISC

Signaling via Trail-R1/2 seems to follow a mechanism similar to the one used by Fas to activate initiator caspases 8 and 10. However, internalization is dispensable for efficient apoptosis induction in type I cells but seems necessary for type II cells (Guicciardi and Gores 2009a). Trail can also initiate pro-survival signaling by a secondary complex, which is initiated at the receptor but works independently afterward. It contains FADD and initiators, but this complex does not support caspase activation. The complex also contains the kinase RIP1, TRAF2, the adaptor TRADD, and IKKγ/NEMO in a manner reminiscent of TNF-R1 signaling. Furthermore, this complex can lead to MAP kinase pathway activation. The role of this complex is not clear because it antagonizes the death pathway, but it may explain why many cancer cells retain Trail receptor expression.

Caspases 8 and 10 Substrates

Initiator caspases 8 and 10 have few substrates. They cleave themselves at the DISC and proteolyze executioner pro-caspases 3 and 7 and Bid (Luthi and Martin 2007). Because both caspases are recruited to the DISC, it is not surprising that some substrates are part of this complex. Indeed, initiators cleave proteins that are part of pro-survival signaling assemblies with a net result of promoting death. For example, caspase 8 proteolyzes RIP1, shifting TNFα signaling toward cell death. TRAF1, which participates with TRAF2 in NFκB signaling, is cleaved by caspase 8. It was shown that the C-terminal fragment of TRAF1 can displace TRAF2 from a TNF-R/TRADD complex and sensitize cells to apoptosis. Consequently, cleavage of TRAF1 would have the same functional role as RIP1 proteolysis.

$FLIP_L$ is cleaved by caspase 8. Its ability to compete with initiators at the DISC, to heterodimerize and activate caspases 8 and 10 (Yu et al. 2009) and even alter caspase 8 substrate preference make it a key player in DISC signaling. To illustrate this, recent work by Oberst and colleagues showed that uncleaved caspase 8-$FLIP_L$ heterodimer abrogates RIP kinase 3-dependent necrosis during development without inducing apoptosis (Oberst et al. 2011), suggesting a different activity of this dimer compared to the cleaved version. Taken together, both the protein composition of the DISC and the specific form of each of its constituents is crucial in determining the substrate repertoire of caspases 8 and 10, and therefore, the cell's fate.

Aside from DISC components, extrinsic initiators cleave few other proteins. HDAC7, a class II histone deacetylase that represses gene transcription, is cleaved by caspase 8, which changes its subcellular localization and its ability to repress gene transcription.

Regulators of Caspase 8/10

Few other caspase 8- and 10-activating platforms have been proposed in recent years. One of them promotes limited activation of caspase 8 and is controlled by the paracaspase MALT1 (mucosa-associated lymphoid tissue; see ▶ MALT1) during lymphocyte proliferation (Kawadler et al. 2008). Independently of its protease domain, MALT1 can associate with caspase 8 and direct its activation. However, the activity of caspase 8 in this complex seems limited to FLIP.

Several proteins regulate extrinsic initiator caspases, among them kinases such as src (sarcoma) and ERK1/2 (Kurokawa and Kornbluth 2009). The specifics of each phosphorylation event will not be discussed here, but the general effect of phosphorylation is to inhibit activity or activation.

The Intrinsic Pathway

Caspase 9 drives an intracellular apoptotic pathway called the intrinsic/mitochondrial pathway (Fig. 4). This route is used in response to various stresses and developmental cues. Indeed, the critical role of caspase 9 during mouse development is exemplified by the fact that knockout mice die perinatally and show severe brain malformation. The intrinsic pathway integrates signals such as DNA damage, reactive oxygen species (ROS) generation, and metabolic cues, and can also be activated by the extrinsic pathway through the cleavage of Bid. The detailed mechanisms by which these stimuli lead to caspase activation are out of the scope of this chapter, but a basic understanding of the major players is necessary. The mitochondrion is central to this pathway in that it amalgamates cues on its outer membrane through a delicate balance of pro- and anti-apoptotic proteins of the Bcl-2 (B-cell lymphoma 2) family. At the resting state, the anti-apoptotic subgroup (A1, Bcl-2, Bcl-W, Bcl-X_L, and Mcl-1) keeps the proapoptotic members Bak, Bax, and Bok in check by forming a heterodimer with them. A third group, named BH3 (Bcl-2 homology 3)-only proteins (Bad, Bid, Bik, Bim, BMF, BNIP3, HRK, Noxa, and Puma), works to destabilize the equilibrium set by the other two groups by either binding to anti-apoptotic members, thus freeing proapoptotic proteins, or by directly binding to proapoptotic members to activate them. Either model leads to mitochondrion outer membrane permeabilization (MOMP) and leakage of key proteins including cyt*c* and Smac (second mitochondria-derived activator of caspases). The latter protein neutralizes IAPs (Fuentes-Prior and Salvesen 2004a) including XIAP, a direct inhibitor of caspases 3, 7, and 9.

Caspase 9 Activation and Regulation

Once released into the cytosol, cyt*c* binds to Apaf-1 (apoptotic peptidase activating factor). In brief, Apaf-1 contains a CARD domain at its N-terminus, a central NOD and C-terminal WD-40 repeats. The latter inhibits the oligomerization of Apaf-1, which is relieved by cyt*c* binding, enabling the formation of a heptameric wheel-shaped structure, the apoptosome (Mace and Riedl 2010a). This complex also requires dATP/ATP ligation to the nucleotide-binding domain (NOD), a feature also found in NLRs. Subsequently, the apoptosome recruits caspase 9 through homophilic CARD interaction into a central hub where it dimerizes. Recent studies have proposed that only a single dimer of caspase 9 is at the apoptosome at any given time (Malladi et al. 2009) and that it is active only when bound to Apaf-1. This creates a molecular timer for executioner caspase activation regulated in part by caspase 9, which stays on the apoptosome. Caspase 9 autoproteolysis is dispensable for the proper execution of apoptosis.

The crystal structure of dimeric caspase 9 has been published. It reveals only one active catalytic unit whereas the other domain has a distorted active site. The biological implication of this half-active dimer remains unclear and may be due to the presence of the inhibitor, which may be fixing the protein in a particular conformation.

Recently, Bratton and Salvesen surveyed regulators of caspase 9 activation and activity in details (Bratton and Salvesen 2010). Therefore, only the most relevant and best-characterized ones will be addressed. First and foremost, cleaved caspase 9 is inhibited by XIAP, which prevents dimerization via its BIR3 (baculovirus inhibitory repeat) domain. The strength of inhibition relies partly on the N-terminus of the cleaved small subunit of caspase 9 that contains a motif reminiscent of the mature Smac N-terminus. Interestingly, caspase 3, but not caspase 7, can remove this epitope by cleaving downstream of it, thus promoting caspase 9 activity by removing the influence of XIAP.

Another way by which caspase 9-driven apoptosis is modulated is through phosphorylation (Kurokawa and Kornbluth 2009). Representative examples include phosphorylation by ERK2 and Akt, both of which prevent caspase 9 activation. Notably, cancer cells rely heavily on the MAP kinase (MAPK) pathway led by ERK1/2 and growth factor phosphatidylinositol 3-kinase (PI3K)-Akt pathway to survive, thus showing a clear path to damper the suicidal tendencies of tumor cells. Interestingly, and contrary to most kinases, c-Abl (Abelson murine leukemia viral oncogene homolog 1)-mediated phosphorylation promotes caspase 9 processing in response to DNA damage.

Caspase 9 Substrates

There are only three well-established caspase 9 substrates: itself and pro-caspases 3 and 7 (Luthi and Martin 2007). By analogy to DISC proteins that are substrates of caspases 8 and 10, proteins that are in

close proximity to the apoptosome may be cleaved by caspase 9. Few other substrates have been proposed (less than 15) for caspase 9, but for many of them, experimental evidences are circumstantial at best.

Based on peptidic substrate library screens, caspase 9 cleaves the preferred LEHD↓ motif (Thornberry et al. 1997). There is no data on residue preference at the prime sites of the scissile bond. Remarkably, none of the reported substrate cleavage sites, including those on pro-caspases 3 and 7 and caspase 9 itself, match the preferred site. Similar to most caspases, the rule of a "good-enough site" seems to prevail. Indeed, no more that twofold improvement can be attained by substituting the primary pro-caspase 7 activation site for the preferred motif recognized by caspase 9 or caspase 8. Even the work-horse of apoptosis, caspase 3, does not have most of its substrates matching its preferred motif, i.e., DEXD↓(S/A/G).

Caspase 2

Caspase 2 is the most conserved member across species and the closest homolog to the unique *C. elegans* caspase ced-3 (Vakifahmetoglu-Norberg and Zhivotovsky 2010). However, it is also the least understood of the initiators. Opinions over its roles in the cell are controversial, with demonstrated pro-survival and apoptotic functions. It is present in the cytoplasm and the Golgi apparatus, and it is the only caspase found in the nucleus of some cell types.

Caspase 2 is activated in response to acute DNA damage by a mechanism involving p53. In this context, p53 promotes the expression of the CARD-containing adaptor RAIDD (RIP1 domain containing adaptor with DD) and PIDD (p53-induced protein with a death domain). Following a two-step autoproteolysis process, which converts PIDD from a pro-survival to a proapoptotic protein, PIDD relocalizes to the nucleus and forms an activation complex with RAIDD called the PIDDosome. However, Bouchier-Hayes and colleagues have localized the PIDDosome only in the cytosol, contradicting previous findings. Irrespective of the definitive location of this complex, it is responsible for the recruitment and activation of caspase 2 by dimerization in a manner reminiscent of other initiator caspase-activating platforms. X-ray crystallization showed that the PIDDosome has a ring-like shape composed of 5 PIDD and 7 RAIDD molecules (Mace and Riedl 2010a). Caspase 2 activation seems to be upstream of mitochondria, but the real requirement for this pathway remains unknown. Interestingly, PIDD-deficient cells display normal caspase 2 cleavage and no abnormalities, which suggests that another pathway can activate this caspase.

Contrary to other caspases, the caspase 2 substrate binding pocket seems to extend beyond the S4 pocket because it is relatively inactive on tetrapeptidic substrates but fairly active on pentapeptides. Caspase 2 substrates are limited compared to other caspases. Unlike the other initiators, caspase 2 cannot process executioner caspases, but it processes itself and cleaves Bid.

Apoptotic Caspases: The Executioners

Caspases 3, 6, and 7 are classified as executioner caspases based on their position in the activation cascade, i.e., downstream of initiator caspases (Fig. 4). Caspase 6 is often omitted from this group. However, because of its crucial role in cleaving nuclear lamins, it earned its status among executioners. They are responsible for the execution of apoptosis by cleaving many cellular substrates critical for the apparition of apoptotic features (Table 1). These caspases are expressed in all cell types and exist as obligate dimers at cellular concentrations.

Unlike the others, executioner caspases possess an N-terminal peptide of 23–28 amino acids instead of a protein-protein interaction domain. This segment is always cleaved during apoptosis. However, the role of this peptide remains unclear. For caspases 3 and 6, the N-peptide seems to silence the enzyme *in cellulo*. Also, the N-peptide removal of caspase 7 allows efficient activation by the serine protease granzyme B (GrB), at least in some cells (Fig. 5).

Cleavage of the linker that separates the large and small subunit of the catalytic domain is the driving force of executioner caspase activation (Fig. 2d). Cleavage occurs at conserved aspartate residues and is performed by initiator caspases or granzyme B. Other proteases can activate executioner caspases, further demonstrating the sole requirement for cleavage of the linker for activation. Proteolysis allows the formation of the substrate binding pocket and the reorientation of the catalytic cysteine. Cleavage is required for the executioners and not for the initiators possibly due to the relatively short length of the

Caspase Family, Table 1 Death substrates cleaved by caspases that result in an apoptotic hallmark

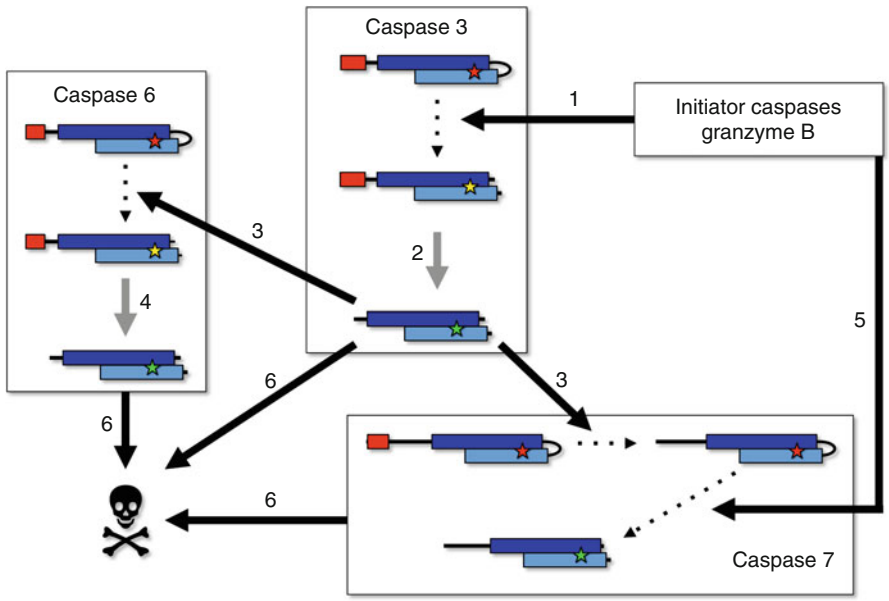
Death substrates	Description	Main role	Hallmark
Vimentin	Intermediate filament networking protein	Structural, cytoskeleton component	Intermediate filament dismantling/ blebbing
iPLA2[a]	Calcium-independent phospholipase A2	Generation of arachidonic acid and lysophosphatidylcholine	Find-me signal, healing response, phagocyte recruitment
Mst-1[b]	Human serin/threonine kinase	Phosphorylate many substrates like JNK, p38 MAPK and H2B histone, Akt1 inhibition	Chromatin condensation via histone H2B phosphorylation
Grasp65[c]	Receptor of GM130	RE and Golgi dynamics	RE and Golgi disruption
Bid[d]	Proapoptotic Bcl-2 family member	Transmit extrinsic death signal to the intrinsic pathway	Extrinsic pathway activation
PARP[e]	Poly(ADP-ribose) polymerase	Signal breaks in double-stranded DNA	PARP cleavage and ATP level maintenance
ICAD[f]	Inhibitor of caspase-activated DNAse	Inhibits and assists the folding of caspase-activated DNAse	Internucleosomal DNA fragmentation
ROCK I/II[g]	Serine/threonine kinase, Rho effector kinase	Phosphorylation of myosin light chain	Membrane blebbing, apoptotic bodies formation, nuclear fragmentation
PKCδ[h]	Protein kinase C, isoform delta	Serine/threonine kinase involved in cell signaling	Amplify apoptosis signal by promoting degradation of Mcl-1
p75 (NDUFS1)	p75 subunit of complex I	Participate in the respiratory chain of mitochondria	Loss of mitochondrial potential and ROS generation
Nuclear lamins	Nuclear structural protein	Nuclear morphology	Chromatin condensation, nuclear fragmentation
Gelsolin	Calcium-dependant regulator of actin filament dynamic	Regulator of actin organization	Cell round-up, matrix detachment
Pannexin 1	Ion channel	ATP and UTP release	Find-me signal

[a]Intracellular phospholipase A2
[b]Mammalian sterile 20-like 1
[c]Golgi reassembly and stacking protein of 65 kDa
[d]BH3 interacting domain death agonist
[e]Poly(ADP-ribose) polymerase
[f]Inhibitor of CAD
[g]Rho-associated protein kinase
[h]Protein kinase C δ

executioner's linker. However, this can hardly be reconciled with the linker of caspase 6, which is longer than that of caspase 8, and still requires cleavage for activation.

Caspase 7 is the best-understood executioner caspase at a structural level. Many structures provided information on executioner activation mechanism and substrate catalysis. The first reported structure of caspase 7 revealed a globular arrangement of two catalytic units and the presence of interdigitation between units by the so-called L2-L2' loops (Fig. 3b). Caspase 3 has a similar structure. The zymogen and the active form of caspase 7 adopt a similar fold. The main differences between them implicate three flexible loops surrounding the unformed catalytic site and substrate binding pocket (Fig. 3a). The unliganded form of caspase 7 exhibits an unformed substrate binding pocket, suggesting that substrate recognition is key to the formation of the fully active enzyme (induced fit process). However, these data remain conflicting, as some groups have reported no differences between unbound and inhibitor-bound caspases 3 and 7. More recently, many studies on the activation mechanism of caspase 6 were published. Globally, conformational changes occurring during the passage from the zymogen to the active state resemble the changes described for caspase 7 (Wang et al. 2010).

As mentioned, the removal of their respective N-terminal peptides seems an important step during *in cellulo* activation of executioners. Once activated, caspase 3 cleaves its own N-terminal peptide and then



Caspase Family, Fig. 5 *Executioner caspases activation cascade*. Within the executioners, a proteolytic cascade exists and is started by the cleavage of pro-caspase 3 by the initiator caspases or, to a lesser extent, granzyme B (step 1). Caspase 3 gains full activity after self-removal of its own N-peptide (step 2). Subsequently, caspase 3 cleaves pro-caspase 6 in its linker and the N-terminal peptide of pro-caspase 7 (step 3). In a manner reminiscent of caspase activation, caspase 6 removes its own N-peptide (step 4). Alternatively, caspase 8 can perform this step. Removal of caspase 7 N-peptide renders this caspase available to initiators and granzyme B for activation (step 5). GrB-mediated cleavage of pro-caspase 7 is one of the fastest proteolytic event known. Afterward, active executioners cleave their repertoire of death substrates (step 6) and cause apoptosis. *Trans* cleavage events are indicated by black arrows whereas gray arrows indicate *cis* proteolysis. The activity status of each protease is indicated by color-coded stars (inactive, *red*; silenced, *yellow*; active, *green*)

cleaves that of caspase 7. Because caspase 3 has higher enzymatic activity than caspase 7 and is present at a higher concentration in cells (~100 nM), it is considered to be the major executioner. Current studies suggest that caspase 6 is activated by caspase 3, not by an initiator caspase.

Caspase activation is regulated by IAPs. Executioner caspases 3 and 7 (and initiator caspase 9) are directly inhibited by XIAP. This IAP, which is often upregulated in cancer cells, provides a unique way to adjust sensitivity to apoptosis, and is responsible for defining type I and type II cells (Jost et al. 2009). Based on work by Scott et al. the second BIR of XIAP uses two binding sites to achieve low nanomolar affinity toward caspase 7 (Scott et al. 2005). Interestingly, the region N-terminal to BIR2 binds in reverse orientation over the substrate binding pocket, whereas the BIR2 domain binds further away from the catalytic site. Through its C-terminal RING (really interesting new gene) domain, XIAP can ubiquitinate caspases and hasten their proteasomal degradation. Moreover, a recent report suggests that executioner caspases and proteasome activity levels are strongly linked (Gray et al. 2010).

Cell Death by Caspases

Many phenotypic hallmarks resulting from caspase activation can be recapitulated, at least in some form, without caspase activity (e.g., DNA fragmentation, mitochondrial depolarization, and cleavage of some death substrates). Caspase activation is thought to be a means to an end more than it is thought to be a part of apoptosis, and this is still being debated. Some have argued that apoptosis should be defined as "caspase-mediated cell death," which contradicts formulations such as "caspase-independent apoptosis," as is often suggested in the literature.

Activation of any of the three executioner caspases is sufficient to induce apoptosis (Gray et al. 2010). For example, overexpression of caspase 7 results in cell

rounding and detachment, PARP and protein kinase C δ (PKCδ) cleavage, and DNA fragmentation without the activation of any other caspases, but no lamin B_1 cleavage and cyt*c* release from the mitochondria. Because the "good" cleavage motif recognized by caspases 3 and 7 is simple (i.e., DXXD↓(G/A/S)), this motif is common in mammalian genomes. Indeed, over 1000 caspase substrates have been identified covering the whole gamut of protein types and cellular functions (Luthi and Martin 2007). Interestingly, only a handful of them other than caspases have been shown to be necessary to produce full-blown apoptosis (Table 1). Notably, genetic studies in mice on the requirement for DNA fragmentation by CAD (caspase-activated DNAse), a hallmark of apoptosis, revealed that this endonuclease is dispensable for apoptosis and proper cell removal by professional phagocytes. Conversely, mice carrying a caspase 3- and 7-resistant PARP, whose cleavage is a characteristic hallmark of apoptosis, are resistant to endotoxic shock and to intestinal and renal ischemia reperfusion. PKCδ cleavage seems essential for apoptosis mediated by some genotoxic reagents. Despite these few examples, it is difficult to clearly establish that a given protein is truly an important death substrate. Here, death substrates are divided into three categories: death substrates in which cleavage (1) is absolutely required, (2) helps the process, or (3) is an accessory to apoptosis (bystander substrates). Thus, for each category – stimulus, cell, or tissue – specificities may exist. For example, cleavage of Bid illustrates group 1 proteins in type II, but not in type I cells, in which proteolysis would only help apoptosis. With the exception of caspases themselves, required cleavage events are quite rare, and most cleavage events are probably of the bystander-type, especially if they occur later during apoptosis. Recently, Timmer and Salvesen have addressed this issue and proposed simple steps, at least in theory, to discriminate true death substrates (likely few) from bystanders (many) (Timmer et al. 2007).

The timeline of hallmark's appearance may be different from one cell to another, may depend on the stimulus that initiates apoptosis, and may be dictated by sensitivity of the assay used. However, it is clear that there are early, intermediate, and late events. The earliest event is likely the exposure of phosphatidyl-serine (PS) on the external leaflet of the plasma membrane, occurring within 1 h in some cells. This "eat-me" signal originates from the downregulation of an amino phospholipid translocase and the activation of scramblases, which are activated by various signals. At the other end of the timescale, the packaging of cell components into small apoptotic bodies would be considered a late event. The other hallmarks find their place somewhere in between early and late events.

Apoptotic Caspases Beyond Death

Recently, acute attention has been paid to non-apoptotic roles of caspases (Yi and Yuan 2009). Caspase 8 is implicated in the immune network by modeling the proliferation and the differentiation of many cell types (monocytes, B and T cells). Reports also ascribe a role to this protease in cell migration and metastasis. Furthermore, caspase 3 activation has been linked to differentiation of some cell types. Skeletal muscles need cleavage of Mst-1 (mammalian sterile 20-like 1) and ICAD (inhibitor of CAD) (Larsen et al. 2010) for myoblast differentiation. In mice, caspases 3 and 7 are implicated in the generation/secretion of factors that promote wound healing and tissue regeneration. This role is concomitant with the execution of apoptosis and seems important for compensatory proliferation during normal tissue apoptosis. Besides, caspase 3 has a role in the maturation of erythrocytes and embryonic stem cell differentiation driven by Nanog proteolysis. Many non-apoptotic roles of caspases are probably hidden by the crude phenotypes of caspase knockout mice, and many other non-apoptotic roles are possibly masked by the compensatory phenotype observed in viable mice lineages.

Caspase and Disease

Multiple attempts to identify mutations in genes encoding caspases in cancers have been made, but no mutation hotspot has emerged. However, in some cancers, mutation rates are relatively high, with many of them having an effect on caspase protein level. Intuitively, any mutation diminishing caspase activity or its expression could contribute to tumorigenesis. However, there is currently little evidence suggesting that caspase gene mutations are early events in tumorigenesis, and the impact of mutations to establish their

relevance in promoting cancer development has not been studied. Nevertheless, at least two publications have suggested that caspases 2 and 8 are tumor suppressors.

Human genomic mutations in extrinsic initiators have been reported and cause divergent but related diseases. For example, an inactivating mutation in human caspase 8 (Arg^{248}->Trp in the large subunit) leads to immunodeficiency, whereas mutation in caspase 10 (Ile^{406}->Leu in the small subunit) produce autoimmune lymphoproliferative syndrome (ALPS). In addition to caspase mutations, cancer cells have many other ways to control caspase activation and activity to escape apoptosis. Indeed, the upregulation of XIAP and some anti-apoptotic Bcl-2 family members are well-known mechanisms by which caspase regulation is altered in tumor cells.

Many pathogens use proteins that target caspases to counteract their action and alter the normal cell response (Best 2008; Faherty and Maurelli 2008). For example, the viral serpin CrmA (cytokine response modifier A) encoded by the cowpox virus is a potent inhibitor of caspases 1 and 8 and granzyme B (a serine protease injected by cytotoxic lymphocytes and natural killer cells into the cytosol of target cells), thus preventing the inflammatory response and apoptosis. Viral FLIP-like protein (vFLIP) encoded by many viruses (e.g., Kaposi's sarcoma-associated herpesvirus) binds the DED-containing proteins FADD and initiator caspases 8 and 10, thus blunting the action of the extrinsic pathway. Viral FLIP also contributes to tumor development by promoting NFκB pathway activation.

Some neurological illnesses are linked to deregulation in caspase activation. In mice, Huntington, which is a caspase 6 substrate, needs cleavage for Huntington disease to occur. Caspase 7 is implicated in spinocerebellar ataxia type 7, a neurological polyglutamine disease. This caspase cleaves Ataxin-7, leading to specific neurological degeneration. A mutation in the cleavage site of DJ-1, normally recognized by caspase 6, is responsible for a subset of familial Parkinson's disease.

Summary

Since their discovery more than 20 years ago, caspases have drawn intense scrutiny. The role of caspases as mediator of inflammation, apoptosis, and skin maturation is relatively well understood, but their involvement in nontraditional processes remains unclear. The mechanisms by which other activities of caspases are selected over the traditionally recognized ones remain elusive.

Despite all the work done, many questions linger. Few of them have been mentioned throughout this chapter. An exciting topic in caspase biology is currently to delineate the substrate repertoire of each of them during apoptosis (Gray et al. 2010) and non-apoptotic processes. A corollary to this is to understand how caspases "know" which subset of proteins needs cleavage in a particular setting. Timmer and Salvesen have proposed that caspases use exosites to "select" their substrates (Timmer et al. 2007). It can be proposed that selectivity toward substrate subsets could be provided by exosite modulation, which are more amenable to regulation than the primary substrate binding pocket. The ability of caspase 10 cleavage status to dictate proteolysis of Bid versus other substrates exemplifies this issue (Wachmann et al. 2010). Irrespective of the death substrate repertoire of each caspases, another outstanding question regards the relevance for apoptosis of a given cleavage event: Is there a need to cleave all ~1,000 proteins for apoptosis to work? It is unlikely, but which cleavage events are important? Finally, caspase therapeutical potential is largely untapped. Although it is not expected that caspase inhibitors will be used for chronic treatments, their acute use can potentially be useful in ischemia reperfusion and acute hepatic failure. Direct caspase activators are, at least for now, more problematic in their design.

Cross-References

▶ MALT1

References

Agard NJ, Maltby D, et al. Mol Cell Proteomics. 2010;9:880–93.
Barnhart BC, Legembre P, et al. EMBO J. 2004;23:3175–85.
Best SM. Annu Rev Microbiol. 2008;62:171–92.
Black RA, Kronheim SR, et al. J Biol Chem. 1989;264:5323–6.
Boatright KM, Salvesen GS. Curr Opin Cell Biol. 2003;15:725–31.
Bratton SB, Salvesen GS. J Cell Sci. 2010;123:3209–14.

Bryant C, Fitzgerald KA. Molecular mechanisms involved in inflammasome activation. Trends Cell Biol. 2009; 19:455–64.
Cho YS, Challa S, et al. Cell. 2009;137:1112–23.
Faherty CS, Maurelli AT. Trends Microbiol. 2008;16:173–80.
Fuentes-Prior P, Salvesen GS. Biochem J. 2004a;384:201–32.
Gray DC, Mahrus S, et al. Cell. 2010;142:637–46.
Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J. 2009;23:1625–37.
Hymowitz SG, Dixit VM. Nat Struct Mol Biol. 2010;17:1289–90.
Jost PJ, Grabow S, et al. Nature. 2009;460:1035–9.
Kawadler H, Gantz MA, et al. Mol Cell. 2008;31:415–21.
Kurokawa M, Kornbluth S. Cell. 2009;138:838–54.
Lamkanfi M. Nat Rev Immunol. 2011;11:213–20.
Larsen BD, Rampalli S, et al. Proc Natl Acad Sci USA. 2010;107:4230–5.
Luthi AU, Martin SJ. Cell Death Differ. 2007;14:641–50.
Mace PD, Riedl SJ. Molecular cell death platforms and assemblies. Curr Opin Cell Biol. 2010a;22:828–36.
Malladi S, Challa-Malladi M, et al. EMBO J. 2009;28:1916–25.
Martinon F, Tschopp J. Cell Death Differ. 2007;14:10–22.
Martinon F, Burns K, et al. Mol Cell. 2002;10:417–26.
Oberst A, Pop C, et al. J Biol Chem. 2010;285:16632–42.
Oberst A, Dillon CP, et al. Nature. 2011;471:363–7.
Oehm A, Behrmann I, et al. J Biol Chem. 1992;267:10709–15.
Park HH, Lo YC, et al. Annu Rev Immunol. 2007;25:561–86.
Pop C, Fitzgerald P, et al. Biochemistry. 2007;46:4398–407.
Salvesen GS, Dixit VM. Proc Natl Acad Sci USA. 1999;96:10964–7.
Schroder K, Tschopp J. The inflammasomes. Cell. 2010;140:821–32.
Scott FL, Denault JB, et al. EMBO J. 2005;24:645–55.
Shikama Y, Yamada M, et al. Eur J Immunol. 2003;33:1998–2006.
Stehlik C, Dorfleutner A. J Immunol. 2007;179:7993–8.
Thome M, Schneider P, et al. Nature. 1997;386:517–21.
Thornberry NA, Rano TA, et al. J Biol Chem. 1997;272:17907–11.
Timmer JC, Salvesen GS, et al. Cell Death Differ. 2007;14:66–72.
Vakifahmetoglu-Norberg H, Zhivotovsky B. Trends Cell Biol. 2010;20:150–9.
Varfolomeev E, Maecker H, et al. J Biol Chem. 2005;280:40599–608.
Wachmann K, Pop C, et al. Biochemistry. 2010;49:8307–15.
Wajant H, Scheurich P. Int J Biochem Cell Biol. 2001;33:19–32.
Wang XJ, Cao Q, et al. EMBO Rep. 2010;11:841–7.
Yi C, Yuan J. Dev Cell. 2009;16:21–34.
Yu JW, Jeffrey PD, et al. Proc Natl Acad Sci USA. 2009;106:8169–74.

Suggested Reading

Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Bazan NG, Blagosklonny MV, Blomgren K, Borner C, Bredesen DE, Brenner C, Castedo M, Cidlowski JA, Ciechanover A, Cohen GM, De Laurenzi V, De Maria R, Deshmukh M, Dynlacht BD, El-Deiry WS, Flavell RA, Fulda S, Garrido C, Golstein P, Gougeon ML, Green DR, Gronemeyer H, Hajnoczky G, Hardwick JM, Hengartner MO, Ichijo H, Jaattela M, Kepp O, Kimchi A, Klionsky DJ, Knight RA, Kornbluth S, Kumar S, Levine B, Lipton SA, Lugli E, Madeo F, Malomi W, Marine JC, Martin SJ, Medema JP, Mehlen P, Melino G, Moll UM, Morselli E, Nagata S, Nicholson DW, Nicotera P, Nunez G, Oren M, Penninger J, Pervaiz S, Peter ME, Piacentini M, Prehn JH, Puthalakath H, Rabinovich GA, Rizzuto R, Rodrigues CM, Rubinsztein DC, Rudel T, Scorrano L, Simon HU, Steller H, Tschopp J, Tsujimoto Y, Vandenabeele P, Vitale I, Vousden KH, Youle RJ, Yuan J, Zhivotovsky B, Kroemer G. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ. 2009;16: 1093–107.
Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J. 2004;384:201–32.
Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J. 2009b;23:1625–37.
Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010;11:621–32.
Mace PD, Riedl SJ. Molecular cell death platforms and assemblies. Curr Opin Cell Biol. 2010b;22:828–36.
Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol. 2010;8:405–13.
Apoptosis, vol 14(4), Apr 2009 (ISSN 1360-8185).

Caspase Recruitment Domain Family, Member 11

► CARMA1

Catenin (Cadherin-Associated Protein), Beta 1 (88kD)

► Beta-Catenin

Catenin Beta

► Beta-Catenin

Catnb

► Beta-Catenin

Cbln1

Keiko Matsuda and Michisuke Yuzaki
Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Synonyms

Precerebellin

Historical Background

In 1984, the cerebellum-specific hexadecapeptide "cerebellin" was identified and shown to be concentrated in the synaptosomal compartment (Slemmon et al. 1984). Although "precerebellin" was originally identified as a precursor of cerebellin, the cerebellin peptide is not flanked by the classical dibasic amino acids observed in many neuropeptide precursors (Urade et al. 1991). In addition, precerebellin clearly belongs to the C1q family, whose members, such as C1q, adiponectin (Adipoq), and collagen X, are secreted and are involved in various intercellular functions (Yuzaki 2008). Indeed, full-length, uncleaved precerebellin is secreted from cerebellar granule cells (Bao et al. 2005; Iijima et al. 2007). Therefore, it is now evident that precerebellin is the actual signaling molecule itself, which should be referred to as Cbln1, although the cerebellin peptide may have additional functions.

Basic Features

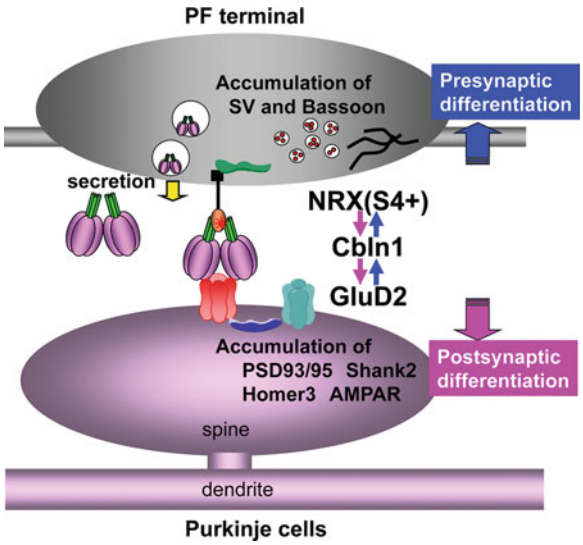
Cbln1 is predominantly expressed in cerebellar granule cells. Analysis of *Cbln1*-null mice revealed two essential functions of Cbln1 at synapses formed between parallel fibers (PFs; axons of granule cells) and Purkinje cells in the cerebellum (Hirai et al. 2005). The number of PF-Purkinje cell synapses is markedly reduced, and as many as 80% of the dendritic spines of Purkinje cells remains uninnervated in *Cbln1*-null mice. In the remaining PF-Purkinje cell synapses in $Cbln1^{-/-}$ mice, the postsynaptic densities (PSDs) are frequently longer than the presynaptic active zones, whereas the length of the active zones completely matches that of PSDs in wild-type mice. In addition, the long-term depression (LTD) of synaptic transmission at PF-Purkinje cell synapses, which is thought to underlie motor coordination and information storage in the cerebellum, is completely abrogated in *Cbln1*-null cerebellum. These unique phenotypes indicate that Cbln1 functions as a synaptic organizer that regulates matching and the maintenance of pre- and postsynaptic structures and proper synaptic functions at PF-Purkinje cell synapses.

Interestingly, *Cbln1*-null mice precisely phenocopy mice lacking a gene encoding the δ2 glutamate receptor (▶ GluD2; ▶ GluRδ2) (Yuzaki 2009). Indeed, ▶ GluD2, which is predominantly expressed in Purkinje cells, has been recently identified as a postsynaptic receptor for Cbln1 (Matsuda et al. 2010). Furthermore, neurexin (NRX), which is located at the presynaptic site of PF-Purkinje cell synapses, has been shown to serve as another receptor for Cbln1 (Uemura et al. 2010; Matsuda and Yuzaki 2011). These findings indicate that Cbln1 released from PFs forms a tripartite complex NRX/Cbln1/▶ GluD2 by binding to its presynaptic receptor NRX and postsynaptic receptor ▶ GluD2 at synaptic junctions and serve as a unique synaptic organizer (Fig. 1).

Cbln1 as a C1q Family Protein

Cbln1 belongs to the C1q family, which is characterized by the globular C1q domain (gC1q) at the C-terminus.All C1q family proteins are thought to form trimers via the C-terminal gC1q domain, and this unit trimer is often further organized into a higher-order multimeric complex via various motifs located at the N-terminus. Indeed, Cbln1 forms a hexamer via the disulfide bond at the N-terminal two cysteine residues (Bao et al. 2005; Iijima et al. 2007). A mutant Cbln1, which could not form a hexamer, does not exhibit any synaptogenic activities (Ito-Ishida et al. 2008). Thus, like other C1q family proteins, such as adiponectin and collagen X, the oligomeric status of Cbln1 is crucial for its functions.

The C1q family proteins including Cbln1 are completely absent in plants (*Arabidopsis thaliana*), yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*), nematodes (*Caenorhabditis elegans*), and insects (*Drosophila melanogaster*). The Cbln family consists of four members, Cbln1–Cbln4, which share 57–79% amino acid similarity with each other. All Cbln proteins are highly conserved among mammals. Indeed, 96–100% of the amino acid sequences of Cbln1–Cbln4 are identical between mice and humans.



Cbln1, Fig. 1 A schematic diagram summarizing synaptic signaling mediated by the tripartite complex NRX/Cbln1/GluD2. Cbln1 serves as a bidirectional synaptic organizer by binding to the presynaptic receptor NRXs containing the splice site #4 (S4+) and the postsynaptic receptor GluD2 at PF-Purkinje cell synapses. At the presynaptic site, NRXs accumulate synaptic vesicles (SVs) and bassoon to induce presynaptic differentiation. At the postsynaptic site, Shank2, PSD93/95, homer3, and AMPA receptors are accumulated by direct or indirect binding to GluD2's C-terminus

In contrast, Cbln3 is absent in frog (*Xenopus laevis*), fish (*Danio rerio*), and birds (*Gallus gallus*). These findings suggest that the Cbln family, especially Cbln3, may have emerged relatively late in the evolution to overcome the requirement for complex synaptic wiring (Yuzaki 2008; Yang et al. 2010).

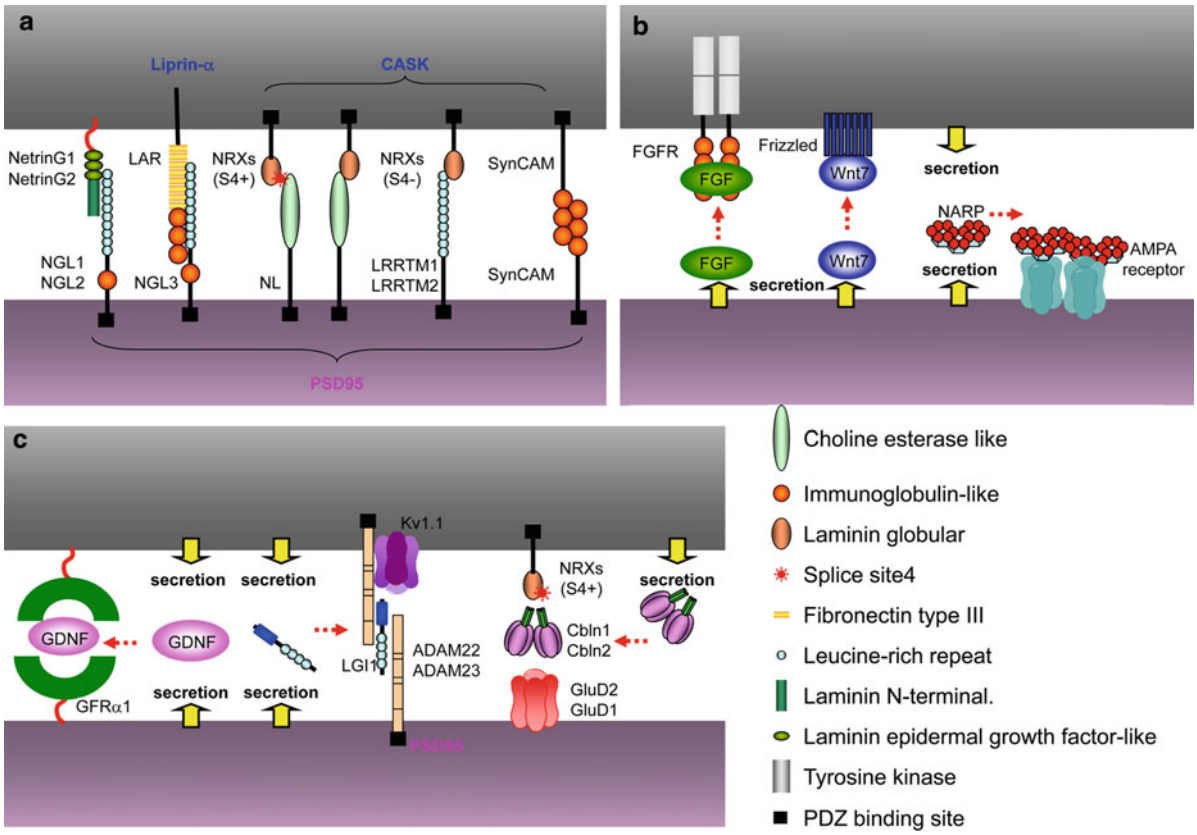
Cbln1, Cbln2, and Cbln4 are expressed in various brain regions outside the cerebellum (Miura et al. 2006). Interestingly, Cbln1 and Cbln2 but not Cbln4 can bind to NRX and induce the presynaptic differentiation of hippocampal and cortical neurons in vitro (Matsuda and Yuzaki 2011). Since Cbln4 is coexpressed with Cbln1 or Cbln2 in many brain regions (Miura et al. 2006), Cbln4 may modulate the synaptogenic activities of Cbln1 and Cbln2 by forming a heteromeric complex. Although ▶ GluD2 is predominantly expressed in the cerebellum, its family protein GluD1 is expressed in various brain regions and binds to Cbln family proteins (Matsuda et al. 2010; Matsuda and Yuzaki 2011). These findings suggest that GluD1 may form a tripartite complex with NRXs and Cbln1/2/4 to exert synaptic functions similar to those achieved by the NRX/Cbln1/▶ GluD2 complex.

A New Comer to the World of Synaptic Organizers

A growing number of synaptic organizers have been identified (Fox and Umemori 2006). They are generally classified into two categories, cell adhesion molecules and secreted factors (Fig. 2). A pair of cell adhesion molecules located at pre- and postsynaptic sites directly link pre- and postsynaptic elements. These include NRXs/neuroligins (▶ NLS (Nuclear Localization Sequence)), NRXs/leucine-rich repeat transmembrane proteins (LRRTMs), synaptic cell adhesion molecules (SynCAMs)/SynCAMs, netrin G–ligand (NGL)/leukocyte common antigen-related (LAR), and NGL/netrin G (Fig. 2a). Upon association, these pairs induce bidirectional synaptic differentiation. On the other hand, most secreted factors, such as Wnt7a, fibroblast growth factors (FGFs), and a pentraxin NARP, work on either the pre- or postsynaptic site, depending on the location of their receptors (Fig. 2b).

In contrast to these conventional synaptic organizers, Cbln1 secreted from PFs is sandwiched between the presynaptic receptor NRXs and the postsynaptic receptor ▶ GluD2 (Figs. 1, 2c). Similarly, glia-derived neurotrophic factor (GDNF) is reported to serve as a synaptic adhesion molecule being sandwiched by its receptor GFRα1 located at both pre- and postsynaptic neurons (Ledda et al. 2007). In addition, leucine-rich glioma inactivated 1 (LGI1) is also secreted from neurons and bind to its pre- and postsynaptic receptors ADAM22 and ADAM23, respectively, to accumulate potassium channels and AMPA receptors at synapses (Fukata et al. 2010). Thus, Cbln1 and these molecules may constitute the third category of synaptic organizers, a sandwich type (Fig. 2c) (Yuzaki 2011).

Cell adhesion molecules, such as cadherins, protocadherins, NRXs/NLs, and NRXs/LRRTMs, generally require extracellular Ca^{2+}. Thus, the second unique feature of the NRX/Cbln1/▶ GluD2 complex is that the binding between these molecules does not require Ca^{2+} (Matsuda and Yuzaki 2011). Indeed, Cbln1 specifically binds to NRXs containing an alternatively spliced exon known as "splice site #4," suggesting that Cbln1-binding properties for NRXs are distinct from those of ▶ NLS (Nuclear Localization Sequence) or LRRTMs. Since synaptic adhesion itself is independent of Ca^{2+}, Ca^{2+}-resistant binding of the NRX/Cbln1/▶ GluD2 complex is consistent with the function of the complex as synaptic glue, connecting pre- and postsynaptic elements.



Cbln1, Fig. 2 A schematic diagram showing three types of synaptic organizers. (**a**) Cell adhesion molecules. Cell adhesion molecules form transsynaptic complexes by their extracellular regions. NGL3 binds to the first two fibronectin type III repeats in LAR. NGL1 and NGL2 bind Netrin-G1 and Netrin-G2 anchored to the plasma membrane by glycosylphosphatidylinositol, respectively. Unlike Cbln1, which specifically binds to NRXs containing the splice site #4 (S4+), LRRTM1/2 binds to NRXs that lack the splice site #4 (S4–). NLs bind NRXs (S4–) and NRXs (S4+). SynCAM forms a homophilic complex through IgG-like domains. (**b**) Secreted molecules. FGF and Wnt7a, secreted from postsynaptic neurons bind to FGFR and Frizzled, respectively, on presynaptic terminals and induce presynaptic differentiation. Narp, secreted from pre- and postsynaptic sites, causes clustering of the AMPA receptors (GluA1 and GluA4 subunits) at the postsynaptic site. (c) Sandwich-type molecules. GDNF binds to GFRα1 located on both pre- and postsynaptic sites. The GFRα1/GDNF/GFRα1 complex induces cell adhesion. LGI1 binds to presynaptic ADAM22 and postsynaptic ADAM23 and accumulate presynaptic potassium channels (Kv1.1) and postsynaptic AMPA receptors, leading to enhanced synaptic transmission. Secretion sites of GDNF and LGI are not well understood. Cbln1 are secreted from presynaptic sites, acting on both pre- and postsynaptic sites by forming the tripartite NRX/Cbln1/GluD2 complex

Summary

Cbln1 is one of the most recently identified synaptic organizers that belong to the C1q family. Unlike other synaptic organizers, a deficiency in Cbln1 causes a severe reduction in the number of synapses between PFs and Purkinje cells in the cerebellum. Cbln1 released from PFs binds to NRXs expressed on the presynaptic PFs and ▶ GluD2 at the postsynaptic site. The NRX/Cbln1/▶ GluD2 tripartite complex is resistant to low extracellular Ca^{2+} levels and serves as a unique sandwich-type synaptic organizer. The mechanism by which the NRX/Cbln1/▶ GluD2 complex activates synapse formation/maintenance remains to be determined in future studies. In addition, the signaling mediated by other family members, such as Cbln2, Cbln4, and GluD1, remains to be clarified in vivo. Interestingly, members of other C1q family, such as the classical complement C1q and C1ql, are also shown to regulate the elimination of synaptic connections (Stevens et al. 2007; Bolliger et al. 2011). Thus, characterization of C1q family

proteins is expected to provide new insights into the mechanisms by which synapses are formed and modified in the CNS.

References

Bao D, Pang Z, Morgan JI. The structure and proteolytic processing of Cbln1 complexes. J Neurochem. 2005;95:618–29.

Bolliger MF, Martinelli DC, Sudhof TC. The cell-adhesion G protein-coupled receptor BAI3 is a high-affinity receptor for C1q-like proteins. Proc Natl Acad Sci USA. 2011;108:2534–9.

Fox MA, Umemori H. Seeking long-term relationship: axon and target communicate to organize synaptic differentiation. J Neurochem. 2006;97:1215–31.

Fukata Y, Lovero KL, Iwanaga T, Watanabe A, Yokoi N, Tabuchi K, et al. Disruption of LGI1-linked synaptic complex causes abnormal synaptic transmission and epilepsy. Proc Natl Acad Sci USA. 2010;107:3799–804.

Hirai H, Pang Z, Bao D, Miyazaki T, Li L, Miura E, et al. Cbln1 is essential for synaptic integrity and plasticity in the cerebellum. Nat Neurosci. 2005;8:1534–41.

Iijima T, Miura E, Matsuda K, Kamekawa Y, Watanabe M, Yuzaki M. Characterization of a transneuronal cytokine family Cbln–regulation of secretion by heteromeric assembly. Eur J Neurosci. 2007;25:1049–57.

Ito-Ishida A, Miura E, Emi K, Matsuda K, Iijima T, Kondo T, et al. Cbln1 regulates rapid formation and maintenance of excitatory synapses in mature cerebellar Purkinje cells in vitro and in vivo. J Neurosci. 2008;28:5920–30.

Ledda F, Paratcha G, Sandoval-Guzman T, Ibanez CF. GDNF and GFRalpha1 promote formation of neuronal synapses by ligand-induced cell adhesion. Nat Neurosci. 2007;10:293–300.

Matsuda K, Yuzaki M. Cbln family proteins promote synapse formation by regulating distinct neurexin signaling pathways in various brain regions. Eur J Neurosci. 2011;33:1447–61.

Matsuda K, Miura E, Miyazaki T, Kakegawa W, Emi K, Narumi S, et al. Cbln1 is a ligand for an orphan glutamate receptor delta2, a bidirectional synapse organizer. Science. 2010;328:363–8.

Miura E, Iijima T, Yuzaki M, Watanabe M. Distinct expression of Cbln family mRNAs in developing and adult mouse brains. Eur J Neurosci. 2006;24:750–60.

Slemmon JR, Blacher R, Danho W, Hempstead JL, Morgan JI. Isolation and sequencing of two cerebellum-specific peptides. Proc Natl Acad Sci USA. 1984;81:6866–70.

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, et al. The classical complement cascade mediates CNS synapse elimination. Cell. 2007;131:1164–78.

Uemura T, Lee SJ, Yasumura M, Takeuchi T, Yoshida T, Ra M, et al. Trans-synaptic interaction of GluRdelta2 and Neurexin through Cbln1 mediates synapse formation in the cerebellum. Cell. 2010;141:1068–79.

Urade Y, Oberdick J, Molinar-Rode R, Morgan JI. Precerebellin is a cerebellum-specific protein with similarity to the globular domain of complement C1q B chain. Proc Natl Acad Sci USA. 1991;88:1069–73.

Yang M, Cagle MC, Honig MG. Identification of cerebellin2 in chick and its preferential expression by subsets of developing sensory neurons and their targets in the dorsal horn. J Comp Neurol. 2010;518:2818–40.

Yuzaki M. Cbln and C1q family proteins: new transneuronal cytokines. Cell Mol Life Sci. 2008;65:1698–705.

Yuzaki M. New (but old) molecules regulating synapse integrity and plasticity: Cbln1 and the delta2 glutamate receptor. Neuroscience. 2009;162:633–43.

Yuzaki M. Cbln1 and its family proteins in synapse formation and maintenance. Curr Opin Neurobiol. 2011;21:215–20.

CBP1

► Regulator of Calcineurin 1 (RCAN1)

CC CKR1

► Chemokine Receptor CCR1

C-CAM

► CEACAMs

CCK(A) Receptor

► Cholecystokinin-1 Receptor

CCK1

► Cholecystokinin-1 Receptor

CCKA

► Cholecystokinin-1 Receptor

CCK-A Receptor

► Cholecystokinin-1 Receptor

CCKAR

▶ Cholecystokinin-1 Receptor

CCK-AR

▶ Cholecystokinin-1 Receptor

CCL3

Carlos Eduardo Repeke[1], Thiago Pompermaier Garlet[2], Ana Paula Favaro Trombone[3] and Gustavo Pompermaier Garlet[1]
[1]OSTEOimmunology lab, Department of Biological Sciences, School of Dentistry of Bauru, São Paulo University – FOB/USP, Bauru, SP, Brazil
[2]Inflammation lab, Department of Pharmacology, School of Medicine of Ribeirão Preto, São Paulo University – FMRP/USP, Ribeirao Preto, SP, Brazil
[3]Pathology lab, Instituto Lauro de Souza Lima - ILSL, Bauru, SP, Brazil

Synonyms

Macrophage inflammatory protein-1 alpha

Historical Background

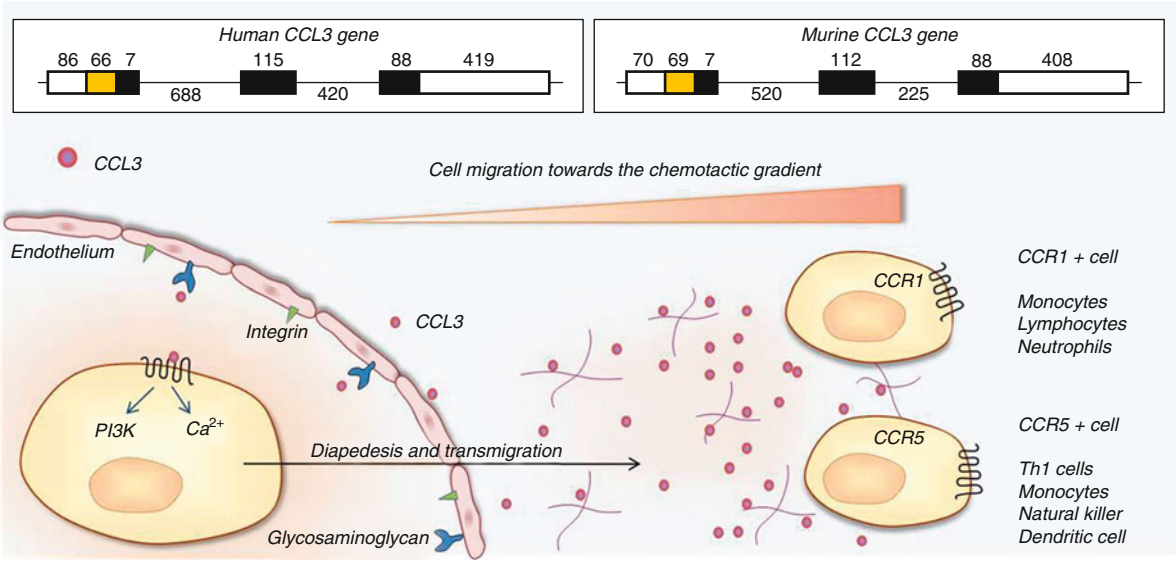
CCL3 was initially described in 1988, as a partially purified 8-kDa protein doublet from conditioned medium of endotoxin-stimulated mouse macrophages. In the view of its prominent proinflammatory chemotactic role, characterized both in vivo and in vitro at that time, this protein was denominated "macrophage inflammatory protein-1 alpha" (MIP-1α). Subsequently, a high nucleotide sequence similarity (69%) was found between the murine MIP-1α cDNA and a reported human cDNA cloned from stimulated lymphocytes, initially called LD78α or GOS19, assuming to be the human counterpart to murine MIP-1α (Wolpe et al. 1988; Menten et al. 2002; Maurer and von Stebut 2004). Interestingly, human and murine MIP-1α has been independently isolated in many laboratories in relatively short time spam, and has been named differently by each group. Similarly, several other cytokines with chemotactic abilities were identified, leading to the introduction of a new classification and nomenclature introduction for the designation of the chemokines, in which, murine MIP-1α and human MIP-1α/LD78α have been renamed CCL3 (Zlotnik and Yoshie 2000).

Collectively, chemokines are defined as small (8–14 KDa) proteins of cytokine family that have a broad range of activities involved in the recruitment and function of specific population of leukocytes at site of inflammation, presenting therefore important roles in the initiation and maintenance of host inflammation. These chemoattractive cytokines are subdivided into four groups based on a cysteine (C) motif, CXC, CC, C, and CX3C (Menten et al. 2002; Maurer and von Stebut 2004; Rollins 1997). In the view chemokine classification, CCL3 is characterized structurally and functionally as an inducible and secreted proinflammatory chemokine of the CC subfamily.

CCL3 Gene and Production

Both murine and human CCL3 are encoded by genes comprising three exons and two introns located on chromosome 11, which code for a 92 amino acids pre-protein. During secretion process a signal peptide of the precursor protein is cleaved off to yield their corresponding mature protein. Mature murine CCL3 contains 69 amino acids and has a theoretical M_r of 7883.9 *Da*. For human CCL3 precursor protein, the recognition site for the signal peptidase may present slight variations (still unclear), and hence the exact length of the mature protein between 66 and 70 residues is still a matter of debate (Fig. 1) (Menten et al. 2002; Hirashima et al. 1992).

While some chemokines expression is characteristically constitutive, CCL3 gene expression is basically inducible in most mature hematopoietic cells, being only very low levels expressed constitutively. In fact, while monocytes and macrophages present just low levels of CCL3 mRNAi, upon stimulation with a relatively wide range of stimuli, including PAMPs (pathogen-associated molecular patterns) such as LPS from Gram-negative bacteria and lipoteichoic acids from Gram-positive bacteria, superantigens such as the lectin phytohemagglutinin (PHA), or innate and adaptive cytokines such as IL-1β



CCL3, Fig. 1 *Schematic illustration of CCL3 structure and function*. The structures of human and murine CCL3 gene comprise exons divided in untranslated sequences (*light boxes*), translated leader sequences (*yellow boxes*) and translated mature protein sequences (*dark boxes*), and two intron sequences presented as *horizontal lines*; the lengths of the segments are indicated in bp. In the CCL3 gene structure, a cell migration toward a chemotactic gradient by diapedesis transmigration and the signaling pathways triggered by CCL3 in leukocyte are represented. On the *right side* of the panel are represented the cells chemoattracted by CCL3 by means of specific chemokine receptors, namely CCR1 and CCR5

and ▶ IFN-γ, a significant CCL3 secretion was observed in human monocytes/macrophages. Neutrophils have been shown to secrete CCL3 when stimulated by microbial antigens. Not only phagocytes, but also lymphocyte populations have been showed to secrete CCL3 when stimulated with PAMPs or superantigens. In addition, it was reported that antigen binding to B cell receptors (BCR) induced CCL3 production by B cells. Natural killer cells were also shown to efficiently produce CCL3 upon stimulation with IL-12 and IL-15. Interestingly, the co-stimulation provided by cytokines increases the stability of the LPS-induced CCL3 mRNA, demonstrating a convergence of proinflammatory signals toward an augmented CCL3 production. In contrast, inhibition of the LPS-mediated CCL3 production in monocytes was reported upon addition of anti-inflammatory and/or Th1-type cytokines such IL-4, IL-10, IL-13, and also by the classic anti-inflammatory and immunosuppressant glucocorticoid dexamethasone. Indeed, IL-10 suppressed the LPS-induced release of CCL3 via an accelerated degradation of CCL3 mRNA (Menten et al. 2002; Maurer and von Stebut 2004; Cook 1996).

CCL3 Receptors

To discover the role of determined chemokine, the researchers started by looking at the receptor usage and the biological responses of the cell types. Chemokine receptors characteristically are G protein-coupled receptors containing seven transmembrane domains that are found predominantly on the surface of leukocytes, being the 19 different chemokine receptors characterized to date expressed by leukocyte subpopulations with distinct degrees of selectiveness and specificness. Indeed, although chemokine receptors share high amino acid identity in their primary sequences, they typically bind a limited number of ligands.

As a CC-family member, CCL3 chemokines mediate their biological effects by binding to cell surface CC chemokine receptors (3×10^4 to 5×10^5 receptors per cell). Receptor binding involves high affinity interactions and a subsequent cascade of intracellular events that rapidly leads to a wide range of target cell functions including chemotaxis, degranulation, phagocytosis, and mediator synthesis. Signal transduction events are initiated by G protein complex leading to its dissociation into Gα and Gβ subunits. Gα induces ▶ phosphoinositide 3-kinase (PI3K) pathway

activation, and Gβγ subunits activate phospholipase C and induce Ca^{2+} influx resulting in the protein kinase C isoform activation. It has also been shown that ▶ MAP kinases as well as the JAK/STAT signaling cascade are involved (Maurer and von Stebut 2004; Proudfoot et al. 2003). The overall effect of chemokine receptor signaling involves the activation of specific cellular mechanisms related to leukocyte migration (such as changes in the avidity of cell adhesion molecules [called integrins] and activation of mechanisms involved in diapedesis) and activation (such as degranulation, polarization, and effector functions) (Maurer and von Stebut 2004).

Human chemokine CCL3 can bind in two specific chemokine receptors, namely, CCR5 and CCR1; while murine CCL3 is able to bind to the CCR1, CCR3, CCR5, and D6 receptors (Fig. 1) (Menten et al. 2002). From a therapeutic point of view it is more interesting to identify the chemokine receptors involved in the control of recruitment of the principal cell type responsible for the initiation and resolution of inflammatory immune responses in lymphoid and peripheral tissues (Horuk 2001).

Analysis of chemokine receptors has revealed using chemokines radiolabeled to demonstrate the presence of specific cell surface binding sites on many cell types. Not different, the first human CC chemokine receptor was isolated using several labeled chemokines, including LD78α. This receptor was designed CC-CKR1, but it has been renamed CCR1 to fit with the new nomenclature (Menten et al. 2002; Zlotnik and Yoshie 2000; Neote et al. 1993). The gene encoding human CCR1 has been mapped to chromosome 3p21, in a cluster with several other chemokine receptor genes (CCR2, CCR3, CCR4, CCR5, CCR8, CCR9, XCR1, CX3CR1). Using polyclonal antibodies to CCR1 the receptor has been shown to be predominantly expressed on monocytes and lymphocytes. On neutrophils, CCR1 can be expressed upon induction with specific cytokines. However, mouse neutrophils express CCR1 constitutively. Furthermore, CCR1 mRNA has been detected in human dendritic cells and eosinophils. CCR1 is expressed in leukocytes from a broad range of species including rhesus, rabbit, rat, and mouse, and there is a high degree of sequence homology among all these sequences. The CCR1 seven-membrane protein consists of 355 amino acid residues and belongs to the peptide subfamily of Class A GPCR family. This chemokine receptor is thought to predominantly signal through Gi/o couple pathway to regulate calcium flux, inhibit adenyl cyclase, and is thought unable to signal via Gq/11 protein. In addition, at least 11 different ligands (chemokines) interact with CCR1, including CCL3 (Cheng and Jack 2008).

In 1996, a second receptor for CCL3 was cloned and, as it was the fifth CC chemokine receptor cloned, named as CC-CKR5 (Samson et al. 1996). This name has been replaced by CCR5 in the new nomenclature; however, it is also called CD195 (Zlotnik and Yoshie 2000). The human CCR5 receptor is encoded by CCR5 gene, located on the short arm at position 21 on chromosome 3. Interestingly, CCR5 is a member of GPCR superfamily and shares 55% identical amino acids with CCR1, the firstly identified CCL3 receptor (Menten et al. 2002). CCR5 is one of the most studied chemokine receptor by the fact that, soon its discovery, CCR5 was shown to function pathologically as a key cell entry co-receptor for HIV-1. Certain population (approximately 20%) has a genetic deletion of a portion of the CCR5 gene (CCR5 Δ32) resulting in a frameshift at amino acid 185 and produces a mutant protein which is not expressed on the cell surface, which may result in distinct functional outcomes regarding inflammatory immune responsiveness (Carrington et al. 1999). In fact, CCR5 play important roles, not only in HIV infection, but also in the elaboration of a specific immune response against a series of pathogens. Lipopolysaccharide, proinflammatory cytokines, and various other stimuli can stimulate the CCR5 expression. This increase of CCR5 expression can influence in the selection of the appropriate effector T cell (i.e., Th1 or Th2) by the way CCR5 is expressed on both Th1 and Th2 lines. Although, it was absent in several Th2 clones markedly influenced by interleukin 2 (Maurer and von Stebut 2004). In addition, CCR5 expression has been detected on primary and secondary lymphoid organs, neurons, capillary endothelial cells, as well as epithelium, endothelium, vascular smooth muscle, fibroblast, Langerhans cells, macrophages, dendritic cells, CD34+ progenitor cells, and thymocytes (CD4+ and CD8+ cells) (Menten et al. 2002; Maurer and von Stebut 2004; Horuk 2001).

In addition to binding to the chemokine receptors, chemokines (including CCL3) characteristically present a carboxyl terminus stretch of positively charged residues that recognize heparan sulfate (HS) glycosaminoglycan (GAGs). Interestingly, chemokines can signal through cognate G protein-coupled receptors

(GPCRs) either at their soluble or immobilized (i.e., glycosaminoglycan associated) states. Recent evidences demonstrate that GAGs are indispensable for immobilization and function of major chemokines required for leukocyte adhesion to and crossing through blood and lymphatic vessels. In fact, chemokines stably immobilized on GAGs at the luminal surface of endothelium prevent their dilution by blood flow, but also to facilitate localized signaling to rolling leukocytes, while GAGs at inflamed tissue contribute to establish a chemotactic gradient that guides the influx of the leukocytes within the tissue. In spite of these versatile functions of HS GAGs in different types of endothelial cells and basement membranes, it is still possible that many extravasation processes involve HS-GAG-independent mechanisms. Interestingly, the presence of HS GAGs on leukocytes do not contribute to their migratory and inflammatory properties, subsets of antigen presenting cells may need to immobilize the chemokines they secrete within particular immune synapses, resulting in local activities essential for adhesion, motility, and survival of the cells involved.

CCL3 Activities

The proinflammatory activities of CCL3 overlap with, but not identical to, the activities of other CC chemokines (Menten et al. 2002; Maurer and von Stebut 2004; Repeke et al. 2010). For example, both CCL3 and ▶ CCL4 induce migration of monocytes and T lymphocytes, but they differ in their effects on different T cell subsets. Thus, while CCL3 is considered primarily chemotactic for B lymphocytes and activated CD8+ T cells, CCL4 is chemotactic for activated CD4+ cells (Fig. 1). Differently of CCL4, CCL3 is able to induce natural killer chemotaxis (Menten et al. 2002; Maurer and von Stebut 2004).

The wide role of CCL3 suggests that this chemokine has an important role in inflammation. In fact, CCL3 participates in the recruitment of monocytes and T cells into the inflamed synovium in rheumatoid arthritis. Furthermore, elevated levels of CCL3 are present in the synovial fluid and tissues of arthritis patients (Patel et al. 2001). High levels of CCL3 are also been detected in the cerebrospinal fluid of patients with bacterial meningitis (Lahrtz et al. 1998). Not different, the upregulation of CCL3 in glomeruli was observed in patients with glomerulonephritis and can be responsible for the part of leukocytes recruitment (Segerer et al. 2000). In addition, CCL3 has been shown to be implicated in many blood cell diseases, such as myeloid leukemia and T cell leukemia (Menten et al. 2002).

To understand the exact role of CCL3, the researchers used embryonic stem cell technology to generate mice homozygous for a disrupted CCL3 gene, called CCL3 knockout (CCL3-KO) or MIP-1KO mice. The homozygous mutant (−/−) mice were born in Mendelian proportions and had no obvious gross or histological abnormalities, indicating that CCL3 is not required for normal development (Cook 1996). The absence of CCL3 in CCL3-KO mice have reduced, but not eliminated influenza virus-induced pneumonitis and presented highly resistence to CVB3-induced myocaditis (Cook 1996). Furthermore, CCL3-KO presented reduced scores of autoimmune encephalomyelitis, arthritis rheumatoid, *Trypanosoma cruzi*, and other inflammatory diseases (Cook 1996; Machado et al. 2005). However, the absence of CCL3 had no significant effect in the ability to decrease the effects on the inflammatory response in some chronic diseases, such as periodontal disease. Although, CCL3 is the most abundantly expressed chemokine in diseased periodontium, its absence probably is supplied by a overlap role of the homologous chemokines CCL4 and ▶ CCL5 (Repeke et al. 2010; Silva et al. 2007).

Summary

In conclusion, CCL3 plays an important role in the induction and maintenance of inflammatory immune responses, in the context of both autoimmune reactions and host defense. In this way, this interest chemokine has shown as an important protein in the body operation, still not appears such as an essential life protein. In addition CCL3 might therefore play an important role in the pathogenesis of inflammatory diseases, such as arthritis, pneumonitis, leukemia, AIDS and others, which would have implications for design of new therapeutic strategies.

References

Carrington M, Dean M, Martin MP, O'Brien SJ. Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. Hum Mol Genet. 1999; 8(10):1939–45.

Cheng JF, Jack R. CCR1 antagonists. Mol Divers. 2008;12(1):17–23.
Cook DN. The role of MIP-1 alpha in inflammation and hematopoiesis. J Leukoc Biol. 1996;59(1):61–6.
Hirashima M, Ono T, Nakao M, Nishi H, Kimura A, Nomiyama H, et al. Nucleotide sequence of the third cytokine LD78 gene and mapping of all three LD78 gene loci to human chromosome 17. DNA Seq. 1992;3(4):203–12.
Horuk R. Chemokine receptors. Cytokine Growth Factor Rev. 2001;12(4):313–35.
Lahrtz F, Piali L, Spanaus KS, Seebach J, Fontana A. Chemokines and chemotaxis of leukocytes in infectious meningitis. J Neuroimmunol. 1998;85(1):33–43.
Machado FS, Koyama NS, Carregaro V, Ferreira BR, Milanezi CM, Teixeira MM, et al. CCR5 plays a critical role in the development of myocarditis and host protection in mice infected with *Trypanosoma cruzi*. J Infect Dis. 2005;191(4):627–36.
Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004;36(10):1882–6.
Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. Cytokine Growth Factor Rev. 2002; 13(6):455–81.
Neote K, DiGregorio D, Mak JY, Horuk R, Schall TJ. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. Cell. 1993; 72(3):415–25.
Patel DD, Zachariah JP, Whichard LP. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. Clin Immunol. 2001;98(1):39–45. Orlando.
Proudfoot AE, Power CA, Rommel C, Wells TN. Strategies for chemokine antagonists as therapeutics. Semin Immunol. 2003;15(1):57–65.
Repeke CE, Ferreira Jr SB, Claudino M, Silveira EM, de Assis GF, Avila-Campos MJ, et al. Evidences of the cooperative role of the chemokines CCL3, CCL4 and CCL5 and its receptors CCR1+ and CCR5+ in RANKL + cell migration throughout experimental periodontitis in mice. Bone. 2010;46(4): 1122–30.
Rollins BJ. Chemokines. Blood. 1997;90(3):909–28.
Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry. 1996; 35(11):3362–7.
Segerer S, Nelson PJ, Schlondorff D. Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies. J Am Soc Nephrol. 2000;11(1):152–76.
Silva TA, Garlet GP, Fukada SY, Silva JS, Cunha FQ. Chemokines in oral inflammatory diseases: apical periodontitis and periodontal disease. J Dent Res. 2007; 86(4):306–19.
Wolpe SD, Davatelis G, Sherry B, Beutler B, Hesse DG, Nguyen HT, et al. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. J Exp Med. 1988;167(2): 570–81.
Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity. 2000; 12(2):121–7.

CCL4

Carlos Eduardo Repeke[1], Thiago Pompermaier Garlet[2], Elcia Maria Silveira[1] and Gustavo Pompermaier Garlet[1]
[1]OSTEOimmunology lab, Department of Biological Sciences, School of Dentistry of Bauru, São Paulo University – FOB/USP, Bauru, SP, Brazil
[2]Inflammation lab, Department of Pharmacology, School of Medicine of Ribeirão Preto, São Paulo University – FMRP/USP, Ribeirao Preto, SP, Brazil

Synonyms

Macrophage inflammatory protein-1 beta

Historical Background

CCL4 was initially described in 1988, as a partially purified 8-kDa protein doublet from conditioned medium of endotoxin-stimulated mouse macrophages. In the view of its prominent proinflammatory chemotactic role, characterized both in vivo and in vitro at that time, this protein was denominated macrophage inflammatory protein-1 (MIP-1). Further biochemical experimentation resulted in the separation and characterization of two distinct but highly related proteins, then renamed as MIP-1α and MIP-1β. Also in 1988, the human equivalent of MIP-1β (Act-2, AT 744) was independently cloned by different groups and similarly, several other cytokines with prominent chemotactic properties were identified, leading to the introduction of a new classification and nomenclature introduction for the designation of the chemokines, in which, murine MIP-1β and human MIP-1β have been renamed CCL4 (Zlotnik and Yoshie 2000).

Collectively, chemokines are defined as small (8–14 kDa) proteins of cytokine family that have a broad range of activities involved in the recruitment and function of specific population of leukocytes at site of inflammation, presenting therefore important roles in the initiation and maintenance of host inflammation. These chemoattractive cytokines are subdivided into four groups based on a cysteine (C)

motif, CXC, CC, C, and CX3C (Rollins 1997; Menten et al. 2002; Maurer and von Stebut 2004). In the view of chemokine classification, CCL4 is characterized structurally and functionally as an inducible and secreted proinflammatory chemokine of the CC subfamily.

CCL4 Gene and Production

Murine CCL4 is encoded by a single gene on chromosome 11, which consists of three exons and two introns (Widmer et al. 1993). In addition, murine CCL4 gene also codes for a pre-protein of 92 amino acids with a theoretical M_r of 7826.9 Da and has a sequence homology of 60% with murine CCL3, a homologous CC family chemokine.

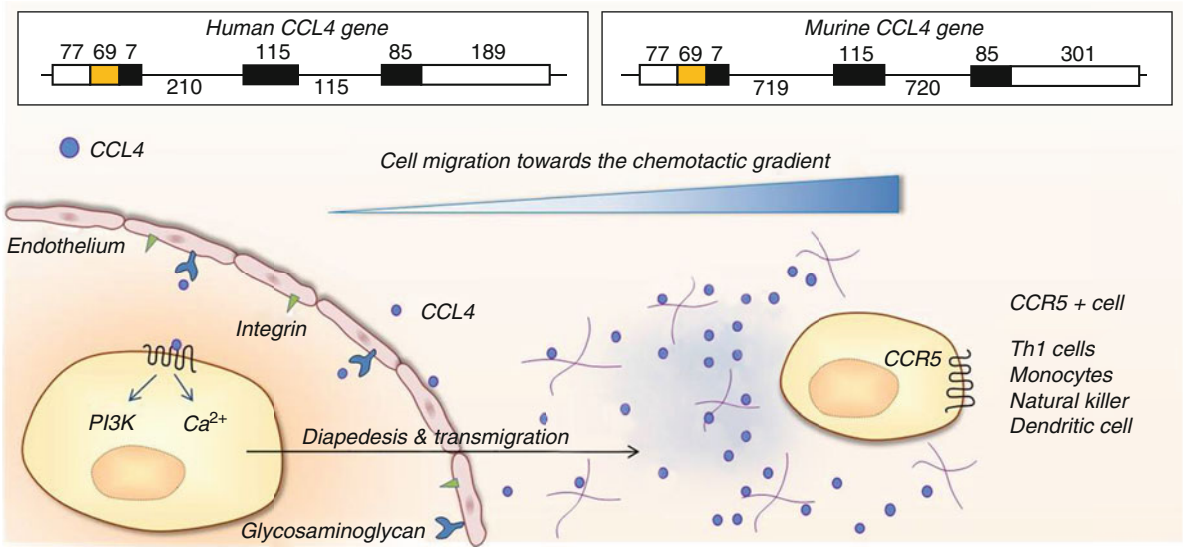
The human CCL4 gene (AT 744.1) is closely linked to human CCL5 gene on chromosome 17. The human CCL4 gene contains three exons and two introns, and codes a pre-protein of 92 amino acids, similar to the murine CCL4 (Fig. 1). The 3′untranslated regions of the murine and human CCL4 cDNAs contain a polyadenylation site (AATAAA) and several AT-rich sequences (Menten et al. 2002; Widmer et al. 1993).

The three-dimensional structure of human CCL4 has been defined by heteronuclear magnetic resonance analysis. It has shown that CCL4 exists as a symmetrical homodimer. Within each human CCL4 subunit (monodimer), the main secondary structure elements comprise a triple-stranded antiparallel β sheet arranged in a Greek key structure on top of which lies an α-helix. The NH_2-terminus consists of an irregular strand and a series of non-classical turns that form a long loop. This loop is followed by a four-residue helical turn, which leads into strand β_1. The two disulfide bridges, present in CCL4, have a left-handed spiral conformation (Menten et al. 2002; Lodi et al. 1994).

About CCL4 cell production, it have been demonstrated that monocytes produce high amounts of CCL4 when stimulated with LPS or IL-7 (Menten et al. 2002; Lukacs et al. 1995). This CCL4 production is counteracted by addition of IL-4, which reduces level of CCL4 mRNA expression. Activated T- and B- cells which have been triggered by antigen binding also secrete CCL4. Furthermore, NK cells are a good source of CCL4 by physiological activation signals, such as cross-linking of their Fc receptors or lysis of target cells (Menten et al. 2002; Oliva et al. 1998). In addition, brain microvessel endothelial cells were found to release CCL4 following stimulation with LPS, TNF-α, IFN-γ, or IL-1β, and pulmonary vascular smooth muscle cells–secreted CCL4 after addition of IL-1β, TNF-α IL-4, IFN-γ, and IL-10 (Menten et al. 2002; Lukacs et al. 1995; Shukaliak and Dorovini-Zis 2000). Finally, dendritic cells have also been shown to produce CCL4 in response to LPS, TNF-α, or CD40 ligand (Menten et al. 2002).

CCL4 Receptors

In 1996, the first receptor for CCL4 was identified as CC-CKR5, the fifth CC chemokine receptor cloned (Samson et al. 1996). This name has been replaced by CCR5 in the new chemokine system nomenclature, however, it is also still called CD195 (Zlotnik and Yoshie 2000). The human CCR5 receptor is encoded by CCR5 gene, located on the short arm at position 21 on chromosome 3. Interestingly, CCR5 is a member of GPCR superfamily and shares 55% identical amino acids with CCR1, the firstly identified CCL3 receptor (Menten et al. 2002). CCR5 is one of the most studied chemokine receptor by the fact that, soon its discovery, CCR5 was shown to function pathologically as a key cell entry co-receptor for HIV-1. Certain population (approximately 20%) has a genetic deletion of a portion of the CCR5 gene (CCR5 Δ32) resulting in a frameshift at amino acid 185 and produce a mutant protein which is not expressed on the cell surface, which may result in distinct functional outcomes regarding inflammatory immune responsiveness (Carrington et al. 1999). In fact, CCR5 plays important roles, not only in HIV infection, but also in the elaboration of a specific immune response against a series of pathogens. Lipopolysaccharide, proinflammatory cytokines, and various other stimuli can stimulate the CCR5 expression. This increase of CCR5 expression can influence in the selection of the appropriate effector T-cell (i.e., Th1 or Th2) by the way CCR5 is expressed on both Th1 and Th2 lines. Although, it was absent in several Th2 clones markedly influenced by IL-2 (Maurer and von Stebut 2004). In addition, CCR5 expression has been detected on primary and secondary lymphoid organs, neurons, capillary endothelial cells, as well as epithelium, endothelium, vascular smooth muscle, fibroblast, Langerhans cells, macrophages, dendritic cells,



CCL4, Fig. 1 *Schematic illustration of CCL4 structure and function*. The structures of human and murine CCL4 gene comprise exons divided into untranslated sequences (*light boxes*), translated leader sequences (*yellow boxes*) and translated mature protein sequences (*dark boxes*) and two intron sequences presented as horizontal lines; the lengths of the segments are indicated in bp. Below CCL4 gene structure, a cell migration toward a chemotactic gradient by diapedesis transmigration and the signaling pathways triggered by CCL4 in leukocyte are represented. On the *right* side of the panel are represented the cells chemoattracted by CCL3 by means of specific chemokine receptors, namely, CCR5

CD34+ progenitor cells, and thymocytes (CD4+ and CD8+ cells) (Fig. 1) (Menten et al. 2002; Maurer and von Stebut 2004; Horuk 2001).

In addition to binding to the chemokine receptors, chemokines (including CCL3) characteristically present a carboxyl terminus stretch of positively charged residues that recognize heparan sulfate (HS) glycosaminoglycan (GAGs). Interestingly, chemokines can signal through cognate G protein coupled receptors (GPCRs) either at their soluble or immobilized (i.e., glycosaminoglycan associated) states. Recent evidences demonstrate that GAGs are indispensable for immobilization and function of major chemokines required for leukocyte adhesion to and crossing through blood and lymphatic vessels. In fact, chemokines stably immobilized on GAGs at the luminal surface of endothelium prevent their dilution by blood flow but also facilitate localized signaling to rolling leukocytes, while GAGs at inflamed tissue contribute to establish a chemotactic gradient that guides the influx of the leukocytes within the tissue. In spite of these versatile functions of HS GAGs in different types of endothelial cells and basement membranes, it is still possible that many extravasation processes involve HS-GAG-independent mechanisms. Interestingly, the presence of HS GAGs on leukocytes do not contribute to their migratory and inflammatory properties, and subsets of antigen-presenting cells may need to immobilize the chemokines they secrete within particular immune synapses, resulting in local activities essential for adhesion, motility, and survival of the cells involved.

CCL4 Activities

When analyzed in vitro in a microchamber chemotaxis assay, CCL4 was shown to be potent lymphocyte chemoattractant. Lymphocyte migration from blood into tissues depends on the integrin-mediated adhesion of lymphocytes to the endothelium. Adhesion requires not only the presence of integrins on the surface of leukocytes, but also the activation of these molecules by chemokines. CCL4 was shown to be effective in augmentation adhesion of T lymphocytes to the integrin vascular cell adhesion molecule 1 (VCAM-1, or CD106) (Menten et al. 2002; Tanaka et al. 1993). In addition, CCL4 is potent chemoattractant for immature dendritic cells, but appears to be totally inactive on basophils (Fig. 1).

A series of studies demonstrate that CCL4 has an important role in inflammation. In fact, CCL4 participates in the recruitment of monocytes and T-cells into the inflamed synovium in rheumatoid arthritis. Furthermore, elevated levels of CCL4 are presented in the synovial fluid and tissues of arthritis patients (Patel et al. 2001). High levels of CCL4 have also been detected in the cerebrospinal fluid of patients with bacterial meningitis (Lahrtz et al. 1998). No difference, the upregulation of CCL4 in glomeruli was observed in patients with glomerulonephritis and can be responsible for the part of leukocytes recruitment (Segerer et al. 2000). Furthermore, CCL4 has been detected during rejection of liver transplant, and CCL4 has been associated with chronic hepatitis C infection (Adams et al. 1996).

Interestingly, since CCL4 shares the binding to CCR5 with the analogous chemokines CCL3 and CCL5, these factors are thought to operate in a complex network that regulates cell traffic in a given tissue. Interestingly, the redundancy (more than one chemokine can ding the same receptor) and the promiscuity (one chemokine can bind to more than one receptor) are characteristics that confer a robustness to the chemokines/chemokine receptors system. Indeed, the robustness is a common feature of many cytokine and growth factor networks that assure their proper performance, since the outputs of these cytokine networks may be retained to a sufficient extent, even if genetic or epigenetic alterations affect qualitatively or quantitatively individual network components. However, robustness, redundancy, and promiscuity also difficult, the study of individual factor roles within these systems, being the role of chemokine receptors, usually more easily unraveled in details than its chemokine ligands.

Summary

In conclusion, CCL4 plays an important role in the induction and maintenance of inflammatory immune responses, in the context of both autoimmune reactions and host defense. In addition, CCL4 might therefore play an important role in the pathogenesis of inflammatory diseases than previously thought, which would have implications for design of new therapeutic strategies.

References

Adams DH, Hubscher S, Fear J, Johnston J, Shaw S, Afford S. Hepatic expression of macrophage inflammatory protein-1 alpha and macrophage inflammatory protein-1 beta after liver transplantation. Transplantation. 1996;61(5):817–25.

Carrington M, Dean M, Martin MP, O'Brien SJ. Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. Hum Mol Genet. 1999;8(10):1939–45.

Horuk R. Chemokine receptors. Cytokine Growth Factor Rev. 2001;12(4):313–35.

Lahrtz F, Piali L, Spanaus KS, Seebach J, Fontana A. Chemokines and chemotaxis of leukocytes in infectious meningitis. J Neuroimmunol. 1998;85(1):33–43.

Lodi PJ, Garrett DS, Kuszewski J, Tsang ML, Weatherbee JA, Leonard WJ, et al. High-resolution solution structure of the beta chemokine hMIP-1 beta by multidimensional NMR. Science. 1994;263(5154):1762–7 (New York, NY).

Lukacs NW, Kunkel SL, Allen R, Evanoff HL, Shaklee CL, Sherman JS, et al. Stimulus and cell-specific expression of C-X-C and C-C chemokines by pulmonary stromal cell populations. Am J Physiol. 1995;268(5 Pt 1):L856–61.

Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004;36(10):1882–6.

Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. Cytokine Growth Factor Rev. 2002;13(6):455–81.

Oliva A, Kinter AL, Vaccarezza M, Rubbert A, Catanzaro A, Moir S, et al. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. J Clin Investig. 1998;102(1):223–31.

Patel DD, Zachariah JP, Whichard LP. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. Clin Immunol. 2001;98(1):39–45.

Rollins BJ. Chemokines. Blood. 1997;90(3):909–28.

Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry. 1996;35(11):3362–7.

Segerer S, Nelson PJ, Schlondorff D. Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies. J Am Soc Nephrol. 2000;11(1):152–76.

Shukaliak JA, Dorovini-Zis K. Expression of the beta-chemokines RANTES and MIP-1 beta by human brain microvessel endothelial cells in primary culture. J Neuropathol Exp Neurol. 2000;59(5):339–52.

Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist U, Shaw S. T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. Nature. 1993;361(6407):79–82.

Widmer U, Manogue KR, Cerami A, Sherry B. Genomic cloning and promoter analysis of macrophage inflammatory protein (MIP)-2, MIP-1 alpha, and MIP-1 beta, members of the chemokine superfamily of proinflammatory cytokines. J Immunol. 1993;150(11):4996–5012.

Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity. 2000;12(2):121–7.

CCL5

Thiago Pompermaier Garlet[1], Carlos Eduardo Repeke[2], Andreia Espíndola Vieira[2], Fernando Queiroz Cunha[1] and Gustavo Pompermaier Garlet[2]
[1]Inflammation lab, Department of Pharmacology, School of Medicine of Ribeirão Preto, São Paulo University – FMRP/USP, Ribeirao Preto, SP, Brazil
[2]OSTEOimmunology Lab, Department of Biological Sciences, School of Dentistry of Bauru, São Paulo University – FOB/USP, Bauru, SP, Brazil

Synonyms

RANTES

Historical Background

CCL5 was initially described in 1988 and this new protein was named for its characteristics: Regulated upon Activation expressed by Normal T cells and presumably Secreted (RANTES) (Schall et al. 1988). A relatively new technology, at the time, was used to indentify RANTES, subtraction cDNA libraries, which help to distinguish genes expressed in one cell and not in another. This genetic approach has since led to the uncovering of close to 50 other chemokines and, in order to clarify the nomenclature of all chemokines and their receptors, a new nomenclature has been introduced, in which, RANTES has been renamed CCL5 (Zlotnik and Yoshie 2000). CCL5 was first discovered in T cell–specific cDNA, and this initial report on CCL5 placed further attention on a new family of chemotactic proteins that proved to be important in influencing a series of biologic and pathological processes (Levy 2009).

Collectively, chemokines are defined as small (8–14 kDa) proteins of cytokine family that have a broad range of activities involved in the recruitment and function of specific population of leukocytes at site of inflammation, presenting therefore important roles in the initiation and maintenance of host inflammation. Four classes of chemokines have been discovered, based on the conserved cysteine (C) residues on the mature protein CXC, CC, C, and CX3C. In the view chemokine classification, CCL5 is characterized structurally and functionally as an inducible and secreted proinflammatory chemokine of the CC subfamily (Conti and DiGioacchino 2001).

CCL5 Gene and Production

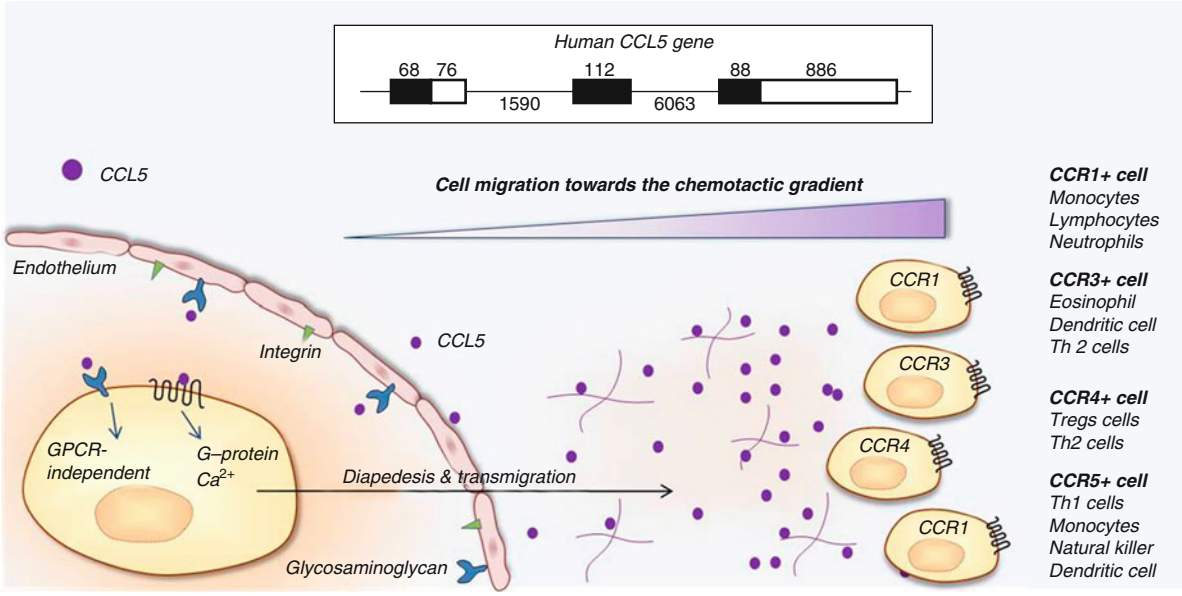
The CCL5 gene is located on the short arm of chromosome 17 (17q11.2); this gene product is predicted to be 10 kDa and, after cleavage of the signal peptide, approximately 8 kDa. Of 68 residues of CCL5, four are cysteines, and there are no sites for N-linked glycosylation (Fig. 1).

Initially, this chemokine was considered a T cell–specific protein; however, it was demonstrated that CCL5 can be produced by many cells in the vertebrate body including platelets, macrophages, eosinophils, and fibroblasts, as well as endothelial, epithelial, and endometrial cells. Interestingly, while stimulation of T cells inhibits the expression of CCL5, whereas as a general rule in the other cells mentioned, synthesis of CCL5 is induced by TNF-α and IL-1α but not by TGFβ, IFNγ, and IL-6 (Graziano et al. 1999).

CCL5 increases the adherence of monocytes to endothelial cells. It selectively supports the migration of monocytes and T lymphocytes expressing the cell surface markers CD4 and UCHL1. These cells are thought to be pre-stimulated helper T cells with memory T cell functions. CCL5, although activating human basophils and causing histamine release from some donor cells, has also been found to inhibit the release of histamine from basophils induced by several cytokines (Graziano et al. 1999). In addition, CCL5 is a potent eosinophil-specific activator of oxidative metabolism, and it also changes the density of eosinophils, making the hypodense, which are thought to represent a state of generalized cell activation. These cells are most often associated with diseases such as asthma and allergic rhinitis (Graziano et al. 1999; Appay and Rowland-Jones 2001).

CCL5 Receptors

Analysis of chemokine receptors using radiolabeled chemokines has the presence of specific cell surface binding sites on many cells type. Interestingly, CCR5 was one of the chemokines used to characterize the first human CC chemokine receptor isolated. This receptor was initially designated CC-CKR1, but it has been renamed CCR1 to fit with the new chemokine system nomenclature (Fig. 1) (Zlotnik and Yoshie 2000; Menten et al. 2002; Neote et al. 1993).



CCL5, Fig. 1 Schematic illustration of CCL5 structure and function. The structures of human and murine CCL5 gene comprise exons divided in untranslated sequences (*light boxes*), translated leader sequences (*yellow boxes*) and translated mature protein sequences (*dark boxes*), and two intron sequences presented as *horizontal lines*; the lengths of the segments are indicated in bp. Below the CCL5 gene structure, a cell migration toward a chemotactic gradient by diapedesis transmigration and the signaling pathways triggered by CCL5 in leukocyte are represented. On the *right side* of the panel are represented the cells chemoattracted by CCL3 by means of specific chemokine receptors, namely, CCR1, CCR3, CCR4, and CCR5

The gene encoding human CCR1 has been mapped to chromosome 3p21, in a cluster with several other chemokine receptor genes (CCR2, CCR3, CCR4, CCR5, CCR8, CCR9, XCR1, CX3CR1). Using polyclonal antibodies to CCR1, the receptor has been shown to be predominantly expressed on monocytes and lymphocytes. On neutrophils, CCR1 can be expressed upon induction with specific cytokines. However, mouse neutrophils express CCR1 constitutively. Furthermore, CCR1 mRNA has been detected in human dendritic cells and eosinophils. CCR1 is expressed in leukocytes from a broad range of species including rhesus, rabbit, rat, and mouse, and there is a high degree of sequence homology among all these sequences. The CCR1 seven-membrane protein consists of 355 amino acid residues and belongs to the peptide subfamily of Class A GPCR family. This chemokine receptor is thought to predominantly signal through Gi/o couple pathway to regulate calcium flux, inhibit adenyl cyclase, and is thought unable to signal via Gq/11 protein. In addition, at least 11 different ligands (chemokines) interact with CCR1, including ▶ CCL3 (Cheng and Jack 2008).

The second CCL5 chemokine receptor cloned is named CCR3, which is primarily expressed by eosinophils and appears to play an important role in regulating the migration of these cells. After the initial report of the first CCR3 clone, the same group showed that eotaxin, an eosinophil-selective CC chemokine and currently named CCL11, was a potent agonist for CCR3. Finally, in 1996, it was demonstrated that the CC chemokine CCL5 was also functional CCR3 agonist. Having noted the importance of CCR3 for eosinophil responses, subsequent studies demonstrate that this receptor and its cognate ligands such as CCL5 are directly involved in Th2-type responses and play an important role in allergy including asthma and atopic dermatitis. Furthermore, CCR3 was shown by several groups to be a HIV-1 co-receptor, and it is expressed on microglial cells of the brain, promoting disease like AIDS dementia, and also expressed in dentritic cells, play a role there in HIV-1 infection.

Another CCL5 chemokine receptor is called CCR4, and it was originally cloned from a human basophilic leukemia cell line library (Power et al. 1995; Horuk 2001). Nowadays, current literature describes that

CCR4 and its ligands are important for regulating immune balance and is known to be expressed selectively on Th2 cells and Treg cells; with special regard to the relationship between CD4 + CD25 + FOXp3+ Treg subset and CCR4 (Horuk 2001; Ishida and Ueda 2006). In a subset of patients with CCR4+ T cell leukemia/lymphoma, the tumor cells themselves function as Treg cells, contributing to tumor survival in the face of host antitumor immune responses. In other types of cancer, the specific ligand chemokines for CCR4 such as CCL5 that are produced by tumor cells and the tumor environment, attract Tregs cells to the tumor, where they create a favorable environment for tumor escape from host immune response (Ishida and Ueda 2006). In addition, CCR4 is known to modulate T cell migration to several sites of inflammation in the body and play a central role in T cell migration to the thymus, and T cell maturation and education (Ishida and Ueda 2006).

In 1996 was cloned the fifth CC chemokine receptor, named CC-CKR5 (Samson et al. 1996). This name has been replaced by CCR5 in the new nomenclature; however, it is also still called CD195 (Zlotnik and Yoshie 2000). The human CCR5 receptor is encoded by CCR5 gene, located on the short arm at position 21 on chromosome 3. Interestingly, CCR5 is a member of GPCR superfamily and shares 55% identical amino acids with CCR1, the firstly identified CCL5 receptor (Menten et al. 2002). CCR5 is one of the most studied chemokine receptor by the fact that, soon its discovery, CCR5 was shown to function pathologically as a key cell entry co-receptor for HIV-1. Certain population (approximately 20%) has a genetic deletion of a portion of the CCR5 gene (CCR5 Δ32) resulting in a frameshift at amino acid 185 and produces a mutant protein which is not expressed on the cell surface, which may result in distinct functional outcomes regarding inflammatory immune responsiveness (Carrington et al. 1999). In fact, CCR5 play important roles, not only in HIV infection, but also in the elaboration of a specific immune response against a series of pathogens. Lipopolysaccharide, proinflammatory cytokines, and various other stimuli can stimulate the CCR5 expression. This increase of CCR5 expression can influence in the selection of the appropriate effector T cell (i.e., Th1 or Th2) by the way CCR5 is expressed on both Th1 and Th2 lines. Although, it was absent in several Th2 clones markedly influenced by interleukin 2 (Maurer and von Stebut 2004). In addition, CCR5 expression has been detected on primary and secondary lymphoid organs, neurons, capillary endothelial cells, as well as epithelium, endothelium, vascular smooth muscle, fibroblast, Langerhans cells, macrophages, dendritic cells, CD34+ progenitor cells, and thymocytes (CD4+ and CD8+ cells) (Menten et al. 2002; Horuk 2001; Maurer and von Stebut 2004).

In addition to the binding to the chemokine receptors, chemokines (including CCL5) characteristically present a carboxyl terminus stretch of positively charged residues that recognize heparan sulfate (HS) glycosaminoglycan (GAGs). Interestingly, chemokines can signal through cognate G protein coupled receptors (GPCRs) either at their soluble or immobilized (i.e., glycosaminoglycan associated) states. Recent evidences demonstrate that GAGs are indispensable for immobilization and function of major chemokines required for leukocyte adhesion to and crossing through blood and lymphatic vessels. In fact, chemokines stably immobilized on GAGs at the luminal surface of endothelium prevent their dilution by blood flow but also to facilitate localized signaling to rolling leukocytes, while GAGs at inflamed tissue contribute to establish a chemotactic gradient that guides the influx of the leukocytes within the tissue. In spite of these versatile functions of HS GAGs in different types of endothelial cells and basement membranes, it is still possible that many extravasation processes involve HS-GAG-independent mechanisms. Interestingly, the presence of HS GAGs on leukocytes do not contribute to their migratory and inflammatory properties, subsets of antigen presenting cells may need to immobilize the chemokines they secrete within particular immune synapses, resulting in local activities essential for adhesion, motility, and survival of the cells involved (Horuk 2001).

On T cells, CCL5 can act by two quite separate signaling pathways. At nanomolar concentrations, CCL5 acts as a typical chemokine, by the GPCR-mediated pathway, as described above. And, at micromolar concentrations, CCL5 also triggers a distinct herbimycim A-sensitive ▶ protein tyrosine kinase (PTK)-mediated pathway (GPCR independent), leading to prolonged calcium influx, hyperphosphorylation, and generalized cell activation. The exact signaling pathways of CCL5 on T cells appear to be very complex and are not yet fully characterized,

especially as the same kinases can be induced within both the GPCR and GPCR-independent signaling pathways (e.g., ZAP-70 and p125). The ability of CCL5 to induce the GPRC-independent pathway and activate leukocytes is a distinct and important feature of the biology of this unusual chemokine. However, because high, and perhaps non-physiological, concentrations of CCL5 are required to demonstrate this phenomenon, its relevance remains unclear (Appay and Rowland-Jones 2001).

CCL5 Activity

CCL5 is a potent chemoattractant for memory T lymphocytes, monocytes, and eosinophils (Fig. 1). It was found highly expressed in activated T lymphocytes, macrophages, fibroblasts, platelets, mesangial cells, epithelial cells, megakaryocytes, and some tumors (Graziano et al. 1999; Appay and Rowland-Jones 2001). The activity of the chemokine CCL5 is not restricted merely to chemotaxis. It is a powerful leukocytes activator, a feature potentially relevant in a range of inflammatory disorders (Conti and DiGioacchino 2001). In fact, increased CCL5 expression has been associated with a wide range of inflammatory disorders and pathologies, including allogeneic transplant rejection, atherosclerosis, arthritis, atopic dermatitis, inflammatory airway disorders such as asthma, delayed-type hypersensitivity reactions, glomerulonephritis, endometriosis, some neurological disorders (such as Alzheimer's disease), and certain malignancies. In all these pathologies, CCL5 is thought to act by promoting leukocytes infiltration to site of inflammation (Appay and Rowland-Jones 2001).

At high concentration, CCL5 is able to induce the activation of T cells. This activation induced by CCL5 is followed by many diverse effects, including T-cell proliferation and apoptosis and the release of proinflammatory cytokines such as Interleukin 2 (IL-2), IL-5, interferon (IFN) –γ, and ▶ CCL4. CCL5-induced activation is not only restricted to T cells, but can also extend to monocytes and neutrophils, where similar dual-signaling pathways are induced (Graziano et al. 1999; Appay and Rowland-Jones 2001). Furthermore, CCL5 not only play a key role in cells activation and inflammatory diseases but also in the immune response to viral infection. In 1995, CCL5 was shown to be the most potent member of a trio CC chemokines (CCL3-5) release by CD8+ T cells that were able to suppress the replication of non-syncitium-inducing (NSI) HIV-1 strains in vitro (Appay and Rowland-Jones 2001; Cocchi et al. 1995).

In the view of the relative unspecificity of CCL5, a modified version of this chemokine was developed by the removal of the two N-terminal residues of CCL5 in order to create a partial agonist of chemokine receptors, and consequently therapeutically interfere with chemokine-based cell migration. This compound, named Met-RANTES, was described to function as a chemotaxis inhibitor and more effectively blocks CCR5-mediated HIV 1 infection of monocytes. Met-RANTES was initially produced during the synthesis of recombinant RANTES by the extension of the product with a single methionine residue, and its treatment was extensively studied in inflammatory bone diseases, such as arthritis rheumatoid (AR) and PD (Doodes et al. 2009; Repeke et al. 2010; Proudfoot et al. 1996). Furthermore, studies demonstrated the ability of met-RANTES treatment to extenuate tissue injury in models of arthritis, renal inflammation, and experimental colitis, among other (Doodes et al. 2009). Similarly, met-RANTES was able to decrease the experimental periodontal disease scores such as alveolar bone loss and inflammatory cell influx (Repeke et al. 2010). Interference with this homologous chemokine production or activity by blocking target receptors represents a potential novel therapeutic strategy in chronic inflammatory disease (Wells et al. 1999).

Summary

In summary, CCL5 plays a key physiological role in T cell migrations and in the inflammation disorders, inducing leukocyte infiltration and activation. CCL5 might therefore play an important role in the pathogenesis of inflammatory diseases than previously thought, which would have implications for design of new therapeutic strategies.

References

Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. Trends Immunol. 2001;22(2):83–7.

Carrington M, Dean M, Martin MP, O'Brien SJ. Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. Hum Mol Genet. 1999;8(10):1939–45.

Cheng JF, Jack R. CCR1 antagonists. Mol Divers. 2008; 12(1):17–23.
Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science. 1995;270(5243):1811–5.
Conti P, DiGioacchino M. MCP-1 and RANTES are mediators of acute and chronic inflammation. Allergy Asthma Proc. 2001;22(3):133–7.
Doodes PD, Cao Y, Hamel KM, Wang Y, Rodeghero RL, Kobezda T, et al. CCR5 is involved in resolution of inflammation in proteoglycan-induced arthritis. Arthritis Rheum. 2009;60(10):2945–53.
Graziano FM, Cook EB, Stahl JL. Cytokines, chemokines, RANTES, and eotaxin. Allergy Asthma Proc. 1999;20(3): 141–6.
Horuk R. Chemokine receptors. Cytokine Growth Factor Rev. 2001;12(4):313–35.
Ishida T, Ueda R. CCR4 as a novel molecular target for immunotherapy of cancer. Cancer Sci. 2006;97(11):1139–46.
Levy JA. The unexpected pleiotropic activities of RANTES. J Immunol. 2009;182(7):3945–6.
Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004;36(10):1882–6.
Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. Cytokine Growth Factor Rev. 2002; 13(6):455–81.
Neote K, DiGregorio D, Mak JY, Horuk R, Schall TJ. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. Cell. 1993; 72(3):415–25.
Power CA, Meyer A, Nemeth K, Bacon KB, Hoogewerf AJ, Proudfoot AE, et al. Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line. J Biol Chem. 1995;270(33):19495–500.
Proudfoot AE, Power CA, Hoogewerf AJ, Montjovent MO, Borlat F, Offord RE, et al. Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. J Biol Chem. 1996;271(5):2599–603.
Repeke CE, Ferreira Jr SB, Claudino M, Silveira EM, de Assis GF, Avila-Campos MJ, et al. Evidences of the cooperative role of the chemokines CCL3, CCL4 and CCL5 and its receptors CCR1+ and CCR5+ in RANKL + cell migration throughout experimental periodontitis in mice. Bone. 2010;46(4):1122–30.
Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry. 1996;35(11):3362–7.
Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, et al. A human T cell-specific molecule is a member of a new gene family. J Immunol. 1988; 141(3):1018–25.
Wells TN, Proudfoot AE, Power CA. Chemokine receptors and their role in leukocyte activation. Immunol Lett. 1999;65(1–2):35–40.
Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity. 2000;12(2): 121–7.

CCN

Satoshi Kubota and Masaharu Takigawa
Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Okayama, Japan

Synonyms

CCN1: Cyr61, CEF10, ßIG-M1, IGFBP10, IGFBP-rP4; CCN2: CTGF, FISP12, HCS24, ßIG-M2, HBGF-0.8, ecogenin, IGFBP8, IGFBP-rP2; CCN3: NOV, IGFBP9, IGFBP-rP3; CCN4: ELM-1, WISP-1; CCN5: rCOP1, WISP-2, CTGF-L, HICP; CCN6: WISP-3

Historical Background

In the early 1990s, three gene products with novel structural similarity were identified. The first member was discovered as a factor induced upon growth factor stimulation and was designated as cysteine-rich 61 (Cyr61), based on its structural characteristics. The second member was purified as a platelet growth factor–related molecule with a mitogenic effect on fibroblasts and, thus, it was initially named connective tissue growth factor (CTGF). Subsequently, a gene with structural similarity to the above two genes was found to be overexpressed in nephroblastomas, which provided the name nephroblastoma-overexpressed (NOV) gene to this third member. Based on these findings, the acronym of the names of these three genes, CCN, was given for the first time as the family name by Bork (1993). Thereafter, the other three members were identified as tumor suppressor–like gene products, or Wnt-inducible secreted proteins, in 1998; however, no CCN-related names were assigned to them (Perbal and Takigawa 2005). As such, a number of different names were confusingly given to the six genes encoding structurally quite similar proteins. In order to solve this problem, a unified nomenclature was proposed at the second International Workshop on the CCN Family of Genes and was approved by major researchers in this field in 2002. This proposal

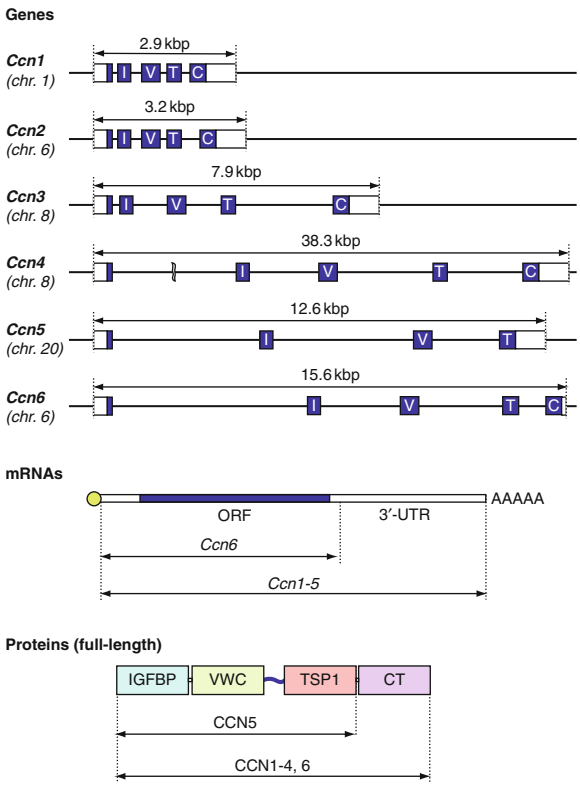
was published in 2003 to encourage comprehensive progress in the research on this novel family of genes and their products (Brigstock et al. 2003).

Structure

In the human genome, the six CCN family members are distributed on five chromosomes. Only *ccn2* and *ccn6* share the same chromosome, number 6, with a significant distance between them. The transcribed area of each gene consists of the first exon with a 5′-untranslated region (UTR) and a small portion encoding the signal peptide for secretion, a few intermediate exons corresponding to the amino-terminal and intermediate modules, and the last exon encoding the carboxy-terminal module with the 3′-UTR, together with the interspersed introns of diverse sizes. It should be noted that the exon-intron boundaries in these genes exactly correspond to those between the modules in their proteins, suggesting that these genes were established through exon shuffling during animal evolution (Kubota and Takigawa 2007a; Chen and Lau 2009). The prototypic gene of the six members is anticipated to be either *ccn1* or *ccn2* since these genes are quite compact with short introns. The genetic promoter for *ccn2* has been profoundly characterized, as described later on; whereas investigation of those promoters in the others is currently not (Leask and Abraham 2006).

Except for *ccn6*, the typical forms of the mRNAs of the CCN family members are characterized by the retention of the 3′-UTR of significant lengths, which suggests the functional significance of these 3′-UTRs. Indeed, the regulatory function of the 3′-UTR of *ccn2* was exclusively analyzed, and its role in posttranscriptional gene regulation was uncovered (Kubota and Takigawa 2007a). Splicing variants lacking 1 or 2 module-encoding exons were reported in the case of *ccn4* and *6* (Perbal and Takigawa 2005).

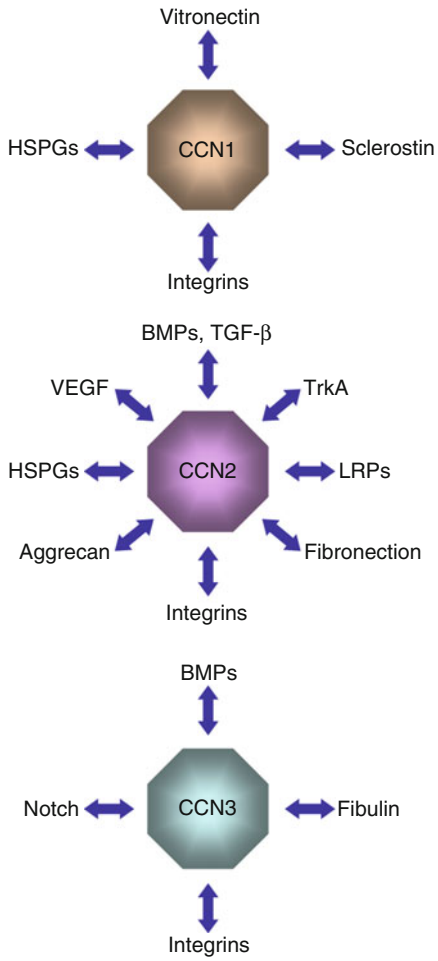
The structures of the resultant proteins encoded by these genes are novel and distinctly analogous. All of the members have four conserved modules referred to as insulin-like growth-factor-binding protein-like (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin type 1 repeat (TSP1), and C-terminal cystine knot (CT) modules, except for CCN5, which lacks the fourth one (Fig. 1). In addition, as many as 30 cysteine residues are present in each member, which also provides the structural basis for the functional property of CCN family proteins. Indeed, 38 cysteine residues are strictly conserved among mammalian CCN1, 2, 3, and 4. Internal VWC and TSP1 modules are connected by an amino acid stretch designated as the "hinge region" having a variety of lengths. This region is generally susceptible to cleavage by proteinases, which occasionally yield subfragments of the corresponding CCN family proteins. In silico prediction suggests remarkable similarities in the tertiary structures among the IGFBP modules of CCN1, 2, 3, and 6 and IGFBP4; among the VWC modules of all members and chordin; among the TSP1 modules of all members and thrombospondin; and among the CT modules of CCN1, 2, and 3 and BMP-7 (Holbourn et al. 2008).



CCN, Fig. 1 Gene and protein structure of the CCN family members. Abbreviations I, V, T, and C represent IGFBP, VWC, TSP1, and CT module-encoding regions, respectively

Molecular action

As summarized above, CCN family proteins comprise four modules, which may be compared to "hands" since all four modules are highly interactive with other molecules. All they have are four hands; however, CCN family proteins can manipulate a number of biomolecules via multiple interactions with these



CCN, Fig. 2 Interaction of CCN family member proteins with other biomolecules. Abbreviations are defined in the text

hands. Therefore, CCN family proteins are able to play central roles in orchestrating the extracellular signaling network in a variety of microenvironments.

The known interacting counterparts of the classical CCN family members are illustrated in Fig. 2. As stated above, such multiple interactions indeed provide the basis of the molecular behavior and multiple effects of CCN family proteins. Owing to the interaction with extracellular matrix (ECM) components and its biological significance, CCN family proteins are frequently called "matricellular proteins." It should be noted that interaction with integrins is a common property of all of the classical members, which eventually induces certain cellular responses, such as migration and adhesion through the adhesion signaling cascade under collaboration with cell-surface proteoglycans (Chen and Lau 2009). Interaction with other receptor molecules on the cell surface, including low-density lipoprotein receptor-related proteins (LRPs), ▶ Notch, and TrkA receptor protein kinase, also promote or modulate intracellular signal transduction (Kubota and Takigawa 2007b; Katsube et al. 2009). Moreover, CCN family proteins are known to modify the signal emission/transduction by other growth factors through direct binding. The effects of CCN2 on the action of transforming growth factor (TGF)-ß, bone morphogenetic protein (BMP)s, and vascular endothelial growth factor (VEGF) are classical examples, whereas interaction with other growth factors and its biological significance are currently being investigated as well. To date, the biological significance of the mutual interaction between CCN family members themselves has also been indicated (Perbal et al. 2010). As a result of such interplay, a number of major intracellular signaling cascades, which include Wnt, JNK, ▶ p38MAPK, ERK, ▶ PI3K, and PKC-mediated pathways, are modulated by CCN family proteins (Chen and Lau 2009).

In other words, CCN family protein function is highly dependent upon the coexisting molecules in the microenvironment. Thus, the same CCN family member may exert apparently opposite biological effects under different local situations, as typically represented by the differential roles of CCN family proteins in the development of malignancies. On the other hand, it is widely recognized that CCN2 and CCN3 are functionally quite counteractive in skeletal development and renal fibrosis (Perbal et al. 2010). The fundamental difference between CCN2 and CCN3 functions may be established by the preference for their functional counterparts, which is determined by the property of their modules.

Physiological Function

Based on the molecular actions stated, several CCN family proteins, particularly three classical ones, are already known to mediate a variety of biological events occurring throughout tissue and organ development, especially in mesenchymal tissues. A major part of these functions, as represented by the angiogenic function common to all of the classical members, is thought to be the outcome of the common and prominent cell biological effects of promoting adhesion, migration, and proliferation of cells of mesenchymal origin, whose promotion is exerted by the integrin-mediated

matricellular action of these proteins (Kubota and Takigawa 2007b; Chen and Lau 2009). Interestingly, CCN5 is the only member that shows functionality opposite to that described above. Probably, this functional property of CCN5 can be ascribed to its structural property of lacking the CT module since binding sites for proteoglycans, such as heparan sulfate proteoglycan (HSPG)s, including perlecan, have been identified in the CT module in CCN2. Although CCN1, 2, and 3 are functionally quite similar in vitro, as stated below, their role in tissue/organ development significantly differs. These differences can be ascribed to their different temporospatial distribution during development, which is enabled by the proper action of their regulatory machinery for gene expression.

CCN1, 2, and 3 are commonly characterized by their angiogenic functions. However, according to the phenotypic analysis of mutant mice, CCN1 appears to play the most critical part in the angiogenic developmental events. In fact, CCN1-null mice display severe defects in embryonic cardiovascular development. Even the disruption of a single *ccn1* locus was reported to cause defects in the atrial septum of the mutant mice. Compared with that of CCN1, the phenotypic change induced by CCN2 or CCN3 deletion is relatively mild; but it should be noted that CCN3 deficiency induces cardiac septal defects in mice (Chen and Lau 2009).

Concerning the classical members, their involvement in skeletal development can be specified as another common property of these molecules, although the quality of contribution differs for each member. CCN1 is known to promote chondrogenesis, whereas CCN2 plays a central role in endochondral ossification by promoting all of the stages of this process. CCN2 is produced predominantly by growth-plate chondrocytes at late differentiation stages, and is supplied and acts on the cells involved in a matricrine manner. The target cells include osteoblasts and vascular endothelial cells in addition to chondrocytes; hence, intramembraneous ossification also requires CCN2 (Kawaki et al. 2008). The regenerative effects of CCN2 on damaged articular cartilage were also confirmed in vivo. These findings are overall supported by the phenotype of CCN2-null mice (Perbal and Takigawa 2005). Such a profound and multiple functionality of CCN2 in osteogenesis is supposed to be based on the interaction of CCN2 with TGF-ß, BMPs, and other growth factors in the microenvironment. The contribution of CCN3 to skeletal development has been also indicated by previous studies on mutant mice. Overexpression of CCN3 in osteoblasts resulted in osteopenia; and, more interestingly, CCN3 deficiency causes severe joint malformation with appendicular skeletal defects. These findings suggest a critical role of CCN3 in joint formation and morphogenesis (Katsube et al. 2009; Chen and Lau 2009).

In addition to these functional characteristics shared by several members, the cell biological activities unique to each member have been revealed through recent investigations, as summarized in Table 1. The most outstanding of them is the action and effects of CCN3 on the hematopoetic system. It has been recently shown that CCN3 is produced by hematopoietic progenitors, regulates hematopoietic stem cell renewal, and induces clonal expansion of progenitors, in which the latter two actions are based on the interaction of CCN3 with Notch and BMPs (McCallum and Irvine 2009; Katsube et al. 2009). In contrast to those of the classical members, the distinct roles of the other three members in certain tissue/organ development are not yet certain, albeit their significant activities are observed in vitro. A contribution of CCN family members to aging is suggested as well. According to a recent study (Perbal et al. 2010), the expression of *ccn3, 4, 5*, and *6* is elevated in human skin upon aging, suggesting their significant roles in biological processes long after development.

Roles in Pathological Conditions and Wound Healing

Up to the present, a vast number of malignant tumor tissues and cells from a variety of origins have shown increased expression of CCN family members. Particularly, cumulative findings indicate the involvement of classical CCN family members in multiple types of malignancies. Indeed, overexpression of *ccn1* is observed in breast cancer, pancreatic tumors, and other tumors, as shown in Table 2. In the case of *ccn2*, basic and clinical investigations have revealed its overexpression in skin cancer, gallbladder cancer, chondrosarcoma, lymphoblastic leukemia, glioma, hepatocellular carcinoma, and melanoma, as well as in other cancers (Table 2), as based on functional evidence (Perbal and Takigawa 2005; Kubota and Takigawa 2007a; Chen and Lau 2009). Also for *ccn3*, osteosarcoma, renal cell and prostate carcinomas, Ewing sarcoma, Wilms' tumor, and rhabdomyosarcoma have been shown to overexpress this particular

CCN, Table 1 Role of CCN family members in developmental processes

	Cell biological activities	Relevant developmental processes	References
CCN1	Endothelial cells: adhesion (↑), migration (↑), proliferation (↑), survival (↑), tube formation (↑)	Angiogenesis Cardiovascular development Chondrogenesis	Perbal and Takigawa (2005); Chen and Lau (2009)
	Chondrocytes: proliferation (↑), early differentiation (↑)		
	Fibroblasts: adhesion (↑), proliferation (↑)		
	Monocytes: adhesion (↑)		
	Platelets: adhesion (↑)		
CCN2	Endothelial cells: adhesion (↑), migration (↑), proliferation (↑), survival (↑), tube formation (↑)	Angiogenesis Endochondral ossification Intramembranous ossification Lactogenic differentiation	Perbal and Takigawa (2005); Kubota and Takigawa (2007a, b); Chen and Lau (2009); Perbal et al. (2010)
	Chondrocytes: proliferation (↑), differentiation (↑)		
	Osteoblasts: proliferation (↑), differentiation (↑)		
	Fibroblasts: adhesion (↑), proliferation (↑), ECM production (↑)		
	Monocytes: adhesion (↑)		
	Platelets: adhesion (↑)		
	Hepatic stellate cells: adhesion (↑)		
	Mesangial cells: apoptosis (↑)		
	Smooth muscle cells: apoptosis (↑)		
	Bone marrow stromal cells: adhesion (↑), migration (↑)		
CCN3	Endothelial cells: adhesion (↑), migration (↑)	Angiogenesis Skeletal morphogenesis Haemopoietic stem cell renewal	Perbal and Takigawa (2005); Chen and Lau (2009)
	Fibroblasts: adhesion (↑)		
	Clonal expansion of naïve cord blood progenitors (↑)		
CCN4	Bone marrow stromal cells: proliferation (↑), osteogenic differentiation (↑)	(unknown)	Ono et al. (2011)
CCN5	Smooth muscle cells: migration (↓), proliferation (↓)	(unknown)	Perbal and Takigawa (2005)
	Osteoblastic cells: differentiation (↓)		
CCN6	(unknown)	(unknown)	

gene. Even 2, 3, or more CCN family members can be overexpressed simultaneously in the same tumor cells. These findings represent the association of CCN family members with malignant tumors. However, whether such overexpression is a causative or inducing factor of malignant tumors, or an anti-tumorigenic response, is not easy to determine; and further experimentation is needed to clarify the situation. Moreover, even if specific effects were experimentally confirmed, the role of CCN1 and CCN2 in tumor development and metastasis still would remain quite controversial, for the forced expression of CCN1 or CCN2 in tumor cells occasionally yields apparently opposite outcome, depending upon the cell type. As discussed above, this functional variation may be conferred by the difference in the composition of the functional counterparts among those cells/tissues. In contrast, experimental evidence indicates the anti-tumorigenic functions of the other four members (Perbal et al. 2010). It is also of great interest that CCN1 induces epithelial-mesenchymal transition (EMT), whereas the loss of CCN6 triggers EMT (Perbal and Takigawa 2005; Chai et al. 2010). The structural basis to account for the difference in behaviors between CCN1/2 and CCN3–6 has not yet been established.

Probably the best-known CCN family-related disorder at present is fibrosis, in which CCN2 is believed to play a central role. Elevated CCN2 gene expression

CCN, Table 2 Involvement of CCN family members in various diseases

	Related disorders		Findings	References
CCN1	Restenosis		Promoter of neointimal hyperplasia	Chen and Lau (2009)
	Nephropathy		Putative glomerular regenerator	Perbal and Takigawa (2005)
	Malignancies	Gastric cancer, ovarian cancer	Enhancer of tumorigenesis	Chen and Lau (2009)
		Non-small cell lung cancer	Inhibitor of tumorigenesis	
		Glioma	Mediator of HGF-mediated growth	Goodwin et al. (2010)
CCN2	Fibrosis	Skin	Inducer of sustained fibrosis	Perbal and Takigawa (2005); Chen and Lau (2009)
		Liver, lung	Mediator of fibrosis	
		Kidney	Inducer of fibrosis and nephropathy	
		Heart	Promoter and marker of fibrosis	Koitabashi et al. (2008)
		Pancreas	Overexpression observed	Kubota and Takigawa (2007a)
	Malignancies	Esophageal and prostate cancers	Promoter of tumorigenesis	Chen and Lau (2009)
		Pancreatic tumor	Promoter of tumor growth	
		Breast cancer	Promoter of bone metastasis	Kubota and Takigawa (2007a)
			Inducer of apoptosis	
		Lung and colorectal cancers	Suppressor of metastasis	Chen and Lau (2009)
		Oral squamous cell carcinoma	Suppressor of malignant phenotype	Kubota and Takigawa (2007a)
	Osteoarthritis		Cartilage regenerator	
	Atherosclerosis		Overexpression observed	
CCN3	Marfan syndrome		Antagonist of fibrillogenesis	Lemaire et al. (2010)
	Malignancies	Melanoma	Repressor of proliferation	Perbal and Takigawa (2005); McCallum and Irvine (2009); Perbal et al. (2010)
			Promoter of metastasis	
		Astrocytoma	Reduced expression observed	
		Glioblastoma	Repressor of proliferation	
CCN4	Malignancies	Melanoma	Inhibitor of tumorigenesis	Chen and Lau (2009)
		Lung cancer	Inhibitor of tumor invasion	
		Gastric cancer	Promoter of metastasis (truncated form from a splicing variant)	Perbal and Takigawa (2005)
CCN5	Transformed fibroblasts		Inhibitor of tumorigenesis	Chen and Lau (2009)
CCN6	Pseudorheumatoid dysplasia		Genetic association	Perbal and Takigawa (2005)
	Inflammatory breast cancer		Suppressor of tumor growth	Chen and Lau (2009)

is observed in most of the fibrotic lesions of various organs. Unlike cancer cases, CCN2 always act positively on fibrosis. In general, such fibrotic changes are hardly reversible; and thus, the establishment of anti-CCN2 molecular therapy is desired. The utility of plasma CCN2 as a diagnostic marker for the fibrotic change in kidney and heart has also been suggested (Kubota and Takigawa 2007a; Koitabashi et al. 2008; Chen and Lau 2009). It is noteworthy that the actions of structurally and functionally similar CCN1 and CCN2 are contrastive in fibrotic kidney disease. CCN2 is known to be a promoter of diabetic nephropathy, whereas CCN1 expressed in podocytes enhances glomerular reconstruction by inhibiting the migration of mesangial cells and promoting neovascularization (Perbal and Takigawa 2005).

Fibrosis can be regarded as a failure in tissue repair after injury. Therefore, it is reasonable for CCN2 to participate in both fibrotic lesion formation and the wound healing process during which transient fibrosis occurs. In contrast, regarding the events in the kidney, CCN1 and CCN2 collaborate therein. Immediately after wounding, CCN2 is rapidly supplied by platelets; and *ccn2* induction is also initiated in the cells around

the injured tissue (Kubota and Takigawa 2007a). During this initial step, both CCN1 and CCN2 support the adhesion of platelets and migration of monocytes (Chen and Lau 2009). After clotting, the migration of fibroblasts and granulation tissue formation are thought to be mediated by the abundant CCN2. The contribution of the other members is currently unknown.

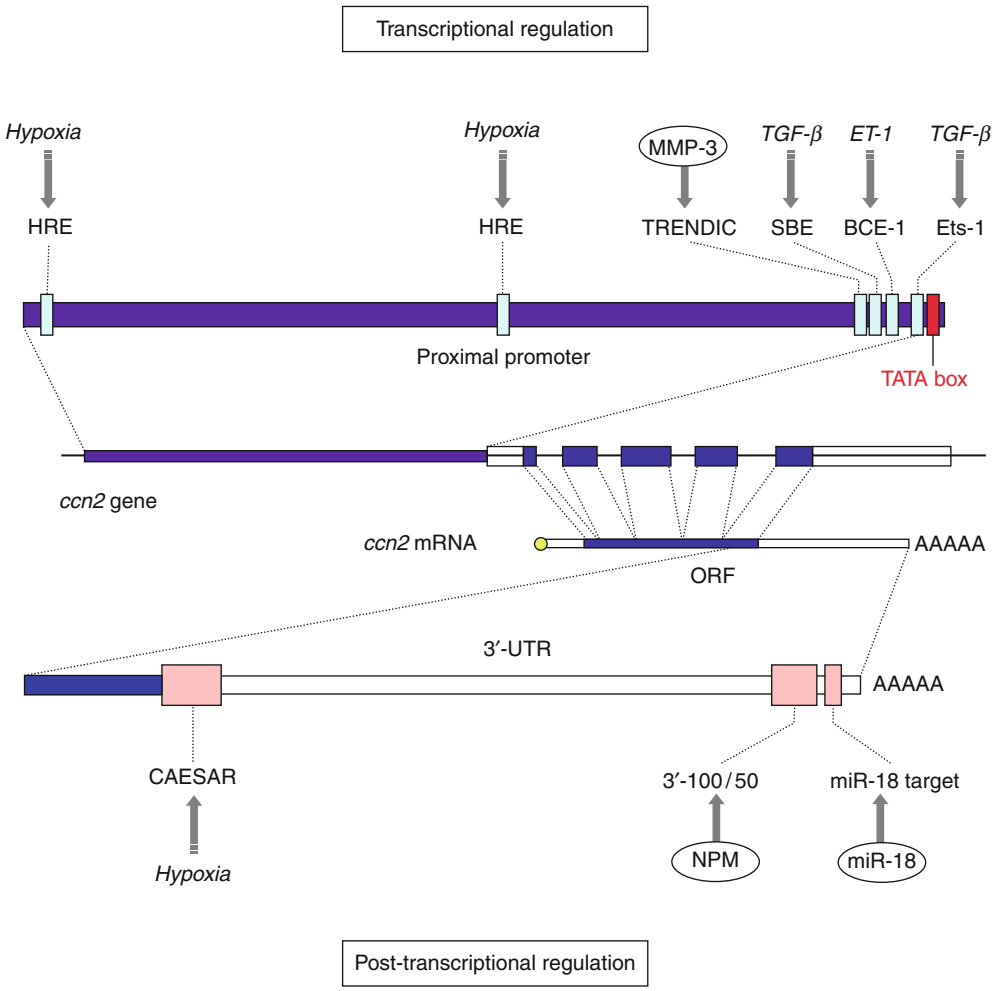
Other specific and relevant association between CCN family members and corresponding disorders is also summarized in Table 2. The genetic linkage between a CCN6 missense mutation and pseudorheumatoid dysplasia in humans is clearly indicated; however, *ccn6*-null mice show no appreciable phenotypic changes. This discrepancy may represent a significant difference between primates and rodents (Chen and Lau 2009).

Gene Regulation

Since the biological outcome caused by CCN family members is highly dependent on the other molecules surrounding them in the microenvironment, it is a critical determinant as to when and where CCN family proteins are produced in vivo. Therefore, the molecular function of each CCN family protein is determined by its gene regulatory system as well as by its molecular architecture itself. Nevertheless, in general, the gene regulatory system of CCN family members has not been sufficiently investigated yet. In fact, little is known about the structure and function of the genetic promoters of *ccn3, 4, 5*, and *6*.

In the case of *ccn1*, several reports have revealed particular regions in the *ccn1* gene that are responsible for the transcriptional upregulation elicited by certain stimuli. A serum response element was identified within a <2 kb region upstream of the TATA-box in murine *ccn1*; and this element was shown to be essential for the transcriptional induction of *ccn1* by serum and platelet-derived growth factor (PDGF) in fibroblasts (Chen and Lau 2009). Also, hypoxic induction of *ccn1* is conducted by well-known mediators of hypoxic gene upregulation, such as hypoxia inducible factor (HIF)-1α (Chen and Lau 2009). This transcriptional regulation is mediated not by a few HIF-1α binding site-like sequences, but through a c-jun/AP-1 binding sequence located at approximately 620 bp upstream of the transcription initiation site in *ccn1*. In addition, transcription of *ccn1* is induced in response to mechanical stretch by the binding of early growth response (Egr)-2 factor to an element located within a 200 bp region upstream of the TATA-box. In contrast to such knowledge about transcriptional regulation, the posttranscriptional regulation of *ccn1* is still left to be explored to date.

Transcriptional and posttranscriptional regulatory systems of *ccn2* have been extensively investigated (Fig. 3), and a number of valuable findings have been reported (Leask and Abraham 2006). According to these studies, the proximal promoter of *ccn2* contains several functional *cis*-regulatory elements. Within the same region and in close proximity, 3 *cis*-elements are located in tandem, all of which mediate *ccn2* induction by TGF-ß, either directly or indirectly. The one closest to the TATA-box and the third one accept the signal from TGF-ß via binding of Ets-1 and Smad transcription factors, respectively, whereas the second one in the middle mediates the signal from endothelin 1 (ET-1), a TGF-ß-inducible extracellular signaling molecule. It was recently revealed that Krupel-like factor 15 (KLF-15) negatively regulates *ccn2* transcription by counteracting Smad 3 signaling (Perbal et al. 2010). This second element was initially referred to as the TGF-ß responsive element (TbRE) since it was characterized by TGF-ß-responsiveness and interaction with nuclear protein(s). Nowadays, the TGF-ß-responsiveness of this element is regarded as being indirect, which is mediated by the induction of ET-1 production; and so, an alternative name, basal control element −1 (BCE-1), has been given. Adjacent to them, another element with a novel protein counterpart has been built into the *ccn2* promoter. This element, named transcriptional enhancer dominant in chondrocytes (TRENDIC), is a target of matrix metalloproteinase 3 (MMP-3), which is widely known to be an extracellular matrix-degrading enzyme. Surprisingly, MMP-3 is distributed also in the nuclei of chondrocytes and drives the transcription of *ccn2*, probably under the collaboration with chromatin remodeling molecules (Perbal et al. 2010). Hypoxia is an effective stimulant to cause the induction of *ccn2* as well as *ccn1* at both transcriptional and posttranscriptional levels. Transcriptional induction of *ccn2* upon hypoxia is mediated by two typical hypoxia responsive element (HRE)s, located relatively distant from the TATA-box in the proximal promoter; and the involvement of forkhead transcription factors, which are also mediators of *ccn1* gene regulation, has been suggested (Chen and Lau 2009; Samarin et al. 2010). Additionally, hypoxic signaling is also mediated by



CCN, Fig. 3 Transcriptional and posttranscriptional gene regulation of a CCN family member, CCN2. Abbreviations are defined in the Gene Regulation subsection in the text

a posttranscriptional element, *cis*-acting element of structure-anchored repression (CAESAR) in the 3′-untranslated region of *ccn2* mRNA.

CAESAR is the first identified posttranscriptional regulatory element in *ccn2*, which is located at the junction of the open reading frame and the 3′-UTR (Leask and Abraham 2006; Kubota and Takigawa 2007a; Chen and Lau 2009). This structured RNA segment exerts dual functions: one is to retain the basal expression level by repressing translation, and the other is to alter the steady-state mRNA level by controlled RNA degradation in response to hypoxia. Subsequently, another element, 3′-100/50, was discovered in the 3′-UTR of chicken *ccn2* mRNA as a regulatory element of mRNA stability in chondrocytes during endochondral ossification. Onto this element, nucleophosmin (NPM)/B23 specifically binds and accelerates the selective degradation of *ccn2* mRNA in the cytoplasm (Perbal et al. 2010). More recently, a functional target of miR-18a, one of the micro RNAs (miRNAs) that posttranscriptionally repress gene expression, was experimentally confirmed (Ohgawara et al. 2009). Of interest, the miR-18a target is located quite close to the 3′-100/50 element, suggesting a mutual interaction between regulatory complexes.

Although genetic targets and precise regulatory mechanisms remain unclarified, a number of other extracellular/intracellular factors are known to regulate *ccn* family gene expression. The expression of the *ccn1* gene is enhanced by angiotensin II, estrogen, the active form of vitamin D, mechanical stress, and UV

light irradiation. CCN2 protein is induced by glucocorticoids, nicotine, inflammatory cytokines, and mechanical stress, occasionally in a cell-type-dependent manner (Kubota and Takigawa 2007a; Chen and Lau 2009; Takeuchi et al. 2010). Repressive regulation of *ccn2* by several miRNAs other than miR-18a has also been reported. It should be noted that TGF-ß enhances the transcription of as many as 4 CCN family members, *ccn1*, *2*, *4*, and *5*, whereas it contrarily represses that of *ccn3*. As represented by the initial names given, *ccn4* and *5* expression is induced by Wnt-1, which led to the discovery of these gene products (Perbal and Takigawa 2005).

Summary

The CCN family consists of six members in vertebrates, all of which are composed of 3–4 highly interactive protein modules. Using these modules as "hands," CCN family proteins harmonize the extracellular signaling network via multiple interactions with molecular counterparts in the microenvironment. Therefore, the function of CCN family proteins is altered by the location and time point of gene expression, both of which are determined by their proper gene regulatory machinery. Physiologically, CCN family proteins enable harmonized development of the tissues involved and thus promote adequate tissue regeneration and wound repair. Conversely, aberrant expression of CCN family genes occasionally causes fibrotic disorders and frequently accompanies malignant transformation and invasion by tumor cells. Based on these unique properties, the medical utility of CCN family proteins and their derivatives is to be expected, and the development of anti-CCN family therapeutic strategy is currently being considered.

References

Bork P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett. 1993;327:125–30.

Brigstock DR, Goldschmeding R, Katsube KI, Lam SC, Lau LF, Lyons K, Naus C, Perbal B, Riser B, Takigawa M, Yeger H. Proposal for a unified CCN nomenclature. Mol Pathol. 2003;56:127–8.

Chai J, Norng M, Modak C, Reavis KM, Mouazzen W, Pham J. CCN1 induces a reversible epithelial-mesenchymal transition in gastric epithelial cells. Lab Invest. 2010;90:1140–51.

Chen CC, Lau LF. Functions and mechanisms of action of CCN matricellular proteins. Int J Biochem Cell Biol. 2009;41:771–83.

Goodwin CR, Lal B, Zhou X, Ho S, Xia S, Taeger A, Murray J, Laterra J. Cyr61 mediates hepatocyte growth factor-dependent tumor cell growth, migration, and Akt activation. Cancer Res. 2010;70:2932–41.

Holbourn KP, Acharya KR, Perbal B. The CCN family of proteins: structure-function relationships. Trends Biochem Sci. 2008;33:461–73.

Katsube K, Sakamoto K, Tamamura Y, Yamaguchi A. Role of CCN, a vertebrate specific gene family, in development. Dev Growth Differ. 2009;51:55–67.

Kawaki H, Kubota S, Suzuki A, Yamada T, Matsumura T, Mandai T, Yao M, Maeda T, Lyons KM, Takigawa M. Functional requirement of CCN2 for intramembranous bone formation in embryonic mice. Biochem Biophys Res Commun. 2008;366:450–6.

Koitabashi N, Arai M, Niwano K, Watanabe A, Endoh M, Suguta M, Yokoyama T, Tada H, Toyama T, Adachi H, Naito S, Oshima S, Nishida T, Kubota S, Takigawa M, Kurabayashi M. Plasma connective tissue growth factor is a novel potential biomarker of cardiac dysfunction in patients with chronic heart failure. Eur J Heart Fail. 2008;10:373–9.

Kubota S, Takigawa M. Role of CCN2/CTGF/Hcs24 in Bone Growth. Int Rev Cytol. 2007a;257:1–41.

Kubota S, Takigawa M. CCN family proteins and angiogenesis: from embryo to adulthood. Angiogenesis. 2007b;10:1–11.

Leask A, Abraham DJ. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci. 2006;119:4803–10.

Lemaire R, Farina G, Bayle J, Dimarzio M, Pendergrass SA, Milano A, Whitfield ML, Lafyatis R. Antagonistic effect of the matricellular signaling protein CCN3 on TGF-beta- and Wnt-mediated fibrillinogenesis in systemic sclerosis and Marfan syndrome. J Invest Dermatol. 2010;130:1514–23.

McCallum L, Irvine AE. CCN3 – A key regulator of the hematopoietic compartment. Blood Rev. 2009;23:79–85.

Ohgawara T, Kubota S, Kawaki H, Kondo S, Eguchi T, Kurio N, Aoyama E, Sasaki A, Takigawa M. Regulation of chondrocytic phenotype by micro RNA 18a: involvement of *Ccn2*/*Ctgf* as a major target gene. FEBS Lett. 2009;583:1006–10.

Ono M, Inkson CA, Kilts TM, Young MF. WISP-1/CCN4 regulates osteogenesis by enhancing BMP-2 activity. J Bone Miner Res. 2011;26:193–208.

Perbal B, Takigawa M. CCN proteins: a new family of cell growth and differentiation regulators. London: Imperial college Press; 2005.

Perbal A, Takigawa M, Perbal B. CCN proteins in health and disease. Dordrecht: Springer; 2010.

Samarin J, Wessel J, Cicha I, Kroening S, Warnecke C, Goppelt-Struebe M. FoxO proteins mediate hypoxic induction of connective tissue growth factor in endothelial cells. J Biol Chem. 2010;285:4328–36.

Takeuchi H, Kubota S, Murakashi E, Zhou Y, Endo K, Ng PS, Takigawa M, Numabe Y. Nicotine-induced CCN2: from smoking to periodontal fibrosis. J Dent Res. 2010;89:34–9.

CCN1: Cyr61, CEF10, ßIG-M1, IGFBP10, IGFBP-rP4

▶ CCN

CCN2: CTGF, FISP12, HCS24, ßIG-M2, HBGF-0.8, Ecogenin, IGFBP8, IGFBP-rP2

▶ CCN

CCN3: NOV, IGFBP9, IGFBP-rP3

▶ CCN

CCN4: ELM-1, WISP-1

▶ CCN

CCN5: rCOP1, WISP-2, CTGF-L, HICP

▶ CCN

CCN6: WISP-3

▶ CCN

CCNA1

▶ Cyclin A

CCNA2

▶ Cyclin A

CCTα

Helena Soares[1,2,3], Sofia Nolasco[2,3,4] and João Gonçalves[2,5]
[1]Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal
[2]Instituto Gulbenkian de Ciência, Oeiras, Portugal
[3]Escola Superior de Tecnologia da Saúde de Lisboa, Lisboa, Portugal
[4]Instituto de Investigação Científica Tropical CIISA, Lisboa, Portugal
[5]Centro de Química e Bioquímica, Lisbon, Portugal

Synonyms

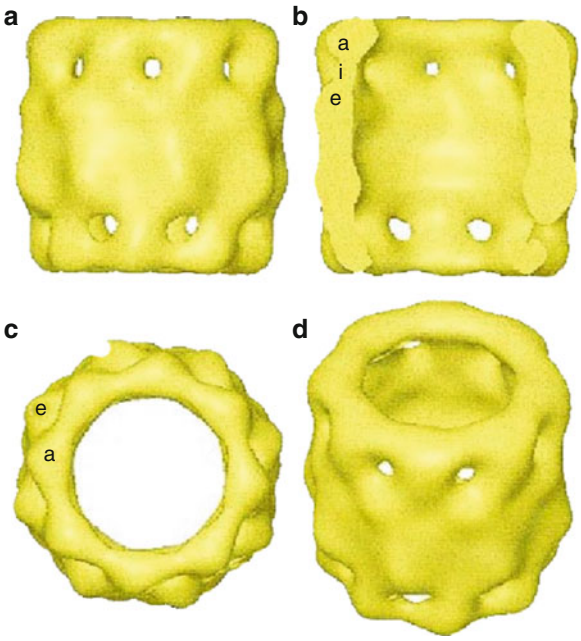
TCP-1 and Cct1

Historical Background

In the cell, the correct folding of many proteins depends on the function of preexisting ones known as Molecular Chaperones (for a review see Hartl and Hayer-Hartl 2009). These, were defined as proteins that bind to and stabilize an otherwise unstable conformation of another protein, and by controlling binding and release, facilitate its correct fate in vivo, be it folding, oligomeric assembly, transport to a particular subcellular compartment, or disposal by degradation. Molecular chaperones do not convey steric information specifying correct folding: instead, they prevent incorrect interactions within and between nonnative peptides, thus typically increasing the yield but not the rate of folding reactions.

Molecular chaperones are ubiquitous and comprise several protein families that are structurally unrelated (Hartl and Hayer-Hartl 2009). The Hsp70s and the Chaperonin families have been extensively studied. **Hsp70** homologs are widespread in prokaryotes and eukaryotes where they occur in the cytosol, mitochondria, chloroplasts, and endoplasmic reticulum. Their expression can be induced by a variety of cellular stresses but they have also essential functions under normal cellular conditions. Chaperonins are large oligomers and have ring-like or toroidal structures with central cavities. The group I chaperonins are found in Eubacteria (the chaperonin of *E. coli* GroEL and

its co-chaperonin GroES), and their close homologs in mitochondria (Hsp60-Hsp10) and chloroplasts (cpn60-cpn10) eukaryotic organelles of endosymbiotic origin. The eukaryotic cytosol also contains a more distantly related chaperonin known as TRiC (TCP-1-ring-complex), TCP-1 complex, cytosolic chaperonin (c-cpn), chromobindin A, or CCT (Chaperonin-containing-TCP-1) that is a member of the group II chaperonins (for review Valpuesta et al. 2002). Although related, both groups of chaperonins are distinct. In the group I chaperonins are homo-oligomeric complexes composed of seven-membered rings, whereas group II chaperonins are hetero-oligomeric complexes consisting of eight- or nine-membered rings. Another important difference between the two groups relies in the fact that group I chaperonins act in combination with a co-chaperonin during protein folding. These co-chaperonins are also oligomeric proteins that cap the central cavity of the chaperonin. The co-chaperonins are absent in chaperonins of group II but their function is mimicked by helical protrusions formed by an extra sequence localized at the tip of the apical domain (for review Hartl and Hayer-Hartl 2009).



CCTα, Fig. 1 Several views of the three-dimensional reconstruction of apo-CCT. ***a***, and ***b***, are side views, with (*b*) representing a cut along the longitudinal axis of the oligomer, where half of the particle has been removed. ***c***, A *top* view and ***d***, a side view tilted 35° toward the viewer. The locations of the apical (a), intermediate (i), and equatorial domains (e) are indicated (figure from Llorca et al. 1999 with permission)

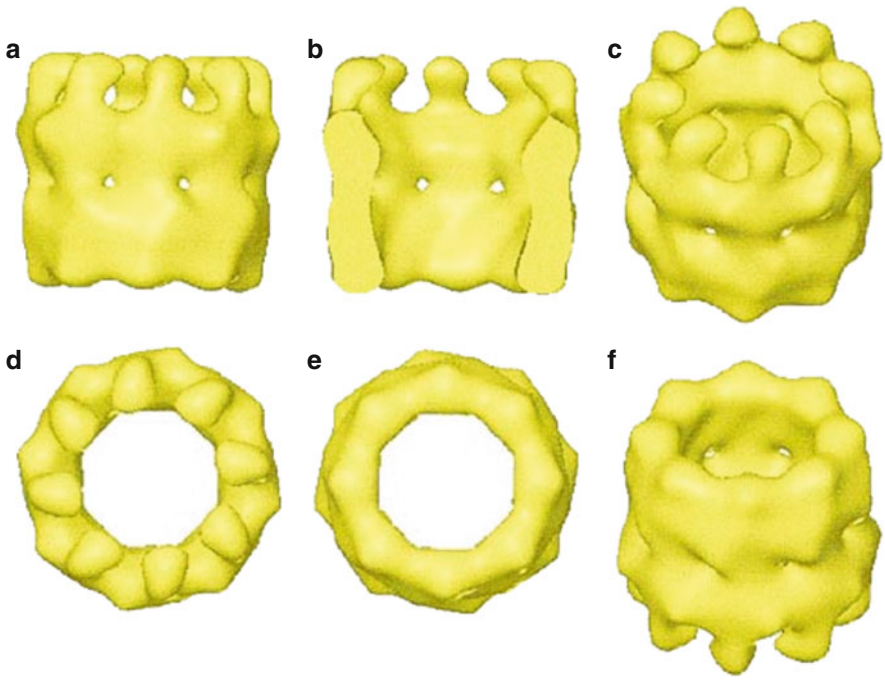
The CCT Complex, Its Interacting Proteins and Folding Mechanism

CCT exists as a hetero-oligomeric complex of about 900 kDa composed of two back-to-back stacked rings of eight different, though related, gene products that were designated as CCTα to CCTζ (for review Kubota et al. 1995). T-complex polypeptide-1 (TCP-1) was the first identified subunit of the chaperonin-containing TCP-1 CCT complex. TCP1 was renamed CCTα, whereas the other CCT subunits were designated by CCTβ, CCTε, CCTδ, CCTγ, CCTη, CCTθ, and CCTζ (Cct1 to Cct8 in yeast). The CCTα encoding gene was first cloned from mouse and humans. The mouse gene is highly expressed in the testis and is located in the mouse t-complex region on chromosome 17, which is involved in the transmission ratio distortion of t-complex-carrying mice (Kubota et al. 1995).

CCT complex forms a barrel-shaped cylinder with a diameter of about 150 Å and a height of 160 Å. Within the complex each subunit interacts with one subunit of the opposite ring and it has been shown that CCTα subunits are opposite, or nearly opposite, to each other in opposing rings (for a review see Valpuesta et al. 2002). The arrangement of subunits in the ring moving clockwise is α/1, ε/5, ζ/6, β/2, γ/3, θ/8, δ/4, and η/7. This unique arrangement in which each subunit occupies a well-defined position seems to be universal throughout eukaryotes (for review Valpuesta et al. 2002).

CCT subunits consist of three domains: an equatorial domain containing an ATP-binding site, an apical domain responsible for target protein interaction, and the intermediate domain connecting the other two. The apical domain contains a helical protrusion, which is involved in opening and closing the central cavity of the chaperonin (Llorca et al. 1999) (Figs. 1 and 2).

Phylogenetic analyses revealed that the sequence differences between CCT subunits from different species are located mainly in their apical domains. Although they contain several highly conserved motifs for ATP binding, the overall amino acid sequence identities are only about 30% (for review Kubota et al. 1995).



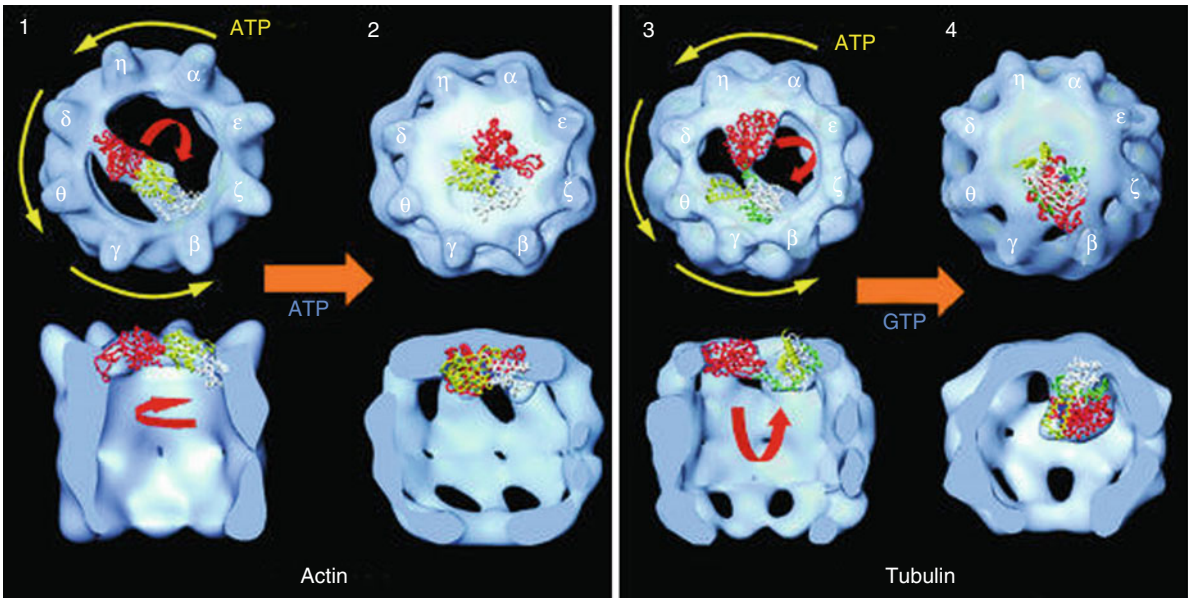
CCTα, Fig. 2 Several views of the three-dimensional reconstruction of ATP-CCT. *a*, and *b*, are side views, with (*b*) representing a cut along the longitudinal axis of the oligomer. *c*, and *f*, are side views tilted 35° toward the viewer showing the *top* and *bottom* ring of the volume in (*a*), respectively. *d*, and *e*, are top views showing the *top* and *bottom* ring of the volume in (*a*), respectively (figure from Llorca et al. 1999 with permission)

Smaller complexes of CCT-subunit subsets have also been detected in several cell types and were proposed to be intermediates in the assembly of the intact chaperonin (for review Valpuesta et al. 2002). Additionally, CCT subunits have functional differences in vivo which is reflected in the fact that the overexpression of each CCT subunit is not able to rescue mutations in the other CCT subunits, indicating CCT-subunits specificity of function (for a review see Gonçalves et al. 2007).

CCT assists the folding of approximately 5–10% of the cellular proteome being tubulins and actin quantitatively the major substrates (for a review see Lundin et al. 2010). Therefore, the chaperonin is implicated in several processes related to cytoskeleton systems, cell cycle control, nuclear pore complex assembly, chromatin remodeling, and protein degradation. Although most of these proteins do not seem to present a sequence signature which identifies them as CCT interacting proteins many present WD40 domains, which contain the WD repeat (a weakly conserved sequence motif of ~31 amino acids ending with conserved tryptophan and aspartate residues) and share a β-propeller fold (for a review see Valpuesta et al. 2002). Among the proteins that require CCT for their biogenesis we find centractin (actin-related protein), septin, von Hippel–Lindau tumor suppressor, histone deacetylases (HDAC3, Set3p, and Hos2p), and cell cycle regulators (Cyclin E, Cdc20p, and Cdc55p) (for reviews see Valpuesta et al. 2002; Dekker et al. 2008). However, not all CCT interacting proteins are chaperonin substrates and are probably involved in the regulation of its activity. For example, proteins such as phosducin-like 1, 2, and 3 (PhLP1-3) bind to CCT in their native forms and were described as modulators of CCT function by competing with CCT substrates or modulating its ATPase activity (for review Willardson and Howlett 2007). In contrast, caveolin-1 (a protein that is a major component of caveolae and membrane lipid rafts) also interacts directly with CCT, modulating the folding of actin through the protein filamin (Doucey et al. 2006).

The whole CCT complex mediates protein folding, driven by ATP binding and hydrolysis. The CCT folding mechanisms have been studied mainly for actin and tubulins.

Newly synthesized tubulins and actin are delivered to the CCT complex by prefoldin, a hetero-hexameric complex also referred to as Gim C, which interacts directly with the chaperonin (for a review see Hartl and Hayer-Hartl; Lundin et al. 2010). Actin and tubulin interact with CCT in a quasi-native conformation



CCTα, Fig. 3 Model of the structural changes undergone by the actin and tubulin molecules during the CCT functional cycle. Docking models with the atomic structures of actin and tubulin have been overimposed to the volume of the same proteins complexed with CCT. Quasi-folded actin (1) and tubulin (3) molecules bind to apo-CCT. The N-terminal domains of both cytoskeletal proteins (*colored red*) bind to CCT with less affinity than the corresponding C-terminal domains. ATP binding induces large movements of the CCT apical domains that seal the cavity. These movements occur sequentially, starting in CCTα, and move in an anticlockwise direction (see *yellow arrows* in 1 and 3). Following this sequence, the N-terminal domains of both actin and tubulin molecules are the first ones to respond to the rearrangement of the apical domains, resulting in their release and movement toward the C-termini, giving rise to a more native conformation (2 and 4). The C-terminal domains bind to CCT with a higher affinity than the N-terminal domains, and this interaction is maintained after nucleotide binding and hydrolysis. Nucleotide release induces the return to the nucleotide-free, open state and the liberation of the folded substrate. Only one of the possibilities for actin and tubulin binding to CCT is shown, although the other fits equally well with the model proposed (figure from Llorca et al. 2001 with permission)

through specific interactions between the apical domains of specific CCT subunits and defined regions of these proteins (Llorca et al. 2001).

It was proposed that actin and tubulin molecules bind to the nucleotide-free (apo-CCT), open conformations of CCT, and are thus stabilized in quasi-native conformations that correspond to a high degree of secondary and tertiary structure (Llorca et al. 2001). On binding ATP, the central ring cavity of the ATP-ring expands slightly and is closed by the helical extensions of the apical domains (Llorca et al., 2001). These movements are coupled to the folding movements of actin and tubulin and force the substrate molecules to achieve their quasi-native structures. Llorca and colleagues (2001) proposed that after ATP binding and hydrolysis the target proteins maintain their interaction with the chaperonin, and the release of the nucleotide leads to the substrate discharge and the regeneration of apo-CCT open conformation (Fig. 3).

CCT: The Cytoskeleton and Cell Cycle Progression

Since the first studies, it was clear that the cytosolic chaperonin CCT was intimately connected with the formation of the cytoskeleton in vivo. For example, in the budding yeast mutations in the CCTα gene caused both abnormal microtubular structures and disruption of actin microfilaments (for review Kubota et al. 1995).

Subsequently, TCP-1/CCTα and CCTζ were identified in centrosomes. Furthermore, the incubation of these microtubule organizing centers with an antibody against TCP-1/CCTα in vitro prevents microtubule growth, indicating that TCP-1/CCTα may assist in microtubule nucleation (for review Brackley and Grantham 2009).

CCTα, CCTγ, CCTζ, and CCTθ subunits associate with microtubules polymerized in vitro behaving as

typical microtubule-associated proteins. In addition, TCP-1/CCTα associates with the microtubules of the manchette, a highly specialized microtubule structure of male germ cells (for review Brackley and Grantham 2009).

In addition, Stephens and Lemieux (1999) reported the progressive appearance of TCP1/CCTα in cilia during the final stages of cilia regeneration in the sea urchin. In agreement, CCTα, CCTε, CCTδ, and CCTη subunits localize at cilia tips, basal bodies, and other complex microtubule structures in the ciliate protozoa *Tetrahymena pyriformis* (Seixas et al. 2003). In, *T. thermophila*, CCTα or CCTδ knockouts caused a loss of cell body microtubules, failure to assemble new cilia, and cell death (Seixas et al. 2010). Additionally, the loss of CCTα subunit activity leads to axoneme shortening and splaying of cilia tips.

Recently, CCT-subunits (CCTα-ε and CCTθ) were shown to form a complex with the chaperonin-like BBS6, BBS10, and BBS12 proteins (vertebrate specific BBS genes) (Seo et al. 2010) required for BBSome assembly. The BBSome is an oligomeric complex of BBS (BBS1-2, BBS4-5, BBS7-9) proteins that have been directly implicated in ciliogenesis by promoting vesicle trafficking to the cilia membrane (Nachury et al. 2007). In fact, several studies have shown that mutations in BBS proteins underlie the Bardel Biedl Syndrome (BBS), a genetically heterogeneous disorder associated with defects in primary cilia (Seo et al. 2010).

The interaction of CCT subunits with membranes was already reported. The adrenal medullary form of CCT (chromobindin A) binds efficiently to chromaffin granule membranes (Kubota et al. 1995). Furthermore, in human erythrocytes, CCTα is translocated to the plasma membrane where it binds by a specific association with actin cytoskeleton proteins following a heat shock (Wagner et al. 2004).

CCT has been also implicated in the assembly of the actin cytoskeleton, by acting at filament ends. In fact, the CCT chaperonin interacts in vitro with filamentous actin and decreases the initial rate of actin polymerization (for review Brackley and Grantham, 2009). Accordingly, the depletion of CCT levels in vivo by CCT-subunit specific siRNA inhibits cell cycle progression and alterations in microtubule and actin cytoskeleton organization and cell motility (for review Brackley and Grantham, 2009).

The fact that CCT subunits do not always colocalize strongly suggest that they might have other functions as free entities or as part of microcomplexes. For example, the CCTα-subunit is more abundant in growing neurites than other CCT-subunits and in yeast the CCTθ subunit appears to play a role in the Ras signaling pathway (for review Kubota et al. 1995; Brackley and Grantham 2009).

The involvement of specific CCT subunits with actin and microtubule networks seems to indicate that they have in fact specific functions outside the whole complex. However, the oligomerization state and the mechanism/s by which CCT-subunits are involved in the assembly and regulation of these dynamic filaments are far from being completely understood and deserve further investigation.

CCT subunits are mainly cytoplasmic; however, CCTα and CCTγ have also been observed to be localized in the nucleus (for a review see Gonçalves et al., 2007). TCP-1/CCTα was also described to associate with heterochromatin in both somatic and germ cells during mammalian spermiogenesis and was suggested that CCT assists in the folding of nuclear matrix proteins and proteins involved in DNA remodeling (for review Brackley and Grantham, 2009). The presence of the whole CCT complex in the nucleus has not yet been demonstrated. However, it has been suggested that CCTα may have a specific nuclear role because it translocates into the nucleus upon the induction of apoptosis (for a review see Gonçalves et al., 2007). Recently, an extensive study of the CCT interactome showed that, besides SET3 and histone deacetylase complex other enzymes responsible for histone post-translational modifications interact with CCT which definitively implicates the chaperonin in chromatin remodeling and consequently in gene expression regulation (Dekker et al. 2008).

Numerous evidences suggest an involvement of CCT in cell cycle-regulated events. For example, reducing CCT levels by siRNA assays in mammalian cultured cells leads to cell cycle arrest (for review Brackley and Grantham, 2009). Additionally, CCT subunits expression is regulated during cell cycle. For example, the expression of TCP-1/CCTα messenger RNA is strongly up-regulated during cell growth especially from the G1/S transition to early S phase (for review Brackley and Grantham, 2009). This idea is supported by observations in the mouse showing that highly proliferative tissues (such as testis, spleen, thymus, and bone marrow) and cultured cells express much higher levels of TCP-1/CCTα and the other CCT subunits than those

with low proliferation rates (such as heart, kidney and lung) (for a review see Gonçalves et al. 2007).

The abundance of CCT varies during the cell cycle which seems to reflect the requirements of the cell for specific cell cycle regulators that are known to interact with such as cyclin E, cdk-2, cdc20 and Plk1. Besides the alterations in abundance it was shown that CCT complexes in M-phase-arrested cells have less ability to bind α- and β-tubulin in vivo and for binding and folding β-actin in vitro when compared with the CCT complexes in S phase-arrested cells (for a review see Gonçalves et al. 2007). Thus, the regulation of CCT activity correlates with a fine tuning of the cell cycle by defining the precise moment of folding/assembly of proteins/complexes involved in cell cycle progression.

CCT in Stress Response and Disease

Many molecular chaperones are stress-inducible, making it interesting that the levels of TCP-1/CCTα decrease in *S. cerevisiae* submitted to hyperthermic stress. However, the levels of TCP1/CCTα were unaffected when mammalian cells were subjected to heat shock (for review Kubota et al. 1995). Nevertheless, it was also shown that TCP-1/CCTα, as well as the other CCT subunits, are up-regulated in several mammalian cell lines during recovery from chemical stress (sodium arsenite or a proline analogue, l-azetidine-2-carboxylic acid) (for a review see Kubota, 2002), suggesting that they respond to protein damage and have a function in the recovery of cells from specific stresses.

Taking into account the different observations concerning the response of CCT genes to stress/environmental stimuli it is plausible that the induction of TCP-1/CCTα by stress conditions may be dependent on the type of stress, cell type, organism, or environmental conditions. Moreover, in some of these studies the corresponding protein levels were not investigated. The mouse TCP-1/CCTα gene contains in its first intron a heat-shock element that is recognized by the heat-shock transcription factors HSF1 and HSF2, whose overexpression in HeLa cells activates the TCP-1/CCTα gene (for review Kubota 2002). However, no significant increase in CCT subunits in HeLa cells was detected by western blot analysis after heat treatment at 42–45°C, whereas **Hsp70** protein was induced (for review Kubota 2002). HSF2 is also known to be important in the tissue-specific and developmental-stage-specific expression of HSPs. This transcription factor is highly expressed in embryos and in testis, and this could indicate that this factor is involved in the regulation of TCP-1/CCTα under non-stress conditions.

Many molecular chaperones, e.g., **Hsp70**, have been extensively implicated in the pathogenesis of misfolding diseases. Indeed, the inability of an essential protein to form its native structure under physiological conditions may be the basis of a variety of human diseases.

In the case of CCT, a relationship has been found with neurodegenerative diseases. The Huntington's disease is caused by the misfolding and aggregation of proteins with expanded polyglutamine (polyQ) repeats. CCT interacts with polyglutamine-expanded variants of the neuronal huntingtin (Htt) protein and prevents their aggregation and the resulting formation of toxic polyQ fibrils. Depletion of CCT enhances polyglutamine aggregation, whereas its overexpression has the opposite effect, preventing neurotoxicity. In this context, this chaperonin may be a key component in the pathway leading to the progression of Htt to pathogenic conformations. Interestingly it was shown that, in this process, the CCT chaperonin acts synergistically with **Hsp70** and Hsp40 on initial oligomer forms of huntingtin promoting the formation of nonpathogenic oligomers (for a review see Broadley and Hartl 2009).

The altered expression of some genes encoding CCT subunits were also reported in other pathological processes. For example the TCP-1/CCTα was shown to present altered patterns of expression in the brain in the early stages of developing Down syndrome (for review Gonçalves et al. 2007).

Increased levels of TCP-1/CCTα were also reported associated with human hepatocellular and colonic carcinoma and in a cisplatin-resistant ovarian cancer cell line (for review Lundin et al. 2010).

Summary

CCT is an abundant cytosolic chaperonin composed of two hetero-oligomeric stacked rings arranged back to back, with a central cavity that interacts with a large number of distinct cellular proteins as shown by the recent characterization of the CCT interactome. Among these interacting proteins some will constitute true substrates while others will be co-chaperones or

regulators. Tubulins and actin are the most prominent cytoskeletal CCT substrates, but proteins involved in various cellular processes, such as cell cycle regulation and chromatin remodeling, have also been described. CCT is therefore a key player in the maintenance of cellular homeostasis. Many evidences, supported by cellular localizations and by the presence of different oligomeric forms of CCT subunits, have also indicated that CCT subunits may play additional functions outside of the chaperonin complex. For example, it has been proposed that CCT subunits behave as microtubule-associated proteins or regulate actin polymerization. Important is also the fact that some CCT subunits have been found in centrosomes, basal bodies, and cilia axonemes and are essential for the maintenance of the ciliary structure. This places the CCTcomplex/CCT subunits in a new cellular compartment and in the middle of the very exciting field of cilia biology. In conclusion, the misregulation of CCTcomplex/CCT subunits might be in the basis of a myriad of human diseases from cancer to ciliopathies.

References

Brackley KI, Grantham J. Activities of the chaperonin containing TCP-1 (CCT): implications for cell cycle progression and cytoskeletal organisation. Cell Stress Chaperones. 2009;14:23–31.

Broadley SA, Hartl FU. The role of molecular chaperones in human misfolding diseases. FEBS Lett. 2009;583:2647–53.

Dekker C, Stirling PC, McCormack EA, Filmore H, Paul A, Brost RL, Costanzo M, Boone C, Leroux MR, Willison KR. The interaction network of the chaperonin CCT. EMBO J. 2008;27:1827–39.

Doucey MA, Bender FC, Hess D, Hofsteenge J, Bron C. Caveolin-1 interacts with the chaperone complex TCP-1 and modulates its protein folding activity. Cell Mol Life Sci. 2006;63:7–8.

Gonçalves J, Nolasco S, Soares H. CCTalpha. AfCS Nat Molecule Pages. 2007. doi:10.1038/mp.a003968.01.

Hartl FU, Hayer-Hartl M. Converging concepts of protein folding in vitro and in vivo. Nat Struct Mol Biol. 2009;16:574–81.

Kubota H. Function and regulation of cytosolic molecular chaperone CCT. Vitam Horm. 2002;65:313–31.

Kubota H, Hynes G, Willison K. The chaperonin containing t-complex polypeptide 1 (TCP-1). Multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. Eur J Biochem. 1995;230:3–16.

Llorca O, Smyth MG, Carrascosa JL, Willison KR, Radermacher M, Steinbacher S, Valpuesta JM. 3D reconstruction of the ATP-bound form of CCT reveals the asymmetric folding conformation of a type II chaperonin. Nat Struct Biol. 1999;6:639–42.

Llorca O, Martín-Benito J, Grantham J, Ritco-Vonsovici M, Willison KR, Carrascosa JL, Valpuesta JM. The 'sequential allosteric ring' mechanism in the eukaryotic chaperonin-assisted folding of actin and tubulin. EMBO J. 2001;20:4065–75.

Lundin VF, Leroux MR, Stirling PC. Quality control of cytoskeletal proteins and human disease. Trends Biochem Sci. 2010;35:288–97.

Nachury MV, Loktev AV, Zhang Q, Westlake CJ, Peränen J, Merdes A, Slusarski DC, Scheller RH, Bazan JF, Sheffield VC, Jackson PK. A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. Cell. 2007;129:1201–13.

Seixas C, Casalou C, Melo LV, Nolasco S, Brogueira P, Soares H. Subunits of the chaperonin CCT are associated with Tetrahymena microtubule structures and are involved in cilia biogenesis. Exp Cell Res. 2003;290:303–21.

Seixas C, Cruto T, Tavares A, Gaertig J, Soares H. CCTalpha and CCTdelta chaperonin subunits are essential and required for cilia assembly and maintenance in Tetrahymena. PLoS ONE. 2010;5:e10704.

Seo S, Baye LM, Schulz NP, Beck JS, Zhang Q, Slusarski DC, Sheffield VC. BBS6, BBS10, and BBS12 form a complex with CCT/TRiC family chaperonins and mediate BBSome assembly. Proc Natl Acad Sci USA. 2010;107:1488–93.

Stephens RE, Lemieux NA. Molecular chaperones in cilia and flagella: implications for protein turnover. Cell Motil Cytoskeleton. 1999;44:274–83.

Valpuesta JM, Martín-Benito J, Gómez-Puertas P, Carrascosa JL, Willison KR. Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT. FEBS Lett. 2002;529:11–6.

Wagner CT, Lu IY, Hoffman MH, Sun WQ, Trent JD, Connor J. T-complex polypeptide-1 interacts with the erythrocyte cytoskeleton in response to elevated temperatures. J Biol Chem. 2004;279:16223–8.

Willardson BM, Howlett AC. Function of phosducin-like proteins in G protein signaling and chaperone-assisted protein folding. Cell Signal. 2007;19:2417–27.

CD103

► Alpha E Integrin

CD160

Armand Bensussan and Anne Marie-Cardine
INSERM U976, Saint Louis Hospital, Paris cedex 10, France

Synonyms

BY55

Historical Background

NK lymphocytes recognize abnormal or aberrant cells through multiple receptors that detect normal host molecules, as well as stress-induced or pathogen-expressed motifs (Lanier 2005). Individual NK cells express both activating and inhibitory receptors, which together drive the specificity toward target cells.

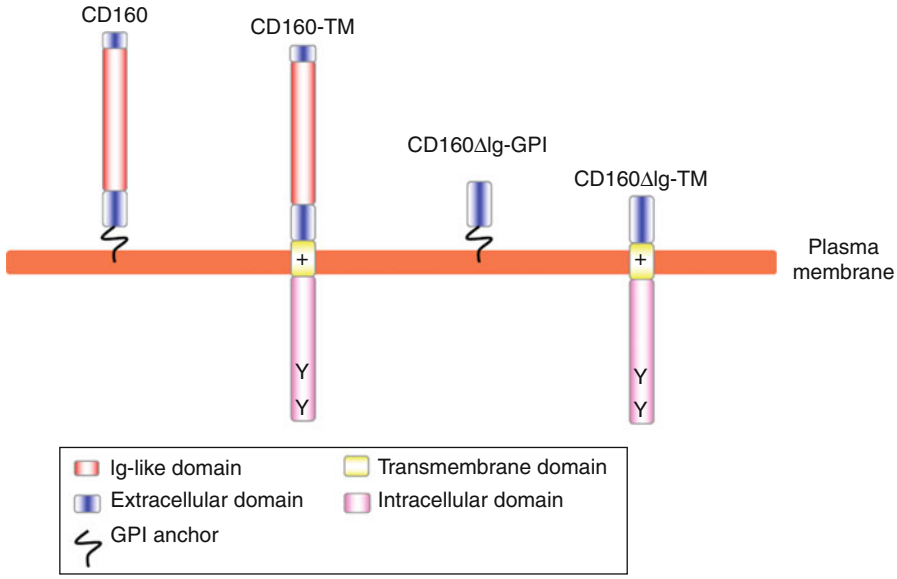
The NK cell inhibitory receptors have been classified into three groups, namely, the heterodimeric CD94/NKG2A, the Ig-like transcript (ILT) receptors, and the members of the killer cell Ig-like receptors (KIRs). All of them bind to classical or nonclassical MHC-class I molecules. A common characteristic of the inhibitory receptors is the presence of immunoreceptor tyrosine-based inhibition motif(s) (ITIM) within their intracellular tail. Following engagement by their ligands, the inhibitory receptors become phosphorylated on the tyrosine residue(s) present in the ITIM(s), creating docking sites for the SH2-domains of the cytoplasmic protein tyrosine phosphatases, SHP1 and SHP2. Their recruitment further results in the down-regulation of the intracellular activation cascade. In contrast, activating receptors recognize a large variety of ligands, mostly distinct from MHC-class I molecules, and exhibit more complex but well-characterized signaling pathways. Natural cytotoxicity receptors (NCRs) and ▶ NKG2D are the major receptors involved in NK cytotoxicity. The NCRs (namely, ▶ NKp46, NKp44, and NKp30) belong to the Ig superfamily and represent non-MHC-class I-specific receptors whose cellular ligands still have to be confirmed (Arnon et al. 2006). In contrast to NKp46 and NKp30, constitutively expressed on circulating NK lymphocytes, NKp44 expression is activation-dependent (Vitale et al. 1998). The NCRs transduce signals through their association with ITAM-containing molecules such as CD3ζ, FcεRIγ, and DAP12 (Moretta et al. 2000; Vivier et al. 2004). Besides the NCRs, NKG2D is a C-type lectin-like receptor shown to recognize the MHC-class I homologues MICA and MICB, and the family of UL16-binding proteins (ULBPs) (Gonzalez et al. 2006). NKG2D uses the transmembrane polypeptide DAP10 for signaling, which interacts with the ▶ PI 3-kinase once phosphorylated. Interestingly NKp80, an additional C-type lectin-like activating receptor exclusively expressed by human NK cells, has been identified. A search for NKp80 ligands led to the identification of activation-induced C-type lectin (AICL) (Welte et al. 2006). However, NKp80 signaling pathway remains enigmatic as this receptor does not contain a transmembrane charged residue (a feature allowing association with ITAM-containing adaptor proteins) or any intracellular consensus activation motifs. Finally, besides these MHC- class Ia/Ib molecule-independent activating receptors, it is important to mention the well-characterized DAP12-associated CD94/NKG2C and KIRs activating isoforms (Lanier et al. 1998; Olcese et al. 1997), although the precise events leading to their specific recruitment still have to be better defined. Here we review our knowledge on a unique NK cell receptor, named CD160, which is expressed by circulating $CD56^{dim}CD16^{bright}CD3^{-}$ NK lymphocytes.

Subheadings

Structure, Specificity, and Function of CD160

CD160 receptor was initially identified by the monoclonal antibody (mAb) BY55 generated following immunization with YT2C2, a human leukemia cell line with NK activity. BY55 mAb was shown to immunoprecipitate an 80 kDa cell surface structure expressed mainly on a subset of circulating NK and T lymphocytes and on all intestinal intraepithelial T lymphocytes (Anumanthan et al. 1998; Maiza et al. 1993). Subsequently, CD160 expression was detected on mast cells (Ortonne et al. 2011), activated endothelial cells (Fons et al. 2006), and found increased in activated T lymphocytes (Abecassis et al. 2007; Nikolova et al. 2009). The increased expression of CD160 during T cell activation was found to be inhibited in $CD4^{+}CD25^{bright}$Fox $P3^{+}$ lymphocytes.

The molecular cloning of CD160 molecule recognized by BY55 mAb revealed that it is a glycosylphosphatidylinositol (GPI)-anchored receptor, unique in its nucleotide and aminoacid sequences, which has a single Ig-like extracellular domain (Anumanthan et al. 1998). The *CD160* gene was found to be located on human chromosome 1q21.1. The mature molecule corresponds to a cysteine rich 134-aminoacids polypeptide, with six cysteines, that has two potential sites for *N*-linked glycosylation. The size predicted for the mature CD160 polypeptide plus two for *N*-linked glycosylations would be approximately 25 kDa indicating

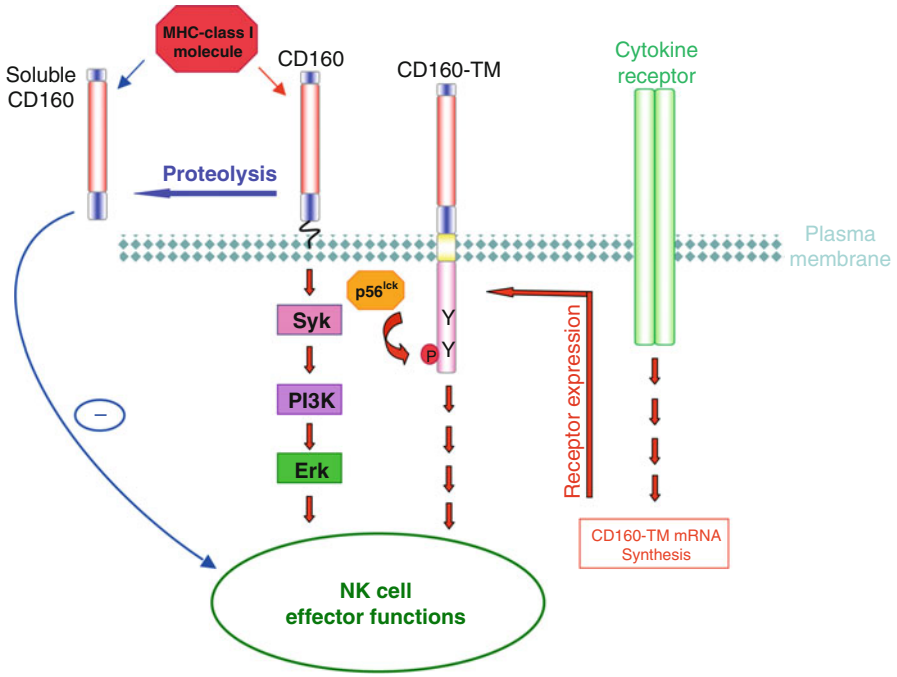


CD160, Fig. 1 Schematic representation of monomeric CD160 isoforms. Two types of receptors can be distinguished according to their mode of insertion to the plasma membrane (GPI-anchored or transmembrane molecule) and the presence or absence of an Ig-like domain within the extracellular part. All isoforms have in common the signal peptide and the membrane proximal moiety. Transmembrane isoforms are characterized by a transmembrane charged lysine residue (+) and the presence of two putative intracellular phosphorylation sites on tyrosine residues (Y)

that the 80 kDa cell surface structure immunoprecipitated by BY55 mAb corresponds to multimers (most likely trimers).

CD160 Ig-like domain is related to a number of Ig superfamily members, with the highest degree of homology being to Ig-V domains of the pregnancy-specific glycoprotein and the first Ig-C2 domain of KIR. This homology is weak, with 29% identity between CD160 and KIR2DL4/CD158d. The 68-aminoacids distance between the Ig cysteines at positions 44 and 112 is more characteristic of an Ig-V domain than an Ig-C domain. The CD160 structure is unusual for an Ig superfamily member as it has an additional cysteine immediately following the 112 Ig cysteine. This dicysteine is found only in few other Ig superfamily members with three Ig-V domains. Similar to CTLA4, CD160 has two cysteines between the Ig cysteines that potentially promote the looping out of a short region between aminoacids 61 and 68. CD160 receptor binds to MHC-class Ia/Ib molecules (Agrawal et al. 1999; Le Bouteiller et al. 2002) and to HVEM (Cai et al. 2008).

Functionally, CD160 behaves as an activating receptor on $CD56^{dim}CD16^{+}$ NK lymphocytes (Fig. 1) as demonstrated by the induction of their cytotoxic potential upon engagement (Le Bouteiller et al. 2002). Furthermore, its engagement with its physiological ligand or with specific mAbs triggers a unique profile of cytokine secretion, with the release of ► TNF-α, ► IFN-γ, and IL-6 (Barakonyi et al. 2004). The CD160-mediated signaling molecules recruited upon specific crosslinking on NK lymphocytes upstream and downstream of PI3-kinase correspond to Syk and Erk, respectively (Rabot et al. 2007). Importantly, in isolated CD160-enriched peripheral blood $CD8^{+}$ T lymphocytes CD160 ligation by itself does not provide activation but enhances their CD3-induced proliferation. Thus, its co-triggering with ► CD3 results in its association with $p56^{lck}$ and tyrosine phosphorylated CD3-ζ chains (Nikolova et al. 2002). Besides its activating receptor function in NK cells and $CD8^{+}$ T lymphocytes, CD160 is found to exhibit inhibitory features in other cell types. Thus, on activated endothelial cells, engagement of CD160 with its physiological ligand HLA-G1 results in inhibition of the endothelial tube formation and induction of apoptosis (Fons et al. 2006). Similarly, CD160 interaction with the herpes virus entry mediator



CD160, Fig. 2 CD160 and CD160-TM signaling in NK cells. Upon ligand recognition, CD160 mediates positive signals with the recruitment of Syk, PI3-kinase, and Erk that result in the generation of an NK cell effector response. Short-time cell activation (addition of cytokines for 1–3 days) leads to the proteolysis of CD160 through a phospholipase-dependent mechanism and to the release of soluble CD160. This latter promotes the down-modulation of NK cell functions, most likely by acting as a blocking molecule that competes with other activating NK cell receptors for the binding of MHC-class I molecules. This step is followed by the synthesis of CD160-TM mRNA and receptor expression at the plasma membrane (day 3–10 following activation). Upon phosphorylation by $p56^{lck}$, CD160-TM initiates a cascade of still undefined activating signals that enhances the NK cell killing activity

(HVEM) is described as inhibiting $CD4^+$ T lymphocytes activation (Cai et al. 2008). However, the mechanisms leading to the arrest of proliferation remain to be elucidated.

In NK lymphocytes or mast cells the GPI-anchored CD160 is down-modulated upon activation with cytokines. Indeed IL-15 treatment converts the membrane-bound receptor to a soluble form through a proteolytic cleavage involving a metalloprotease (Giustiniani et al. 2007). The CD160 transcript remains highly synthesized during the process of protein shedding, its synthesis being even induced in $CD56^{bright}$ NK cells upon interleukin-treatment. The soluble CD160 that is released by NK lymphocytes or mast cells impairs the MHC-class I-specific cytotoxic $CD8^+$ T lymphocytes and NK cells function. This observation is most likely related to the ability of soluble CD160 to interact with MHC-class I molecule (Giustiniani et al. 2007) and to eventually compete with activating NK receptors for ligand binding, resulting in the down-modulation of NK cell cytotoxic activity (Fig. 1).

CD160 Transmembrane Isoform (CD160-TM): Structure and Function

Later on, three additional RNA encoding putative CD160 isoforms were identified in NK cells. These transcripts originate from the alternative splicing of the *CD160* gene, and their translation would lead to the synthesis of proteins sharing high level of homology with CD160 (Fig. 2; Giustiniani et al. 2009). The first one corresponds to a CD160 mRNA that lacks the coding region for the Ig domain; this deletion would therefore result in a GPI-anchored molecule devoid of extracellular Ig domain (CD160ΔIg-GPI). The second transcript presents a high level of identity with the CD160ΔIg-GPI coding sequence and would lead to a CD160 molecule with no extracellular Ig domain

but possessing a transmembrane and an intracellular domain (CD160ΔIg-TM). The third mRNA is the larger one and codes for a putative receptor having the complete extracellular moiety of CD160, as well as a transmembrane and an intracellular part, and would thus corresponds to a transmembrane isoform of the original GPI-anchored molecule (CD160-TM). CD160-TM encompasses a transmembrane charged aminoacid, a feature usually found in activating KIRs and allowing their interaction with DAP10 or DAP12, as well as two potential tyrosine phosphorylation sites within its intracellular domain. The two transcripts coding for the GPI-linked molecules are detected in resting NK cells, while the synthesis of the transmembrane receptors mRNA is only induced upon NK cell activation (Giustiniani et al. 2009). While the proper expression of the two isoforms lacking an Ig domain cannot be assessed because of a lack for the appropriate antibodies, the translation of the CD160-TM isoform is specifically detected in activated NK cells but not activated T lymphocytes, mast cells, or endothelial cells. In fact, the appearance of CD160-TM at the plasma membrane parallels the disappearance of the GPI-bound isoform that occurs through a phopholipase-dependent proteolysis (Giustiniani et al. 2007).

Biochemical analyses further reveal that CD160-TM is expressed as a multimeric molecule with an apparent molecular weight of 100 kDa. Its cellular ligands remain to be identified, but given the complete homology of its extracellular domain with the one of CD160, they may share the same ligands, namely, MHC-class I molecules and HVEM. However, conformational changes induced by the presence of a transmembrane and an intracellular domain may also drive a different ligand specificity for CD160-TM. This possibility is sustained by the observation that CD160-TM isoform is not recognized by the anti-CD160 mAb BY55 (Giustiniani et al. 2009).

Functionally, CD160-TM behaves as an activating receptor on NK cells (Fig. 1). Indeed, its triggering leads to the generation of cytotoxic activity toward target cells and enhances NK cells degranulation. This activating function is under the control of an intracellular tyrosine residue located within the intracellular domain of CD160-TM and requires the expression of the Src-family kinase $p56^{lck}$, as assessed by the loss of function upon tyrosine mutation or kinase depletion (Giustiniani et al. 2007). However, the proteic intermediates involved in the downstream signaling pathways remain to be identified.

Conclusions

Among the NK receptors, CD160 and the NK cell lineage highly restricted CD160-TM present unique structural and functional characteristics. Work performed so far suggests that the tightly regulated expression of CD160 isoforms might be an important step in the cascade of events leading to a specific and efficient NK cell response. The existence of at least a GPI-linked and a transmembrane CD160 receptor, each expressed at different stage of NK cell activation and signaling through a distinct intracellular pathway, together with the generation of a soluble form of CD160 that mediates immunoregulatory functions open new perspectives regarding the cellular events involved in the regulation of NK cell functions. Finally, while the transmembrane CD160 receptor does not exist in mice, the GPI-anchored CD160 is expressed but only as a dimeric molecule (Maeda et al. 2005). Therefore, it remains to consider the functional specificity of CD160 according to its various structural features.

Summary

CD160 is a multimeric Ig-like 134-aminoacid glycoprotein expressed as a GPI-anchored receptor on $CD56^{dim}CD16^{+}$ circulating NK lymphocytes, T lymphocyte subsets, endothelial cells, and mast cells. Upon activation, only in NK lymphocytes, an additional transmembrane CD160 isoform, with two putative tyrosine phosphorylation sites within its cytoplasmic tail, is expressed. Both isoforms share identical extracellular region with an Ig-like V-domain, that is weakly homologous to the one of KIR2DL4/CD158d, and behave as activating NK receptors upon engagement. MHC-class I molecules have been shown to bind with low affinity to CD160 in mice and humans. The two receptors are described as participating in the generation of an appropriate NK cell effector response. Thus, while the transmembrane CD160 isoform is exclusively expressed on NK cells upon activation, the GPI-anchored receptor is cleaved and the resulted soluble CD160 molecule exhibits immunoregulatory function.

References

Abecassis S, Giustiniani J, Meyer N, Schiavon V, Ortonne N, Campillo JA, et al. Identification of a novel CD160+ CD4+ T-lymphocyte subset in the skin: a possible role for CD160 in skin inflammation. J Invest Dermatol. 2007;127:1161–6.

Agrawal S, Marquet J, Freeman GJ, Tawab A, Bouteiller PL, Roth P, et al. Cutting edge: MHC class I triggering by a novel cell surface ligand costimulates proliferation of activated human T cells. J Immunol. 1999;162:1223–6.

Anumanthan A, Bensussan A, Boumsell L, Christ AD, Blumberg RS, Voss SD, et al. Cloning of BY55, a novel Ig superfamily member expressed on NK cells, CTL, and intestinal intraepithelial lymphocytes. J Immunol. 1998;161:2780–90.

Arnon TI, Markel G, Mandelboim O. Tumor and viral recognition by natural killer cells receptors. Semin Cancer Biol. 2006;16:348–58.

Barakonyi A, Rabot M, Marie-Cardine A, Aguerre-Girr M, Polgar B, Schiavon V, et al. Cutting edge: engagement of CD160 by its HLA-C physiological ligand triggers a unique cytokine profile secretion in the cytotoxic peripheral blood NK cell subset. J Immunol. 2004;173:5349–54.

Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. Nat Immunol. 2008;9:176–85.

Fons P, Chabot S, Cartwright JE, Lenfant F, L'Faqihi F, Giustiniani J, et al. Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and by direct binding to CD160 receptor expressed by endothelial cells. Blood. 2006;108:2608–15.

Giustiniani J, Marie-Cardine A, Bensussan A. A soluble form of the MHC class I-specific CD160 receptor is released from human activated NK lymphocytes and inhibits cell-mediated cytotoxicity. J Immunol. 2007;178:1293–300.

Giustiniani J, Bensussan A, Marie-Cardine A. Identification and characterization of a transmembrane isoform of CD160 (CD160-TM), a unique activating receptor selectively expressed upon human NK cell activation. J Immunol. 2009;182:63–71.

Gonzalez S, Groh V, Spies T. Immunobiology of human NKG2D and its ligands. Curr Top Microbiol Immunol. 2006;298:121–38.

Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225–74.

Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity. 1998;8:693–701.

Le Bouteiller P, Barakonyi A, Giustiniani J, Lenfant F, Marie-Cardine A, Aguerre-Girr M, et al. Engagement of CD160 receptor by HLA-C is a triggering mechanism used by circulating natural killer (NK) cells to mediate cytotoxicity. Proc Natl Acad Sci USA. 2002;99:16963–8.

Maeda M, Carpenito C, Russell RC, Dasanjh J, Veinotte LL, Ohta H, et al. Murine CD160, Ig-like receptor on NK cells and NKT cells, recognizes classical and nonclassical MHC class I and regulates NK cell activation. J Immunol. 2005;175:4426–32.

Maiza H, Leca G, Mansur IG, Schiavon V, Boumsell L, Bensussan A. A novel 80-kD cell surface structure identifies human circulating lymphocytes with natural killer activity. J Exp Med. 1993;178:1121–6.

Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. Immunol Today. 2000;21:228–34.

Nikolova M, Marie-Cardine A, Boumsell L, Bensussan A. BY55/CD160 acts as a co-receptor in TCR signal transduction of a human circulating cytotoxic effector T lymphocyte subset lacking CD28 expression. Int Immunol. 2002;14:445–51.

Nikolova M, Lelievre JD, Carriere M, Bensussan A, Levy Y. Regulatory T cells differentially modulate the maturation and apoptosis of human CD8+ T-cell subsets. Blood. 2009;113:4556–65.

Olcese L, Cambiaggi A, Semenzato G, Bottino C, Moretta A, Vivier E. Human killer cell activatory receptors for MHC class I molecules are included in a multimeric complex expressed by natural killer cells. J Immunol. 1997;158:5083–6.

Ortonne N, Ram-Wolff C, Giustiniani J, Marie-Cardine A, Bagot M, Mecheri S, et al. Human and mouse mast cells express and secrete the GPI-anchored isoform of CD160. J Invest Dermatol. 2011; 131: 916–24.

Rabot M, El Costa H, Polgar B, Marie-Cardine A, Aguerre-Girr M, Barakonyi A, et al. CD160-activating NK cell effector functions depend on the phosphatidylinositol 3-kinase recruitment. Int Immunol. 2007;19:401–9.

Vitale M, Bottino C, Sivori S, Sanseverino L, Castriconi R, Marcenaro E, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med. 1998;187:2065–72.

Vivier E, Nunes JA, Vely F. Natural killer cell signaling pathways. Science. 2004;306:1517–9.

Welte S, Kuttruff S, Waldhauer I, Steinle A. Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction. Nat Immunol. 2006;7:1334–42.

CD191

► Chemokine Receptor CCR1

CD230

► Prion Protein (PRNP)

CD247

► CD3ζ

CD266

▶ Fn14

CD280

▶ MRC2

CD283

▶ Toll-Like Receptor 3

CD283 Antigen

▶ Toll-Like Receptor 3

CD284

▶ TLR4, Toll-Like Receptor 4

CD3

Sumit Deswal[1,2] and Wolfgang W. A. Schamel[1,3,4]
[1]Max Planck Institute of Immunobiology and Faculty of Biology, Biology III, University of Freiburg, Freiburg, Germany
[2]Spemann Graduate School of Biology and Medicine, Freiburg, Germany
[3]Centre for Biological Signaling Studies (BIOSS), University of Freiburg, Freiburg, Germany
[4]Centre of Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Freiburg, Germany

Synonyms

CD3γ; CD3δ; CD3ε; T3

Introduction

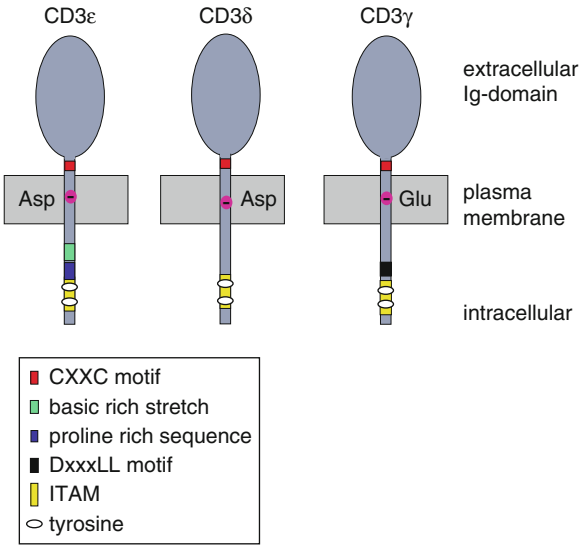
CD3 is a complex of three type 1 transmembrane (TM) proteins expressed exclusively in T cells: CD3γ, CD3δ, and CD3ε. ▶ CD3ζ (▶ CD247) is described in a separate chapter due to significant differences compared to CD3γ, CD3δ, and CD3ε. The CD3γ and CD3δ chains are glycoproteins, each of which forms a heterodimer with a non-glycosylated CD3ε chain. These chains associate with TCRαβ and a CD3ζζ dimer to form the αβ T cell antigen receptor (TCR-CD3) complex in αβ T cells (Figs. 1 and 2). The CD3 chains and CD3ζζ also associate with pTα and TCRβ to form the pre-TCR-CD3 and with TCRγδ to form the γδTCR-CD3 in the case of pre-T cells and γδ T cells, respectively. Each of the CD3 subunits possesses an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail which becomes phosphorylated upon antigen binding to TCRαβ. Hence, the TCR-CD3 is responsible for activation of a T cell upon antigen encounter, and thus, initiation of the immune response. In addition, CD3 and CD3ζ subunits bring TCRαβ to cell surface. As the CD3 subunits, CD3γ, CD3δ, and CD3ε, are expressed exclusively on T cells, they are used as T cell markers in clinical and basic research studies using flow cytometry.

Historical Background

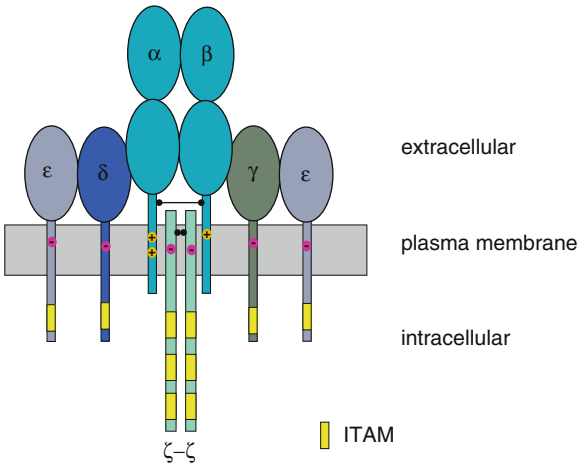
In the late 1970s, monoclonal antibodies against T cells were generated. One such antibody, OKT3, was generated in 1979 by P. Kung and G. Goldstein (Kung and Goldstein 1979). Later it was recognized that OKT3 binds to proteins involved in T cell activation and therefore the proteins were named T3. With the introduction of the immunological CD nomenclature in 1982, the proteins were renamed CD3. Now it is known that OKT3 binds to properly folded CD3ε expressed on the T cell surface and the interaction has been characterized in detail using X-ray crystallography.

Genomic Organization and Protein Structure

The CD3 genes are clustered in a 50-kb region of chromosomes 11 and 9 in humans and mice, respectively (Tunnacliffe et al. 1988). CD3δ is located in the



CD3, Fig. 1 Schematic representation of the CD3 subunits. CD3ε, CD3γ, and CD3δ are shown with their structural features highlighted



CD3, Fig. 2 The TCR-CD3 complex. CD3γ and CD3δ, each form a heterodimer with CD3ε. These heterodimers associate with TCRαβ and CD3ζζ to generate the TCR-CD3 complex that is expressed on T cells of the immune system. TCRαβ are the ligand-binding subunits, while the CD3 chains aid in receptor assembly, transport to the cell surface, and in signal transmission. Important for assembly are the potentially charged amino acids in the TM region of the TCR-CD3

central position of the cluster and flanked on either side by the oppositely transcribed CD3γ and CD3ε gene loci. CD3γ is less than 2 kb apart from CD3δ, and CD3ε is located about 22 kb downstream of CD3δ. CD3δ is encoded by five exons, whereas seven exons encode CD3γ and seven or eight exons encode CD3ε depending on the presence of two and three miniexons in human and mouse, respectively (Fig. 3). A model for the evolution of the CD3 gene family predicts that a single CD3 gene first duplicated to form CD3γ/δ and CD3ε, and a second duplication of the CD3γ/δ gene subsequently generated the CD3γ and CD3δ genes as found in mammals (Gobel and Dangy 2000).

At protein level, the CD3γ, CD3δ, and CD3ε subunits share a similar structure, comprised of an extracellular immunoglobulin (Ig) domain, a stalk region, a TM region of 27 amino acids, and a cytoplasmic domain of 45–55 amino acids. The Ig domains of the CD3εγ and CD3εδ dimers have been characterized by X-ray crystallography and nuclear magnetic resonance (NMR). Like all proteins that enter the secretory pathway, the CD3 chains contain an N-terminal signal peptide that targets protein translation to the endoplasmic reticulum (ER). Since this peptide is cleaved co-translationally, it is not present in the mature proteins.

The structure of these subunits can be described as follows.

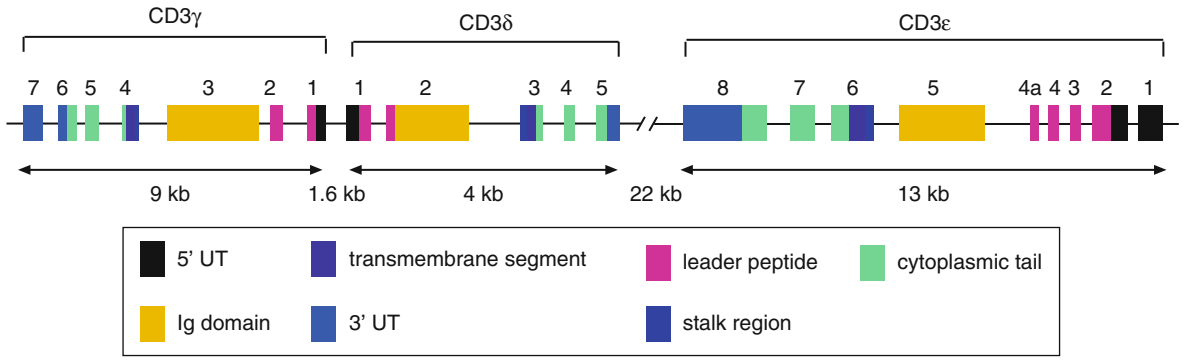
Ectodomains

The solution structure of the Ig domains of the mouse CD3εγ heterodimer showed that CD3ε and CD3γ both have C2-set Ig domains that interact with each other to form an unusual side-to-side dimer configuration. Crystal structures of the Ig domains of human CDεγ in combination with the OKT3 Fab fragment and human CD3εδ in combination with a single-chain fragment of the anti-CD3ε antibody UCHT1 have been solved (Arnett et al. 2004; Kjer-Nielsen et al. 2004). In contrast to mouse, human CD3ε contains a C1-set Ig fold. Human CD3γ has the C2-set Ig fold. Although the sequence identity between CD3ε and CD3γ is only 20%, the subunits share significant structural homology. Human CD3δ contains a C1-set Ig fold (Fig. 4).

In addition, CD3ε possesses several negatively charged residues in the N-terminal region before the Ig domain, which can be cleaved by metalloproteases. This results in two CD3ε isoforms with different molecular weights and isoelectric points.

Stalk Region

CD3ε, CD3γ, and CD3δ molecules each contain a CXXC motif in their short stalk region connecting the Ig-like domains to their TM regions. Some reports have suggested that the CXXC motif is involved in the dimerization of CD3ε to CD3γ or to CD3δ, or in the



CD3, Fig. 3 Genomic organization of the human CD3 cluster. Approximate sizes of CD3 genes and the intervening sequences are shown

binding of CD3 dimers to TCRαβ. However, other studies did not find significant impairment of assembly either of CD3 dimers or of the TCR-CD3 complex by mutating those cysteines in CD3ε. Molecular dynamics studies have suggested that the β strands of CD3ε become more rigid upon anti-CD3 antibody binding. This stiffening effect can be transmitted to the stalk region. The CXXC motif seems to participate in the transmission of the stiffening effect to the TM and cytoplasmic parts. In accordance with this, mutation of these cysteines in CD3ε prevented both the transmission of an antigen-induced conformational change to the cytoplasmic tail of CD3ε and the activation of T cells (Martinez-Martin et al. 2009). Thus, the stalk region might be important in transmitting the information that antigen has bound from TCRαβ to the cytoplasmic tails of CD3.

Transmembrane Region

CD3ε and CD3δ each possess an aspartic acid residue in its TM region, while CD3γ possesses a glutamic acid residue. These four acidic amino acids, along with two acidic residues of CD3ζζ dimer and three basic TM residues on TCRαβ (Fig. 1), play an important role in the assembly of TCRαβ with CD3εγ, CD3εδ, and CD3ζζ dimers. However, to date it is not known whether these TM residues are charged or not.

Cytoplasmic Region

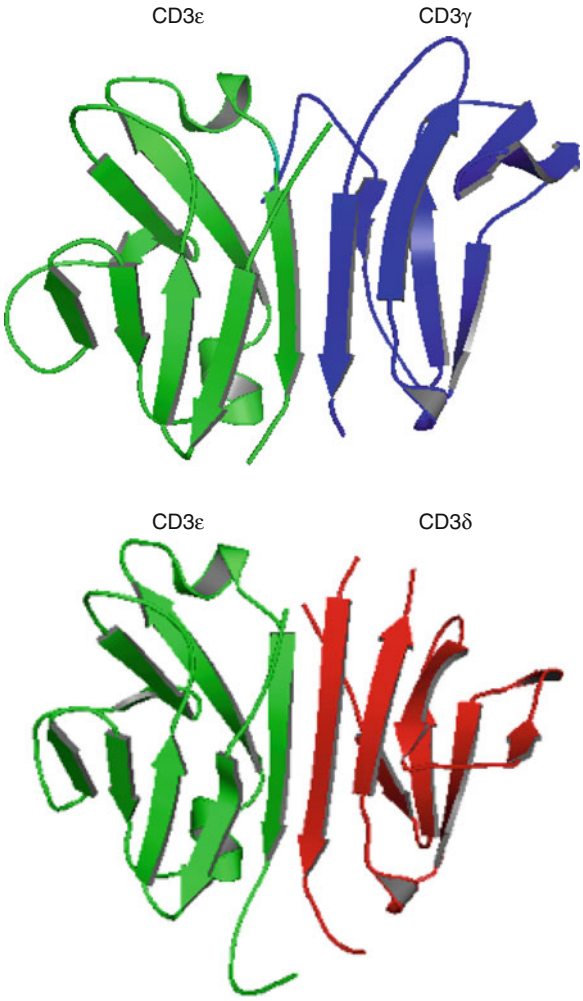
In the cytoplasmic portion, the CD3 subunits possess the signaling motifs discussed below.

Immunoreceptor Tyrosine-Based Activation Motifs

ITAMs have a consensus sequence of Yxx(L/I)$x_{6–8}$ Yxx(L/I) (Reth 1989). This motif was also known as TAM, ARH1, ARAM, YXXL, and Reth motif and occurs in a variety of proteins that include CD3ε, CD3δ, CD3γ, ▶ CD3ζ (3 ITAMs on each ▶ CD3ζ chain), Ig-α, Ig-β, FcεR1β, FcεR1γ, DAP12, and some viral proteins, e.g., the LMP2A protein from the Epstein–Barr virus. The ITAM tyrosine residues of CD3 are necessary for ITAM signaling activity and, when both are phosphorylated, bind to the tandem Src homology 2 (SH2) domains of ▶ ZAP70. Each TCR-CD3 complex contains 10 ITAMs. The exact role of each ITAM is still not defined due to the possibility of redundancy. The non-conserved amino acid sequences within the ITAMs, particularly at positions Y + 1 and Y + 2, might facilitate the association of signaling proteins through SH2 domain-containing domains. Indeed, in addition to ZAP70, phosphorylated ITAMs bind to Syk, Fyn, Lck, p85, Shc, ▶ SHIP, and other signaling proteins. The first tyrosine of the CD3ε ITAM lies within a proline-rich region. CD3 proteins do not contain non-ITAM tyrosines.

Di-Leucine-Based Motif

The di-leucine-based motif (DxxxLL) of CD3γ binds to the clathrin-coated vesicle adaptor proteins AP-1 and AP-2 and is involved in down-regulation of the TCR-CD3 (Dietrich et al. 1994). Phosphorylation of serine 126 induces a conformational change in the context of complete TCR-CD3. The DxxxLL motif is then exposed for AP-1/2 binding, a mechanism required for down-regulation. In an incompletely assembled TCR-CD3 lacking ▶ CD3ζ, the DxxxLL motif is directly accessible and recognized by AP-1 at the trans-Golgi network, leading to sorting of TCRαβCD3εγεδ to lysosomes for degradation. In contrast, in the fully assembled TCR-CD3 complex,



CD3, Fig. 4 Crystallographic structures of the immunoglobulin domains of CD3εγ and CD3εδ. Crystal structures were obtained from the protein data bank (human CD3γε heterodimer in complex with antibody OKT3 Fab fragment, PDB ID: 1SY6 and human CD3δε heterodimer in complex with UCHT1 single-chain antibody fragment, PDB ID, 1XIW) and figures generated using the software MacPymol. For simplicity, the antibody fragments are omitted

the DxxxLL motif is inaccessible, and the receptor follows the default transport route to the plasma membrane. Thus, ▶ CD3ζ masks the CD3γ DxxxLL motif in the fully assembled TCR-CD3 and this effect relies more on the length of the cytoplasmic tail than on the primary sequence (Lauritsen et al. 2004).

Proline-Rich Sequence

Proline-rich sequences (PRS) bind to Src homology 3 (SH3) domains. In CD3ε, the evolutionarily conserved PRS (RPPPVPNPDYEP) binds to the first SH3 domain of the adaptor protein Nck upon a conformational change induced by antigen binding to TCR-CD3 (Gil et al. 2002). The precise function of this interaction is not clear. According to some observations, the CD3ε PRS is necessary for peptide-MHC-induced phosphorylation of CD3ε, and for recruitment of protein kinase Cθ to the immune synapse in differentiated cytotoxic T lymphocytes. It is speculated that the PRS amplifies weak TCR signals by promoting synapse formation and CD3ε phosphorylation. This interaction is also used as a readout for the CD3 conformational change. In addition, Eps8L1 (epidermal growth factor receptor pathway substrate 8-related protein 1) binds to the CD3ε PRS but the functional consequence of this interaction has yet to be determined.

Basic Rich Stretch

The basic rich stretch (BRS, also called phospholipid binding motif) locates to the juxtamembrane portion of the CD3ε cytoplasmic tail (Deford-Watts et al. 2009). Positively charged residues of the BRS enable this part of CD3ε to bind to acidic phospholipids in vitro. The BRS is also shown to be important for localization of CD3ε to the immunological synapse and binding to signaling proteins GRK2 (G protein-coupled receptor kinase2) and CAST (CD3ε associated signal transducer).

Serine Residues

Unique to the cytoplasmic tail of CD3γ are three serine residues which represent potential phosphorylation sites that can subsequently interact with proteins containing phospho-serine binding domains such as 14-3-3, WW, and MH2 domains.

Retention Motif

All subunits of the TCR-CD3 contain ER retention/retrieval motifs. These motifs are important for sequential assembly of the TCR-CD3 components and assure that only a fully assembled TCR-CD3 is expressed on the cell surface. Different subunits contain different numbers of retention motifs. The CD3ε retention motif (an elongated α-helix followed by βI' turn and contains three closely spaced residues, tyrosine, leucine, and arginine) seems to be dominant as the CD3δε and CD3γε heterodimers reach the cell surface when the CD3ε retention motif is mutated. The CD3ε retention motif is overridden only when CD3ζ is incorporated in the TCR-CD3 complex.

Assembly and Membrane Organization

Assembly in the ER begins with the formation of CD3εδ and CD3εγ heterodimers. CD3εδ then associates with TCRα using the potentially charged TM residues (see above). CD3εγ dimers associate with TCRβ in a similar manner (Alarcon et al. 1988). Once TCRαβCD3εγεδ has formed, the CD3ζζ homodimer is the last subunit to join. The CD3ζζ homodimer requires the aspartic acids at position six in the TM regions of CD3ζ for interaction with the arginine residue in TCRα and upon its incorporation, the whole TCR-CD3 complex is transported to the plasma membrane (Sancho et al. 1989). In addition, the ectodomains of CD3δ and CD3γ play a role in selective association of CD3εδ to TCRα and CD3εγ to TCRβ.

No structural data have been obtained for the entire TCR-CD3 complex yet. However, mutagenesis studies suggest that CD3εγ and CD3εδ are located on the same side in the TCR-CD3 complexes, so that the other side can mediate homotypic TCRαβ interactions. Using electron microscopy and Blue Native-PAGE studies, it was shown that TCR-CD3s exist both as monovalent receptors with a stoichiometry of TCRαβCD3εγεδζζ (see below) and pre-clustered multimers of the monovalent receptor (Schamel et al. 2005).

Functions

Together with ▶ CD3ζ, the CD3 subunits are the main signaling units in the TCR-CD3. Upon antigen binding to TCRαβ, a conformational change in CD3ε exposes the PRS where the adaptor protein Nck can bind as discussed earlier. Antigen binding also leads to the phosphorylation of the ITAM tyrosines by the Src family kinases Lck and Fyn, which results in association of ▶ ZAP70 to phospho-tyrosines with its tandem SH2 domains. ▶ ZAP70 phosphorylates the adaptor protein ▶ LAT on several sites, which act as the hub for initiation of downstream signaling. The CD3 subunits also regulate surface TCRαβ expression. Knock-out mice and humans that lack individual CD3 chains show impairment in TCR-CD3 expression. CD3δ associates with the co-receptor CD8 and couples the TCR-CD3 to CD8 for signaling by the CD8-associated kinase Lck (Doucey et al. 2003) and for enhanced apparent affinity to peptide-MHC. As CD3 subunits are also associated with the pre-TCR-CD3 on developing thymocytes, CD3-mediated signaling is required toward progression of the double-positive thymocytes and TCRα gene rearrangement. Pro-T cells express low levels of CD3 possibly in association with calnexin in the absence of TCRαβ at their surface. Anti-CD3ε antibody-induced cross-linking of the CD3 molecules on pro-T cells of RAG-$1^{-/-}$ mice in vivo induces differentiation of these pro-T cells into pre-T cells. This suggests that CD3 has a functional role in pro-T cells.

Various transfection studies, genetic knock-out mice, and natural mutations have revealed the importance of individual CD3 subunits in T cell development. In the absence of intact CD3ε, thymocytes do not progress beyond the $CD44^{-/low}CD25^{+}$ triple-negative stage in the CD3ε knock-out mouse. CD3γ is essential for development of both the αβ and γδ T cell lineages in mice, whereas human patients lacking CD3γ develop αβ and γδ T cells in which CD3γ is replaced by CD3δ. CD3δ-deficient mice develop γδ T cells but no αβ T cells. Human patients lacking CD3δ, on the other hand, fail to develop either αβ or γδ T cells (Dadi et al. 2003). These observations can be explained by the distinct stoichiometries of human and mouse γδTCR-CD3s. While the stoichiometry of human γδTCR-CD3 is TCRγδCD3εγεδζζ, mouse γδTCR-CD3 has a stoichiometry of TCRγδCD3εγεγζζ (Siegers et al. 2007). Also the individual CD3 subunits have partially different roles in T cell development at distinct stages of development. CD3ε-deficiency blocks the T cell development at a triple-negative stage whereas CD3δ is more important for positive selection as evident by the mice lacking CD3δ in which T cells progress from a $CD4^{-}CD8^{-}$ double-negative to a $CD4^{+}CD8^{+}$ double-positive stage but fail to undergo positive selection.

Pathophysiological and Clinical Roles

Absence of individual CD3 subunits causes immunodeficiencies of varying severity. As discussed earlier, CD3δ- and CD3ε-deficient humans fail to develop either αβ or γδ T cells, resulting in severe combined immunodeficiency (SCID) (Dadi et al. 2003; Soudais et al. 1993). Deficiency in CD3γ results in a milder form of SCID, since T cells are present in which CD3γ is replaced by CD3δ (Arnaiz-Villena et al. 1992). Table 1 summarizes the effect of the deficiencies in individual CD3 subunits in human and mouse.

CD3, Table 1 Immunological disorders associated with different CD3-deficiencies in human and mouse

Human				
Deficiency	Presence of αβ T cells	Presence of γδ T cells	Phenotype	References
CD3γ	+	+	Mild immunodeficiency	Recio et al. (2007)
CD3δ	–	–	SCID	Dadi et al. (2003)
CD3ε	–	–	SCID	de Saint Basile et al. (2004)
Mouse				
Deficiency	Presence of αβ T cells		Presence of γδ T cells	References
CD3γ	–		–	Haks et al. (1998)
CD3δ	–		+	Dave et al. (1997)
CD3ε	–		–	Malissen et al. (1995)

In addition, anti-CD3 antibodies are used in the clinic for treatment of various immunological disorders. In fact, OKT3 was the first monoclonal antibody to be approved by the US Food and Drug Administration (FDA) in 1986 for clinical use. OKT3 is an immunosuppressant given to reduce acute rejection in patients with organ transplants such as allogeneic renal, heart, and liver transplants. Anti-CD3 antibodies are also used for the treatment of T cell acute lymphoblastic leukemia. Immediately after administration of anti-CD3 antibodies, T cells are depleted from circulation. OKT3 appears to kill CD3 positive cells by inducing Fc-mediated apoptosis, antibody-mediated cytotoxicity, and complement-dependent cytotoxicity. In addition, removal of the TCR-CD3 from the cell surface by internalization is thought to be a mechanism of action. Due to their mitogenic activity and release of cytokines, Fc receptor (FcR)-binding antibodies such as OKT3 induce flu-like symptoms in treated patients. Thus, in 1994 the first humanized non-FcR-binding antibodies specific for human CD3 were produced and by late 1990s successfully used in the clinic. Teplizumab and Visilizumab are some of the non-FcR-binding anti-CD3 antibodies used to date (Chatenoud and Bluestone 2007).

Summary

The three CD3 subunits CD3γ, CD3δ, and CD3ε form an integral part of the TCR-CD3 complex and, together with ▶ CD3ζ, provide TCRαβ (and TCRγδ) with the signal transmission ability upon antigen binding. The CD3 proteins are type 1 TM proteins that form dimers, CD3εγ and CD3εδ, containing diverse cytoplasmic signaling motifs for inducible interaction with kinases, adaptor proteins, and other signaling proteins. In addition, CD3 is important for the assembly and surface transport of the TCR-CD3. Thus, CD3 subunits are crucial for both normal T cell development and their function in the periphery. Lack of any of these subunits causes immunodeficiencies. Anti-CD3 antibodies are being used in the clinic as immunosuppressants to reduce acute transplant rejection and in the treatment of T cell acute lymphoblastic leukemia.

References

Alarcon B, Berkhout B, et al. Assembly of the human T cell receptor-CD3 complex takes place in the endoplasmic reticulum and involves intermediary complexes between the CD3-gamma.delta.epsilon core and single T cell receptor alpha or beta chains. J Biol Chem. 1988;263(6):2953–61.

Arnaiz-Villena A, Timon M, et al. Brief report: primary immunodeficiency caused by mutations in the gene encoding the CD3gamma subunit of the T-lymphocyte receptor. N Engl J Med. 1992;327:529–33.

Arnett KL, Harrison SC, et al. Crystal structure of a human CD3-epsilon/delta dimer in complex with a UCHT1 single-chain antibody fragment. Proc Natl Acad Sci USA. 2004;101(46):16268–73.

Chatenoud L, Bluestone JA. CD3-specific antibodies: a portal to the treatment of autoimmunity. Nat Rev Immunol. 2007;7(8):622–32.

Dadi HK, Simon AJ, et al. Effect of CD3delta deficiency on maturation of alpha/beta and gamma/delta T-cell lineages in severe combined immunodeficieny. N Engl J Med. 2003;349:1821–8.

Dave VP, Cao Z, et al. CD3 delta deficiency arrests development of the alpha beta but not the gamma delta T cell lineage. EMBO J. 1997;16(6):1360–70.

de Saint Basile G, Geissmann F, et al. Severe combined immunodeficiency caused by deficiency in either the delta or the epsilon subunit of CD3. J Clin Invest. 2004;114(10):1512–7.

Deford-Watts LM, Tassin TC, et al. The cytoplasmic tail of the T cell receptor CD3 epsilon subunit contains a phospholipid-binding motif that regulates T cell functions. J Immunol. 2009;183(2):1055–64.

Dietrich J, Hou X, et al. CD3 gamma contains a phosphoserine-dependent di-leucine motif involved in down-regulation of the T cell receptor. EMBO J. 1994;13(9):2156–66.
Doucey MA, Goffin L, et al. CD3 delta establishes a functional link between the T cell receptor and CD8. J Biol Chem. 2003;278(5):3257–64.
Gil D, Schamel WW, et al. Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. Cell. 2002;109(7):901–12.
Gobel TW, Dangy J-P. Evidence for a stepwise evolution of the CD3 family. J Immunol. 2000;164:879–83.
Haks MC, Krimpenfort P, et al. The CD3gamma chain is essential for development of both the TCRalphabeta and TCRgammadelta lineages. EMBO J. 1998;17(7):1871–82.
Kjer-Nielsen L, Dunstone MA, et al. Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3. Proc Natl Acad Sci USA. 2004;101(20):7675–80.
Kung P, Goldstein G, et al. Monoclonal antibodies defining distinctive human T cell surface antigens. Science. 1979;206(4416):347–9.
Lauritsen JP, Bonefeld CM, et al. Masking of the CD3 gamma di-leucine-based motif by zeta is required for efficient T-cell receptor expression. Traffic. 2004;5(9):672–84.
Malissen M, Gillet A, et al. Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. EMBO J. 1995;14(19):4641–53.
Martinez-Martin N, Risueno RM, et al. Cooperativity between T cell receptor complexes revealed by conformational mutants of CD3epsilon. Sci Signal. 2009;2(83):ra43.
Recio MJ, Moreno-Pelayo MA, et al. Differential biological role of CD3 chains revealed by human immunodeficiencies. J Immunol. 2007;178(4):2556–64.
Reth M. Antigen receptor tail clue. Nature. 1989;338:383.
Sancho J, Chatila T, et al. T-cell antigen receptor (TCR)-alpha/beta heterodimer formation is a prerequisite for association of CD3-zeta 2 into functionally competent TCR.CD3 complexes. J Biol Chem. 1989;264(34):20760–9.
Schamel WW, Arechaga I, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. J Exp Med. 2005;202:493–503.
Siegers GM, Swamy M, et al. Different composition of the human and the mouse gammadelta T cell receptor explains different phenotypes of CD3gamma- and CD3delta-immunodeficiencies. J Exp Med. 2007;204(11):2537–44.
Soudais C, de Villartay J-P, et al. Independent mutations of the human CD3epsilon gene resulting in a T cell receptor/CD3 complex immunodeficiency. Nat Genet. 1993;3:77–81.
Tunnacliffe A, Olsson C, et al. Organization of the human CD3 locus on chromosome 11. Eur J Immunol. 1988;18(10):1639–42.

CD314

► NKG2D

CD335

► NKp46

CD38

Leopoldo Santos-Argumedo
Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), México, DF, México

Synonyms

ADP-ribosyl cyclase; cADPr hydrolase; Cyclic ADP-ribose hydrolase; T10 antigen

Historical Background

As with many molecules, CD38 was first identified in the early 1980s through the pioneering work of E. L. Reinherz and S. F. Schlossman. It was identified by the monoclonal antibody OKT10, and for this reason, the molecule was initially called T10 antigen (Reinherz et al. 1980). Through the production of many monoclonal antibodies, this research team identified numerous molecules associated with the T cell receptor (TCR) and many others that eventually produced lineage markers initially used to define clusters of differentiation (CDs). Several years later, using a cloning system designed by B. Seed and A. Aruffo, D. G. Jackson and J. I. Bell cloned and identified the T10 antigen (Jackson and Bell 1990). *This molecule had been defined as CD38 by the panel of the 4th International Conference on Human Leukocyte Differentiation Antigens*. The preliminary interest in this molecule was due to the distinctive discontinuous expression revealed during the maturation of both T and B lymphocytes. The identification and cloning of murine CD38 opened new avenues to investigate its biological role not only because a closer look was feasible using rodents but also because it was possible to manipulate its expression to analyze its roles in many different biological processes.

CD38 Gene and Protein

CD38 was the prototype of a new family of molecules that includes both CD157 and the enzyme ADP-rybosil cyclase, identified in the sea slug *Aplysia californica* (GeneCards®; Human Genome Organisation (HUGO)). Throughout the years, other members have been cloned or predicted in mammals, birds, amphibian, and fish (Fig. 1) (Malavasi et al. 2008). Due to the homology, chromosome location, and gene organization between CD38 and CD157, it is thought that both molecules came from a common ancestor that was duplicated around 300 million years ago. The CD38 gene is located on chromosome 4p15 in humans and chromosome 5 (23.85 cM) in mice. The gene spans more than 80 kb and consists of eight exons and seven introns. Intron 1 is about 37 kb and interrupts the 5-prime coding region. Exon 1, the largest exon, encodes intracytoplasmic, transmembrane, and membrane proximal (33 aa) regions (GeneCards®; Human Genome Organisation (HUGO); OMIM ®). Exons 2–8 are smaller and encode the rest of the extracellular domains. Gene expression is regulated by retinoic acid, TNF (Tumor necrosis factor), and IFNβ among other factors (Malavasi et al. 2008; OMIM ®).

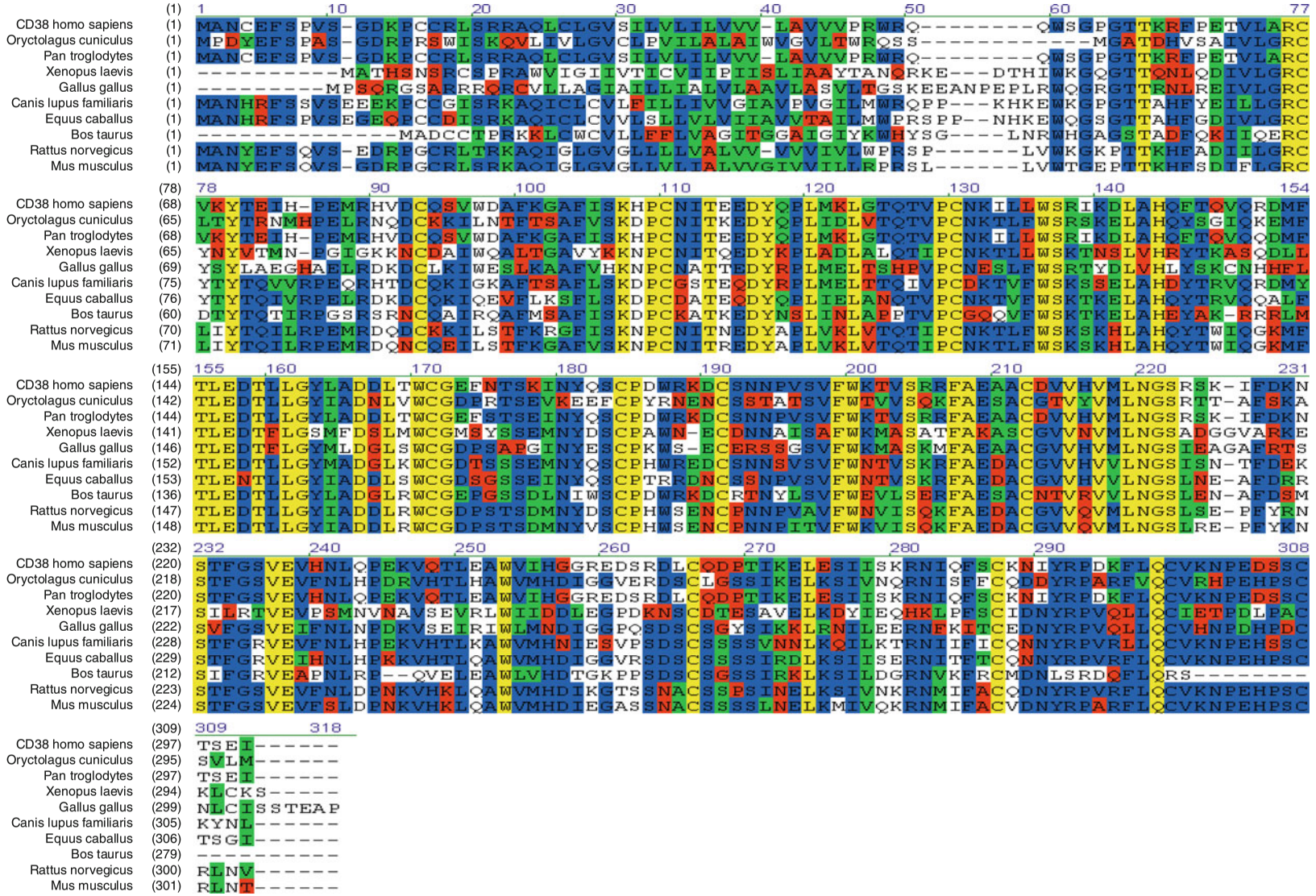
CD38 protein is a 45 kDa type II glycoprotein. It contains an intracytoplasmic (∼21 aa), transmembrane (∼22 aa), and extracellular (∼257 aa) region. The extracellular domain contains four glycosylation sites and is divided into two domains: the NH2 (amino membrane proximal) domain, which is comprised of a bundle of five α-helices and two short β-strands (residues 45–118 and 144–200) and the COOH (membrane distal) domain, which is comprised of four parallel β-sheets, two long, and two short α-helices (residues 119–143 and 201–300). The NH2 and COOH domains are connected by a hinge region composed of three peptide chains (residues 118–119, 143–144, and 200–201). CD38 is an ectoenzyme whose catalytic activity depends on the disulfide bonds that stabilize the conformation of the enzyme (Lee 2006).

CD38 Distribution

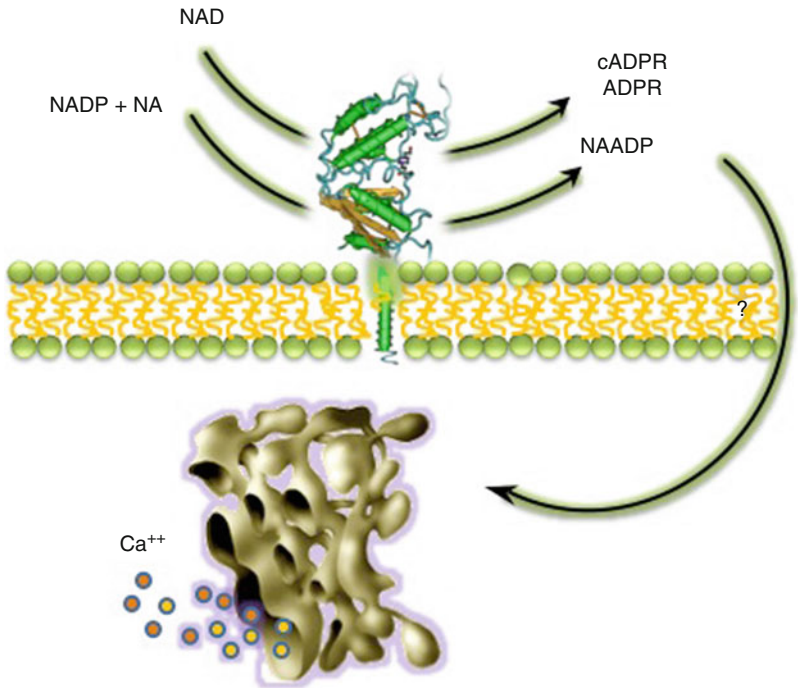
Historically, CD38 was first identified as a leukocyte marker, expressed in both T and B lymphocytes. However, CD38 is widely distributed in myeloid, bone marrow, pancreas, prostate, brain, kidney, muscle, and eye cells (GENATLAS; Higashida et al. 2007; Human Genome Organisation (HUGO); Malavasi et al. 2008; Okamoto et al. 1997; OMIM ®). Some reports of the distribution outside the immune system have found CD38 in the cytoplasm and the nucleus of these cells (Malavasi et al. 2008; Okamoto et al. 1997). In lymphocytes, the expression of CD38 is tightly regulated, and its expression fluctuates during the different stages of the development and differentiation of these cells (Campana et al. 2000; Malavasi et al. 2008).

CD38 as an Enzyme

The analysis of the CD38 sequence showed a low homology (∼30%) with an enzyme cloned from the sea slug *Aplysia*; however, the gene structure was similar, and more importantly, 10 out of 12 cysteines were fully conserved indicating that CD38 may have a similar conformation and function. As predicted, CD38 showed enzymatic activity within its extracellular domain (Howard et al. 1993). CD38 uses nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) plus nicotinic acid to produce cyclic ADP ribose (cADPr), ADP ribose (ADPr), and nicotinic acid adenine dinucleotide phosphate (NAADP). This finding is important because it highlights that cADPr, and later NAADP, can mobilize Ca++ by an IP3-independent mechanism. The Ca++ mobilization requires ryanodine sensitive channels (RyR) (Lee 2006; Schuber and Lund 2004). Conversely, one of the difficulties with this finding is that the substrates are not usually found in the extracellular milieu, and the products of the enzymatic reaction reach the internal compartments, where these ryanodine Ca++ compartments are located. Mechanisms have been suggested that resolve these discrepancies, including specific channels that transport the products of the enzymatic reaction inside the cell. A second suggestion elaborated in several papers is that the CD38 active site may also face the cytosol or that there may even be a cytosolic isoform; however, neither genetic nor clear biochemical evidence for these isoforms has yet been published. An intermediate argument is that CD38 may be found in vesicles, but still the substrate would need to enter the vesicle and the products would need to be transported into the cytosol. For immune cells, it has been suggested that inflammation, including cell destruction, may provide



CD38, Fig. 1 Alignment of CD38 from ten species. Indicating the conserved amino acids, the colors indicate black = non-similar; red = weakly similar; green = similar; blue = conservative; and yellow = identical; respectively

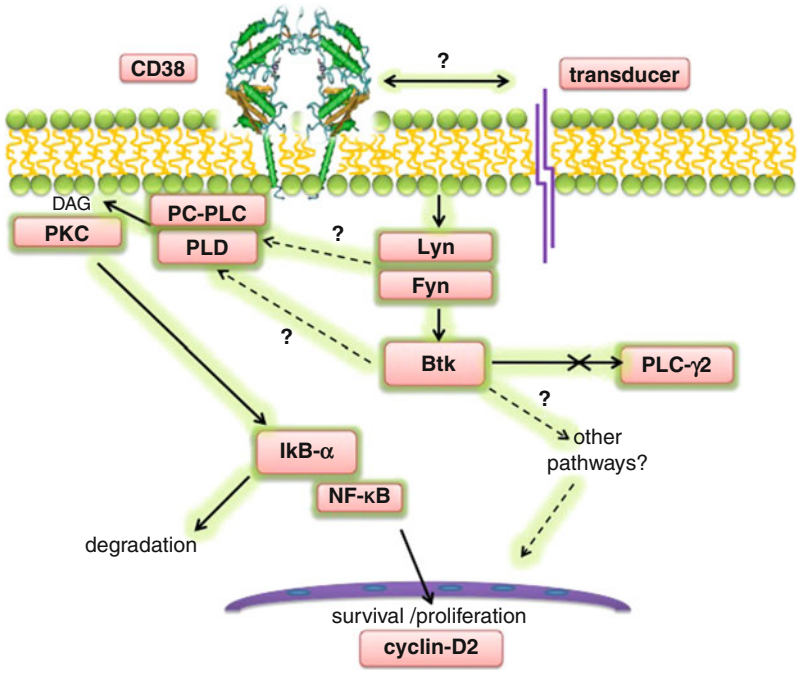


CD38, Fig. 2 CD38 as an enzyme. CD38 uses nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) plus nicotinic acid (NA) to produce cyclic ADP ribose (cADPr), ADP ribose (ADPr), and nicotinic acid adenine dinucleotide phosphate (NAADP). NAADP and cADPr mobilize Ca++ by an IP3-independent mechanism

the necessary substrates, but the transfer of the products still requires the identification and characterization of specific channels that facilitate their transport through the plasma membrane (Schuber and Lund 2004). Although enzymatic activity has been described as important for the functions of CD38 in immunity, CD38 has also demonstrated a variety of functions outside the immune system. For example, CD38 has been shown to participate in the regulation of insulin and oxytocin secretion and to have roles in diabetes and social behavior (Higashida et al. 2007; Okamoto et al. 1997). It has also been shown that CD38 participates in resorption of bone. All these activities depend on the enzymatic activity of this molecule (Fig. 2) (Malavasi et al. 2008; Schuber and Lund 2004).

CD38 as a Receptor

Most of the work characterizing CD38 as a receptor has come from studies using agonistic antibodies (usually monoclonal) for the activation of lymphocytes. The first set of evidence used the monoclonal antibody (mAb) A10/1B4. This mAb induced the proliferation of human peripheral blood mononuclear cells (PBMC). Stimulation of CD38 in human T lymphocytes induced the phosphorylation of different substrates (CD3-ε, PLC-γ1, c-Cbl, ZAP-70, LAT, and Shc) and the initiation of canonical signaling pathways shared by other receptors, such as CD3, even though there are differences between stimulation through CD38 and TCR (Malavasi et al. 2008). Mature B lymphocytes also respond to CD38 crosslinking through proliferation and differentiation (Lund 2006; Moreno-Garcia et al. 2005). Agonistic antibodies include clones of rat anti-mouse mAbs NIM-R5, Ab 90, and CS/2. Interestingly, both human and murine B lymphocytes respond differentially to the engagement of CD38 depending on their maturation status. Thus, immature B cells become apoptotic, while more mature cells proliferate and differentiate (Campana et al. 2000; Moreno-Garcia et al. 2005). This dual response has also been described for stimulation through the B cell antigen receptor (BCR). The signaling pathways involved with these responses in B cells have not been completely elucidated, but they include the recruitment and activation of BTK (Bruton's Tyrosine Kinase), as well several canonical proteins previously described in activation via the BCR. Interestingly, the signaling of murine B cells through CD38 seems to be



CD38, Fig. 3 CD38 as a receptor in murine B lymphocytes. CD38, probably associates with some transduction signaling subunits, activates Lyn and Fyn, which in turn activates Btk. Btk does not activate PLC-γ2 and instead may participate in the activation of PC-PLC/PLD. Activation of PC-PLC/PLD then promotes the production of DAG, activation of PKC, and NF-kB translocation to nucleus, providing signals that promote cell survival, cyclin-D2 expression, and cell proliferation

independent of PLCγ2 participation and IP3 production (Moreno-Garcia et al. 2005). To date, this represents the main difference between the activation triggered by CD38 and the activation triggered by BCR, whose requirement of PLCγ2 seems to be essential. There is, however, an increase of cytosolic Ca++ that has been linked to the enzymatic activity of CD38, as described earlier. Protein Kinase C (PKC) recruitment and activation, which require diacylglycerol (DAG), are downstream of the CD38/BTK pathway, and other phospholipases must participate to generate this metabolite. Both phosphorylcholine-PLC (PC-PLD) and PLD have been shown to participate in the generation of DAG, but the connections among CD38, BTK, and these two phospholipases are still missing (Fig. 3) (Moreno-Garcia et al. 2005).

The cytoplasmic tail of CD38 is very short and does not contain canonical motifs involved in signal transduction. There is some literature suggesting a direct link between CD38 and Lck; however, most of the studies assessing the receptor properties of CD38 support an indirect association with the transduction machinery. One of the strongest arguments against a direct link is the finding that the cytoplasmic tail is unnecessary for signaling, suggesting instead that CD38 associates laterally with other molecules to transmit its signal. For T lymphocytes, it has been described that CD38 uses the CD3/TCR module, while there is evidence of participation of both the BCR and the complex CD19/CD21/CD81 in B cells. For NK cells, monocytes, and dendritic cells studies indicate the modules CD16/CD81, MHC-II/CD81, and CD11b/CD81/CD83, respectively (Lund 2006; Malavasi et al. 2008).

As described previously, some functions of CD38 activity may be attributed to its enzymatic activity; however, there is also clear evidence that enzymatically dead mutants can transmit signals. Therefore, for at least for some biological functions of CD38, its receptor activities are completely independent of its enzymatic activity (Lund 2006).

The only CD38 ligand identified so far is CD31, a molecule present in endothelial cells as well several leukocytes and platelets. There is at least one other possible candidate described in murine dendritic cells that may participate in the engagement of CD38 present on the surface of T and B lymphocytes. This is an interesting possibility because it has been shown that CD38 is recruited to the immunological synapse during the interaction of T lymphocytes with antigen presenting cells (Malavasi et al. 2008).

CD38 in Health and Disease

For more than a decade, CD38 has been used to classify tumors from patients suffering from different types of leukemia. CD38 on CD8 T cells have also been used extensively to evaluate AIDS progression in patients infected with HIV. During the mid-1990s, it was clear that CD38 had a dual function as both an enzyme and a receptor, but there was no association between its dysfunction and any disease. After the development of CD38 deficient mice (CD38−/−), it was possible to evaluate its function in the general health of these rodents. From the results of various studies, it was possible to speculate about human diseases in which CD38 may be involved. The first results indicated that although CD38−/− mice have no major defects in hematopoiesis, they have difficulties in the production of antibodies. Later, it was shown that these mice also have deficiencies in innate immunity, especially in the ability of their neutrophils to migrate to sites of infection. Additionally, dendritic cells from CD38−/− mice showed deficiencies in migration, affecting the priming of specific T cells and, as a consequence, affecting T-B cell collaboration for the adequate production of antibodies. Although these deficiencies are mild, they have an important impact, making the animals more prone to some infections (Lund 2006; Malavasi et al. 2008).

Outside of the immune system, the results from CD38−/− mice have also been very enlightening. For example, it was demonstrated that these mice have lower insulin levels than their wild-type counterparts. Because it is known that NAD is important in controlling insulin secretion, these results can be linked to CD38 enzymatic activity. This effect can be explained by the CD38-dependent production of cADPr that controls the Ca++ release from internal stores, which regulates insulin secretion (Okamoto et al. 1997). There is evidence in the literature about the role of CD38 in the development of human diabetes, and it is clear that CD38 is one of the many factors controlling the development of this complex and multigenic disease. Related to the control of caloric metabolism, CD38−/− mice are also shown to be resistant to developing obesity when fed high fat diets, in contrast with their wild-type counterparts that became significantly obese; this result is an exciting phenomenon that has yet to be fully explained.

Finally, CD38 has also been implicated in the regulation of oxytocin secretion, which has an important role in social behavior. CD38−/− female mice have defects in maternal nurturing and male animals fail to develop social memory. The role of enzymatic activity of CD38 in this phenomenon was demonstrated with the administration of enzyme antagonists in wild-type mice to mimic the effect of the absence of CD38. Therefore, it is possible to speculate that some human neurodevelopmental disorders may relate to the defective expression or function of CD38, thus opening the possibility for a pharmacological approach to treat these diseases (Higashida et al. 2007).

Summary

Despite the fact that there are more than 20 years since the initial description and cloning of CD38, its biological role is just emerging, revealing unexpected surprises. Most studies investigate the role of CD38 in leukocytes, but knowledge of its role outside of the immune system is growing greatly and unveiling many of its undisclosed roles. Due to its wide distribution in organisms, it is reasonable to expect more discoveries about the biological roles of this molecule, and there is still much work to be done to fully understand the many ways in which CD38 participates in the control of many diverse biological activities.

References

Campana D, Suzuki T, Todisco E, Kitanaka A. CD38 in hematopoiesis. Chem Immunol. 2000;75:169–88.

GENATLAS. http://genatlas.medecine.univ-paris5.fr/fiche.php?n=3802.

GeneCards®. http://www.genecards.org/cgi-bin/carddisp.pl?gene=cd38.

Higashida H, Salmina AB, Olovyannikova RY, Hashii M, Yokoyama S, Koizumi K, Jin D, Liu HX, Lopatina O, Amina S, Islam MS, Huang JJ, Noda M. Cyclic ADP-ribose as a universal calcium signal molecule in the nervous system. Neurochem Int. 2007;51:192–9.

Howard M, Grimaldi JC, Bazan JF, Lund FE, Santos-Argumedo L, Parkhouse RM, Walseth TF, Lee HC. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. Science. 1993;262:1056–9.

Human Genome Organisation (HUGO). http://www.genenames.org/data/hgnc_data.php?hgnc_id=1667.

Jackson DG, Bell JI. Isolation of a cDNA encoding the human CD38 (T10) molecule, a cell surface glycoprotein with an

unusual discontinuous pattern of expression during lymphocyte differentiation. J Immunol. 1990;144:2811–5.
Lee HC. Structure and enzymatic functions of human CD38. Mol Med. 2006;12:317–23.
Lund FE. Signaling properties of CD38 in the mouse immune system: enzyme-dependent and -independent roles in immunity. Mol Med. 2006;12:328–33.
Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, Vaisitti T, Aydin S. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. Physiol Rev. 2008;88:841–86.
Moreno-Garcia ME, Lopez-Bojorques LN, Zentella A, Humphries LA, Rawlings DJ, Santos-Argumedo L. CD38 signaling regulates B lymphocyte activation via a phospholipase C (PLC)-gamma 2-independent, protein kinase C, phosphatidylcholine-PLC, phospholipase D-dependent signaling cascade. J Immunol. 2005;174:2687–95.
Okamoto H, Takasawa S, Nata K. The CD38-cyclic ADP-ribose signalling system in insulin secretion: molecular basis and clinical implications. Diabetologia. 1997;40:1485–91.
OMIM ® - Online Mendelian Inheritance in Man. CD38 ANTIGEN; CD38. http://www.ncbi.nlm.nih.gov/omim/107270.
Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF. Discrete stages of human intrathymic differentiation: analysisof normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc Natl Acad Sci USA. 1980;77:1588–92.
Schuber F, Lund FE. Structure and enzymology of ADP-ribosyl cyclases: conserved enzymes that produce multiple calcium mobilizing metabolites. Curr Mol Med. 2004;4:249–61.

CD38 Negative Kinase 2

► ACK1

CD39

► E-NTPDase Family

CD3γ

► CD3

CD3δ

► CD3

CD3ε

► CD3

CD3ζ

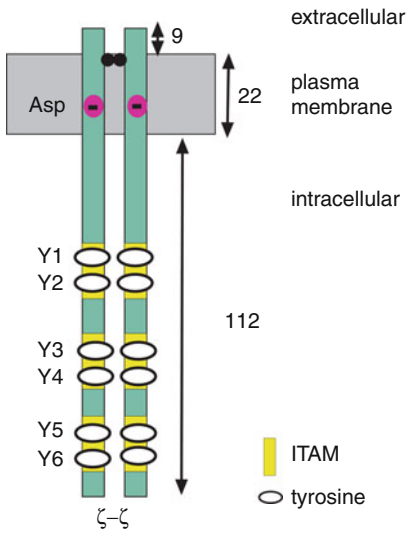
Sumit Deswal[1,2] and Wolfgang W. A. Schamel[1,3,4]
[1]Max Planck Institute of Immunobiology and Faculty of Biology, Biology III, University of Freiburg, Freiburg, Germany
[2]Spemann Graduate School of Biology and Medicine, Freiburg, Germany
[3]Centre for Biological Signalling Studies (BIOSS), University of Freiburg, Freiburg, Germany
[4]Centre of Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Freiburg, Germany

Synonyms

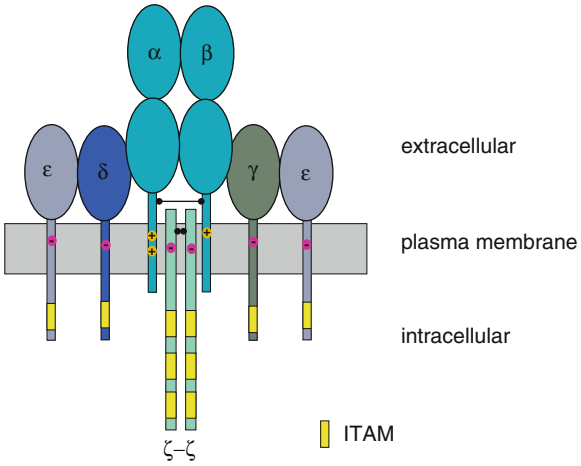
ζ; CD247; TCRζ

Introduction

CD3ζ is a homodimer-forming type 1 transmembrane (TM) protein and is part of the T-cell antigen receptor (TCR-CD3) complex along with TCRαβ, CD3γε, and CD3δε dimers expressed on the surface of T cells (Figs. 1 and 2). T cells are an important component of the vertebrate adaptive immune system that are activated via TCR-CD3 by the peptides generated from infectious agents and presented on major histocompatibility complex (MHC) molecules on the surface of cells. CD3ζ possesses a small extracellular part, a TM region, and a long cytoplasmic part that contains three immunoreceptor tyrosine-based activation motifs (ITAMs), which correspond to the six tyrosines that get phosphorylated upon antigen binding to the extracellular part of TCRαβ. Phosphorylation subsequently activates several downstream signaling cascades. Hence, CD3ζ plays a vital role in the activation of a T cell. CD3ζ is also part of the pre-TCR-CD3 in pre-T cells and the γδTCR-CD3 in γδ T cells that contains TCRγδ instead of TCRαβ. In addition, CD3ζ



CD3ζ, Fig. 1 Schematic representation of CD3ζ. CD3ζ possesses a small nine amino acid ectodomain, a TM region, and a long cytoplasmic domain (112 amino acids in human) with three ITAMs, each of which contains two tyrosines. The two CD3ζ chains are joined by a disulphide bond at the border of the extracellular and the TM regions



CD3ζ, Fig. 2 Structure of the TCR-CD3 complex. The CD3ζζ dimer is a component of the TCR-CD3 complex, which in addition contains CD3γε, CD3δε, and TCRαβ and is expressed on T cells of the immune system. TCRαβ are the ligand binding subunits, while the CD3 chains aid in receptor assembly, transport to the cell surface, and in signal transmission. Important for assembly are the potentially charged amino acids in the TM region of the TCR-CD3

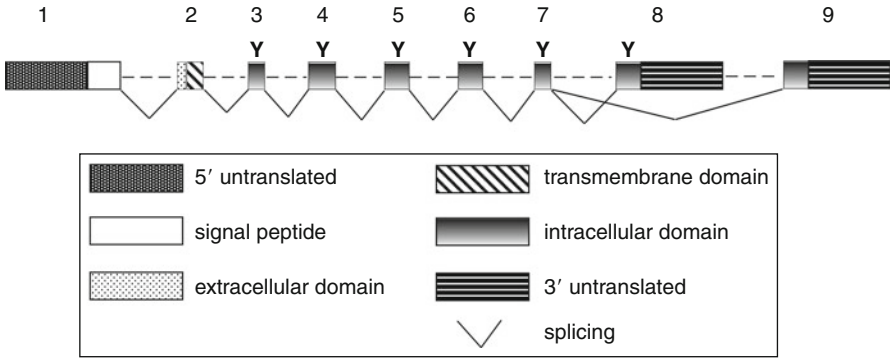
is also expressed in other cell types and as a member of other receptor complexes. In experimental studies, CD3ζ is often tagged with fluorescent proteins for the study of TCR-CD3 dynamics in the immune synapse.

Historical Background

CD3ζ was first discovered in T cells as a component of the TCR-CD3 in 1985 (Samelson et al. 1985) (and was the last subunit of TCR-CD3 to be discovered) and as a part of the pre-TCR-CD3 complex where it was shown to play an important role in T cell development. Later, it was also found to be a component of the activating receptors NK-cell protein 46 (▶ NKp46), NKp30, and the low affinity Fc receptor for IgG (FcγRIII), which are expressed by NK cells (Lanier et al. 1989). It was recently found expressed in retinal ganglion cells, where it regulates neuronal development (Xu et al. 2010).

Evolution, Genomic Organization, and Protein Structure

The CD3ζ gene is evolutionarily related to other ITAM-containing proteins as highlighted by analysis of its exon–intron organization. The nucleotide sequence corresponding to each of the three CD3ζ ITAMs is encoded by two exons that are interrupted by a phase 0 intron at the same position (one amino acid after the first tyrosine of the ITAM). This indicates that these repeated motifs probably derive from triplication of an ancestral pair of exons, the product of which participates in the intracellular signaling by binding to tandem SH2 domain–containing proteins. Such exon–intron structure is also present in most of the other sequences coding for ITAMs. Therefore, a primitive two-exon set has probably undergone multiple rounds of duplication and transposition through evolution to give rise to the gene for CD3ζ by triplication and to various Ig-like (Ig-α, Ig-β, CD3γ, CD3δ and CD3ε) or non-Ig-like (FcεRI-β, FcεRI-γ, DAP-12/KARAP) ectodomain-containing proteins through exon shuffling. It is speculated that the CD3ζ, FcεRI-γ, and DAP-12/KARAP polypeptide set might have branched off from the ancestor of the CD3εγδ set and lost the exon corresponding to the extracellular Ig domain. Therefore, CD3ζ probably shares both a common origin (an ancestral two-exon set) and a common function (recruiting SH2-containing signaling proteins) with the other ITAM-containing proteins found associated with immunoreceptors (Malissen 2003).



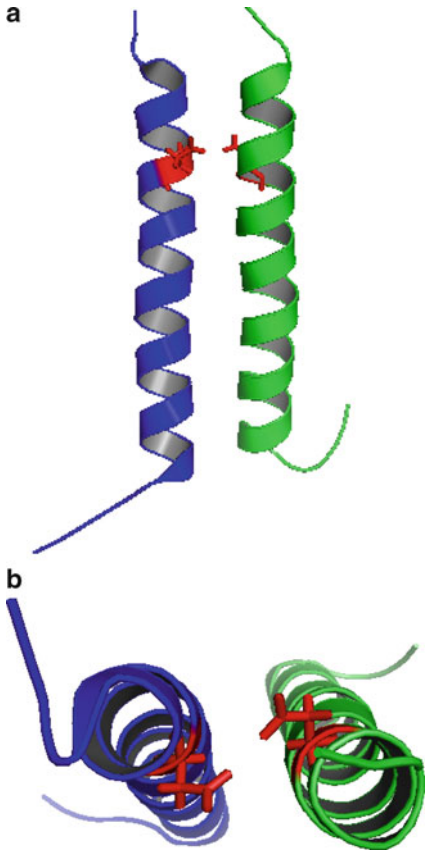
CD3ζ, Fig. 3 Genomic organization of the human CD3ζ gene. The gene encoding CD3ζ is localized to the distal part of chromosome 1 and comprises a total of nine exons. CD3ζ is translated from the first eight exons while in an alternatively spliced form, CD3η, exon 8 is replaced with exon 9. The nucleotides location corresponding to each ITAM tyrosine is marked by Y in exons 3–8

However, the CD3ζ chain differs from other TCR-CD3 subunits in its genetic organization, chromosomal localization, and protein structure. The gene encoding the CD3ζ chain is found in the distal part of chromosome 1 in humans and mice. There is an alternatively spliced form, CD3η, in which exon 8 (zeta specific exon) is replaced with exon 9 (η specific exon) (Ohno and Saito 1990) (Fig. 3). In some circumstances, the TCR-CD3 complex can contain heterodimers of CD3ζ/η or FcRγ. For example, following activation of the γδ T cells, FcRγ is expressed and is included in the γδTCR-CD3 complex (Hayes and Love 2002), and in $CD4^{-}CD8^{-}$ double negative $NK1.1^{+}$ T cells, FcRγ forms a heterodimer with CD3ζ which may drive these cells along NK cell lineage (Curnow et al. 1995).

At its N-terminus CD3ζ contains a signal peptide that targets protein translation to the endoplasmic reticulum. Since this peptide is cleaved co-translationally, it is not present in mature CD3ζ. Unlike the other ▶ CD3 subunits, CD3ζ has a very short ectodomain of nine amino acids and a long cytoplasmic tail (112 amino acids in human and 113 in mouse) and does not belong to the Ig supergene family. The short CD3ζ ectodomain is buried within the TCR-CD3 complex and its length, but not primary amino acid sequence, is highly conserved across orthologs. When this domain is artificially enlarged, the resulting TCR-CD3 complex is distorted leading to a hyperactive phenotype and enhanced T cell activation (Minguet et al. 2008). So far, only the three-dimensional structure of the TM part of the CD3ζζ dimer has been solved by NMR studies (Call et al. 2006), which showed that the TM domain of CD3ζ adopts an α-helical structure (Fig. 4). The TM dimer interface is composed of both hydrophobic packing interactions and intermolecular hydrogen bonds and a potentially charged aspartic acid on each CD3ζ. A disulfide bond between the cysteine residues in the TM portion stabilizes the dimer, although it is not absolutely required for dimerization or assembly with the TCR. In its cytoplasmic domain, CD3ζ contains three ITAMs, which get phosphorylated upon antigenic stimulation. In contrast, CD3η contains 155 amino acids and only two ITAMs in its cytoplasmic domain.

Assembly and Membrane Organization

Only a fully assembled TCR-CD3 complex is transported to the T cell plasma membrane, while individual subunits are most likely degraded in the endoplasmic reticulum. Thus, in T cells lacking CD3ζ, a TCRαβCD3εγεδ complex is only expressed at low levels on cell surface. CD3ζ is the last subunit to be associated with TCR-CD3 complex and may only assemble to the other subunits in the Golgi, where it shields the lysosomal targeting sequence in CD3γ. Each of the CD3ζ, CD3ε, and CD3δ molecules possess an ionizable aspartic acid in their TM region, while CD3γ possesses a glutamic acid residue. Together, this gives six acidic residues in the TM region of the TCR-CD3 complex. TCRβ and TCRα possess one and two basic residues, respectively, in their TM



CD3ζ, Fig. 4 NMR structure of CD3ζ transmembrane region. The TM domain of CD3ζ adopts an α-helical structure. The NMR structure was downloaded from protein data bank (PDB ID: 2HAC) and images prepared using the software MacPymol. The acidic residue aspartic acid is shown in the stick form. (**a**) is rotated by approximately 90° compared to (**b**)

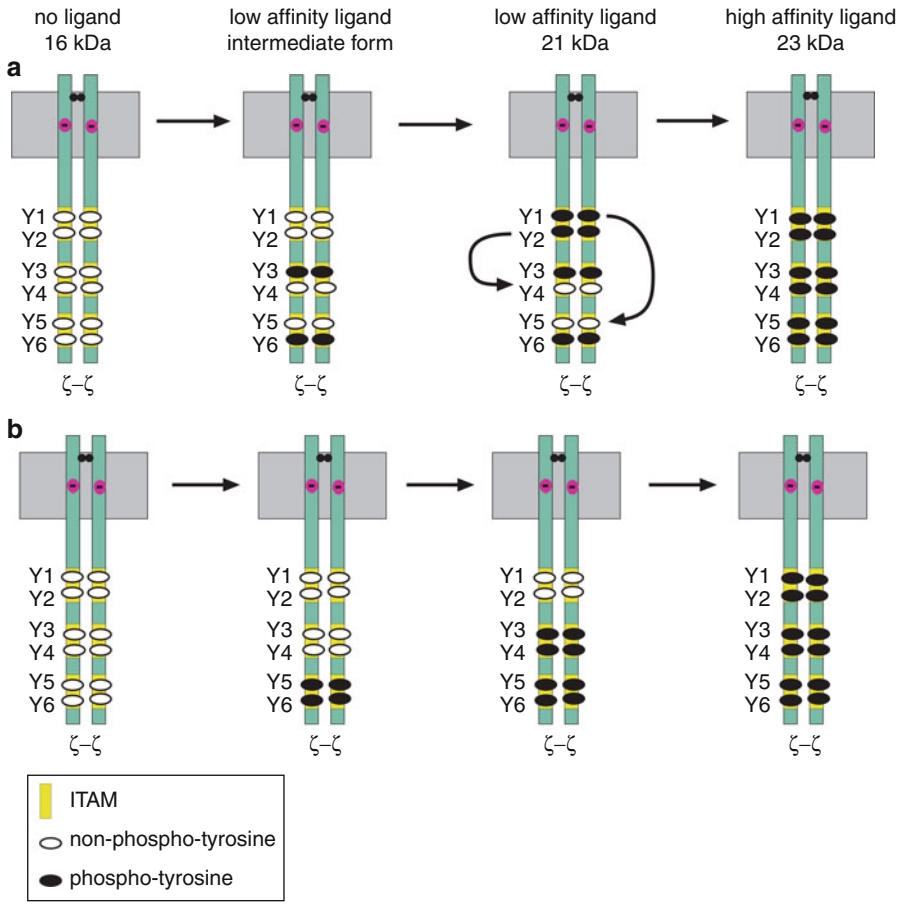
region. This gave rise to the speculation that each TCR-CD3 complex might contain two TCRαβ dimers to balance the six acidic residues in the CD3εγεδζζ. However, several studies have now confirmed a stoichiometry of TCRαβCD3εγεδζζ (Schamel et al. 2005). Indeed, assembly of each of the three CD3 dimers with TCRαβ involves a trimeric interface between one basic and two acidic TM residues where CD3ζ associates with TCRα. So the basic TCR–CD3 complex is monovalent, which then associates with other TCR-CD3 complexes to give rise to TCR-CD3 nanoclusters. The exact role of these TCR-CD3 nanoclusters is still a question under study but it might provide the T cell with high sensitivity to detect minute numbers of antigenic peptides (Schamel et al. 2005).

Phosphorylation and Internalization of CD3ζ

Following receptor ligation, CD3ζ is the most heavily tyrosine phosphorylated subunit of the TCR-CD3. The phosphorylation pattern of CD3ζ has been a topic of intense debate. In Western blot experiments, several forms of CD3ζ can be observed depending upon its phosphorylation status. Among these, forms with an apparent molecular weight of 16, 21, and 23 kDa are the most prominent in reducing SDS-PAGE. The 16 kDa form represents non-phosphorylated CD3ζ. The 21 kDa form most likely represents CD3ζ phosphorylated at four tyrosines and is generated upon stimulation of T cells by low affinity self pMHC ligands or altered peptide ligands. The 23 kDa form results from phosphorylation of all the six tyrosines upon stimulation with high affinity foreign pMHC ligands. The order of phosphorylation of the different tyrosines and which four tyrosines are phosphorylated in 21 kDa form is not clear, as the results from different studies are contradictory. According to one study (Kersh et al. 1998), tyrosine 3 (Y3) and tyrosine 6 (Y6) are phosphorylated in resting T cells (Fig. 5a), most likely by the interaction of TCRαβ with low affinity self-peptides presented on MHC. Upon stimulation by high affinity foreign peptides on MHC, the phosphorylation of tyrosines follows a specific order as indicated in Fig. 5a. In a second study (van Oers et al. 2000), a different order of phosphorylation was proposed (Fig. 5b). The four membrane distal tyrosines are phosphorylated by low affinity self-pMHC interactions (the 21 kDa form of CD3ζ) and all tyrosines are phosphorylated only upon high affinity interaction with foreign pMHC (the 23 kDa form) as in the earlier study.

CD3ζ has not been reported to undergo serine or threonine phosphorylation.

How the binding of pMHC ligand to the ectodomains of TCRαβ transmits the signal to the cytoplasmic tail of CD3ζ has not been resolved yet. In aqueous solution, the cytoplasmic tail of CD3ζ is unstructured, whereas in the presence of liposomes it exhibits a helical secondary structure. This lipid binding-dependent conformational change in CD3ζ could be one activation mechanism. In the pre-activation state when CD3ζ is bound to the inner leaflet of the plasma membrane, it is resistant to phosphorylation by the ► Src family tyrosine kinases. TCR-CD3 engagement by pMHC ligands might force cytoplasmic tails



CD3ζ, Fig. 5 Phosphorylation pattern of CD3ζ. After different stimulation of the TCR-CD3, several distinct molecular weight forms of CD3ζ can be distinguished by reducing SDS-PAGE. There are two models (a and b) that explain to which phosphorylation forms they correspond. (**a**) In one model, tyrosine 3 (Y3) and tyrosine 6 (Y6) are phosphorylated in resting T cells. Upon stimulation by antigen, the phosphorylation of tyrosines follows a specific order as indicated. Only the high affinity agonist ligand results in full phosphorylation and generates 23 kDa CD3ζ form. (**b**) In another model, CD3ζ undergoes a stepwise phosphorylation that is initiated at Y6. Tyrosines 3–6 are phosphorylated by low affinity self-peptide-MHC interactions (21 kDa form) and all tyrosines are phosphorylated upon high affinity interaction with foreign peptide loaded on MHC (23 kDa)

to be released from the membrane, which now are accessible for phosphorylation by the kinases (Aivazian and Stern 2000). Another possibility is that the TCR-CD3 is in equilibrium between open and closed conformations. CD3ζ tyrosines are only accessible for phosphorylation in the open conformation. In resting state, equilibrium favors the closed, inaccessible conformation. pMHC binding stabilizes the open conformation, hence makes the tyrosines accessible for phosphorylation (Minguet and Schamel 2008).

TCR-CD3 is constitutively internalized and recycled to the plasma membrane in naïve and activated T cells. Phosphorylation of TCR-CD3 upon activation by antigen binding leads to enhanced TCR-CD3 down regulation from surface. Cell surface TCR-CD3s lacking CD3ζ are endocytosed more rapidly than completely assembled receptors. CD3ζ may stabilize TCR-CD3 expression on cell surface by blocking access to the internalization motifs on other CD3 subunits (D'Oro et al. 2002). On the other hand phosphorylated CD3ζ targets internalized TCR-CD3 for ubiquitin-dependent degradation. Src-like adapter protein (SLAP) binds to internalized and phosphorylated CD3ζ via its SH2 domain in the endosomal compartment and mediates recruitment of the E3 ubiquitin ligase CBL (Casitas B-lineage lymphoma; also known as c-CBL), resulting in ubiquitylation of CD3ζ, thereby targeting the TCR-CD3 for

degradation. A recent study suggests that phosphorylated CD3ζ accumulates in endosomes (Yudushkin and Vale 2010). This endosomal CD3ζ remained signaling competent and could possibly help to sustain long term signaling in T cells.

Functions

The TCR-CD3 complex plays a critical role in the immune response by activating the T cells which then help in the activation of B cells by releasing helper cytokines (helper T cells) or kill the target cell directly by inducing apoptosis. CD3ζ has two important functions: (1) assembly of the TCR-CD3 complex in the ER/Golgi and transport to the cell surface, as a TCR-CD3 complex lacking CD3ζ is mostly subjected to lysosomal degradation, (2) signaling, as the TCRαβ or TCRγδ dimer itself lacks the signaling motifs and relies on the CD3ε, CD3γ, CD3δ, and CD3ζ components for intracellular signaling. Each CD3ζ contains six tyrosines, helping in signal amplification. The tyrosines of the ITAM are phosphorylated by the Src family kinases Lck and Fyn. This leads to the recruitment of the tyrosine kinase ► ZAP70 to the phosphotyrosines via its tandem SH2 domains. This initiates further downstream signaling and ultimately activation of the T cell. A similar sequence of events takes place in the signal transduction downstream of pre-TCR-CD3 in the developing pre-T cells. The signals are required for TCRα gene arrangement and further development of these cells. In addition, CD3ζ is expressed on pro-T cells. In pro-T cells CD3ζ was phosphorylated upon anti-CD3ε antibody stimulation, although no direct association between CD3ζ and other CD3 subunits was observed. CD3ζ-deficient mice have a decreased number of peripheral T cells, as will be discussed later.

Several studies have been performed to explore the role of the individual ITAMs in the different CD3 proteins in T-cell development and function. According to one study, together the ITAMs in CD3ε, CD3γ, and CD3δ can provide normal TCR-CD3 signal transmission in mature, peripheral T cells, and CD3ζ ITAMs play mainly a role in positive selection in the thymus (Pitcher et al. 2005b). However, in another study, expression of the mutant CD3ζ, which lacked Y1 and Y2, so that the 23 kDa form of CD3ζ could not be generated (Fig. 5), partially impaired negative selection and promoted the emergence of potentially autoreactive T cells (Pitcher et al. 2005a). Furthermore, contradicting results were obtained in a comprehensive study of all CD3 ITAMs (Holst et al. 2008). Mice with fewer than seven ITAMs (any of the seven) developed a lethal, multiorgan autoimmune disease due to breakdown in central tolerance. The proliferation potential of cells was directly proportional to the number of ITAMs, whereas cytokine production was independent of the ITAM number. Thus, a high number of ITAMs in TCR-CD3 (independent of which subunit) amplifies the signaling that effects proliferation and ensures negative selection to prevent autoimmunity.

In resting human T cells, a portion of CD3ζ associates with the actin cytoskeleton. This interaction, mediated by a sequence in the C-terminus of CD3ζ, may be involved in the localization of the TCR-CD3 into lipid raft structures and/or in TCR-CD3 recycling. Some viral proteins, such as simian immunodeficiency virus Nef, bind the CD3ζ and downmodulate TCR-CD3. Such interaction seems to have evolved as an immune escape strategy for the virus. CD3ζ is also shown to interact with the transferrin receptor (TfR) and plays a role in T-cell activation via TfR stimulation. The TfR/CD3ζ complex is expressed on the cell surface independent of the expression of the other subunits of the TCR-CD3, and activation of this complex might be a signal-amplifying mechanism for T cells. Phosphorylated CD3ζ can bind to several SH2 domain–containing proteins which include adaptor proteins Shc and Grb2 and the p85 subunit of PI3K. SLAP-2, an SH2 domain–containing protein related to SLAP, binds to CD3ζ upon ligand binding and is a negative regulator of downstream signaling. CTLA-4, another negative regulator of the T-cell activation, binds to phospho-CD3ζ and prevents accumulation of the TCR-CD3 in lipid rafts upon antigen binding.

In addition, CD3ζ is also expressed in cells other than T cells, for example, NK cells and neurons. In NK cells, CD3ζ is associated with NK FcγRIII (CD16) and may be necessary for efficient cell surface expression of this receptor complex. Activation of NK cells with an anti-FcγRIII antibody induces tyrosine phosphorylation of CD3ζ and FcγRIII-associated CD3ζ might be downregulated in patients with cancer due to chronic inflammation (Eleftheriadis et al. 2008). CD3ζ is also associated with NKp46 and NKp30 receptors on NK cells, and its phosphorylation is required for

transmission of activating signals upon antigen binding to these receptors. CD3ζ is also expressed in retinal ganglion cells and brain neurons, where it regulates neuronal development by reducing the size of the dendritic arbor.

Pathophysiological Roles

Under normal circumstances, only the fully assembled TCR-CD3 complex is displayed on the T-cell surface. Partially assembled TCR-CD3s are retained in the endoplasmic reticulum or targeted for degradation. However, certain pathologies might be associated with downregulation of CD3ζ. In these cases, despite the absence of CD3ζ, normal TCR-CD3 numbers are expressed on the surface of T cells, for example, the tumor infiltrating T cells (Baniyash 2004). Downregulation of CD3ζ is also reported in autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis, and infectious diseases such as HIV and leprosy (Baniyash 2004). These reports have given rise to speculation over a role for downregulation of CD3ζ chain as an immune escape mechanism induced by various pathologies. Pregnancy is also associated with suppression of immunity, including downregulation of CD3ζ expression (Taylor et al. 2002). There are several mutations identified in the human CD3ζ gene (listed in Table 1) that are associated with primary immunodeficiencies and systemic lupus erythematosus. In mouse, elimination of the CD3ζ gene from the genome results in a drastically reduced thymus due to the lack of T cells and T-cell precursors. These cells express low level of a TCRαβCD3εγεδ complex. The number of single positive cells detected in the thymus is very low, which could be because of a failure to transition from double positive to single positive cells. In contrast to the thymus, spleen and lymph nodes contain large numbers of TCR-CD3^{low} T cells (Malissen et al. 1993). Also, the number of γδ intestinal intraepithelial T lymphocytes are not significantly affected by deletion of the CD3ζ (Ohno et al. 1993). These studies indicate that CD3ζ is required for T-cell development but it is not absolutely essential.

CD3ζ, Table 1 Immunological disorders associated with mutations in CD3ζ

Mutation	Immunological disorder	Reference
Q70X	Primary immnunodeficiency	(Rieux-Laucat et al. 2006)
−76 T insertion in the promotor	SLE	(Nambiar et al. 2001)
344 bp insertion in 3′ UTR	SLE	(Nambiar et al. 2001)
Splice mutation deleting of exon 7	SLE	(Takeuchi et al. 1998)

Summary

CD3ζ is a type 1 TM protein and a subunit of the TCR−CD3 and pre-TCR-CD3 complexes. It forms a dimer and is necessary for assembly and expression of these receptors on the cell surface. CD3ζ possesses three ITAMs with six tyrosines, which are phosphorylated upon receptor engagement. Once phosphorylated, these tyrosines recruit SH2 domain–containing proteins for downstream signaling which ultimately leads to the development of pre-T cells in the thymus, and activation of mature T cells in the periphery, which then initiate an immune response. The phosphorylation pattern of the six CD3ζ tyrosines is still under debate, but it is commonly accepted that all tyrosines are phosphorylated only by the high affinity agonist ligands, whereas low affinity ligands lead to partial phosphorylation without T-cell activation. CD3ζ is also part of several other receptors on T cells and NK cells. It is also expressed and has a function in neurons. Downregulation of CD3ζ has been noticed in tumors and autoimmune and infectious diseases, and further investigations of the molecular mechanism of this downregulation might offer better therapeutic options for treatment of these diseases.

References

Aivazian D, Stern LJ. Phosphorylation of T cell receptor zeta is regulated by a lipid dependent folding transition. Nat Struct Biol. 2000;7(11):1023–6.

Baniyash M. TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response. Nat Rev Immunol. 2004;4(9):675–87.

Call ME, Schnell JR, et al. The structure of the zetazeta transmembrane dimer reveals features essential for its assembly with the T cell receptor. Cell. 2006;127(2):355–68.

Curnow SJ, Boyer C, et al. TCR-associated zeta-Fc epsilon RI gamma heterodimers on CD4-CD8- NK1.1+ T cells selected by specific class I MHC antigen. Immunity. 1995;3(4):427–38.

D'Oro U, Munitic I, et al. Regulation of constitutive TCR internalization by the zeta-chain. J Immunol. 2002;169(11):6269–78.
Eleftheriadis T, Kartsios C, et al. Chronic inflammation and CD16+ natural killer cell zeta-chain downregulation in hemodialysis patients. Blood Purif. 2008;26(4):317–21.
Hayes SM, Love PE. Distinct structure and signaling potential of the gamma delta TCR complex. Immunity. 2002;16(6):827–38.
Holst J, Wang H, et al. Scalable signaling mediated by T cell antigen receptor-CD3 ITAMs ensures effective negative selection and prevents autoimmunity. Nat Immunol. 2008;9(6):658–66.
Kersh EN, Shaw AS, et al. Fidelity of T cell activation through multistep T cell receptor zeta phosphorylation. Science. 1998;281(5376):572–5.
Lanier LL, Yu G, et al. Co-association of CD3 zeta with a receptor (CD16) for IgG Fc on human natural killer cells. Nature. 1989;342(6251):803–5.
Malissen B. An evolutionary and structural perspective on T cell antigen receptor function. Immunol Rev. 2003;191:7–27.
Malissen M, Gillet A, et al. T cell development in mice lacking the CD3-zeta/eta gene. EMBO J. 1993;12(11):4347–55.
Minguet S, Schamel WWA. A permissive geometry model for TCR-CD3 activation. Trends Biochem Sci. 2008;33(2):51–7.
Minguet S, Swamy M, et al. The short length of the extracellular domain of zeta is crucial for T cell antigen receptor function. Immunol Lett. 2008;116(2):195–202.
Nambiar MP, Enyedy EJ, et al. Polymorphisms/mutations of TCR-zeta-chain promoter and 3′ untranslated region and selective expression of TCR zeta-chain with an alternatively spliced 3′ untranslated region in patients with systemic lupus erythematosus. J Autoimmun. 2001;16(2):133–42.
Ohno H, Saito T. CD3 zeta and eta chains are produced by alternative splicing from a common gene. Int Immunol. 1990;2(11):1117–9.
Ohno H, Aoe T, et al. Developmental and functional impairment of T cells in mice lacking CD3 zeta chains. EMBO J. 1993;12(11):4357–66.
Pitcher LA, Mathis MA, et al. Selective expression of the 21-kilodalton tyrosine-phosphorylated form of TCR zeta promotes the emergence of T cells with autoreactive potential. J Immunol. 2005a;174(10):6071–9.
Pitcher LA, Mathis MA, et al. The CD3 gamma epsilon/delta epsilon signaling module provides normal T cell functions in the absence of the TCR zeta immunoreceptor tyrosine-based activation motifs. Eur J Immunol. 2005b;35(12):3643–54.
Rieux-Laucat F, Hivroz C, et al. Inherited and somatic CD3zeta mutations in a patient with T-cell deficiency. N Engl J Med. 2006;354(18):1913–21.
Samelson LE, Harford JB, et al. Identification of the components of the murine T cell antigen receptor complex. Cell. 1985;43(1):223–31.
Schamel WW, Arechaga I, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. J Exp Med. 2005;202:493–503.
Takeuchi T, Tsuzaka K, et al. TCR zeta chain lacking exon 7 in two patients with systemic lupus erythematosus. Int Immunol. 1998;10(7):911–21.
Taylor DD, Sullivan SA, et al. Modulation of T-cell CD3-zeta chain expression during normal pregnancy. J Reprod Immunol. 2002;54(1–2):15–31.
van Oers NS, Tohlen B, et al. The 21- and 23-kD forms of TCR zeta are generated by specific ITAM phosphorylations. Nat Immunol. 2000;1(4):322–8.
Xu HP, Chen H, et al. The immune protein CD3zeta is required for normal development of neural circuits in the retina. Neuron. 2010;65(4):503–15.
Yudushkin IA, Vale RD. Imaging T-cell receptor activation reveals accumulation of tyrosine-phosphorylated CD3 {zeta} in the endosomal compartment. Proc Natl Acad Sci USA. 2010;107(51):22128–33.

CD40

Gail A. Bishop[1], Dima A. Decker[1] and Bruce S. Hostager[2]
[1]Department of Microbiology & Internal Medicine, The University of Iowa and VA Medical Center, Iowa City, IA, USA
[2]Department of Pediatrics, University of Iowa, Iowa City, IA, USA

Synonyms

Bp50; CDW40; MGC9013; p50; TNFRSF5

Historical Background

CD40, a 50 kDa transmembrane member of the tumor necrosis factor (TNF) receptor (TNFR) superfamily of molecules, plays a key role in adaptive immune responses. This includes contact-mediated signals to B cell from activated T lymphocytes, as well as costimulatory interactions between T cells and other antigen-presenting cells (APC), such as dendritic cells (DC) and macrophages. CD40-mediated interactions between B and T cells contribute to the development of an optimal humoral memory response (Bishop and Hostager 2001a).

CD40 lacks intrinsic enzymatic activity and therefore depends on cytoplasmic adaptor molecules referred to as TNF receptor associated factors (TRAFs) for delivery of signals to the cytoplasm. CD40 engagement on B cells stimulates the binding of TRAFs to the CD40 cytoplasmic domain and the stimulation of kinase activity and gene expression pathways that lead to a variety

of responses including B cell survival and expansion, immunoglobulin (Ig) production, isotype switching and affinity maturation, B cell memory, and the production of various cytokines (Bishop and Hostager 2001a, 2003; Graham et al. 2010).

CD40 was identified in 1984 as an antigen associated with transitional cell carcinoma of the human urinary bladder. CD40-specific mAbs bind in vitro transformed B lymphocytes and 5–10% of peripheral lymphocytes (Koho et al. 1984). The first signaling role of the CD40 receptor was suggested by experiments in which an anti-human CD40 agonistic mAb induced in human B-lineage lymphoid cells the tyrosine phosphorylation of cellular proteins, activation of phospholipase Cγ2, and activation of specific Ser/Thr protein kinases (Uckun et al. 1991). The natural ligand for CD40, now known as CD154, was discovered via the complementary methods of in vitro mouse cell experiments and the study of a human immunodeficiency disease, X-linked Hyper IgM syndrome (HIGM). Plasma membrane fractions from activated T helper (Th) cells induce B cell cycle entry, a process that requires de novo protein synthesis. Ultimately, the identity of the Th membrane protein responsible was elucidated by the cloning of CD154 in 1992 (previously referred to as CD40L, gp39, TRAP, or TRAM) (Schönbeck and Libby 2001; Graham et al. 2010).

It was subsequently discovered that defects in CD154 expression or its CD40 binding site cause HIGM, in which patients are susceptible to recurrent extracellular bacterial infections as well as certain intracellular pathogens. HIGM does not affect T cell–independent immune responses, but features a decrease in the production of "switched" isotypes of IgG, IgA, and IgE, as well as defects in antigen presentation. CD40- and CD154-deficient mice display defects in T cell–mediated immunity, including immune responses to parasites, due to defects in T cell–APC interactions (Bishop and Hostager 2001b; Bishop 2004). CD40- and CD154-deficient mice also display defective germinal center formation and B cell memory development in response to antigenic challenge. Together, these findings show that CD40/CD154 interactions are needed for successful T-dependent B cell activation, promoting B cell proliferation, antibody production, isotype switching, germinal center formation, B cell memory, upregulation of costimulatory and adhesion molecules, and cytokine production (Bishop and Hostager 2003; Graham et al. 2010).

Although CD40–CD154 interactions are important for appropriate immune responses, recent studies have revealed a more diverse role of this receptor and its ligand in physiological and pathological processes due to its broad expression pattern. While constitutive expression of CD40 is generally restricted to B cells, dendritic cells, and macrophages, it has also been reported to be expressed under specific conditions on epithelial cells, monocytes, basophils, eosinophils, T cells, vascular endothelium, neuronal cells, and smooth muscle cells. Additional studies have also demonstrated CD40 expression on fibroblasts, keratinocytes, and platelets. CD154, originally thought to only be expressed on CD4+ T helper cells has also been shown to have inducible expression on CD8+ T cells, mast cells, basophils, eosinophils, epithelial cells, monocytes, fibroblasts, NK cells, and platelets. Further studies also reveal that low constitutive levels of CD154 are expressed and can be upregulated after cell activation on endothelial cells, smooth muscle cells, marcrophages, and dendritic cells (Graham et al. 2010).

This unique receptor–ligand pair has been implicated in chronic inflammation and immune responses in transplantation rejection and diseases including cancer (both in tumor-promoting and growth-inhibitory roles depending on the tumor), Grave's disease, type 1 diabetes, atherosclerosis, neuroinflammatory disease, systemic lupus erythematosus, psoriasis, arthritis, and inflammatory bowel disease (Schönbeck and Libby 2001; Peters et al. 2009). Therefore, a detailed understanding of CD40 signaling pathways in normal immunity and disease is important for manipulation of this important player for potential therapeutic benefits.

CD40 and TRAFs

Although CD40 itself lacks intrinsic enzymatic activity, it delivers a variety of important signals to cells. Due to space constraints, this review will focus upon studies performed in B lymphocytes; the reader is referred to other recent reviews of CD40 function in other cell types (Bishop 2009). Human CD40's short 62 amino acid (aa) cytoplasmic (CY) domain has a highly conserved protein sequence, which is almost identical in human and mouse (73 aa long CY). The human CD40 CY domain does not contain tyrosine residues, and thus is devoid of motifs for tyrosine kinases or phosphatase-binding motifs common to other immune receptors. The CY domain does contain serine and threonine residues

which could serve as sites of phosphorylation, and initial structure–function studies determined that T234 in human (T254 in mouse) CD40 plays an important role in growth inhibition of certain transformed B and T cell lines (Bishop and Hostager 2003). However, phosphorylation of the CY domain of CD40 has not been shown to have a major functional role. Rather, proximal signaling events appear largely dependent upon specific sequences in the cytoplasmic domain of CD40 that mediate binding to adapter proteins called TNFR-associated factors (TRAFs) (Bishop and Hostager 2001b).

Although death receptors, such as CD95, do not appear to bind TRAFs, most other members of the TNFR superfamily (TNFRSF) utilize distinct but overlapping sets of TRAFs to transduce intracellular signals. TRAFs 1 and 2 were isolated as proteins physically associating with the CY tail of the tumor necrosis factor receptor type II (TNFR-2/CD120b). In the same year, TRAF3 was initially dubbed CD40-binding protein, as it was first isolated via association with both mouse and human CD40. Within several years TRAFs 4–6 were isolated (Bishop and Hostager 2003; Bishop 2004). CD40 directly binds TRAFs 2, 3, 5, and 6, and also can recruit TRAF1 via a heterotypic interaction with TRAF2. TRAFs 1, 2, and 6 play important positive roles in CD40-mediated signaling, while TRAF3 plays an inhibitory role, and TRAF5 appears to play only a modest role (Bishop and Hostager 2001b; Bishop et al. 2003). CD40 has three known TRAF binding sites: (a) a membrane proximal region for direct TRAF6 binding; (b) a more medial site for overlapping binding of TRAFs 1, 2, 3, and 5; and (c) a second distal TRAF2-binding region (Graham et al. 2010).

All TRAFs contain a conserved C-terminal domain which mediates direct binding to TNFRSF members (Bishop and Hostager 2001b). The six known TRAFs also contain a coiled-coil, leucine-zipper domain known as TRAF-N, shown to mediate homo- and hetero-trimerization of TRAFs. All TRAF molecules except TRAF1 have a zinc-binding Really Interesting New Gene (RING) domain and multiple zinc finger domains at the N-terminus. The RING domain appears to mediate TRAF involvement with the process of ubiquitin modification, an important regulatory mechanism for TRAFs and TNFRSF signaling (Bishop 2004). Removal of the zinc RING domains abolishes TRAF-mediated signaling and creates a "dominant negative" (DN) molecule (Bishop and Hostager 2003).

To fully understand the complex details of CD40-mediated signaling pathways in B cells, the specific contributions of each TRAF, and their interactive functions in CD40 signaling, must be elucidated. There are many approaches to determine TRAF roles. The utilization of DN TRAF molecules which can still associate with CD40, but no longer initiate downstream signals, can provide hints about the biological roles of their full-length counterparts in CD40 signaling. A similar alternative to this approach is the use of CD40 molecules that contain mutations in the CY domain for the study of TRAF binding properties. Application of these approaches in cell lines and transgenic mouse models has yielded useful information about TRAF association and CD40 functions. However, DN versions of TRAFs (especially TRAFs 2 and 3) can alter the binding of other TRAFs due to the overlapping nature of the TRAF 1, 2, 3, and 5 binding site of CD40, a concern that also arises for mutations of the CD40 CY tail in this region (Bishop and Hostager 2001a, 2003; Graham et al. 2010).

Many studies have used the 293 transformed epithelial cell line in which both CD40 and certain TRAFs (wild type and DN) have been transiently overexpressed. However, 293 cells are not a physiologically relevant cell type to determine CD40 functions specific to immune cells. Additionally, studies in such systems do not always accurately reflect binding requirements seen in the cells' physiological levels of both CD40 and relevant signaling proteins (Bishop and Hostager 2001b, 2003; Bishop 2004).

The application of gene targeting by homologous recombination, in which the expression of specific gene products has been disrupted, has facilitated the creation of mice deficient in many known signaling proteins. This powerful technology provides many valuable new insights into the function of various proteins. However, because many signaling molecules mediate multiple pathways involved in normal development and physiology, removal of such proteins from the entire animal results in early lethality of many of these strains. This approach has limited the usefulness in studies of receptor signaling in mature cell types expressing CD40, as has been the case for mice deficient in TRAFs 2, 3, and 6. Although these first efforts to understand TRAFs yielded many clues about TRAF binding and function, much of the information gained from these approaches cannot be clearly interpreted (Bishop and Hostager 2001a; Bishop 2004; Graham et al. 2010).

Advances in gene targeting techniques led to the production of "conditional" knockout mice, in which a gene flanked by bacterial recombinase recognition sequences is removed from specific cell types in the targeted mouse by breeding with a strain expressing the recombinase behind a promoter specific for expression in the desired cell type(s). This technology holds great promise in gaining new insights into TRAF function; however, it is a high-cost, time-consuming, and labor-intensive process (Bishop et al. 2007).

To overcome these obstacles, TRAF deficient B cell lines were created by targeting the TRAF genes via homologous recombination. This system allows transfection of the cells with mutant CD40 molecules as well as wild-type and mutant TRAFs, and avoids the interpretation complications of mutant TRAF or CD40 molecules affecting other TRAFs. This approach has revealed valuable information about roles of TRAF proteins in CD40-mediated signals in B cells. Through information gained from the various complementary approaches, a more detailed picture of CD40-mediated molecular signaling events is emerging (Graham et al. 2010).

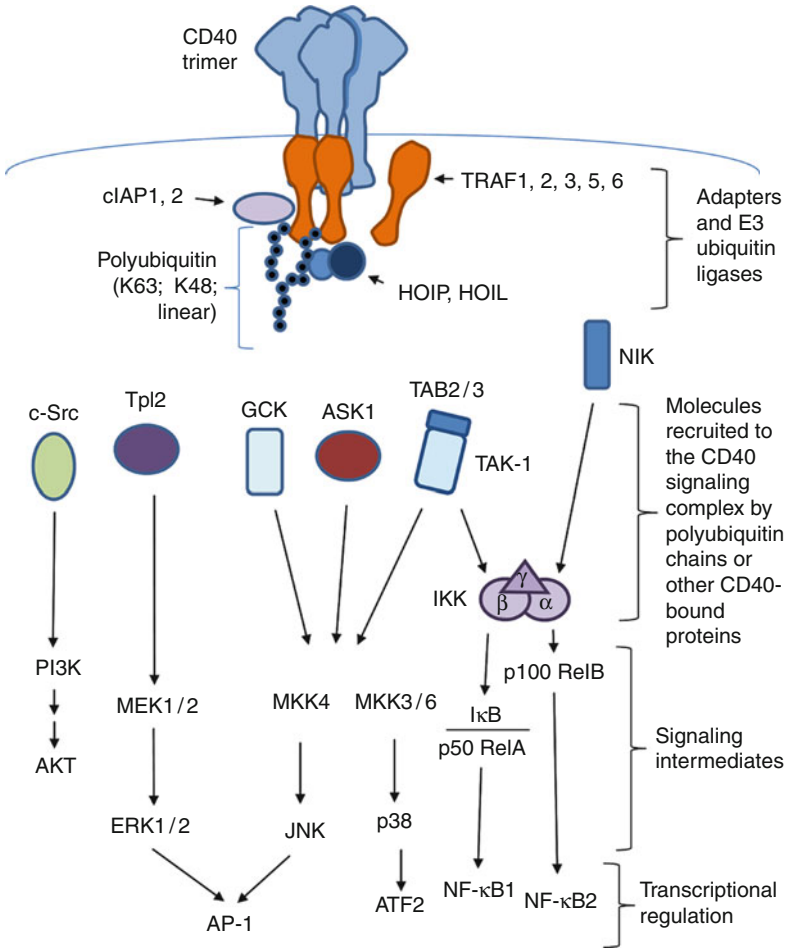
TRAF Recruitment and Degradation

Following ligation, CD40 redistributes into membrane lipid rafts and recruits TRAFs to the membrane signaling complex (Bishop and Hostager 2001b; Bishop 2004). The degree of receptor aggregation regulates downstream TRAF-dependent signaling in a complex manner. Most CD154-mediated signals can be initiated by agonistic antibodies, which can be used as a ligand substitute in many signals. However, CD40-mediated production of interleukin-6 (IL-6) in B cells requires a membrane-bound form of CD154. CD154 ligation leads to enhanced activation of c-Jun amino-terminal kinase (JNK) which binds to and activates c-Jun. The pairing of c-Jun with the protein c-Fos results in the formation of the transcription factor AP-1. The cooperation of AP-1, nuclear factor-kappa B (NF-κB), and C/EBP-β leads to induction of Il-6 gene transcription. Strength and duration of CD40 engagement in B cells can also alter TRAF binding. Agonistic antibody binding to CD40 is sufficient to initiate TRAF2 and 3 recruitment, but CD154-mediated engagement is required for optimal recruitment of TRAF6 (Bishop 2004). This may explain the CD154-dependent nature of CD40-mediated IL-6 production, which is itself TRAF6-dependent (Hostager 2007).

The Zn-binding RING domains of TRAFs 2 and 3 are required for optimal raft recruitment, as their removal or incubation with a membrane-permeable Zn-chelating agent inhibits this process, suggesting that the TRAF RING domain interacts with an unidentified raft-localized protein (Bishop and Hostager 2001b; Bishop et al. 2002). Following recruitment to CD40, TRAFs 2 and 3 are polyubiquitinated and degraded. CD40 association does not mediate degradation of TRAFs 1 or 6. Studies have also shown that TRAF6 association requires greater CD40 cross-linking and its recruitment to rafts is slower than that of TRAFs 2 and 3, a recruitment process that might be enhanced by the degradation of TRAFs 2 and 3 (Bishop 2004). TRAFs 2 and 6 have activity as E3 ubiquitin ligases in various contexts (Bishop et al. 2007).

CD40 signals need to be tightly regulated because CD40 plays a central role in the activation of multiple facets of immune responses. CD40-mediated degradation is important in limiting downstream signaling activation, for dysregulation of CD40 or CD40-like signals can contribute to autoimmunity and/or malignancies. The Epstein-Barr virus key transforming latent membrane protein (LMP1), is a CD40 mimic that provides an amplified and sustained signal. Unlike CD40, however, LMP1 appears able to bypass the activation of negative regulatory mechanisms that otherwise help to control TRAF activity. Avoidance of these regulatory mechanisms may contribute to the oncogenic activity of the virus (Graham et al. 2009).

CD40 signaling is tightly regulated by ubiquitination (Ub) of TRAFs 2, 3, and 6. Ub events appear to be critical for the proper assembly of the CD40 signaling complex and for the signals which it delivers (Fig. 1). This two-phase transduction mechanism starts with the covalent modification of signaling proteins with branched polyubiquitin chains in which ubiquitin molecules are covalently attached at lysine 63 (K63) of the preceding ubiquitin in the chain. This type of ubiquitin chain has been associated with the activation of various signaling pathways. TRAF2 and TRAF6 appear to undergo self-modification with K63-linked polyubiquitin, a RING-dependent process that mediates recruitment of TRAF3 and cellular Inhibitor of Apoptosis Proteins 1 and 2 (cIAP1 and cIAP2). TRAF2 and TRAF6 subsequently serve as E3 ubiquitin ligases for cIAP1/2 leading to their K63 polyubiquitination (K63 polyUb) and activation. These K63



CD40, Fig. 1 CD40 signaling pathways. Molecules shown as symbols are those for which there is evidence of direct interactions with CD40, members of the TRAF family, or polyubiquitin. See text for details of these interactions

polyubiquitin chains may provide docking sites for a number of additional components of the signaling complex including important MAPKKK (Karin and Gallagher 2009). These proteins include the Inhibitor of Kappa B (IκB) Kinase γ (IKKγ; also called NF-κB essential modulator – NEMO) and the transforming growth factor-β-activated kinase (TAK1), whose recruitment is mediated by the TAK1-binding proteins 2 and 3 (TAB2 and TAB3) (Hostager 2007). TRAF2 also mediates recruitment of MEKK1 (MAPK/ERK kinase kinase 1), a MAPKKK (Karin and Gallagher 2009). A newly identified component, heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1L) interacting protein (HOIP), may also be recruited by K63-linked polyubiquitin chains. HOIP, likely in a complex with another ubiquitin ligase, HOIL-IL, may serve to promote the activation of IKKγ through the addition of linear polyubiquitin to proteins in the CD40 signaling complex (Hostager et al. 2010).

The second phase of CD40-mediated signal transduction targets TRAFs 2 and 3 for proteasomal degradation by chains of Ub molecules linked through lysine at position 48 (K48). CD40 regulation was first observed when a decrease in the amount of TRAF2 in B cell lysates over time was noted during CD40 signaling; a similar trend was later observed for TRAF3. CD40-induced TRAF2 and TRAF3 degradation has been associated with the two Ub ligases cIAP1 and cIAP2 (Bishop and Hostager 2001b). After cIAP1/2 are activated by K63 polyUb as stated above, they may act as E3 ligases for TRAF3, promoting its K48 polyUb and proteasomal degradation (Karin and Gallagher 2009; Graham et al. 2010). TRAF3 is a negative regulator of CD40-mediated signals and acts as a brake on MAPK activation. TRAF3 degradation is thought to release signaling complexes into the cytosol where MAPKKKs can undergo autophosphorylation and activate downstream events (Graham et al. 2009, 2010).

Other ubiquitin ligases also interact with CD40 via TRAF interactions and include Casitas B lymphoma-b (Cbl-b), an E3 ubiquitin ligase that has been implicated in negatively regulating CD40 signaling. Cbl-b is recruited to CD40 via TRAF2, and Cbl-b deficient splenic B cells display decreased CD40-stimulated TRAF2 and TRAF3 Ub and degradation (Graham et al. 2010). Negative regulatory proteins such as A20 are also recruited to sites of polyUb and are likely involved in switching off signaling at the appropriate time, as A20 inhibits NF-κB activation (Bishop 2004).

Early CD40 Signals

Clustering of CD40 upon engagement leads to TRAF aggregation and the subsequent activation of downstream kinase cascades, including a variety of proteins in mitogen-activated protein kinase (MAPK) and stress-activated protein kinase (SAPK) pathways. There are several distinct CD40-signaling pathways linked to TRAF proteins. These pathways lead to the activation of SAPK/MAPKs including JNK, p38, NF-κB, and ERK1/2 (Fig. 1; Bishop 2004).

CD40-mediated activation of SAPK/MAPK JNK and p38 has been attributed to various kinases including members of the MAPKKK family. Mechanisms that have been confirmed in B cells include interactions of germinal center kinase (GC kinase) and related enzymes with the TRAF domain of TRAF2, which contribute to the induction of JNK activation. TRAF2 may also activate another MAPKKK, ASK-1 (apoptosis-signaling kinase 1) which is also upstream of JNK and p38 (Bishop 2004). Other MAPKKKs associated with JNK and p38 activation in B cells include MEKK1 and the TRAF6-dependent TAK-1. MAPKKs that have been demonstrated to participate in CD40-mediated activation of JNK and p38 include MKK4 and MKK3/6 respectively. The E2 ubiquitin-conjugating enzyme Ubc13 is also required for CD40-mediated activation of the JNK and p38 pathways (Karin and Gallagher 2009; Graham et al. 2010).

CD40 signaling activates both the canonical (NF-κB1) and noncanonical (NF-κB2) pathways. Direct TRAF–protein interactions for NF-κB1 activation include the TRAF6-dependent activation of TAK1. TAK1 can mediate activation of the downstream IKK complex which phosphorylates IκB proteins. This leads to their polyUb and proteasome-mediated degradation, followed by release and nuclear translocation of NF-κB1 which is composed of p50 and the transcription factor protein encoded by v-rel reticuloendotheliosis viral oncogene homolog A gene (RelA) to activate transcription. Unlike the rapid response of the canonical NF-κB1 pathway, the noncanonical NF-κB2 pathway requires new protein synthesis and is activated with much slower kinetics (Bishop and Hostager 2001a, b). In the NF-kB2 pathway, p100 is processed to p52, which complexes with the transcription factor protein encoded by v-rel reticuloendotheliosis viral oncogene homolog B gene (RelB) and translocates to the nucleus. It was initially thought that the first kinase to initiate TRAF-mediated NF-κB activation was NF-κB -inducing kinase (NIK) a serine-threonine MAPKKK. NIK activates the IKK complex, and although NIK enhances NF-κB activation in 293 cells, there is no direct evidence that it specifically mediates CD40-mediated NF-κB activation in B lymphocytes (Bishop and Hostager 2003). It is important to note that the interactions of TRAFs with NIK have mostly been demonstrated under non-physiological conditions in which both TRAFs and candidate kinases were transiently overexpressed in epithelial cells (Bishop et al. 2003). One study suggests that NIK is degraded rapidly in non-stimulated splenic B cells (and is therefore difficult to detect) and that CD40 engagement leads to stabilization of NIK via accumulation of newly synthesized protein that can finally activate the noncanonical NF-κB pathway (Karin and Gallagher 2009).

Another MAPK regulated by CD40-mediated signaling is the extracellular signal-regulated kinase (ERK). There are many factors upstream of ERK activation, including MAPKAPK-2, implicated in ERK and p38 activation and MEK1/2 phosphorylation, an event that requires tumor progression locus 2 (Tpl2) (Bishop et al. 2007). CD40 signals can also activate the Src-family kinase Lyn, and the phosphorylation of both phosphatidylinositol 3-kinase (PI3K)/Akt and phospholipase Cγ2 in human B cells (Bishop 2004). CD40-mediated signals have been shown to activate the serine-threonine kinase Pim-1 in mouse B cells and Janus kinase JAK3 in a human B cell line (Bishop et al. 2003; Graham et al. 2010).

Kinases are not the only proteins believed to interact with TRAFs during early CD40-mediated signals. The adaptor proteins Act1 and the B-cell scaffold protein with ankyrin repeats (BANK) have been identified as negative regulators of CD40-mediated signaling. Act1 (an adaptor protein that interacts with TRAF3) and BANK both negatively regulate

CD40-mediated kinase activation. Act1-deficient primary mouse splenic B cells display increased CD40-mediated ERK and NF-κB activation. CD40-mediated activation of another kinase, Akt, is also enhanced in BANK-deficient mouse splenic B cells, resulting in enhanced B cell proliferation and survival. However, it is important to note that a different Act1-deficient mouse strain shows only increases in Interleukin-17 (IL-17) production without an impact on CD40 signaling. Similar to the elusive kinase NIK, Act1's role in regulating CD40 signaling needs further investigation (Graham et al. 2010).

Intermediate CD40 Signals

CD40-mediated signaling to B cells causes increased production of surface molecules, lymphokines, and immunoglobulins, and plays a critical role in the activation of Ig isotype switching. Upstream of these events, CD40 can affect transcriptional regulation of the involved genes (Bishop and Hostager 2003). NF-κB mediated transcriptional regulation participates in a large number and variety of cellular functions, so studies of mice deficient in the various proteins associated with NF-κB transcription (RelA, RelB, p52, p50) cannot provide clearly interpretable information on the role of NF-kB in CD40-mediated effects. B cells from these mice develop in an altered environment and are not ideal models. An inducibly expressed mutant form of the inhibitory protein IκBα that cannot be phosphorylated and degraded in mouse B cell lines revealed that CD40-mediated NF-κB1 activation is critical for some, but not all, CD40 effector functions (Bishop and Hostager 2001b). Functions dependent on NF-κB1 include upregulation of the costimulatory CD80 molecule, Pim-1 kinase activation, IgM secretion, and isotype switching to IgE. There is also a partial dependence upon NF-κB1 for upregulation of CD23, CD95, and CD54. NF-κB1-independent functions include upregulation of LFA-1 and CD11a, and JNK activation (Bishop and Hostager 2001b; Bishop 2004).

Basal levels of NF-κB1 subunits in the nucleus, together with induced AP-1 and C/EBP-β activation, are required for maximal CD40-incuded IL-6 promoter activity as well as maximal IL-6 protein production in B cells (Bishop 2004). An important contributor to this cooperation is also the need for CD40-mediated activation of c-Jun, which can form the transcription factor AP-1 by pairing with c-Fos. However, the protein complex binding of the canonical AP-1 site in the mouse IL-6 promoter can be mediated by a transcriptionally active c-Jun homodimer. In addition to regulating IL-6, AP-1 and C/EBP-β transcription factors also play a role in CD40-mediated B cell IgM secretion and CD80 surface expression (Baccam et al. 2003; Bishop 2004).

Additional transcription factors that contribute to CD40-dependent functions include the B cell-specific activator protein (BSAP) and Stat6. In B cells, these factors appear to play roles in the transcription of the germ line ε gene that precedes class switch recombination to IgE. Furthermore, Stat6 and NF-κB can interact, with a potential contribution toward the synergy between CD40 and IL-4 signals in the induction of germline ε transcription. NF-AT, Stat5, and E2F are also activated in B cells following CD40 ligation. However, the causative roles of each of these factors in B cell functions have not been fully explored. Both the germline ε and CD23 promoters may contain yet unidentified regulatory elements specific to CD40-mediated gene expression (Bishop and Hostager 2001a, b, 2003; Bishop 2004).

Late CD40 Signals

Several biological functions of CD40 in the immune response were discussed above. The production of cytokines and chemokines is also an important function of B cells. These factors help regulate many CD40-mediated processes, including Ig switching, and antigen presentation. CD40 signaling has been shown to induce the production of various cytokines and chemokines including IL-2, IL-6, IL-7, IL-10, IL-12, IL-15, IL-17, IFNγ, lymphotoxin-α (TNF-β), and TNF-α (Graham et al. 2010). As discussed above, CD40-mediated signals play an important role in antigen presentation, a process that requires the upregulation of the costimulatory molecules CD80, CD86, and MHCII. The CD40-mediated upregulation of adhesion molecules including CD23, CD30, CD54, Fas, ICAM, and LFA-1 (Graham et al. 2010) is also important for enhancing T cell–B cell interactions during an immune response.

Summary

CD40 engagement results in downstream signaling events that include the activation of MAPK/SAPK kinase cascades and many transcription factors that enhance and regulate a number of biological processes.

Studies in both mouse and human cells from many laboratories have established that CD40 signaling pathways play a key role in mediating adaptive immune responses and are implicated in the pathogenesis and progression of many inflammatory and autoimmune diseases, as well as malignancies. A more accurate and detailed understanding of CD40-mediating molecular signaling may offer many new molecular targets for the development of new vaccines and new molecular therapies for the treatment of immune-mediated disorders. However, much remains to be learned, including how CD40 regulates transcriptional activation of a variety of genes. Other questions include the specific contributions of each of the known TRAFs and the specific mechanisms mediating their regulatory interactions with CD40. TRAFs can initiate signaling cascade via directly interacting with receptors, or alternatively, with intracellular signaling proteins to regulate downstream pathways. The activities of the TRAF proteins involved in CD40 signaling are, as yet, only partially defined. It is also quite likely that additional signaling molecules both directly and indirectly associate with the CY domain of CD40; CD40-mediated signaling cascades can also interact with other receptors' signaling pathways. Addressing these questions will elucidate CD40 functions and also provide important principles governing the regulation of signaling by other TRAF-utilizing receptors.

References

Baccam M, Woo SY, Vinson C, Bishop GA. CD40-mediated transcriptional regulation of the IL-6 gene in B lymphocytes: involvement of NF-kappa B, AP-1, and C/EBP. J Immunol. 2003;170:3099–108.

Bishop GA. The multifaceted roles of TRAFs in the regulation of B-cell function. Nat Rev Immunol. 2004;4:775–86.

Bishop GA, editor. The many faces of CD40: multiple roles in normal immunity and disease. Sem Immunol. 2009;21:255–312.

Bishop GA, Hostager BS. Molecular mechanisms of CD40 signaling. Arch Immunol Ther Exp. 2001a;49:129–37.

Bishop GA, Hostager BS. Signaling by CD40 and its mimics in B cell activation. Immunol Res. 2001b;24:97–109.

Bishop GA, Hostager BS. The CD40-CD154 interaction in B cell-T cell liaisons. Cytokine Growth Factor Rev. 2003;14:297–309.

Bishop GA, Hostager BS, Brown KD. Mechanisms of TNF receptor-associated factor (TRAF) regulation in B lymphocytes. J Leukoc Biol. 2002;72:19–23.

Bishop GA, Haxhinasto SA, Stunz LL, Hostager BS. Antigen-specific B-lymphocyte activation. Crit Rev Immunol. 2003;23:149–97.

Bishop GA, Moore CR, Xie P, Stunz LL, Kraus ZJ. TRAF proteins in CD40 signaling. Adv Exp Med Biol. 2007;597:131–51.

Graham JP, Moore CR, Bishop GA. Roles of the TRAF2/3 binding site in differential B cell signaling by CD40 and its viral oncogenic mimic, LMP1. J Immunol. 2009;183:2966–73.

Graham JP, Arcipowski KM, Bishop GA. Differential B lymphocyte regulation by CD40 and its viral mimic, latent membrane protein 1. Immunol Rev. 2010;237:1–22.

Hostager BS. Roles of TRAF6 in CD40 signaling. Immunol Res. 2007;39:105–14.

Hostager BS, Fox DK, Whitten D, Wilkerson CG, Eipper BA, Francone VP, et al. HOIL-1L interacting protein (HOIP) as an NF-kappaB regulating component of the CD40 signaling complex. PLoS ONE. 2010;5:e11380.

Karin M, Gallagher E. TNFR signaling: ubiquitin-conjugated traffic signals control stop-and-go for MAPK signaling complexes. Immunol Rev. 2009;228:225–40.

Koho H, Paulie S, Ben-Aissa H, Jónsdóttir I, Hansson Y, Lundblad ML, et al. Monoclonal antibodies to antigens associated with transitional cell carcinoma of the human urinary bladder. I. Determination of the selectivity of six antibodies by cell ELISA and immunofluorescence. Cancer Immunol Immunother. 1984;17:165–72.

Peters AL, Stunz LL, Bishop GA. CD40 and autoimmunity: the dark side of a great activator. Semin Immunol. 2009;21:293–300.

Schönbeck U, Libby P. The CD40/CD154 receptor/ligand dyad. Cell Mol Life Sci. 2001;58:4–43.

Uckun FM, Schieven GL, Dibirdik I, Chandan-Langlie M, Tuel-Ahlgren L, Ledbetter JA. Stimulation of protein tyrosine phosphorylation, phosphoinositide turnover, and multiple previously unidentified ser/thr-specific protein kinases by the pan-BCR CD40/Bp50 at discrete developmental stages of human B-cell ontogeny. J Biol Chem. 1991;266:17478–85.

CD43

Maria Elena Bravo-Adame, Monserrat Alba Sandoval-Hernandez, Oscar Arturo Migueles-Lozano and Yvonne Rosenstein
Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Synonyms

Leukosialin; Sialophorin, galactoglycoprotein

Historical Background

CD43, also called sialophorin or leukosialin was first identified as a defective molecule of leukocytes and

platelets of patients affected with the Wiskott-Aldrich syndrome. It was thought that expression of this heavily glycosylated, mucin-type membrane protein was restricted to hematopoietic cells. However, recent advances in the field, evidence that CD43 is present in non-lymphoid tissues, particularly tumor cells. Here we reviewed the most important features about this molecule, highlighting the recent advances that have contributed to our understanding of the roles of CD43.

Gene Expression and Protein Structure

CD43 is a type I cell surface glycoprotein abundantly expressed on almost all hematopoietic cells, except for erythrocytes and resting B cells. Although initially considered an immune cell molecule, recent reports evidence a much broader distribution, as epithelial cells, and a range of tumors of epithelial origin are CD43 positive. Expression of CD43 has also been documented in the normal brain and in the uterus. The gene encoding human CD43 is located in chromosome 16; it has two transcription initiation sites and it is composed of two exons and a single intron within the untranslated 5′ region, although the entire protein is encoded only by the second exon. Alternative polyadenylation signals generate two mRNAs that differ from each other in the length of the 3′ untranslated region (Pallant et al. 1989).

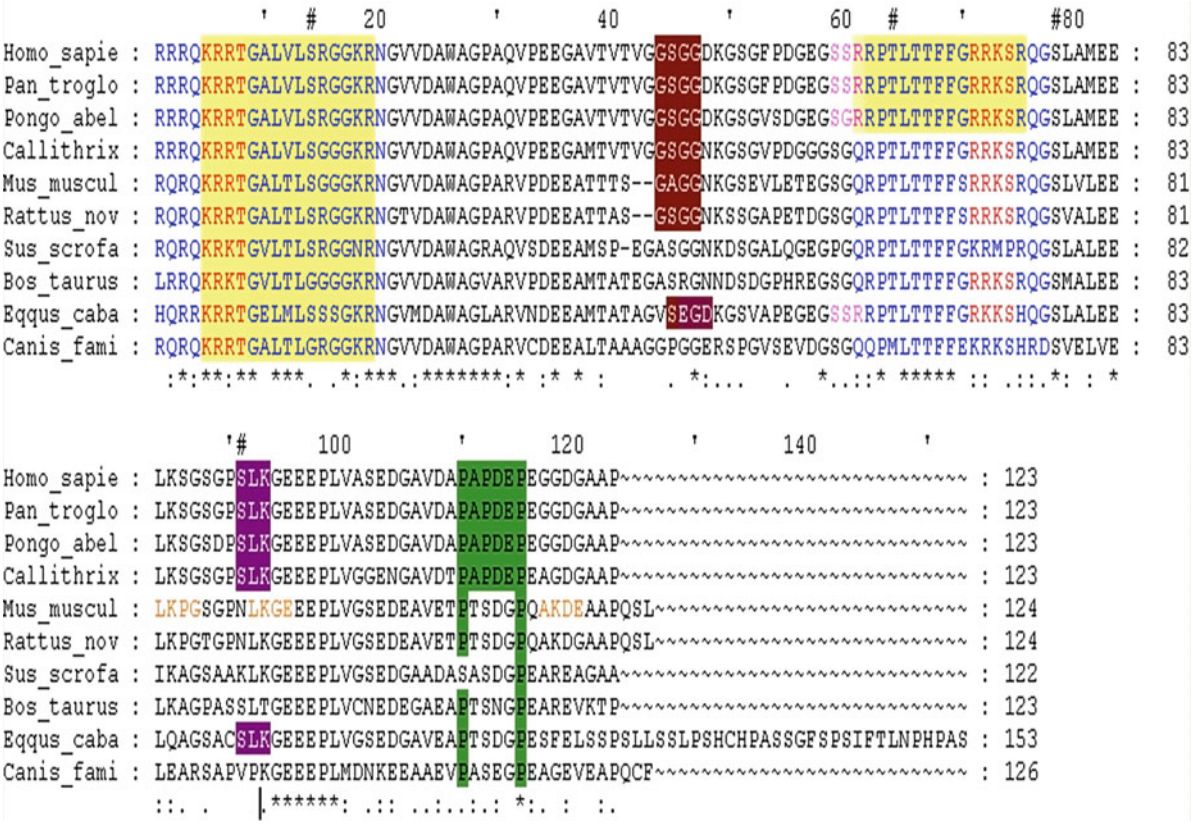
CD43 is a bulky and extended cell surface mucin that protrudes 45 nm from the cell surface. Its 239 amino acids long extracellular domain comprises five tandem repeats of 18 amino acids each (^{116}Ile–^{205}Ser), rich in serines and threonines modified by O-GalNAc glycosylation. As a result of the timely controlled activity of core 2 β-1,6-N-acetylglucosaminyltransferase (C2GnT), two isoforms of CD43 that define cell interaction affinities and functional cell states have been characterized. A 115 kDa isoform is expressed preferentially in thymocytes, resting CD4 T lymphocytes and monocytes, and a 130 kDa isoform is detected in resting CD8, CD4 activated T lymphocytes, neutrophils, platelets, B lymphocytes and macrophages, as well as tumor cells. The low molecular isoform contains almost exclusively the tetrasaccharide NeuAc(α2-3)-Gal(β1-3)[(NeuAc(α2-6)]GalNAc (Core1) and the high molecular isoform exhibits mainly the branched hexasaccharide NeuAc(α2-3)-Gal(β1-3[NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal-NAc (Core2) (Fukuda 2002). The intracellular domain has 123 amino acids with a highly conserved sequence among primates, mouse, and rat; it includes different motifs (Fig. 1) that allow CD43 to participate in different signaling pathways, supporting a functional role for this molecule.

CD43 Expression is Tightly Controlled

The levels and relative abundance of CD43 on the cell surface are controlled through several mechanisms: expression regulation, molecular density, or processing, evidencing a regulatory role for CD43 in cell function.

Aside from a tissue-specific gene transcription regulation, the relative abundance of CD43 at a specific location of the cell membrane is tightly controlled. As T lymphocytes and neutrophils migrate to inflammatory lesions, CD43, together with PSGL-1, ICAM-3 and CD44, moves from the leading edge to the rear end (uropod) of the cell through its association with members of the ERM family of molecules, while chemokine receptors relocate at the leading edge. Likewise, and through a similar mechanism, during the encounter of an antigen-specific T cell with the appropriate antigen-presenting cell, CD43 is excluded from the immunological synapse (intimate contact zone between the two cells), and relocates partially to the distal pole, where it plays a yet to define role. Interestingly, it is excluded from the inhibitor natural killer (NK) cell synapse, but not from the activating one (Reviewed in Ostberg et al. 1998; Aguilar-Delfín et al. 2006).

In addition, CD43 expression can be regulated by proteolysis and shedding. Processing of CD43 has been described in human neutrophils, murine T lymphocytes, as well as in human carcinoma cell lines (Lopez et al. 1998; Seo and Ziltener 2009; Andersson et al. 2004). The enzymatic cleavage of CD43 by a γ secretase-dependent intramembrane processing mechanism leads to the shedding of the extracellular domain, known as galactoglycoprotein and present in high concentrations in normal serum, and to the translocation of the intracytoplasmic domain to the nucleus, where it has been found to protect cells from apoptotic signals, promoting cell survival (Andersson et al. 2004; Seo and Ziltener 2009). Lastly,



CD43, Fig. 1 Multiple alignment of the intracellular region of CD43. Sequences are the reported on NCBI or were obtained by a BLAST analysis and picking the hits with at least 80% identity. The *Pan troglodytes, Pongo abelii, Rattus novergicus, Canis familiaris, Callithrix jacchus* and *Equus caballus* sequences are computational predictions. Two binding sites for ERM family proteins (ezrin, radixin, moesin) are highly conserved between species (blue letters, aminoacids 1–20 and 61–77). Two nuclear localization signals (NLS, yellow-shaded residues), comprising residues 5–19 and 61–75 of the intracellular domain respectively, overlap with the ERM binding domains. Potential phosphorylation sites are also located within the NLS and ERM binding regions. Serine/threonine residues experimentally confirmed to be phosphorylated in human, and well conserved, are marked with a "#" symbol. Red colored letters represent predicted cAMP- and cGMP-dependent protein kinase phosphorylation sites (residues 5–8 and 71–74). Pink letters and purple shaded letters tag predicted Protein kinase C phosphorylation pattern sites (residues 59–61 and 91–93). Between the two ERM binding domains, potential casein kinase II phosphorylation sites are predicted (brown shaded letters, residues 44–47, and 45–48 in Equus caballus). In addition, three potential SUMO modification consensus sequence sites (ψKXE) located close to the C terminus (orange letters) have been identified in the murine CD43 protein (residues 84–87, 92–95, 117–120, as you can see the middle one is well conserved across species). Finally, a proline rich region (green shaded letters, residues 110–115) at the C-terminus allows SH3-domain containing proteins to bind and participate in CD43 signaling pathways (Reviewed in Aguilar-Delfin et al. 2006; Seo and Ziltener 2009)

secretion of intracellularly stored CD43 is yet another mechanism through which CD43 expression levels are regulated: following LPS exposure, the intracellular levels CD43 present in intestinal epithelial cells have been found to augment, and correlate with an important secretion of CD43 to the intestinal lumen, underscoring a role for this mucin as a defense mechanism against infection in the intestinal epithelium (Amano et al. 2001).

A Multifunctional Protein

The abundance of CD43, its elongated structure, and the sequence homology of its intracytoplasmic domain between species indicate that this molecule transduces information from the cell surface to the intracellular milieu in order to modulate the decisions of the cell. In line with the fact that CD43 has been found to partner with a seemingly ever-growing list of membrane

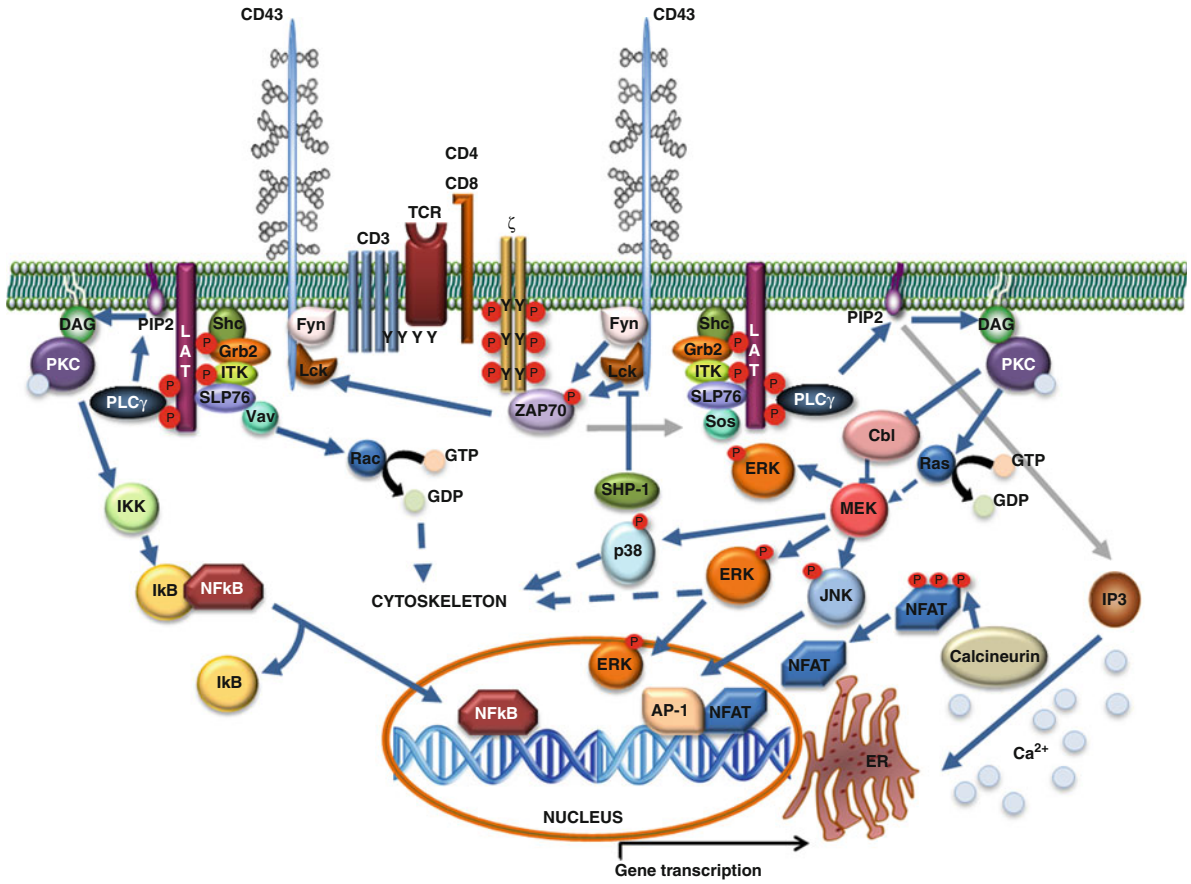
molecules, multiple, but often opposing functions have been described for CD43. The capacity of CD43 to transduce activation signals that regulate these multiple functions relies on its intracytoplasmic domain. Depending on the cell, and most probably on the ligand it encounters, CD43 must activate different signaling pathways that ultimately fine-tune the decision-making process of the cell, although little is known about this.

The net negative charge of CD43, resulting from the elevated glycosylation and sialic acid content, led to first propose that the role of this abundantly expressed cell surface mucin was to provide the cells with a repulsive force to limit cell contacts. Further attempts to understand the role of CD43 have been undertaken in vivo, with the $CD43^{-/-}$ mice, in different disease models where T lymphocytes are pivotal. In a model of vaccinia virus infection, despite the fact that CD8-dependent cytotoxic response is more robust in the knockout mice, viral clearance is less efficient in these mice as compared to wild-type animals (Manjunath et al. 1995). Likewise, infiltration of naïve CD8+ T cells to the brain following intracranial infection with Lymphocytic Choriomeningitis Virus (LCMV) is impaired in $CD43^{-/-}$ mice, resulting in prolonged survival of the animals. In an experimental model of autoimmune encephalomyelitis (EAE), $CD43^{-/-}$ mice have a lower incidence rate of the disease and milder symptoms due to a defective migratory capacity of antigen-specific CD4 T cells to the central nervous system. In addition, and consistent with a reduced inflammation, these animals have higher levels of IL-4 rather than of IFNγ. The LCMV model also provides evidence that, in addition to regulate the migration of T lymphocytes, CD43 participates in the contraction phase of the response, as antigen-specific CD8+ T cells numbers remain higher in the knockout mice, due to enhanced levels of ▶ Bcl-2 and hence decreased apoptosis (Onami et al. 2002; Ford et al. 2003). Altogether, these results highlight a positive role of CD43 in regulating the migration of naïve T cells to inflammation sites, and in regulating cell number and homeostasis. This in turn determines the development and the outcome of the disease and correlates with the fact that anti-CD43 antibodies prevent the migration of T cells to pancreatic islands, and the development of diabetes (Johnson et al. 1999). However, all these studies were done in the mixed background BL6.129, and care should be taken in the interpretation of the data, as a mixed genetic background can influence the outcome of the experiments. Also, it should be taken into account that these animals are completely deficient for CD43. It will be interesting to valuate the function of this mucin in mice where CD43 deficiency is limited to a specific cell type.

In addition to interact with cytoskeletal elements and to participate in directing cell migration, CD43 regulates cell–cell interactions and activation (Fig. 2). In vitro, CD43 engagement has been shown to induce homotypic cell adhesion, dendritic cell maturation, monocyte respiratory burst, increased T and B cell proliferation, and cytokine and chemokine secretion in NK cells, mast cells, dendritic cells, and T cells. CD43 engagement can induce apoptosis of Jurkat T cells and bone marrow cells; however, constitutive expression of CD43 in B cells promotes cell survival. Only expressed on a small proportion of naive B cells, CD43 expression is upregulated upon stimulation through the BCR or pokeweed mitogen and it contributes to cell proliferation; moreover, forced expression of CD43 was shown to inhibit B cell G1 arrest, extending B cell survival. Consistent with the fact that expression of CD43 on B cell lymphomas correlates with a bad prognosis, uncontrolled proliferation and enhanced survival capacity, two hallmarks of cancer cells, are positively regulated by CD43 in B cells (Reviewed in Aguilar-Delfín et al. 2006 and Pedraza-Alva and Rosenstein 2007).

Multiple Ligands for a Multifunctional Protein

The diversity of the physiological ligands identified for CD43 evidences a dynamic role for this cell-surface mucin in regulating intercellular interactions. The fact that ICAM-1 and MHC-1 molecules have been found to act as receptors for CD43 (Reviewed in Pedraza-Alva and Rosenstein 2007), together with the recent finding that CD43 interacts with LFA-1 and CD147 in two distinct complexes (Khunkaewla et al. 2008), indicates that CD43 plays a role in cell–cell adhesion, in concert with, and probably regulating, other adhesion molecules. Consistent with this possibility, splenocytes and thymocytes from CD43-deficient mice exhibit enhanced homotypic adhesion to ICAM-1 and fibronectin (Manjunath et al. 1995). Paradoxically, in contrast with this anti-adhesive function, a number of anti-CD43 mAbs block homotypic cell–cell interactions or lymphoid cell binding to lymph



CD43, Fig. 2 The signaling pathway of CD43 has been best characterized in T cells, where it functions as a co-receptor of the TCR and its co-stimulatory function is independent of CD28 (Sperling et al. 1995). Additional to its role as a costimulatory molecule, CD43 also signals by itself. CD43 engagement in human T cells induces its association to the ▶ Src family kinases Lck and Fyn, through the interaction of their SH3 domains and the proline-rich region of CD43. This then leads to the phosphorylation of the CD3 ς chain and the assembly of macromolecular complexes that include adaptor proteins such as Shc, Grb2, ▶ SLP-76, and the guanine exchange factor ▶ Vav. These signaling complexes promote ERK MAPK activation, leading to regulation of actin cytoskeleton and a positive feedback loop for Lck signaling as a result of an ERK-dependent serine phosphorylation of Lck, which inhibits its association to the phosphatase SHP-1. CD43 engagement also induces calcium fluxes and Protein kinase C activation, necessary for Cbl serine phosphorylation and its interaction with 14-3-3. Moreover, T cell pre-stimulation by the CD43 co-receptor molecule before TCR engagement inhibits the TCR-dependent c-Cbl tyrosine phosphorylation and interaction with the adapter molecule Crk-L and promotes Cbl-b ubiquitination and degradation in a Protein kinase C θ dependent manner. The inhibition of these E3 ubiquitin ligases results in prolonged tyrosine phosphorylation and delayed degradation of ▶ ZAP-70 and of the CD3 ς chain, leading to enhanced MAPK activation and robust T cell response. These data indicate that CD43-mediated signals lower the threshold for T cell activation by restricting the c-Cbl and Cbl-b inhibitory effects on TCR signaling (Reviewed in Pedraza-Alva and Rosenstein 2007)

nodes and high endothelial venules (Reviewed in Ostberg et al. 1998, and in Aguilar-Delfín et al. 2006). Moreover, E-selectin has been found to interact with CD43, favoring neutrophil and B cell leukemia cell lines rolling, further evidencing the participation of CD43 as a pro-adhesive molecule that regulates the infiltrating ability of cells (Matsumoto et al. 2008; Nonomura et al. 2008). CD43 also interacts with Siglec-1 (sialoadhesin), a regulator of lymphoid and myeloid cell adhesion, probably consolidating physical contacts between cells. On the contrary, the putative association of CD43 to human serum albumin inhibits neutrophil function by limiting cell spreading as well as CD43 proteolysis by elastase (Reviewed

in Aguilar-Delfín et al. 2006). Altogether, these data support the idea that CD43 triggers signaling pathways that modulate other conventional adhesion mechanisms.

In human dendritic cells, galectin-1-CD43 interaction primes the cells and results in cytokine production, upregulation of metalloproteases, and increased migration, through signaling pathways that depend on calcium fluxes as well as on Syk and Protein kinase C activation. However, through its interaction with galectin-1, CD43 promotes T cell apoptosis, possibly by recruiting galectin-1 molecules to the cell surface and making them accessible to CD7, which is directly responsible for galectin-1-induced cell death (Hernandez et al. 2006; Fulcher et al. 2009). Interestingly, through the interaction of membrane nucleolin with capped CD43, macrophages recognize apoptotic cells (Miki et al. 2009).

The myriad of ligands underscores a regulatory role for CD43, ultimately favoring homeostasis of the immune system. It is clear that, since most of these molecules are also ligands for other receptors, and different functions have been associated to each of them, CD43 participates in the fine-tuning of cell fate. However, information about the cellular responses and the intracellular signals that result from the interaction of CD43 with each of its ligands is very fragmentary, and this is undoubtedly a question that needs to be addressed.

CD43 is a Pathogen Recognition Receptor

The complex glycosylation pattern of CD43 functions as bait for multiple pathogens. During HIV infection, as a result of increased Core2 enzyme activity, more O-Core2 glycans and lactosamine residues decorate CD43, ultimately leading infected cells to apoptosis, presumably as a result of the interaction of galectin-1 with CD43 and CD45. Moreover, it was suggested that this pathway can contribute to the death of infected as well as noninfected cells, and thus to a decrease in T cell counts (Lanteri et al. 2003). What is more, the combination of CD43 and TCR-▶ CD3 complex signals lowers the signaling threshold for HIV LTR-driven gene activity, promoting the activation of NFκB and ▶ NFAT, ultimately favoring viral replication (Reviewed in Pedraza-Alva and Rosenstein 2007). In addition, the alternative glycosylation of CD43 promotes the production of autoantibodies against hyposialylated isoforms, although their role in HIV pathogenesis is not understood.

Influenza A virus (IAV) was also found to interact with polymorphonuclear leukocytes through the specific interaction of CD43 with the IAV hemagglutinin. In addition to lessen the oxidative burst of these cells, IAV modifies the expression level of adhesion-related molecules: while it reduces that of CD43 and L- and P-selectins, it augments that of integrins CD11b and CD11c, subtly modulating the adhesive interactions of polymorphonuclear cells (Hartshorn and White 1999). It is presently not known if this is the result of the signals transduced by the CD43–hemagglutinin A interaction or if it reflects the activation of additional, CD43-independent pathways.

Several pathogenic bacteria also use CD43 as a docking molecule. Recent reports have defined Cpn60.2 (Hsp65, GroEL), a molecular chaperone and an adhesin of the capsule of *Mycobacterium tuberculosis* as a ligand for CD43 (Hickey et al. 2010). This is consistent with the fact that, on macrophages, CD43 was previously identified as a receptor for *M. tuberculosis*, taking part in bacillus uptake and limiting its intracellular growth through the production of TNF α (Fratazzi et al. 2000). *Streptococcus gordonii*, the causative agent of infective endocarditis, has also been found to specifically interact with CD43 through Has, another adhesin (Ruhl et al. 2000). More recently, CD43 was reported to be the substrate of StcE, a metalloprotease that cleaves mucin-like glycoproteins, and is secreted by enterohemorrhagic *Escherichia coli*, the enteric pathogen that causes hemorrhagic colitis. The net balance of this proteolytic activity is the cleavage of the extracellular domain of CD43 from neutrophils, increased oxidative burst, but impaired neutrophil motility (Szabady et al. 2009).

In addition, CD43 is a counter-receptor for the trans-sialidase of *Trypanosome cruzi*, the causal agent of Chagas disease. This enzyme transfers sialic acid from β-galactopyranosil donors to β-galactopyranosil acceptors with α2–3-linkages providing the parasite with a disguise to avoid recognition by the host immune system. Furthermore, it restores the glycosylation status of CD43 to a Core1 glycans expression pattern, similar to that of resting T cells, thus compromising the T cell-dependent response, ensuring parasite replication. Interestingly, *T. cruzi* produces large amounts of an inactive and soluble form of the trans-sialidase that is also

recognized by CD43, although the biological significance of this is not understood presently (Freire-de-Lima et al. 2010).

On non-lymphoid cells, CD43 retains the capacity to function as a pathogen recognition receptor (PRR). Together with the glycolipid GM1, CD43 has been described as a receptor for the heat-labile enterotoxin of enterotoxigenic *E. coli* in the microvilli of enterocytes (Zemelman et al. 1989). Interestingly, in CaCo2 cells differentiated into enterocytes, CD43 is not exposed on the cell surface, but stored in intracellular granules from where it is released and secreted to the extracellular compartment upon exposure of the cells to bacterial lipopolysaccharides (Amano et al. 2001).

Altogether, these data suggest that through the pathogen receptor function of CD43, PAMPS initiate signal transduction pathways that modulate the activation of target cells and the onset of an inflammatory response and inflammation-related diseases. A better knowledge of the specific role of CD43 in the pathogen recognition and the cell-surface association processes will provide a clue to identify key target molecules both on the pathogen and on the host's cells.

CD43 and Noninfectious Diseases: A Poorly Understood Function

In addition to function as a PRR, CD43 has been associated to diverse diseases. CD43 was first described as the molecule responsible for the Wiskott-Aldrich Syndrome (WAS), an immunodeficiency characterized by frequent infections, skin eczema, decreased platelet numbers, and defective T-cell function (Remold-O'Donnell et al. 1984). However, it was later demonstrated that this X-linked disease was caused by mutations in the gene encoding WASP, a protein involved in signal transduction from cell receptors to the actin cytoskeleton. Yet, the link between WASP and CD43 is still unclear.

Although the participation of CD43 in several autoimmune diseases is documented, we are still lacking the full picture regarding the mechanisms of pathogenesis associated to this. Decreased expression of CD43 on the cell surface of synovial fluid polymorphonuclear cells of rheumathoid arthritis patients (Humbria et al. 1994) while an enhanced expression on the T cells infiltrating the synovium of osteoarthritis patients (Sakkas et al. 1998) have been reported. In systemic lupus erythematosus (SLE) patients, anti-CD43 and anti-galectin-1 autoantibodies, together with high levels of soluble galectin-1 were found to correlate with the time of disease evolution and low levels of complement (Montiel et al. 2010). A role for CD43 in Type I diabetes has been suggested, as preventing T cell migration and possibly that of other CD43+ cells such as monocytes and dendritic cells from bloodstream to inflammation sites (lymph nodes, pancreas, and salivary glands) with exogenous anti-CD43 antibodies, inhibits the progression of the disease (Johnson et al. 1999). Thus, reflecting the dual nature of CD43, anti-CD43 antibodies could function in two ways: favoring the inflammatory response, exacerbating the disease (SLE and arthritis), or preventing the establishment of the disease (diabetes).

CD43: A New "Kid on the Block" in Tumor Development

CD43 function has been mostly studied in hematopoietic cells and hence in an immunological context. However as mentioned, this molecule is no longer considered solely as an immune protein. It has been detected in normal and transformed tissues. In addition to hematopoietic tumors, non-hematopoietic tumor-derived cells from breast, lung, colon, bladder, cervix, and prostate express CD43 (Reviewed in Aguilar-Delfin et al. 2006) pointing to a role for this molecule in cell transformation, raising the question as to whether CD43 could be used as a marker for malignancy and prognosis.

The contribution of CD43 to tumor development is slowly emerging. CD43 is expressed in approximately 30% of low-grade B-cell lymphomas and 90% of T-cell lymphomas, B-cell lymphoblastic lymphomas, and of T-cell lymphoblastic lymphomas (Leong et al. 2003). Since CD43 expression in B cells is restricted to early B-cell ontogeny and activation of peripheral B cells, expression of CD43 suggests a state of malignancy. In fact, when expressed in a $CD43^{-}$ murine B-cell lymphoma, CD43 was found to provide the cells with increased viability and proliferation when cultured under serum deprivation conditions. Interestingly, expression of a CD8/CD43 chimera did not result in cell proliferation, indicating that the intracellular domain of CD43 is not sufficient to protect the cells from apoptosis and to promote cell cycle entry and, that the interaction of the extracellular domain of the

molecule with its ligands generates intracellular signals necessary for cell survival and proliferation (Misawa et al. 1996). Accordingly, T-cell lymphomas that test positive for the expression of the c-Maf protooncogene, also express CD43 together with high levels of cyclins D1 and D2 (Murakami et al. 2007). Moreover and consistent with these findings, in the absence of the tumor suppressors ▶ p53 and/or ARF, overexpression of CD43 in a human carcinoma cell line protects the cells from FAS-mediated apoptosis, ultimately favoring cell proliferation (Kadaja et al. 2004). In addition, it is now clear that the proteolytic cleavage of the CD43 cytoplasmic domain through a regulated intramembrane proteolysis (RIP) process exposes a nuclear localization signal that promotes the translocation of the truncated protein to the nucleus, where it interacts with ▶ beta-catenin, resulting in increased expression of the c-Myc and cyclin D1 genes (Andersson et al. 2004). Furthermore, CD43 was recently shown to participate in tumor cell-peritoneum interaction, through a metalloprotease-dependent mechanism activated by the interaction with ICAM-1, one of its putative ligands (Alkhamesi et al. 2007). Altogether, these data demonstrate that CD43 participates in the coordinated regulation of cell adhesion and cell motility as well as in the control of cell cycle entry, ultimately favoring cell transformation, tumor formation, and invasiveness. A deeper understanding of the CD43-mediated signals involved in tumor cell proliferation or survival will be critical for the rational development of drugs aimed to reduce malignancy.

Summary

CD43 is a mucin that modulates the response of all cells of the immune system as well as that of other cells. Because of its elongated structure and its abundance on the cell surface, it serves as an antenna that senses the environment, and prepares the cells for future actions. CD43 signals help the cell progress toward differentiation and maturation, as it regulates cell migration and cell contact. In addition, through the interaction with its numerous physiological and pathological ligands, CD43 actively participates in the onset of an inflammatory response and of inflammation-related diseases, underscoring the possibility to consider this molecule as a potential therapeutic target. An effort should be undertaken to identify those regions of the extracellular domain that are important for ligand recognition as well as to understand the rules that dictate selectivity of the different isoforms of CD43 for a given ligand. Unraveling the signals that follow interaction with a given ligand will help to untangle the multiple intracellular signals generated through this molecule. Understanding the role soluble CD43 is also a challenge. A better comprehension of the functions regulated by the interaction of CD43 with each of its multiple ligands in different cells in diverse contexts of activation will provide a sharper picture of the roles this molecule plays in cell physiology in different organs, under normal and pathological scenarios.

References

Aguilar-Delfin I, Fierro NA, Rosenstein Y. CD43. In: UCSD-nature molecule pages. 2006. doi:0.1038/mp.a000001.01.

Alkhamesi NA, Roberts G, Ziprin P, Peck DH. Induction of proteases in peritoneal carcinomatosis, the role of ICAM-1/CD43 interaction. Biomark Insights. 2007;2:377–84.

Amano J, Morimoto C, Irimura T. Intestinal epithelial cells express and secrete the CD43 glycoform that contains core 2 O-glycans. Microbes Infect. 2001;3(9):723–8.

Andersson CX, Fernandez-Rodriguez J, Laos S, Sikut R, Sikut A, Baeckstrom D, Hansson GC. CD43 has a functional NLS, interacts with beta-catenin, and affects gene expression. Biochem Biophys Res Commun. 2004;316(1):12–7.

Ford ML, Onami TM, Sperling AI, Ahmed R, Evavold BD. CD43 modulates severity and onset of experimental autoimmune encephalomyelitis. J Immunol. 2003;171(12):6527–33.

Fratazzi C, Manjunath N, Arbeit RD, Carini C, Gerken TA, Ardman B, et al. A macrophage invasion mechanism for mycobacteria implicating the extracellular domain of CD43. J Exp Med. 2000;192(2):183–92.

Freire-de-Lima L, Alisson-Silva F, Carvalho ST, Takiya CM, Rodrigues MM, DosReis GA, et al. Trypanosoma cruzi subverts host cell sialylation and may compromise antigen-specific CD8+ T cell responses. J Biol Chem. 2010;285(18):13388–96.

Fukuda M. Roles of mucin-type O-glycans in cell adhesion. Biochim Biophys Acta. 2002;1573(3):394–405.

Fulcher JA, Chang MH, Wang S, Almazan T, Hashimi ST, Eriksson AU, et al. Galectin-1 co-clusters CD43/CD45 on dendritic cells and induces cell activation and migration through Syk and protein kinase C signaling. J Biol Chem. 2009;284(39):26860–70.

Hartshorn KL, White MR. Influenza A virus up-regulates neutrophil adhesion molecules and adhesion to biological surfaces. J Leukoc Biol. 1999;65(5):614–22.

Hernandez JD, Nguyen JT, He J, Wang W, Ardman B, Green JM, Fukuda M, Baum LG. Galectin-1 binds different CD43 glycoforms to cluster CD43 and regulate T cell death. J Immunol. 2006;177(8):5328–36.

Hickey TB, Ziltener HJ, Speert DP, Stokes RW. Mycobacterium tuberculosis employs Cpn60.2 as an adhesin that binds

CD43 on the macrophage surface. Cell Microbiol. 2010;12(11):1634–47.
Humbria A, Diaz-Gonzalez F, Campanero MR, Arroyo AG, Laffon A, Gonzalez-Amaro R, et al. Expression of L-selectin, CD43, and CD44 in synovial fluid neutrophils from patients with inflammatory joint diseases. Evid soluble form L selectin synovial fluid Arthritis Rheum. 1994;37(3):342–8.
Johnson GG, Mikulowska A, Butcher EC, McEvoy LM, Michie SA. Anti-CD43 monoclonal antibody L11 blocks migration of T cells to inflamed pancreatic islets and prevents development of diabetes in nonobese diabetic mice. J Immunol. 1999;163(10):5678–85.
Kadaja L, Laos S, Maimets T. Overexpression of leukocyte marker CD43 causes activation of the tumor suppressor proteins p53 and ARF. Oncogene. 2004;23(14):2523–30.
Khunkaewla P, Schiller HB, Paster W, Leksa V, Cermak L, Andera L, Horejsí V, Stockinger H. LFA-1-mediated leukocyte adhesion regulated by interaction of CD43 with LFA-1 and CD147. Mol Immunol. 2008;45(6):1703–11.
Lanteri M, Giordanengo V, Hiraoka N, Fuzibet JG, Auberger P, Fukuda M, et al. Altered T cell surface glycosylation in HIV-1 infection results in increased susceptibility to galectin-1-induced cell death. Glycobiology. 2003;13(12):909–18.
Leong AS, Cooper K, Leong FJ. Manual for diagnostic antibodies for immunohistology. 2nd ed. London: Greenwich Medical Media Ltd; 2003.
Lopez S, Seveau S, Lesavre P, Robinson MK, Halbwachs-Mecarelli L. CD43 (sialophorin, leukosialin) shedding is an initial event during neutrophil migration, which could be closely related to the spreading of adherent cells. Cell Adhes Commun. 1998;5(2):151–60.
Manjunath N, Correa M, Ardman M, Ardman B. Negative regulation of T-cell adhesion and activation by CD43. Nature. 1995;377(6549):535–8.
Matsumoto M, Shigeta A, Miyasaka M, Hirata T. CD43 plays both antiadhesive and proadhesive roles in neutrophil rolling in a context-dependent manner. J Immunol. 2008;181(5):3628–35.
Miki Y, Itoh T, Hirano K, Eda S, Hayashi A, Yamanaka M, Beppu M. Clearance of oxidatively damaged cells by macrophages: recognition of glycoprotein clusters by macrophage-surface nucleolin as early apoptotic cells. Biol Pharm Bull. 2009;32(4):564–72.
Misawa Y, Nagaoka H, Kimoto H, Ishii Y, Kitamura K, Tsunetsugu-Yokota Y, Shibuya M, Takemori T. CD43 expression in a B cell lymphoma, WEHI 231, reduces susceptibility to G1 arrest and extends survival in culture upon serum depletion. Eur J Immunol. 1996;26(11):2573–81.
Montiel JL, Monsivais-Urenda A, Figueroa-Vega N, Moctezuma JF, Burgos-Vargas R, Gonzalez-Amaro R, Rosenstein Y. Anti-CD43 and anti-galectin-1 autoantibodies in patients with systemic lupus erythematosus. Scand J Rheumatol. 2010;39(1):50–7.
Murakami YI, Yatabe Y, Sakaguchi T, Sasaki E, Yamashita Y, Morito N, et al. c-Maf expression in angioimmunoblastic T-cell lymphoma. Am J Surg Pathol. 2007;31(11):1695–702.
Nonomura C, Kikuchi J, Kiyokawa N, Ozaki H, Mitsunaga K, Ando H, et al. CD43, but not P-selectin glycoprotein ligand-1, functions as an E-selectin counter-receptor in human pre-B-cell leukemia NALL-1. Cancer Res. 2008;68(3):790–9.
Onami TM, Harrington LE, Williams MA, Galvan M, Larsen CP, Pearson TC, et al. Dynamic regulation of T cell immunity by CD43. J Immunol. 2002;168(12):6022–31.
Ostberg JR, Barth RK, Frelinger JG. The Roman god Janus: a paradigm for the function of CD43. Immunol Today. 1998;19(12):546–50.
Pallant A, Eskenazi A, Mattei MG, Fournier RE, Carlsson SR, Fukuda M, Frelinger JG. Characterization of cDNAs encoding human leukosialin and localization of the leukosialin gene to chromosome 16. Proc Natl Acad Sci USA. 1989;86(4):1328–32.
Pedraza-Alva G, Rosenstein Y. CD43: One molecule, many tales to recount. Signal Transduction. 2007;7:372–85.
Remold-O'Donnell E, Kenney DM, Parkman R, Cairns L, Savage B, Rosen FS. Characterization of a human lymphocyte surface sialoglycoprotein that is defective in Wiskott-Aldrich syndrome. J Exp Med. 1984;159(6):1705–23.
Ruhl S, Cisar JO, Sandberg AL. Identification of polymorphonuclear leukocyte and HL-60 cell receptors for adhesins of Streptococcus gordonii and Actinomyces naeslundii. Infect Immun. 2000;68(11):6346–54.
Sakkas LI, Scanzello C, Johanson N, Burkholder J, Mitra A, Salgame P, et al. T cells and T-cell cytokine transcripts in the synovial membrane in patients with osteoarthritis. Clin Diagn Lab Immunol. 1998;5(4):430–7.
Seo W, Ziltener HJ. CD43 processing and nuclear translocation of CD43 cytoplasmic tail are required for cell homeostasis. Blood. 2009;114(17):3567–77.
Sperling AI, Green JM, Mosley RL, Smith PL, DiPaolo RJ, Klein JR, Bluestone JA, Thompson CB. CD43 is a murine T cell costimulatory receptor that functions independently of CD28. J Exp Med. 1995;182(1):139–46.
Szabady RL, Lokuta MA, Walters KB, Huttenlocher A, Welch RA. Modulation of neutrophil function by a secreted mucinase of *Escherichia coli* O157:H7. PLoS Pathog. 2009;5(2):e1000320.
Zemelman BV, Chu SH, Walker WA. Host response to Escherichia coli heat-labile enterotoxin via two microvillus membrane receptors in the rat intestine. Infect Immun. 1989;57(10):2947–52.

CD45 (PTPRC)

Pauline Johnson, Asanga Samarakoon, Amy E. Saunders and Kenneth W. Harder
Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada

Synonyms

B220; CD45R; Ly 5.2; Ly5.1; protein tyrosine phosphatase receptor type C; T200; The leukocyte common antigen (LCA)

Historical Background

CD45 was first identified by antisera and then by monoclonal antibodies (mAbs) as a major lymphocyte cell surface glycoprotein. CD45 is leukocyte specific, expressed on all hematopoietic cells except red blood cells and this has led to the clinical use of pan-specific CD45 mAbs to identify leukocytes and cells of hematopoietic origin. Some mAbs identify specific isoforms of CD45 that are expressed in a cell type and developmentally regulated manner and are referred to as CD45R mAbs. CD45 isoforms range from 180 to 220 kDa and cloning of the CD45 gene revealed that these isoforms arise by alternative splicing of at least three exons (ABC) that encode a region near the amino terminus of the extracellular domain of CD45 (Thomas 1989; Johnson et al. 1997). The B220 (CD45RABC) isoform of CD45 is expressed primarily on B cells and the CD45RA and RB isoforms are expressed on T cells and downregulated on T cell activation whereas the CD45RO isoform, which does not include any of the alternatively spliced exons, is upregulated on T cell activation. Thus CD45 isoform expression is a useful parameter to monitor the activation state of the T cell and has been particularly useful for human cells. The PTPRC gene, which encodes CD45, was cloned in the 1980s, revealing significant sequence identity of the cytoplasmic domain of CD45 with the protein tyrosine phosphatase, PTP1B. This led to the identification of CD45 as a receptor-like protein tyrosine phosphatase (Trowbridge and Thomas 1994). The phosphatase activity of CD45 was subsequently shown to be important for antigen receptor signaling in T and B lymphocytes where it regulates the phosphorylation state and activity of specific Src family kinases (Alexander 2000). In addition, work with CD45-deficient cells and mice has implicated CD45 in regulating leukocyte adhesion, cytokine signaling, and immune receptor signaling involving Fc, NK, and Toll-like receptors.

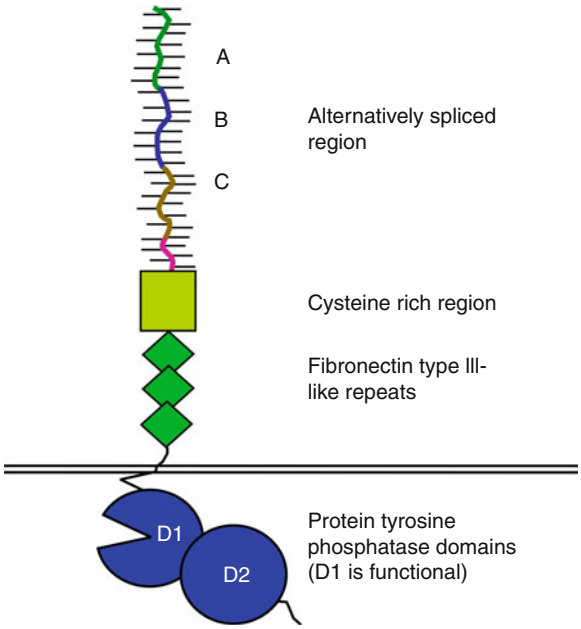
CD45 is a Transmembrane Protein Tyrosine Phosphatase

The PTPRC gene is conserved across several vertebrate species, from zebra fish, lamprey, and shark to chickens, cows, and humans. It spans over 50 MB and is located on chromosome 1q31-32 in humans and chromosome 1 at 74 cM in mice. Further information on the *PTPRC* gene, the protein sequences from the different species, and the crystallographic structure of the cytoplasmic domain of CD45 can be found at the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov). CD45 is a type I transmembrane protein, which is abundantly expressed on leukocytes. By electron microscopy, CD45 has a globular cytoplasmic domain of 12 nm and a rod-like extracellular domain of 28 nm, which extends to 51 nm when the alternatively spliced exons are included (Johnson and Felberg 2000). This makes CD45 one of the largest molecules on the lymphocyte membrane, being considerably larger than the cell surface molecules involved in antigen recognition that are estimated to extend approximately 8 nm from the cell surface. CD45 is heavily glycosylated with 25% of its mass attributed to carbohydrate. N-linked glycosylation sites are interspersed throughout the extracellular domain whereas O-linked sites are enriched in the amino terminal and alternatively spliced regions. The rest of the extracellular domain is relatively cysteine rich and may contain up to three fibronectin type III-like domains (Fig. 1).

CD45 has a single transmembrane region and a large intracellular domain of over 700 amino acids that has been shown by crystallographic studies to contain two protein tyrosine phosphatase domains. Only the membrane proximal phosphatase domain (D1) has catalytic activity but the second domain is required for optimal activity (Johnson and Felberg 2000). Since basal phosphotyrosine levels in naive lymphocytes are low, CD45 is thought to be a constitutively active phosphatase, whose activity can be regulated by its level of expression and by access to substrate. No specific ligands have been identified for CD45 but the carbohydrates of CD45 can bind lectin molecules such as galectin 1, which cluster CD45 and modulate its activity. Serine phosphorylation and the interaction of CD45 with other proteins (such as CD45AP, the CD45 associated protein) may also regulate its ability to dephosphorylate substrates, but these mechanisms are not well understood.

CD45 is a Major Regulator of the Src Family Kinases, Lck and Lyn

The ▶ Src family tyrosine kinase, Lck, was identified as a key substrate for CD45 in T cells and Lyn was

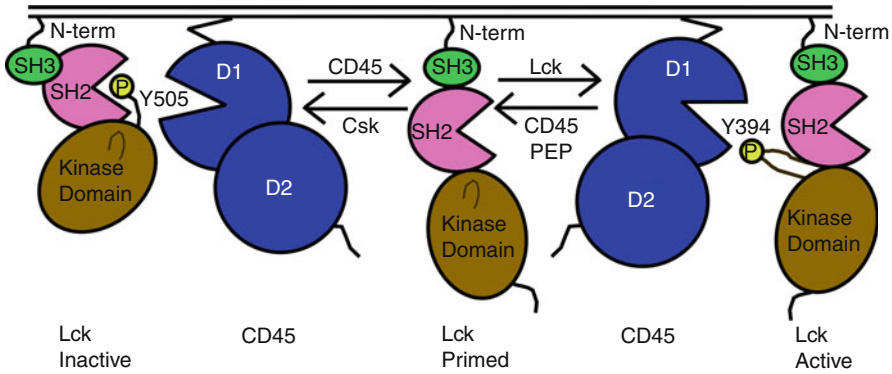


CD45 (PTPRC), Fig. 1 *Schematic of CD45 structure*. CD45 is a type I transmembrane glycoprotein. The extracellular domain is relatively large and heavily glycosylated with several N-linked carbohydrate sites interspersed throughout the region whereas the majority of O-linked glycosylation sites are localized in the amino terminal region and the alternatively spliced regions (designated *A*, *B*, and *C*) of CD45. The cytoplasmic region of CD45 consists of a membrane proximal wedge region followed by two protein tyrosine phosphatases (*D1* and *D2*), with the catalytic cysteine residing in *D1*, and then a short C-terminal region

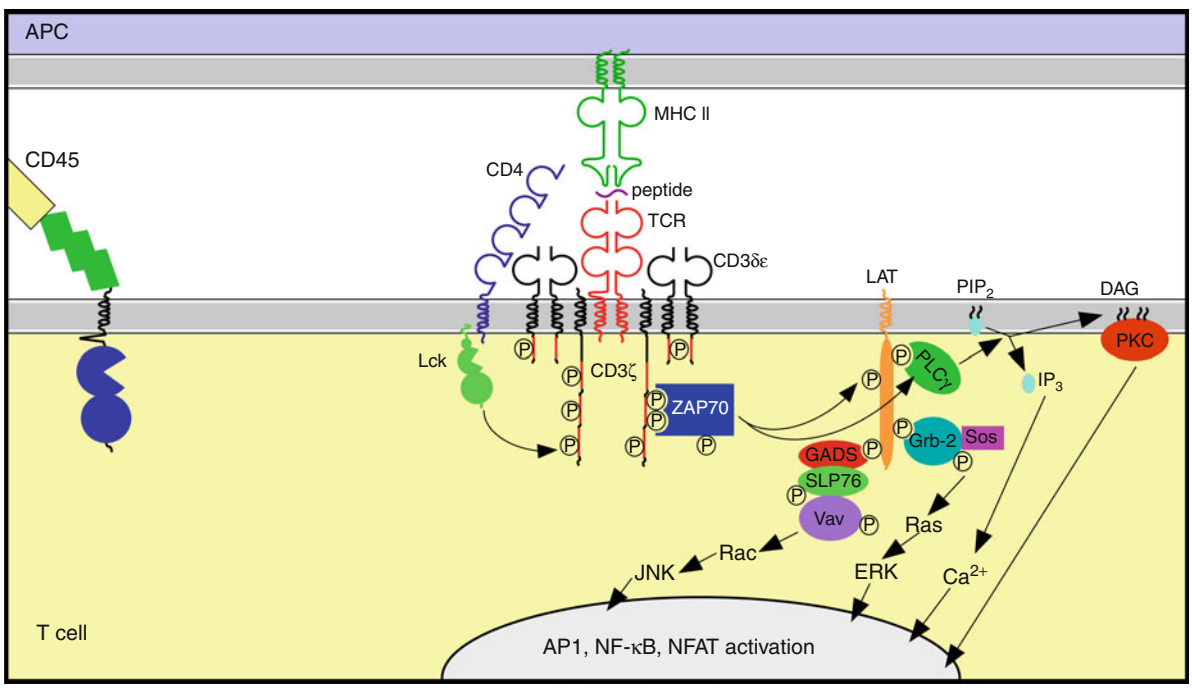
identified as a major substrate in B cells. Fyn may also be a CD45 substrate in T cells, but is less affected by the absence of CD45. In macrophages, the Src family tyrosine kinases Lyn and Hck have been identified as substrates for CD45 (Alexander 2000; Hermiston et al. 2003; Saunders and Johnson 2010). The Src family tyrosine kinases are regulated by two major tyrosine phosphorylation sites: a negative regulatory site close to the C-terminus of the protein and a positive regulatory site in a loop close to the active site of the kinase which, when phosphorylated, moves the loop away from the catalytic site thereby allowing substrate access (Williams et al. 1998). CD45 was shown to dephosphorylate Lck at the negative regulatory site in T cells, thus priming Lck for activation. However, subsequent work showed that CD45 could also dephosphorylate the positive regulatory site that is normally autophosphorylated in the active kinase. Thus CD45 has the ability to both upregulate and downregulate Lck activity in T cells and has the ability to maintain Lck in the primed, dephosphorylated state (Fig. 2). Although CD45 has been identified as a significant regulator of Src family kinase phosphorylation in hematopoietic cells, it is not the only regulator, as other tyrosine phosphatases such as CD148 can dephosphorylate the negative regulatory site and SHP-1 and Lyp/PEP can dephosphorylate the positive regulatory site (Hermiston et al. 2009).

CD45 and T Cell Antigen Receptor Signaling

The distribution of CD45 in the membrane, as well as the recruitment of Lck to the membrane either by myristoylation or by association with the CD4 or CD8 transmembrane proteins influences the ability of CD45 to access and dephosphorylate Lck. The basal activity of CD45 in naïve T cells primes Lck so it is ready to effectively participate in T cell receptor (TCR) signaling. Recognition by the TCR of peptide antigen presented by MHC class I or II molecules together with the binding of CD4 or CD8 co-receptor molecules to MHC molecules brings Lck into close proximity with the TCR associated ▶ CD3 chains. Antigen recognition is thought to induce clustering of these TCR complexes, which excludes CD45 and allows Lck to phosphorylate the ▶ CD3 chains at specific immunoregulatory tyrosine activation motifs (ITAMs) (van der Merwe and Dushek 2011). This results in the recruitment of the SH2 domain–containing tyrosine kinase ▶ Zap-70 that further propagates the phosphorylation cascade and the recruitment of signaling proteins (Tomlinson et al. 2000 and Fig. 3). Signaling from these microclusters leads to T cell activation and proliferation. These microclusters also aggregate to form the center of an immune synapse (cSMAC), which is surrounded by a ring of adhesion molecules referred to as the pSMAC (Dustin et al. 2010). CD45 is dynamically regulated during this process; it is initially excluded from the microclusters and the initial cSMAC then accumulates at the cSMAC at later time points, where it may play a role in downregulating Lck kinase activity and terminating the phosphorylation-induced signals. In the absence of CD45, Lck is constitutively phosphorylated in T cells, which leads to inefficient TCR signaling and



CD45 (PTPRC), Fig. 2 *Regulation of Src family kinase phosphorylation by CD45*. CD45 can dephosphorylate both the negative (Y505) and positive (Y394) regulatory tyrosines of the Src family kinase, Lck in T cells. In its dephosphorylated state, Lck is maintained in a primed state. CD45 acts reciprocally to the ▶ Csk kinase to dephosphorylate Lck at Y505. This releases the intracellular binding of phosphorylated Y505 to the SH2 domain to create an open, primed Lck. Lck either autophosphorylates or transphosphorylates Y394 which displaces the loop from the catalytic site, and creates an active kinase. CD45, as well as other phosphatases such as PEP, downregulate Lck activity by dephosphorylating Y394



CD45 (PTPRC), Fig. 3 *Function of CD45 in TCR signaling*. In the unactivated T cell, CD45 dephosphorylates Lck on the negative regulatory site and maintains Lck in a primed, dephosphorylated state. On engagement of peptide-MHC on the antigen-presenting cell (APC) by the TCR, costimulatory molecules (CD4 or CD8) are recruited along with Lck and signaling clusters are formed that exclude CD45. Active Lck then phosphorylates tyrosine residues in the ITAMs on CD3 molecules (δ, ε and ζ), which leads to the recruitment and phosphorylation of the kinase ZAP-70. Activated ZAP-70 then phosphorylates signaling molecules and initiates a downstream signaling cascade that leads to T cell activation. Later in the signaling process, TCR clusters form an immune synapse and CD45 moves into the synapse where it is thought to inactivate Lck and terminate TCR signaling

an increased threshold for T cell activation. This disrupts both positive and negative selection in the thymus, leading to reduced numbers of peripheral T cells in CD45-deficient mice that do not respond appropriately on antigen recognition (Alexander 2000; Hermiston et al. 2003; Saunders and Johnson 2010).

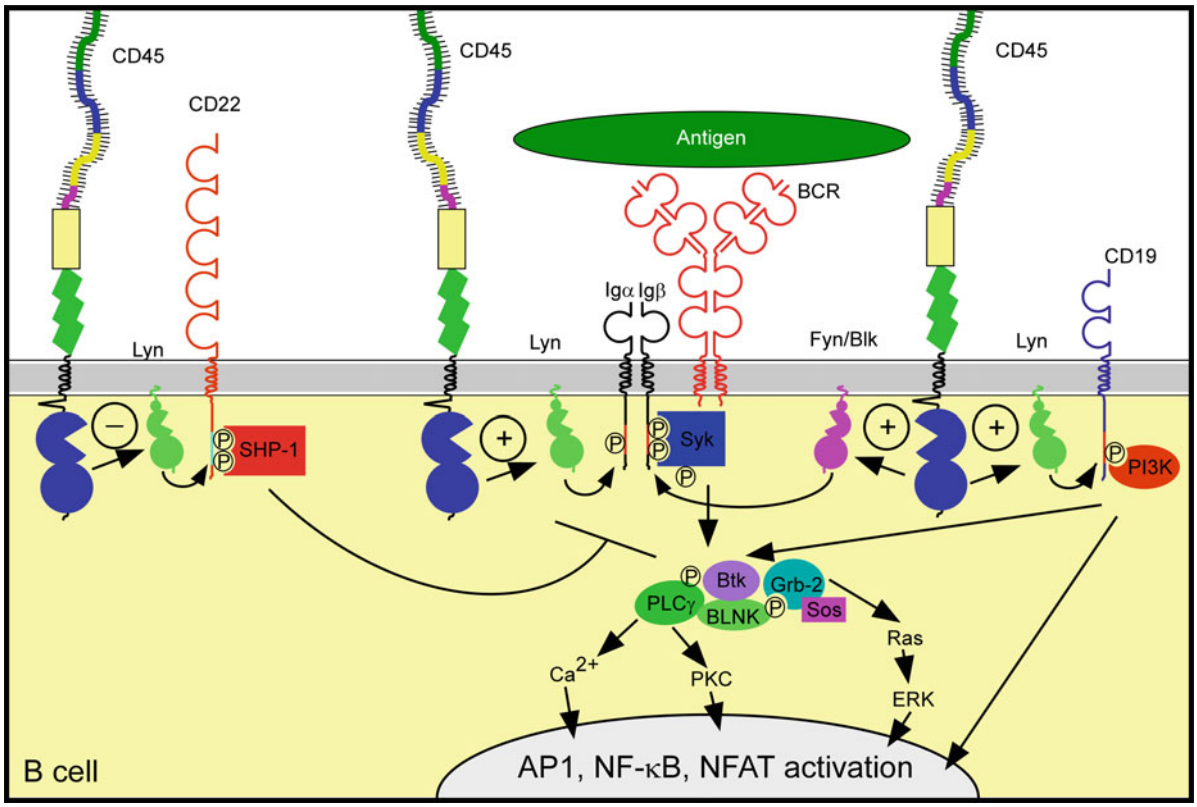
CD45 and B Cell Antigen Receptor Signaling

B cell antigen receptor (BCR) signaling, like TCR signaling, requires protein tyrosine kinase cascades to propagate signals required for B cell development and function. However the BCR is less dependent on CD45 and Src family kinases and is instead more dependent on the Syk tyrosine kinase. Syk is not as dependent on CD45 for activation as some BCR signaling events do occur in the absence of CD45. As a result, B cell development occurs in CD45-deficient mice but with increased numbers of immature IgM^{hi} cells and marginal zone B cells in the spleen and decreased numbers of circulating mature B cells (Hermiston et al. 2005). In CD45-deficient B cells, Syk and Btk are activated to some extent, Lyn is hyperphosphorylated and Fyn and Blk are not activated upon BCR engagement. This results in Igα and Igβ tyrosine phosphorylation but reduced ERK phosphorylation and diminished calcium signaling, which is insufficient to induce B cell proliferation in response to IgM cross-linking and points to a role for CD45 in enhancing BCR signaling strength. This reduced BCR signaling, as well as reduced phosphorylation of the costimulatory molecule, CD19, implicates CD45 in the positive regulation of Lyn, Fyn, and Blk (Fig. 4). However, CD22 was hyperphosphorylated in a CD45-deficient B cell line and Lyn was hyperphosphorylated at both the negative and positive regulatory sites and was more active in an in vitro assay, suggesting that CD45 may also negatively regulate Lyn (Huntington and Tarlinton 2004; Saunders and Johnson 2010). In B cells and myeloid cells, Lyn has the unique ability to phosphorylate immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in key negative regulatory molecules such as CD22, PIR-B, and FcγRIIB1 (Xu et al. 2005; Scapini et al. 2009). This non-redundant role for Lyn became evident through studies of Lyn-deficient mice where B cells are hyperresponsive to BCR ligation and resistant to signal inhibition induced by inhibitory receptor BCR co-ligation. Lyn-deficient mice also showed diminished tyrosine phosphorylation of key inhibitory phosphatases such as SHP-1 and SHIP-1. Many of these effects were opposite to those observed in CD45-deficient B cells, consistent with the loss of CD45 having an activating effect on Lyn. This view is strengthened by analysis of Lyn gain-of-function (Lyn^{up}) mice, which have enhanced BCR-induced calcium mobilization and tyrosine phosphorylation of both stimulatory (PLCγ, Syk) and inhibitory proteins, impaired B cell proliferation, and reduced circulating B cells in vivo. Thus there are many similarities between the CD45-deficient and Lyn^{up} B cells, with the minor differences perhaps being explained by the effect of CD45 on Blk and Fyn. It is also possible that CD45 has a dual effect on Lyn in B cells, downregulating its unique role activating inhibitory receptors and promoting Lyn's role in BCR signaling (Fig. 4), but exactly how this occurs is not known. Overall, the data indicate a clear role for CD45 in regulating BCR-induced signaling by regulating Lyn kinase.

Regulation of Additional Signaling Pathways by CD45 in Leukocytes

CD45 regulates other signals that are associated with ITIM or ITAM containing signaling molecules such as the Fc and NK receptors (Hermiston et al. 2009; Saunders and Johnson 2010). These receptors associate with ITAM-containing signaling proteins such as DAP12, FcRγ and ▶ CD3ζ chains, which are phosphorylated by Src family kinases and recruit Syk or ▶ Zap-70; thus, a similar pattern to antigen receptor regulation by CD45 is thought to exist. CD45 is required for IgE-mediated degranulation and IgE-mediated anaphylaxis in mast cells; however, FcRγ-mediated events such as IgG-mediated phagocytosis and antibody-dependent cytotoxicity in NK cells are not CD45 dependent. Interestingly, cytokine production induced by cross-linking NK cell receptors is CD45 dependent and correlates with impaired calcium mobilization and Syk and ERK activation. Thus the effect of CD45 may depend on the relative importance of Src family kinases in the response.

CD45 can also regulate cytokine signaling in hematopoietic cells (Saunders and Johnson 2010). CD45 can either upregulate or downregulate IFNα signaling in



CD45 (PTPRC), Fig. 4 *Function of CD45 in BCR and inhibitory protein* (*CD22*) *signaling*. Antigen binding cross-links the BCR, which activates the Src family kinases (Lyn, Fyn, and Blk) to phosphorylate ITAMs on Igα and Igβ. This leads to the recruitment and activation of Syk, which can then phosphorylate signaling molecules propagating a downstream signal that leads to B cell activation and proliferation. CD45 dephosphorylates and positively regulates these Src family kinases involved in BCR signal transduction. However, Lyn also negatively regulates BCR signaling by phosphorylating ITIMs present on inhibitory receptors such as CD22. This recruits the SHP-1 tyrosine phosphatase which downregulates BCR signaling. The data suggest that in this situation, CD45 negatively regulates Lyn activity, thereby promoting BCR signaling. Thus the net effect of CD45 is a partial inhibitory effect on BCR signaling

T cells and downregulates IL-3-induced proliferation in bone marrow–derived mast cells. CD45 can also negatively regulate erythropoietin-stimulated bone marrow progenitors to produce erythroid colony-forming units (CFU), although others see no difference in CFU after IL-3 stimulation. Penninger's group showed that the Janus kinase, JAK2, was hyperphosphorylated in IFNα-stimulated CD45-deficient thymocytes, Jurkat T cells, and in IL-3-induced bone marrow–derived mast cells. This led the authors to conclude that CD45 is a JAK phosphatase (Penninger et al. 2001). Interestingly, Lyn-deficient mice have increased splenic CFUs in response to IL-3, GM-CSF, and CSF-1, illustrating that Lyn may also negatively regulate cytokine signaling (Hibbs and Harder 2006). Thus it is possible that CD45 may activate Lyn to downregulate cytokine signaling by phosphorylating inhibitory receptors. The receptors recruit inositol (SHIP-1) and tyrosine (SHP-1) phosphatases that inhibit cytokine signaling through the attenuation of the PI3K pathway or by dephosphorylation of Janus kinases, respectively.

In CD45-deficient macrophages, autophosphorylation and activation of Hck and Lyn kinases leads to dysregulated $\alpha_M\beta_2$-mediated adhesion. In CD45-deficient T cells, enhanced $\alpha_5\beta_1$ integrin- and CD44-mediated adhesion leading to enhanced signaling and sustained Src family kinase activity is observed. Thus the negative regulation of Src family kinases by CD45 also impacts leukocyte adhesion.

In dendritic cells, CD45 can modulate pro-inflammatory cytokine production in response to Toll-like receptor (TLR) stimulation. The effect depends on the type of TLR activated and may be explained by a differential effect of CD45 on the ▶ MyD88 dependent and independent TLR signaling pathways. Although Btk as well as Hck and Lyn have been implicated in TLR signaling, tyrosine phosphorylation is not considered a major component of the TLR signaling pathway and exactly how signals from these kinases are integrated into the TLR signaling pathway is not well understood. TLR signals can also be modulated by signals derived from other receptors such as integrins, cytokine, and inhibitory receptors, raising the possibility that CD45 may also impact TLR signaling by modulating this cross talk (Johnson and Cross 2009).

Summary

CD45 is a protein tyrosine phosphatase, conserved throughout the evolution of vertebrates. CD45 is leukocyte specific and dephosphorylates specific Src family kinases, namely, Lck and Fyn in T cells and Lyn and Hck in B cells and myeloid cells. As CD45 can both positively and negatively regulate Src family kinases, it is challenging to determine whether Src kinase substrates are also direct CD45 substrates or whether their phosphorylation state is indirectly determined by the effect of CD45 on the kinase. Although the lack of CD45 in leukocytes significantly affects the phosphorylation state and activity of these Src family kinases, it is not the only regulator of these kinases. Specific regulators of Src family kinases may operate in specific locations under specific circumstances or some may have overlapping roles. Understanding when and where CD45 regulates Src family kinases will also provide a better understanding of immune cell activation. One of the main functions of CD45 is to help maintain specific Src family kinases in a primed, dephosphorylated state, preventing both hyperactivation and inactivation, which both lead to severe immune dysfunction. Indeed, the loss of CD45 in humans and mice results in severe combined immunodeficiency, illustrating the importance of CD45 in leukocyte function.

References

Alexander D. The CD45 tyrosine phosphatase: a positive and negative regulator of immune cell function. Semin Immunol. 2000;12:349–59.

Dustin ML, Chakraborty AK, Shaw AS. Understanding the structure and function of the immunological synapse. Cold Spring Harb Perspect Biol. 2010;2:a002311.

Hermiston M, Xu Z, Weiss A. CD45: a critical regulator of signaling thresholds in immune cells. Annu Rev Immunol. 2003;21:107–37.

Hermiston ML, Tan AL, Gupta VA, Majeti R, Weiss A. The juxtamembrane wedge negatively regulates CD45 function in B cells. Immunity. 2005;23:635–47.

Hermiston M, Zikherman J, Zhu JW. CD45, CD148, and Lyp/Pep: critical phosphatases regulating Src family kinase signaling networks in immune cells. Immunol Rev. 2009;228:288–311.

Hibbs ML, Harder KW. The duplicitous nature of the Lyn tyrosine kinase in growth factor signaling. Growth Factors. 2006;24:137–49.

Huntington ND, Tarlinton DM. CD45: direct and indirect government of immune regulation. Immunol Lett. 2004;94:167–74.

Johnson P, Cross JL. Tyrosine phosphorylation in immune cells: direct and indirect effects on toll-like receptor-induced proinflammatory cytokine production. Crit Rev Immunol. 2009;29:347–67.

Johnson P, Felberg J. CD45: a key regulator of Lck and T cell activation. Mod Asp Immunobiol. 2000;1:156–9.

Johnson P, Maiti A, Ng DHW. CD45: A family of leukocyte specific cell surface glycoproteins. In: Herzenberg LA, Weir DM, Herzenberg LA, Blackwell C, editors. Weir's Handbook of experimental immunology. 5th ed. Malden: Blackwell; 1997. p. 62.1–.16.

Penninger JM, Irie-Sasaki J, Sasaki T, Oliveira-dos-Santos AJ. CD45: new jobs for an old acquaintance. Nat Immunol. 2001;2:389–96.

Saunders AE, Johnson P. Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. Cell Signal. 2010;22:339–48.

Scapini P, Pereira S, Zhang H, Lowell CA. Multiple roles of Lyn kinase in myeloid cell signaling and function. Immunol Rev. 2009;228:23–40.

Thomas ML. The leukocyte common antigen family. Annu Rev Immunol. 1989;7:339–69.

Tomlinson MG, Lin J, Weiss A. Lymphocytes with a complex: adapter proteins in antigen receptor signaling. Immunol Today. 2000;21:584–91.

Trowbridge IS, Thomas ML. CD45: An emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. Annu Rev Immunol. 1994;12:85–116.

van der Merwe PA, Dushek O. Mechanisms for T cell receptor triggering. Nat Rev Immunol. 2011;11:47–55.

Williams JC, Wierenga RK, Saraste M. Insights into Src kinase functions: structural comparisons. Trends Biochem Sci. 1998;23:179–84.

Xu Y, Harder KW, Huntington ND, Hibbs ML, Tarlinton DM. Lyn tyrosine kinase: accentuating the positive and the negative. Immunity. 2005;22:9–18.

CD45R

► CD45 (PTPRC)

CD47

David D. Roberts[1], David R. Soto-Pantoja[1] and Jeff S. Isenberg[2]
[1]Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
[2]Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh School of Medicine and the Vascular Medicine Institute of the University of Pittsburgh, Pittsburgh, PA, USA

Synonyms

Integrin-associated protein; Integrin-associated signal transducer; Ovarian cancer antigen OA3; Rh-related antigen

Historical Background

CD47 was first identified in 1987 as an antigen that is missing in red blood cells of patients with Rhesus (Rh)-null hemolytic anemia (see the following reviews for CD47 source references unless otherwise cited: (Brown and Frazier 2001; Frazier et al. 2010)). Loss of CD47 is not the primary cause of this disease, but CD47 closely associates with the Rh complex on red blood cells. Independently, the same protein was identified as an antigen, OA3, that is overexpressed in ovarian carcinoma and as a protein that co-purified with certain integrins and named integrin-associated protein (IAP). IAP and OA3 were shown in 1994 to be identical to CD47. CD47 is a type I integral membrane protein with an extracellular immunoglobulin variable (IgV)-like domain, five membrane-spanning segments, and a short alternatively spliced carboxy-terminal cytoplasmic tail. CD47 is widely expressed in higher vertebrates. In addition to its lateral association with certain integrins and the Rh complex, three extracellular ligands have been identified for CD47: thrombospondin-1 (TSP1), SIRPα (also known as ► Src homology 2 domain containing protein tyrosine phosphatase substrate-1 or SHPS1), and SIRPγ. TSP1 is a secreted protein and is the most studied ligand for regulating signaling through CD47. SIRPs are integral membrane proteins with important signaling functions, so CD47 is technically a counter-receptor for SIRPs. Most studies of SIRP-CD47 interactions regard CD47 as the ligand that regulates signal transduction through SIRPα (Barclay and Brown 2006), which will not be discussed here. However, there is growing evidence that this signaling is bidirectional (Sarfati et al. 2008), and more research is needed to clarify the role of SIRPs as signaling ligands for CD47.

Early studies of CD47 function as a signaling receptor focused on its role in integrin activation, and peptides derived from TSP1 that bind to CD47 were shown to enhance the activation of specific integrins. Ligation of CD47 by these peptides in different cell types also stimulated calcium influx and changes in cAMP levels, ► MAP kinase activities, and Giα activation. The central function of CD47 in cardiovascular physiology was first revealed by an enhanced angiogenic response to nitric oxide (NO) in muscle explants from TSP1 and CD47 null mice. TSP1 binding to CD47 inhibited NO-mediated activation of soluble guanylate cyclase (sGC) in endothelial cells. Subsequent studies extended this inhibition of NO signaling to other vascular cells, including smooth muscle cells, platelets, and T lymphocytes. Functional studies showed that this inhibitory signaling by TSP1 through CD47 acutely controls vascular tone, blood pressure, and platelet hemostasis (Isenberg et al. 2009). Subsequent biochemical studies expanded the intracellular targets of this inhibitory signaling to include cGMP-dependent protein kinase (cGK), endothelial nitric oxide synthase (eNOS, NOS3), and vascular endothelial growth factor receptor-2 (VEGFR2) (Bauer et al. 2010; Kaur et al. 2010). Thus, CD47 signaling is now recognized as a highly redundant inhibitor of the NO/cGMP signaling cascade in vascular cells.

A second major function that has emerged for CD47 is to control cell survival. Early studies showed that some CD47 antibodies can induce apoptosis of T cells. Studies in mice lacking CD47 have extended this more broadly to survival of ischemic stress, ischemia reperfusion, and radiation injury. Mechanistic studies show some involvement of the NO/cGMP cascade and regulation of SIRPα-mediated phagocytosis, but

additional pathways have been identified through which CD47 signaling controls mitochondrial-dependent cell death.

Proximal Targets of CD47

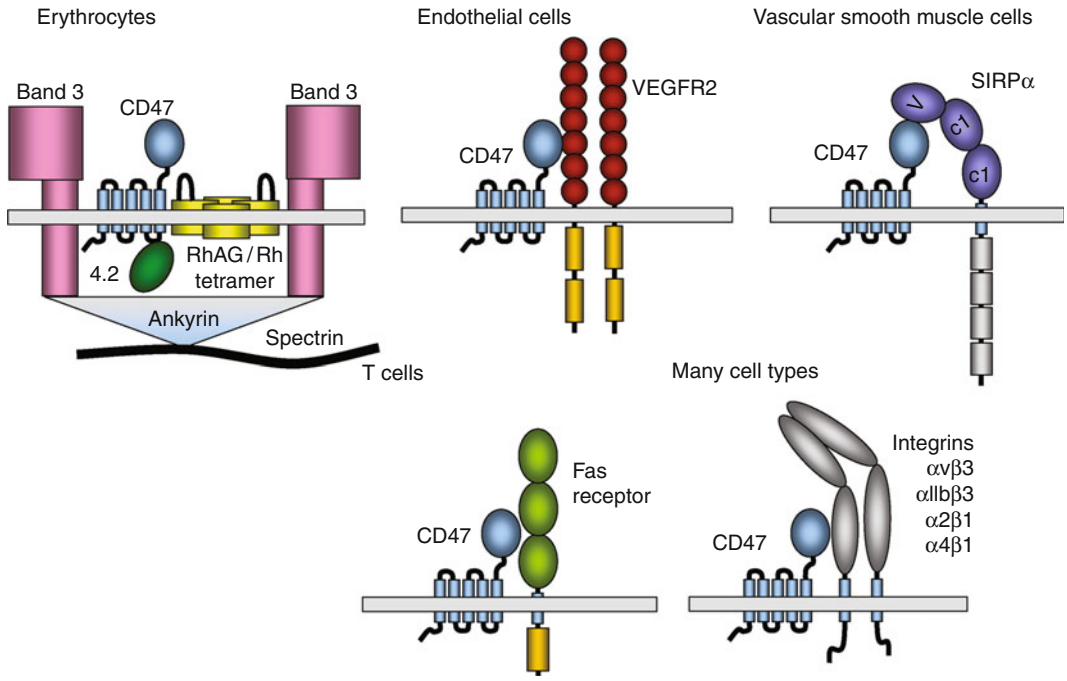
CD47 has a only a short C-terminal cytoplasmic tail, so signal transduction through CD47 is generally believed to be mediated by its interactions with other cellular proteins, either laterally in the plasma membrane or by recruiting cytoplasmic proteins to the complex of CD47 with its membrane binding partners (Fig. 1). The first direct interaction partner identified for CD47 was the integrin $\alpha_v\beta_3$ based on their co-purification. This is a lateral interaction between two integral membrane proteins, and the extracellular IgV domain of CD47 is necessary and sufficient to mediate integrin binding and to activate αvβ3 to bind its ligand vitronectin. Ligation of CD47 by TSP1 or certain TSP1 peptides leads to activation of this and some other integrins, including $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, and $\alpha_4\beta_1$. These integrins were also shown to interact with CD47 based on co-immunoprecipitation, although it is not clear that all of these integrins are direct binding partners of CD47. In red blood cells, the Rh complex replaces integrins as the major lateral binding partner of CD47. Although the composition of this complex is well known, the proximal interactions of CD47 within the complex remain unclear, and potential effects of CD47 ligands on functions of the Rh complex remain to be determined. SIRPα has been implicated as a lateral binding partner of CD47 in smooth muscle cells (Fig. 1), but the alternative possibility that the extracellular domain of SIRPα is shed and then binds to CD47 on the same cell has not been excluded. In T cells, CD47 laterally associates with Fas, and this association is increased by Fas ligation. Finally, colocalization, co-precipitation, and fluorescence resonance energy transfer studies recently identified VEGFR2 as a proximal binding partner of CD47 (Kaur et al. 2010). This interaction is specifically disrupted when VEGF and TSP1 bind simultaneously to their respective receptors.

Several cytoplasmic binding partners of CD47 have been identified. CD47 was shown to coprecipitate with some heterotrimeric G proteins. Ligation of CD47 leads to dissociation of Giα from Gβγ. The ability of cyclodextrin to dissociate Giα from CD47 suggests that this interaction is not direct. Yeast two-hybrid studies revealed that protein linking IAP and cytoskeleton-1 (PLIC-1, ubiquilin-1) and the related PLIC-2 (ubiquilin-2) are proximal cytoplasmic ligands of CD47. The latter may be a general negative regulator of G protein–coupled receptor endocytosis (N'Diaye et al. 2008). Another yeast 2-hybrid screen identified Bcl2 homology 3-only protein 19 kDa interacting protein-3 (BNIP3) as an interaction partner of CD47 (Lamy et al. 2003).

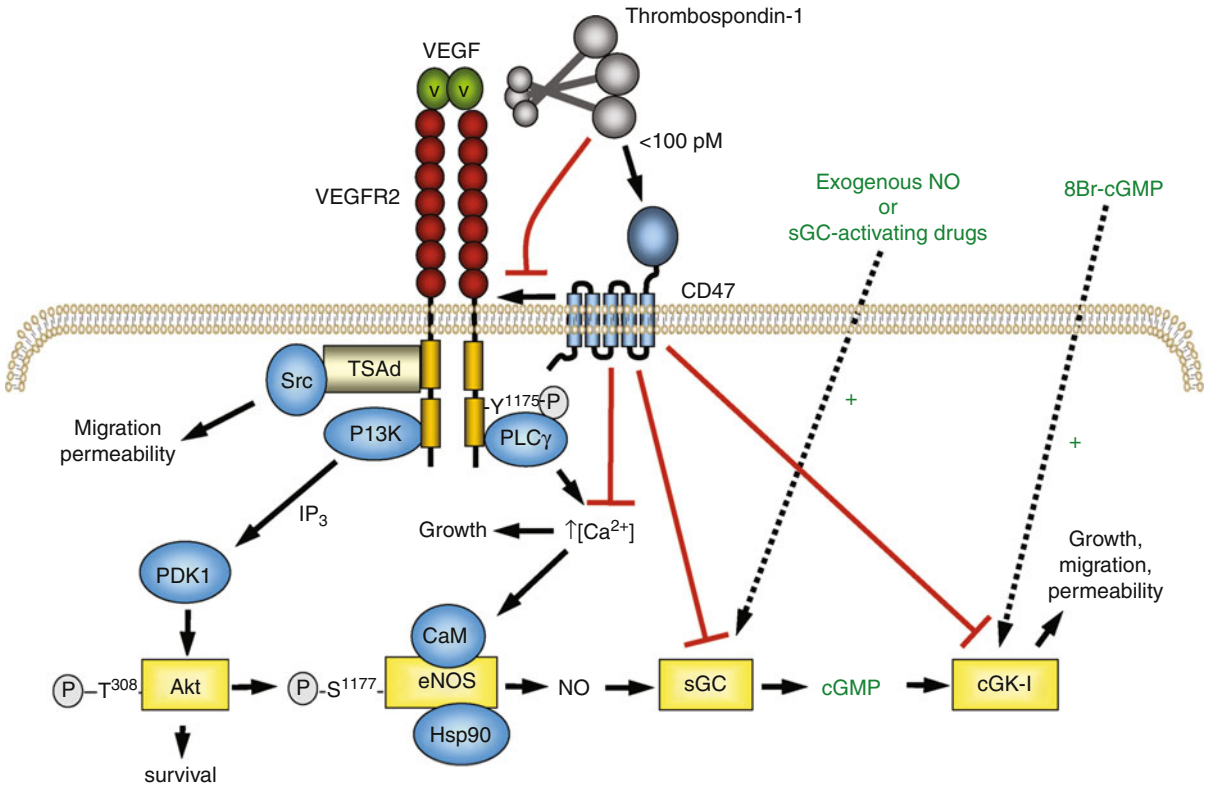
NO/cGMP Pathway

NO is a central regulator of cardiovascular function, hemostasis, immunity, and neural processing. Three NO synthases (eNOS, nNOS, and iNOS) convert L-arginine to L-citrulline and NO. NO freely traverses cell membranes to rapidly activate its intracellular targets and so can mediate intercellular signaling between endothelium and arterial smooth muscle. NO binds to heme proteins, including ▶ sGC, which is the primary sensor of physiological NO levels (Fig. 2). NO binding increases cGMP synthesis, which in turn activates cGMP-dependent protein kinases and cyclic nucleotide-gated ion channels to suppress inflammation and platelet aggregation, inhibit leukocyte recruitment, dilate blood vessels, and stimulate angiogenesis. A majority of cardiovascular diseases are associated with loss of endogenous NO production and decreased sensitivity to NO. Conversely, several cardiovascular therapeutics enhance NO signaling, including nitroglycerine and other nitrovasodilators and phosphodiesterase inhibitors. NO signaling can be modulated through control of NO production and phosphodiesterases that degrade cGMP. However, CD47 signaling mediates a more profound control of this pathway.

TSP1 binding to CD47 potently limits sGC activation by NO in endothelial and vascular smooth muscle cells and platelets. TSP1 also potently inhibits sGC activation by heme-dependent and nonheme-dependent chemical activators such as YC-1 and Riocuguat (BAY 63-2521) in vascular cells (Miller et al. 2010). Physiological circulating concentrations of TSP1 in blood (0.1–0.2 nM) are sufficient to inhibit sGC activation. This is consistent with the observation that endothelial and vascular smooth muscle cells and platelets from TSP1 and CD47 null mice have elevated basal levels of cGMP. In wounds and some chronic



CD47, Fig. 1 Membrane complexes containing CD47



CD47, Fig. 2 Thrombospondin-1 interaction with CD47 redundantly inhibits VEGFR2 and NO/cGMP signaling

disease states, much greater levels of TSP1 can be demonstrated in tissues and blood. These findings suggest that TSP1 functions in both health and disease to inhibit NO-stimulated activation of sGC. At higher concentrations (>10 nM), TSP1 also inhibits sGC activation via engaging the cell receptor CD36, but this inhibitory activity still requires the presence of CD47.

TSP1 limits activation of cGK in platelets stimulated by NO or by cell permeable analogs of cGMP (Fig. 2). Furthermore, assay of cGK activity using a defined peptide substrate in lysates of platelets pretreated with TSP1 showed inhibition of in vitro activation of the enzyme by cGMP. Therefore, cGK is a second direct target of CD47 signaling (Fig. 2).

eNOS is the major NO synthase expressed in vascular endothelium. eNOS is activated by hormonal or mechanical stimulation of the endothelium through modulation of intracellular calcium and phosphorylation of eNOS. TSP1 also inhibits eNOS activation in endothelial cells, thereby limiting endothelial-dependent arterial dilation (Bauer et al. 2010). This inhibitory activity on eNOS requires interaction with endothelial CD47. Importantly, CD47 null mice are hypotensive at rest compared to wild type controls, demonstrating a role for the cell surface receptor as a mediator of arterial tone and vascular resistance. In vivo circulating TSP1 was found to limit endothelial activation and associated decreases in blood pressure. Thus, physiologic levels of circulating TSP1 function as a hypertensive by limiting eNOS activation and endogenous NO production, and in this capacity TSP1 functions to support blood pressure. Targeting the TSP1-CD47 ligand receptor interaction can acutely lower blood pressure, suggesting possible therapeutic opportunities in the treatment of systemic hypertension.

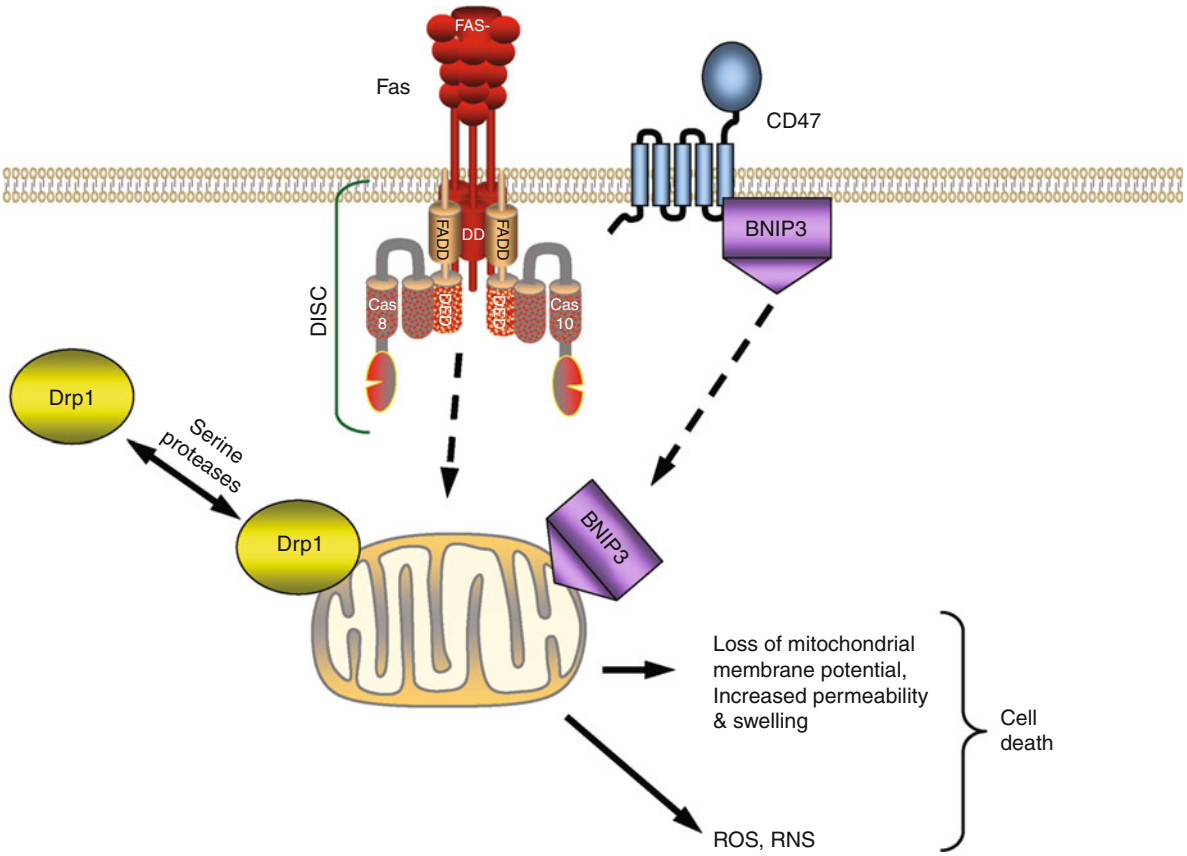
Phosphorylation of eNOS activates the enzyme and can be stimulated in endothelial cells by VEGF binding to its receptor VEGFR2 (Fig. 2). Recently, CD47 was found to interact with VEGFR2 in endothelial cells (Kaur et al. 2010). Ligation of CD47 in the presence of VEGF dissociates CD47 from VEGFR2 and prevents autophosphorylation of VEGFR2 at Tyr^{1175}. This is a necessary phosphorylation for downstream signaling. Therefore, CD47 ligation by TSP1 can globally inhibit signaling downstream of VEGFR2, which includes Src kinase and phospholipase Cγ pathways important for cell migration and proliferation in addition to blocking Akt and subsequent eNOS activation (Fig. 2).

Cell-Survival Signaling

Antibody ligation of certain epitopes on CD47 induces death of activated T cells independent of Fas and TNFR signaling (Sarfati et al. 2008). Cell death induced via CD47 does not involve DNA fragmentation, suggesting activation of CD45 and HLA class I signaling. Moreover, Jurkat T cells lacking CD47 are relatively resistant to Fas-mediated death but are efficiently killed by Fas ligand or anti-Fas antibody upon re-expression of CD47 (Sarfati et al. 2008). Cells lacking CD47 exhibit impaired downstream responses to Fas activation, including caspase activation, poly-(ADP-ribose) polymerase cleavage, cytochrome C release from mitochondria, loss of mitochondrial membrane potential, and DNA cleavage (Fig. 3). Treatment with an anti-Fas antibody induces an association of Fas with CD47. Antibody binding to CD47 enhances Fas-dependent apoptosis in Jurkat T cells and in primary mouse T cells. The expression of CD47 causes Fas to cluster and associate with the extracellular IgV of CD47, affecting Fas function upstream of caspase activation.

Cell death involving CD47 is not exclusive to T cells. Treatment with an immobilized monoclonal antibody or TSP1 induces apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cell clones from patients. As observed in T cells, CLL cell death occurred without nuclear features such as chromatin condensation and DNA fragmentation. However, cytoplamic changes, such as cell shrinkage, and decrease in mitochondrial membrane potential were observed (Sarfati et al. 2008). Moreover, electron microscopy revealed swelling of the mitochondria, indicating an increase in permeability of the mitochondrial membrane. This indicates that CD47 may regulate mitochondrial function to control cell survival. In activated T-cells, treatment with the CD47 antibody B6H12 disrupts mitochondrial transmembrane potential, followed by the release of reactive oxygen species (Roue et al. 2003). This dysfunction in the mitochondria is not accompanied by the release of cytocrome C or AIF, indicating an alternate mechanism for programmed cell death (Roue et al. 2003).

One possible mediator of CD47-dependent cell death is BNIP3, a member of the Bcl2 interacting proteins that translocates to the mitochondria to induce apoptosis (Lamy et al. 2003). BNIP3 contains a BH3 domain and a transmembrane domain that are associated with pro-apoptotic functions. BNIP3 interacts



CD47, Fig. 3 CD47 regulation of cell survival

with the transmembrane domains of CD47 (Lamy et al. 2003). Antisense suppression of BNIP3-inhibited, CD47-mediated induction of apoptosis, indicating that binding of antibody or peptides mimicking TSP1 induces BNIP3 to translocate from the plasma membrane to the mitochondria to execute cell death (Lamy et al. 2003). This regulation of mitochondrial function by CD47 implicates other forms of cell death.

CD47 ligation also regulates dynamin-related protein 1 (Drp1), a mayor regulator of type III programmed cell death (Bras et al. 2007). CD47 ligation induces Drp1 translocation from the cytosol to mitochondria, a process controlled by chymotrypsin-like serine proteases. Once in mitochondria, Drp1 blocks the mitochondrial electron transport chain, which dissipates the mitochondrial transmembrane potential, increases reactive oxygen species generation, and produces a drop in ATP levels (Bras et al. 2007). This process is reversed upon ligation of CD47 in conjunction of with Drp1 blockade, suggesting that this protein is essential for the CD47 regulation of mitochondrial function and induction of type III–programmed cell death.

CD47 also controls cell survival independent of controlling programmed cell death by allowing senescent or damaged cells to escape phagocytosis. CD47 is up-regulated in a number if cancers, including ovarian and bladder carcinomas and myeloid leukemia and in migrating hematopoietic progenitors. Increased CD47 expression correlates with an ability to evade phagocytosis by macrophages and cytolysis by NK cells (Jaiswal et al. 2010). Conversely, CD47 expression limits survival of many cells types under conditions of stress (Lamy et al. 2003; Isenberg et al. 2008b). In fixed ischemic stress generated by surgically creating myocutaneous flaps, tissue blood flow and perfusion were dramatically increased in CD47 null animals (Isenberg et al. 2008). These animals experienced 100% tissue survival, whereas WT flaps underwent close to 50% necrosis, indicating that CD47 enhances tissue necrosis during ischemic injury (Isenberg et al. 2008). Similar observations have been made for other

fixed ischemic insults or ischemia/reperfusion injuries in mice, rats, and pigs (Isenberg et al. 2008). In a model of cerebral focal ischemia, CD47 null mice show reduced brain damage after ischemic insult. These activities of CD47 to limit tissue survival are based on its inhibition of NO/cGMP-mediated maintenance of tissue perfusion and vascular remodeling. In addition, CD47 expression promotes neuroinflamation by promoting MMP-9 upregulation, neutrophil extravasation, allowing the progression of ischemic brain injury and death (Jin et al. 2009). Treatment with antibodies to CD47 or antisense morpholino oligonucleotides that suppress CD47 mRNA translation increase recovery from ischemic insults in several animal models (Isenberg et al. 2008). Together, these data suggest that therapeutic blockade of CD47 could be clinically useful to restore blood flow and improve tissue survival after ischemic injuries.

The cytoprotective effects of decreased CD47 expression extend to ionizing radiation (IR) injuries. IR acutely damages cellular DNA and other cellular macromolecules, eliciting stress responses that ultimately lead to cell death. CD47 null and TSP1 null mice show enhanced skin, muscle, and bone marrow preservation after radiation injury (Isenberg et al. 2008). Vascular cells cultured from the null mice showed enhanced cell-survival and proliferative capacity after IR injury, demonstrating that this acute radioprotection of vascular cells is cell-autonomous (Isenberg et al. 2008). In a follow-up study, treatment with a CD47-binding peptide or antisense suppression of CD47 protected human endothelial cells in vitro and protected soft tissue, bone marrow, and tumor-associated leukocytes in irradiated hindlimbs of mice (Maxhimer et al. 2009). Although NO is a known radioprotectant, enhanced NO signaling is not sufficient to account for radioprotection by CD47 blockade. Treatment with DETA/NO or a cell permeable cGMP analog does not result in a significant survival advantage to irradiated endothelial cells (Maxhimer et al. 2009). Conversely, inhibition of endogenous NO production does not prevent the protective effects of CD47 morpholino (Maxhimer et al. 2009). Therefore, the radioprotection observed by CD47 targeting occurs in a largely NO-independent manner.

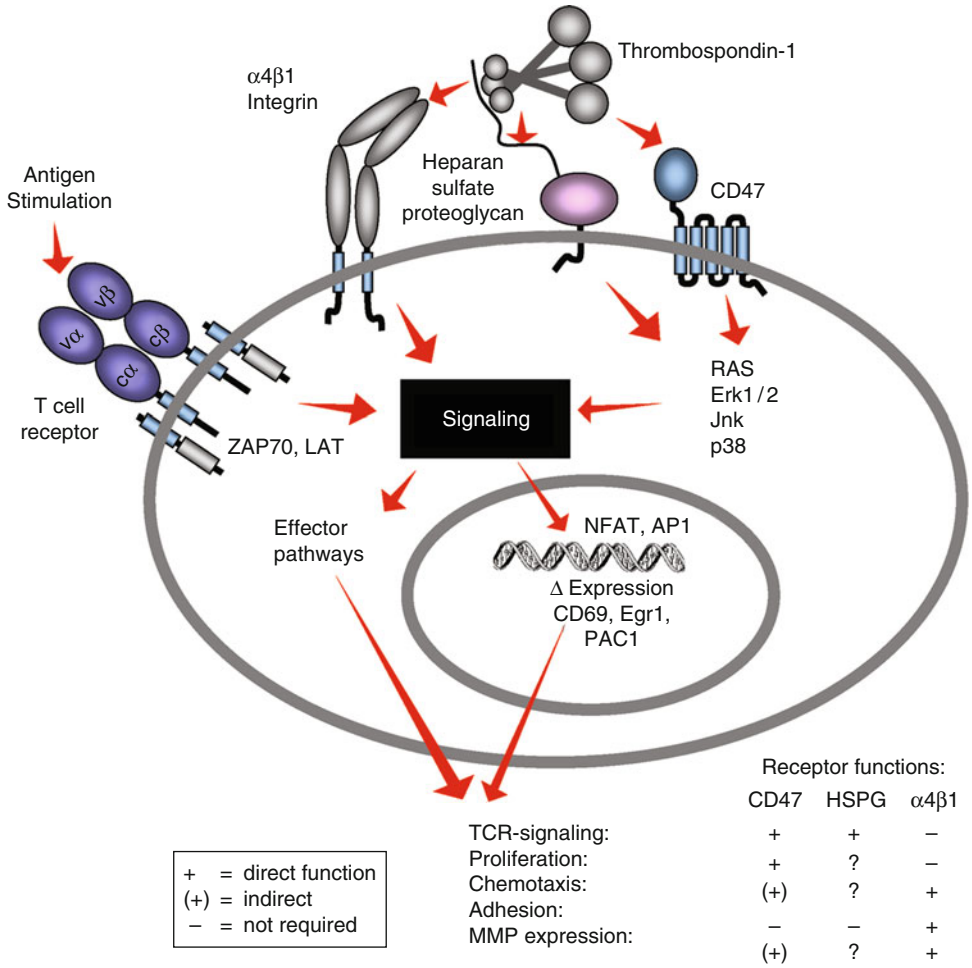
Because therapeutic irradiation is employed in treating a majority of cancer patients, the suppression of CD47 was studied in tumor-bearing mice in combination with radiation treatment. Treatment with IR alone resulted in the expected tumor regrowth delay. However, CD47 morpholino treatment followed by IR dramatically delayed tumor regrowth (Maxhimer et al. 2009). In vitro, CD47 blockade conferred minimal radioprotection to tumor cells, suggesting that suppression of CD47 enhances tumor growth delay after irradiation through enhancing host antitumor immunity (Maxhimer et al. 2009). Although details of this mechanism remain to be elucidated, it is clear that the expression of CD47 is a critical regulator of survival for several cell types. This regulation is important for normal tissue homeostasis, such as clearance of aging erythrocytes and limiting inflammation, but is also essential in the protection of tissues subjected to stress or pathologies that dysregulate programmed cell death.

T-Cell Signaling and Differentiation

Ligation of CD47 in T cells results in transient activation of Ras and the MAP kinases ERK, JNK, and p38 (Wilson et al. 1999). However, engaging CD47 in conjunction with TCR signaling globally inhibits T-cell activation (Sarfati et al. 2008). This occurs downstream of the TCR signaling molecules ▶ LAT and ▶ ZAP70 (Fig. 4). This inhibitory signaling limits induction of interleukin-2 and CD69. Because the CD47 ligand TSP1 also regulates T-cell behavior via integrin and proteoglycan receptors, T-cell responses integrate signals from these three receptors. Signaling through CD47 also drives T cells toward Treg differentiation. As discussed above, CD47 signaling can induce T-cell apoptosis, and CD47-deficient mice consequently exhibit T cell–mediated inflammatory responses due to maintaining excessive numbers of activated inflammatory cells (Lamy et al. 2007).

Other Second Messengers

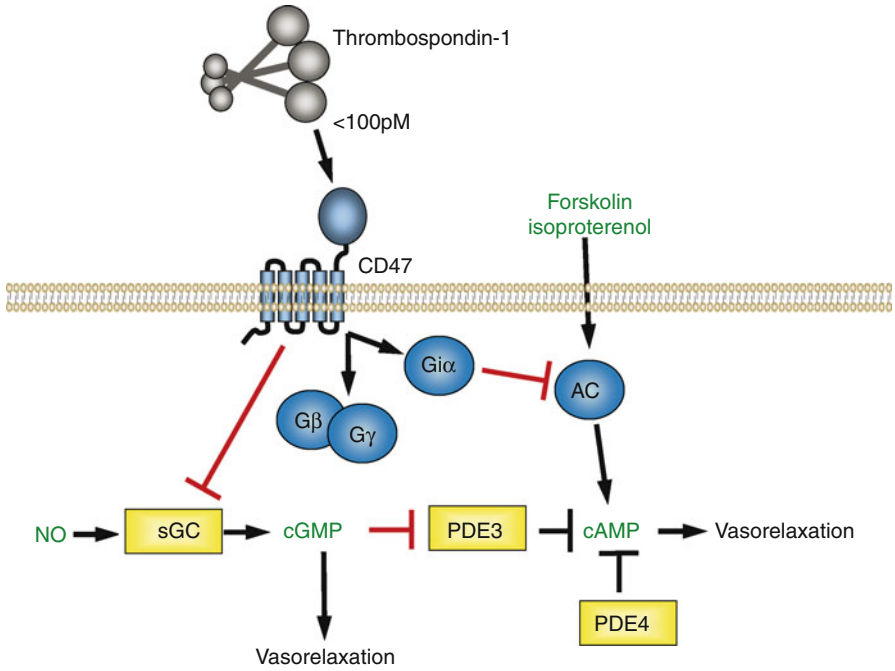
Cyclic AMP is a ubiquitous second messenger that promotes relaxation of airway and arterial smooth muscle cells, in part through lowering intracellular calcium. In platelets, cAMP inhibits aggregation. These activities of cAMP parallel those of cGMP, and the levels of both intracellular second messengers trend in the same direction. cAMP is produced by the enzyme ▶ adenylyl cyclase when activated by G protein–coupled receptor signaling. cAMP is



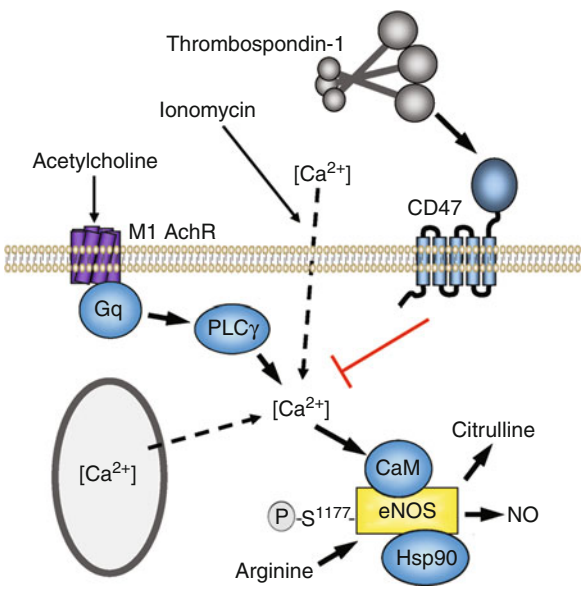
CD47, Fig. 4 CD47 regulation of T-cell signaling

hydrolyzed by several phosphodiesterases (PDEs), which are themselves controlled through cGMP-dependent cross talk. CD47 ligation alters cAMP levels in several cell types. In thyroid cells, a TSP1 peptide specific for CD47 maintains cAMP levels. Conversely, in T cells, vascular smooth muscle cells, and platelets CD47 ligation lowers cAMP levels, in part, through cGMP-mediated control of PDEs. cAMP levels are inherently higher in vascular cells, skeletal and cardiac muscle from TSP1, and CD47 null mice, suggesting that in the absence of ligand engagement CD47 modulates cellular cAMP (Isenberg et al. 2009). Ligation of CD47, though both NO-dependent and NO-independent signaling, inhibits vascular smooth muscle cell cAMP (Yao et al. 2010). In vascular smooth muscle cells, the TSP1-CD47 axis inhibits direct activation of adenylyl cyclase and, in this manner, blocks cAMP-stimulated vasorelaxation. These latest findings support a role for CD47 in limiting both cGMP and cAMP driven vasorelaxation (Fig. 5).

Calcium is another critical regulator in intracellular signal transduction. Fluxes in intracellular calcium regulate cell contraction, migration, and growth. Through its G protein–coupled receptor-like activity and/or integrin interactions, CD47 has been associated with changes in calcium in neurons, T cells, mast cells, and both melanoma and prostate tumor cells (Frazier et al. 2010). In the cardiovascular system, endogenous NO production by eNOS requires calcium, and fibronectin-stimulated increases in endothelial calcium can



CD47, Fig. 5 Thrombospondin-1 interaction with CD47 regulates crosstalk between cGMP and cAMP in vascular smooth muscle



CD47, Fig. 6 Thrombospondin-1, via CD47, inhibits agonist driven calcium flux and eNOS activation

be blocked by a CD47 antibody. Both ionomycin and the physiologic activator acetylcholine increase eNOS-stimulated production of NO by increasing cytoplasmic calcium levels. TSP1 and a CD47-specific recombinant domain of TSP1 inhibit activator-based calcium fluxes and eNOS activation in endothelial cells (Fig. 6) (Bauer et al. 2010).

Summary

CD47 is a ubiquitously expressed transmembrane receptor for two members of the SIRP/SHPS family and TSP1. Signaling between CD47 and SIRPα is bidirectional, but signaling through CD47 in response to SIRPα ligation remains poorly characterized. TSP1 binding to CD47 alters its lateral interactions with several integrins, VEGFR2, Fas, and possibly SIRPα. This results in altered signaling through each of these receptors to modulate cell adhesion, survival, contractility, proliferation, and motility. Downstream signaling through CD47 controls levels of the second messengers calcium, cAMP, and cGMP in a cell-specific manner. In vascular cells, NO is a major physiological target of CD47 signaling. CD47 signaling redundantly controls both synthesis and effector pathways downstream of NO, enabling it to control both intracellular and intercellular signaling between vascular cells. This signaling has physiological roles in angiogenesis, cardiovascular dynamics, and hemostasis. Antagonism of NO signaling by CD47 also limits tissue responses to ischemic and radiation stress.

References

Barclay AN, Brown MH. The SIRP family of receptors and immune regulation. Nat Rev. 2006;6:457–64.

Bauer EM, Qin Y, Miller TW, Bandle RW, Csanyi G, Pagano PJ, et al. Thrombospondin-1 supports blood pressure by limiting eNOS activation and endothelial-dependent vasorelaxation. Cardiovasc Res. 2010;88:471–81.

Bras M, Yuste VJ, Roue G, Barbier S, Sancho P, Virely C, et al. Drp1 mediates caspase-independent type III cell death in normal and leukemic cells. Mol Cell Biol. 2007;27:7073–88.

Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. Trends Cell Biol. 2001;11:130–5.

Frazier WA, Isenberg JS, Kaur S, Roberts DD. CD47. Nature signaling gateway 2010. http://www.signaling-gateway.org/molecule/query?afcsid=A002870. Accessed 16 Feb 2010.

Isenberg JS, Frazier WA, Krishna MC, Wink DA, Roberts DD. Enhancing cardiovascular dynamics by inhibition of thrombospondin-1/CD47 signaling. Curr Drug Targets. 2008a;9:833–41.

Isenberg JS, Maxhimer JB, Hyodo F, Pendrak ML, Ridnour LA, DeGraff WG, et al. Thrombospondin-1 and CD47 limit cell and tissue survival of radiation injury. Am J Pathol. 2008b;173:1100–12.

Isenberg JS, Qin Y, Maxhimer JB, Sipes JM, Despres D, Schnermann J, et al. Thrombospondin-1 and CD47 regulate blood pressure and cardiac responses to vasoactive stress. Matrix Biol. 2009;28:110–9.

Jaiswal S, Chao MP, Majeti R, Weissman IL. Macrophages as mediators of tumor immunosurveillance. Trends Immunol. 2010;31:212–9.

Jin G, Tsuji K, Xing C, Yang YG, Wang X, Lo EH. CD47 gene knockout protects against transient focal cerebral ischemia in mice. Exp Neurol. 2009;217:165–70.

Kaur S, Martin-Manso G, Pendrak ML, Garfield SH, Isenberg JS, Roberts DD. Thrombospondin-1 inhibits vascular endothelial growth factor receptor-2 signaling by disrupting its association with CD47. J Biol Chem. 2010. doi:10.1074/jbc.M110.172304.

Lamy L, Ticchioni M, Rouquette-Jazdanian AK, Samson M, Deckert M, Greenberg AH, et al. CD47 and the 19 kDa interacting protein-3 (BNIP3) in T cell apoptosis. J Biol Chem. 2003;278:23915–21.

Lamy L, Foussat A, Brown EJ, Bornstein P, Ticchioni M, Bernard A. Interactions between CD47 and thrombospondin reduce inflammation. J Immunol. 2007;178:5930–9.

Maxhimer JB, Soto-Pantoja DR, Ridnour LA, Shih HB, DeGraff WG, Tsokos M, et al. Radioprotection in normal tissue and delayed tumor growth by blockade of CD47 signaling. Sci Transl Med. 2009;1:3ra7.

Miller TW, Isenberg JS, Roberts DD. Thrombospondin-1 is an inhibitor of pharmacological activation of soluble guanylate cyclase. Br J Pharmacol. 2010;159:1542–7.

N'Diaye EN, Hanyaloglu AC, Kajihara KK, Puthenveedu MA, Wu P, von Zastrow M, et al. The ubiquitin-like protein PLIC-2 is a negative regulator of G protein-coupled receptor endocytosis. Mol Biol Cell. 2008;19:1252–60.

Roue G, Bitton N, Yuste VJ, Montange T, Rubio M, Dessauge F, et al. Mitochondrial dysfunction in CD47-mediated caspase-independent cell death: ROS production in the absence of cytochrome c and AIF release. Biochimie. 2003;85:741–6.

Sarfati M, Fortin G, Raymond M, Susin S. CD47 in the immune response: role of thrombospondin and SIRP-alpha reverse signaling. Curr Drug Targets. 2008;9:842–50.

Wilson KE, Li Z, Kara M, Gardner KL, Roberts DD. β1 integrin- and proteoglycan-mediated stimulation of T lymphoma cell adhesion and mitogen-activated protein kinase signaling by thrombospondin-1 and thrombospondin-1 peptides. J Immunol. 1999;163:3621–8.

Yao M, Roberts DD, Isenberg JS. Thrombospondin-1 inhibition of vascular smooth muscle cell responses occurs via modulation of both cAMP and cGMP. Pharmacol Res. 2010. doi:10.1016/j.phrs.2010.10.014.

CD49a

► Integrin α1 (ITGA1)

CD49b

► Integrin α2 (ITGA2)

CD49d

► Integrin Alpha 4 (Itga 4)

CD51

► Integrin Alpha V (ITGAV)

CD53

Pedro A. Lazo[1] and Ramiro Barcia[2]
[1]Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain
[2]Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Santiago de Compostela, Lugo, Spain

Synonyms

CD53 antigen; Cell surface glycoprotein CD53; Leukocyte surface antigen CD53; MOX44; OX44; Ox-44; Tetraspanin-25; Tspan25l; Tspan-25

Historical Background

CD53 is a member of the tetraspanin family of hydrophobic membrane-spanning proteins. Tetraspanins form microdomains on the cell surface that can interact with many different proteins implicated in signaling. CD53 has no known extracellular ligand. The specific function of CD53 has not yet been defined, but CD53 has been shown to modulate cell adhesion, migration, cell proliferation, and survival. Ligation of CD53 with antibodies protects cells from apoptosis; this effect is mediated by phosphorylation and activation of Akt, increased levels of Bcl-Xl, decreasing the amount of Bax, and reducing caspase activation. In mesangial cells, CD53 ligation stimulates the induction of DNA synthesis via the MEK-Erk pathway. CD53 ligation induces calcium mobilization, activation of PKCα, and expression of the inducible nitric oxide synthase genes resulting in increased nitric oxide production. But all these effects may be a consequence of the tetraspanin complex on which CD53 is integrated in specific cell types (Yunta and Lazo 2003b).

Protein Structure

The CD53 protein belongs to the tetraspanin family (Horejsi and Vlcek 1991). Tetraspanins are highly hydrophobic and have four transmembrane domains, but a specific function for these proteins has not yet been identified (Hemler 2003, 2005; Maecker et al. 1997). Tetraspanins can organize into membrane microdomains, known as the tetraspanin web, that interact with many different proteins on the cell surface (Boucheix and Rubinstein 2001; Yunta and Lazo 2003b). Thus, tetraspanin proteins may function as facilitators of other cellular functions (Hemler 2005). CD53 has two extracellular loops, a short (EC1) and a long one (EC2), that are maintained by disulfide bonds (Fig. 1). The structure of the EC2 determines the type of tetraspanin protein (Seigneuret et al. 2001).

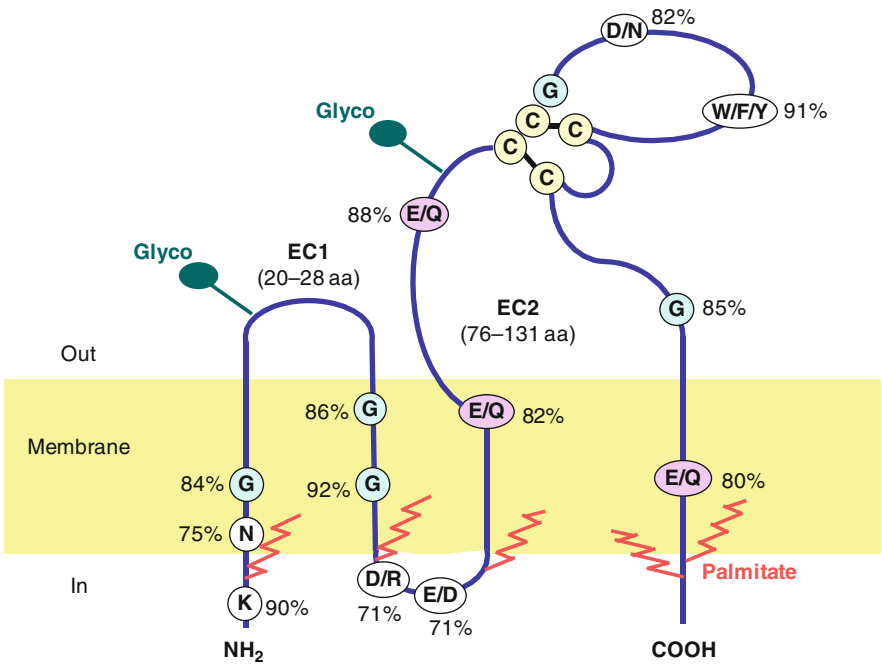
Protein Function

Most of the functional data associated with CD53 was obtained by CD53 ligation with antibodies. Ligation induces homotypic adhesion in lymphoma cell lines (Cao et al. 1997; Lazo et al. 1997; Yunta and Lazo 2003a). CD53 ligation induces phosphorylation of Akt, increased Bcl-Xl level, reduction of Bax, and activation of caspases, thus protecting cells against apoptosis induced by serum deprivation (Yunta and Lazo 2003a).

CD53 ligation induces a burst of DNA synthesis and initiation of the cell cycle through activation of the MEK-Erk pathway in mesangial cells. However, additional stimuli are required for completion of the cell cycle (Yunta et al. 2003); in the U937 monocytic cell line, CD53 ligation blocked proliferation (Stonehouse et al. 1999). In macrophages ligation of CD53 induces nitric oxide production through calcium mobilization, activation of PKCα, and expression of the inducible nitric oxide (iNOS) synthase gene (Bosca and Lazo 1994). Calcium mobilization has also been detected in human monocytes and B cells (Olweus et al. 1993). CD53 appears to play an adaptor or scaffold protein that influences adhesion, proliferation, and survival. Additional studies are required to elucidate the precise role for CD53 in these and other cellular processes.

Tetraspanin Web and CD53 Protein Interactions in Plasma Membranes

CD53 interacts with other tetraspanins forming the tetraspanin web (Hemler 2005; Yunta and Lazo 2003b). The web composition may vary depending on the pattern of tetraspanin expression in particular cell types. No ligand of any tetraspanin is known, and the web is likely to function as a facilitator of other signals. Tetraspanins interact with several types of proteins, although it is not clear which component of the web is modulating cell signaling. The same types of molecules are found in these complexes, independently of the specific tetraspanin studied, complicating efforts to understand the precise identity and stoichiometry of components within the tetraspanin web. As many of the interactions described below were identified by co-immunoprecipitation, it is not necessarily clear with which components CD53 interacts directly. Thus, unless otherwise noted, these interactions probably take place within the tetraspanin web, but not necessarily with CD53. In the tetraspanin web in human cells, CD53 interacts with other tetraspanins CD9, CD37, ▶ CD81, CD82, and CD151 determined by use of different detergents



CD53, Fig. 1 Structure of human CD53 antigen. Extracellular loops (EC1 and EC2). Cysteine disulfide bonds in EC2, conserved residues among tetraspanins (*circles*), and glycosylation and palmitoylation sites are indicated (Lazo 2007)

(Angelisova et al. 1990; Hemler 2003). Palmitoylation contributes to the interaction with CD81 and CD53 (Charrin et al. 2002). CD53 interacts with class II antigens of the major histocompatibility complex (MHC) or human leukocyte antigen (HLA) systems in murine and human cells (Angelisova et al. 1994; Damjanovich et al. 1998; Szollosi et al. 1996); these molecules are within 2–10 nm range since fluorescence could be transmitted between HLA (MHC class I molecules and at least one DR, DQ), CD20 and CD53, and CD81 and CD82. The simultaneous energy transfer from CD20, CD53, CD81, and CD82 to DR suggests that all these molecules are in a single complex (Szollosi et al. 1996). Several associated molecules were identified by atomic force microscopy. Nonrandom co-localization of MHC class I and II; intercellular adhesion molecule-1 (ICAM-1); the T-cell receptor (TCR)–► CD3–CD4 complex; the CD81, CD82, and CD53 tetraspanins; and the α, β and γ subunits of the IL-2 receptor was detected in a lymphoma cell line (Damjanovich et al. 1998).

CD53 directly interacts in these microdomains with several integrins (ITG) containing the β_1 chain. The presence or absence of particular integrin α chains varies depending on cell type, but integrin $\alpha_4\beta_1$ coprecipitated with CD53 in cell lines (Mannion et al. 1996).

Tetraspanin Web and CD53 Interactions with Intracellular Signaling

Several intracellular signaling molecules interact with tetraspanins. PKC can be associated with CD9, CD53, CD81, CD82, and CD151. Although formation and maintenance of tetraspanin-PKC complexes is not dependent on integrins, tetraspanins can act as linker molecules that bring PKC into proximity with specific integrins. The specificity for PKC association probably resides in the cytoplasmic tails or the first two transmembrane domains of tetraspanins, and CD53 has been found to interact with PKCα (Zhang et al. 2001). CD53 crosslinking induces effects mediated by PKCα, such as nitric oxide production by the inducible nitric oxide synthase (iNOS) (Bosca and Lazo 1994), and homotypic cell adhesion in a B cell lymphoma (Lazo et al. 1997). Also CD53, CD21, CD19, CD81, and CD82 interact with γ-glutamyl transpeptidase (GGT), a membrane protein involved in recycling extracellular glutathione and regulation of intracellular redox potential (Nichols et al. 1998). This might be relevant for cell sensitivity to radiation mediated, which is affected by the redox state. CD53 is overexpressed 20–50 fold in murine B cells that are resistant to radiotherapy and apoptosis (Voehringer et al. 2000). CD53 coprecipitates with GGT in lymphocytes from patients with rheumatoid arthritis that are

also resistant to apoptosis (Pedersen-Lane et al. 2007). CD53 can protect cells from apoptosis by inducing AKT survival signals (Yunta and Lazo 2003a). CD53, CD9, and CD81 have been detected in budding HIV-1 particles in infected macrophages (Deneka et al. 2007).

CD53 Gene Expression and Regulation

Human CD53 is restricted to normal B cells during B-cell development (Barrena et al. 2005b). The promoter of the human *CD53* gene contains two sites that are recognized by Sp1 and PU.1 transcription factors. These sites are essential for its expression in different cell types, but play different roles, in some are activators and in other repressors (Hernandez-Torres et al. 2001). Other transcription factors regulating CD53 expression are the B-cell factor (EBF-1), which is required for B-cell differentiation (Mansson et al. 2007); and 1,25-Dihydroxyvitamin D3 (1,25-(OH)2 D3), which by its receptor, modulates the expression of CD53 and induces monocytic differentiation of HL-60 leukemic cell (Brackman et al. 1995).

CD53 Protein Level Regulation and Pathology

Neutrophil activation with ▶ TNF-α, PDGF, N-formyl-methionyl-leucyl-phenylalanine, or phorbol esters downregulated surface expression of CD53; which involves a proteolytic mechanism triggered by PKC and leads to a complete loss of surface antigen, but the effect is transitory lasting a few hours (Mollinedo et al. 1998). CD53 surface expression was also downregulated in leukocytes from patients with myelodysplastic syndromes (MDSs), and correlates with the activation status of these cells (Kyriakou et al. 2001).

In humans, the only phenotype reported associated to CD53 loss corresponds to a family with three affected members in which CD53 is not detected in B cells, T cells, or neutrophils, and present a syndrome characterized by recurrent bacterial and viral infections of heterogeneous origin, which resembles a leukocyte adhesion defect (Mollinedo et al. 1997).

CD53, along with CD81, CD63, SAS, and CD82, were expressed at high levels in over 90% of tested Burkitt lymphoma (BL) cell lines. There are no major differences in tetraspanin expression pattern among sporadic or endemic tumors, type of translocation, or Epstein-Barr virus status, suggesting that the cell of origin for these tumors is the same (Ferrer et al. 1998). Neutrophils aging in vitro resulted in a significant increase of CD53 and CD63 expression (Beinert et al. 2000).

In human B-cell malignancies CD53 is expressed at very high levels in malignancies that are well differentiated, like multiple myeloma, while its expression is much lower in less differentiated malignancies, such as diffuse large B-cell lymphomas or follicular lymphomas (Barrena et al. 2005b). The expression of CD53 in combination with CD81 can be used to discriminate two different tumors in a single patient. Combined expression of CD53 and CD81 distinguishes B-chronic lymphocytic leukemia (B-CLL) from splenic marginal zone B-cell lymphoma (SMZL) in the same patient (Barrena et al. 2005a).

Summary

CD53 is one of the least characterized members of the tetraspanin family. CD53 is integrated in the tetraspanin web, and sends intracellular signals, directly or indirectly via PKC, AKT, and γ-glutamyl transpeptidase (GGT). Its expression can affect cell survival, resistance to apoptosis, and radiation sensitivity. In B-cell development CD53 is a marker of mature B cells. In human B-cell malignancies, the pattern of tetraspanin expression identifies the developmental stage of the corresponding tumors. The pattern of tetraspanins can be used to differentiate B-cell malignancies in an individual patient with several lymphoid tumors.

References

Angelisova P, Vlcek C, Stefanova I, Lipoldova M, Horejsi V. The human leucocyte surface antigen CD53 is a protein structurally similar to the CD37 and MRC OX-44 antigens. Immunogenetics. 1990;32:281–5.

Angelisova P, Hilgert I, Horejsi V. Association of four antigens of the tetraspans family (CD37, CD53, TAPA-1, and R2/C33) with MHC class II glycoproteins. Immunogenetics. 1994;39:249–56.

Barrena S, Almeida J, Yunta M, Lopez A, Diaz-Mediavilla J, Orfao A, Lazo PA. Discrimination of biclonal B-cell chronic lymphoproliferative neoplasias by tetraspanin antigen expression. Leukemia. 2005a;19:1708–9.

Barrena S, Almeida J, Yunta M, Lopez A, Fernandez-Mosteirin N, Giralt M, Romero M, Perdiguer L, Delgado M, Orfao A, et al. Aberrant expression of tetraspanin molecules in B-cell chronic lymphoproliferative disorders and its correlation with normal B-cell maturation. Leukemia. 2005b;19:1376–83.

Beinert T, Munzing S, Possinger K, Krombach F. Increased expression of the tetraspanins CD53 and CD63 on apoptotic human neutrophils. J Leukoc Biol. 2000;67:369–73.

Bosca L, Lazo PA. Induction of nitric oxide release by MRC OX-44 (anti-CD53) through a protein kinase C-dependent pathway in rat macrophages. J Exp Med. 1994;179:1119–26.

Boucheix C, Rubinstein E. Tetraspanins. Cell Mol Life Sci. 2001;58:1189–205.

Brackman D, Lund-Johansen F, Aarskog D. Expression of leukocyte differentiation antigens during the differentiation of HL-60 cells induced by 1,25-dihydroxyvitamin D3: comparison with the maturation of normal monocytic and granulocytic bone marrow cells. J Leukoc Biol. 1995;58:547–55.

Cao L, Yoshino T, Kawasaki N, Sakuma I, Takahashi K, Akagi T. Anti-CD53 monoclonal antibody induced LFA-1/ICAM-1-dependent and -independent lymphocyte homotypic cell aggregation. Immunobiology. 1997;197:70–81.

Charrin S, Manie S, Oualid M, Billard M, Boucheix C, Rubinstein E. Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation. FEBS Lett. 2002;516:139–44.

Damjanovich S, Matko J, Matyus L, Szabo Jr G, Szollosi J, Pieri JC, Farkas T, Gaspar Jr R. Supramolecular receptor structures in the plasma membrane of lymphocytes revealed by flow cytometric energy transfer, scanning force- and transmission electron-microscopic analyses. Cytometry. 1998;33:225–33.

Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. J Cell Biol. 2007;177:329–41.

Ferrer M, Yunta M, Lazo PA. Pattern of expression of tetraspanin antigen genes in Burkitt lymphoma cell lines. Clin Exp Immunol. 1998;113:346–52.

Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. Annu Rev Cell Dev Biol. 2003;19:397–422.

Hemler ME. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol. 2005;6:801–11.

Hernandez-Torres J, Yunta M, Lazo PA. Differential cooperation between regulatory sequences required for human CD53 gene expression. J Biol Chem. 2001;276:35405–13.

Horejsi V, Vlcek C. Novel structurally distinct family of leucocyte surface glycoproteins including CD9, CD37, CD53 and CD63. FEBS Lett. 1991;288:1–4.

Kyriakou D, Alexandrakis MG, Kyriakou ES, Liapi D, Kourelis TV, Mavromanolakis M, Vlachonikolis I, Eliakis P. Reduced CD43 expression on the neutrophils of MDS patients correlates with an activated phenotype of these cells. Int J Hematol. 2001;73:483–91.

Lazo PA. Functional implications of tetraspanin proteins in cancer biology. Cancer Sci. 2007;98:1666–77.

Lazo PA, Cuevas L, Gutierrez del Arroyo A, Orue E. Ligation of CD53/OX44, a tetraspan antigen, induces homotypic adhesion mediated by specific cell-cell interactions. Cell Immunol. 1997;178:132–40.

Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. FASEB J. 1997;11:428–42.

Mannion BA, Berditchevski F, Kraeft SK, Chen LB, Hemler ME. Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associated with integrin alpha 4 beta 1 (CD49d/CD29). J Immunol. 1996;157:2039–47.

Mansson R, Lagergren A, Hansson F, Smith E, Sigvardsson M. The CD53 and CEACAM-1 genes are genetic targets for early B cell factor. Eur J Immunol. 2007;37:1365–76.

Mollinedo F, Fontan G, Barasoain I, Lazo PA. Recurrent infectious diseases in human CD53 deficiency. Clin Diagn Lab Immunol. 1997;4:229–31.

Mollinedo F, Martin-Martin B, Gajate C, Lazo PA. Physiological activation of human neutrophils down-regulates CD53 cell surface antigen. J Leukoc Biol. 1998;63:699–706.

Nichols TC, Guthridge JM, Karp DR, Molina H, Fletcher DR, Holers VM. Gamma-glutamyl transpeptidase, an ectoenzyme regulator of intracellular redox potential, is a component of TM4 signal transduction complexes. Eur J Immunol. 1998;28:4123–9.

Olweus J, Lund-Johansen F, Horejsi V. CD53, a protein with four membrane-spanning domains, mediates signal transduction in human monocytes and B cells. J Immunol. 1993;151:707–16.

Pedersen-Lane JH, Zurier RB, Lawrence DA. Analysis of the thiol status of peripheral blood leukocytes in rheumatoid arthritis patients. J Leukoc Biol. 2007;81:934–41.

Seigneuret M, Delaguillaumie A, Lagaudriere-Gesbert C, Conjeaud H. Structure of the tetraspanin main extracellular domain. A partially conserved fold with a structurally variable domain insertion. J Biol Chem. 2001;276:40055–64.

Stonehouse TJ, Woodhead VE, Herridge PS, Ashrafian H, George M, Chain BM, Katz DR. Molecular characterization of U937-dependent T-cell co-stimulation. Immunology. 1999;96:35–47.

Szollosi J, Horejsi V, Bene L, Angelisova P, Damjanovich S. Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. J Immunol. 1996;157:2939–46.

Voehringer DW, Hirschberg DL, Xiao J, Lu Q, Roederer M, Lock CB, Herzenberg LA, Steinman L. Gene microarray identification of redox and mitochondrial elements that control resistance or sensitivity to apoptosis. Proc Natl Acad Sci USA. 2000;97:2680–5.

Yunta M, Lazo PA. Apoptosis protection and survival signal by the CD53 tetraspanin antigen. Oncogene. 2003a;22:1219–24.

Yunta M, Lazo PA. Tetraspanin proteins as organisers of membrane microdomains and signalling complexes. Cell Signal. 2003b;15:559–64.

Yunta M, Rodriguez-Barbero A, Arevalo MA, Lopez-Novoa JM, Lazo PA. Induction of DNA synthesis by ligation of the CD53 tetraspanin antigen in primary cultures of mesangial cells. Kidney Int. 2003;63:534–42.

Zhang XA, Bontrager AL, Hemler ME. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. J Biol Chem. 2001;276:25005–13.

CD53 Antigen

▶ CD53

CD56

▶ NCAM1

CD66

▶ CEACAMs

CD72

Hsin-Jung Wu[1], Natarajan Muthusamy[2] and Subbarao Bondada[3]
[1]Department of Immunobiology, University of Arizona, Tuscon, AZ, USA
[2]Department of Hematology, Ohio State Comprehensive Cancer Center, Ohio State University, Columbus, OH, USA
[3]Department of Microbiology, Immunology and Molecular Genetics, Markey Cancer Center, University of Kentucky, Lexington, KY, USA

Synonyms

CD72 antigen; Ly-19; Ly-32; Lyb-2; Ly-m19

CD72, a Historical Background

B lymphocytes express several surface molecules other than the B cell receptors (BCR) that function as markers of differentiation as well as molecules that can fine-tune BCR signaling. Current research has identified a function for most antigens that were previously classified as markers of lymphocyte differentiation. ▶ CD72 is one such molecule that was originally discovered as a marker of B cell differentiation using conventional serological and classical genetic techniques. Subsequently generation of monoclonal antibodies helped its definition as a molecule that can affect B cell growth and differentiation on its own or in the context of BCR signaling. Initially Sato and Boyse described an activity in sera from C3H.I mice immunized with 1.29 ascites tumor cells that reacted only with a subset of spleen cells after it was absorbed to remove an unknown reactivity and subsequently the cell type in the spleen was identified as the B cell (Sato and Boyse 1976). Early studies identified ▶ CD72 as an antigen expressed on early pre-B and mature B cells but not on plasma cells. This serum was designated as anti-Lyb2 and the antigen identified by the antiserum was given the name Lyb 2.1. Genetic studies demonstrated that Lyb2 was specified by a gene on chromosome 4 that was closely linked to the *Ly-19* and *Ly-32* loci. Later biochemical studies established that antibodies to Lyb2, Ly19, and Ly32 were all recognizing the same molecule (Wu and Bondada 2002). The Lyb 2.1 allele was expressed in a subset of mouse strains such as C57Br, C58J, C58L, CBA/J, and DBA/2 mice as defined by reactivity with this antiserum and the allele in BL/6 mice as Lyb 2.2 (Sato and Boyse 1976). Two monoclonal antibodies, 9-6-1 and 10.1.D2 were generated by Yakura et al. and Subbarao et al., respectively, which allowed an analysis of functional properties of Lyb 2 and recognition that it was not simply a differentiation marker but affected B cell function (Wu and Bondada 2002). These and other monoclonal antibodies demonstrated that there are four alleles for the *Lyb2* locus in the mouse (Wu and Bondada 2002). After molecular cloning of the gene for Lyb2 and the characterization of the human analogue, the name Lyb2 was changed to ▶ CD72 to be consistent with the nomenclature for other cluster differentiation antigens (Nakayama et al. 1989). The human analogue was subsequently identified both by molecular cloning and by isolation of monoclonal antibodies. Two recent reviews have summarized the function of murine and human ▶ CD72 molecules and their ligands (Mizui et al. 2009; Wu and Bondada 2009). Despite its original discovery as a B cell-specific molecule, new functions for this molecule are being discovered such as in NK cells and mast cells (Kataoka et al. 2010; Alcón et al. 2009). This entry will review the molecular nature and function of the ▶ CD72 molecule in the context of B lymphocyte activation and differentiation.

► CD72 is a 45 kDa type II glycoprotein of 359 amino acids in human (354 in mouse) that exists as a dimer on the cell surface (trimers have also been identified) (Wu and Bondada 2009; Robinson et al. 1993). The oligomerization may involve the alpha-helical coiled-coil stalk region in the extracellular domain which is known to facilitate receptor oligomerization. The presence of 11 cysteines in the extracellular domain and two each in the transmembrane and cytoplasmic domain may also have a role in the formation of ► CD72 oligomers. Remarkably all the cysteines except the residue at position 178 are highly conserved among all the mammalian ► CD72 proteins (human, chimpanzee, mouse rat, dog, porcine, and bovine). The cysteines thought to form the intramolecular disulfide bridge are also conserved between the mammalian and the avian forms of ► CD72. There is a single N-linked glycosylation site at position 136 in the mouse sequence, which is also conserved in the mammalian isoforms. The C-terminal domain has a c-type lectin-like structure similar to CD23 and the asialoglycoprotein found on natural killer cells, but the nature of the carbohydrate moiety recognized by ► CD72 has not been defined (Nakayama et al. 1989). Similar lectin-like domain is found in ► CD72 molecules from human, mouse, rat, chimpanzee, dog, and chicken. Surprisingly, sequence homology analysis predicts that the c-type lectin domain in the bovine isoform of ► CD72 shows less homology to other species and may be truncated (http://www.ncbi.nlm.nih.gov/sites/homologene/1350). The alleles of ► CD72 found in mouse strains are considered complex alleles as they exhibit multiple differences in amino acid sequence which are more often found in the extracellular domain (Wu and Bondada 2002). Alternate splicing may be involved in the ► CD72 polymorphisms in the mouse. The intracellular domain of ► CD72 exhibits greater conservation among different mouse strains and across the species. The cytoplasmic domain has three tyrosines that are potential phosphorylation sites and their role in ► CD72 signaling will be discussed further in the later sections.

Early studies showed that antibodies to ► CD72 inhibited T-dependent antibody responses to the particulate antigen, sheep erythrocytes (SRBC) suggesting that ► CD72 may be involved in cell-cell interactions or as a receptor for cytokines (Yakura et al. 1982). Accordingly it was demonstrated that biotinylated membrane CD5 isolated from T cells bound to ► CD72 expressing cell lines leading to the proposal that CD5 and ► CD72 might form a ligand receptor pair (Wu and Bondada 2002). However, subsequent studies with fusion proteins of CD5 and the Fc region of human Ig failed to detect binding of such proteins to ► CD72 expressing cells suggesting that ► CD72 ligand may be distinct from CD5 (Bikah et al. 1998). In 2000 Kumanogoh et al. provided unequivocal evidence that ► CD72 binds to CD100, a member of semaphorin family of molecules (Sema 4D) that is expressed frequently in the nervous system but also in the immune system (Kumanogoh and Kikutani 2004). This finding and the generation of ► CD72 knockout mice (Pan et al. 1999) have helped advance the understanding of the physiological role of ► CD72.

Functional Role of CD72

► CD72 ligation induces blast transformation and proliferation of purified murine B cells which was initially interpreted to suggest that ► CD72 provides a positive signal to B cells (Subbarao and Mosier 1983). Anti-► CD72 antibodies inhibited T-dependent antibody response to SRBC, which required the antibodies to be present at early stages of B cell activation with the SRBC (Yakura et al. 1982). One interpretation of these studies was that anti-► CD72 enhances polyclonal B cell expansion at the expense of antigen-specific B cells reducing the antibody response to SRBC. Accordingly anti-► CD72 was shown to enhance polyclonal B cell activation induced by T-helper cell lines. Subsequently, several studies have shown that ► CD72 ligation synergizes with B cell receptor (BCR) signaling, which would have predicted an expansion of antigen-specific B cells (Wu et al. 2001; Wu and Bondada 2002). In the light of the discovery that ► CD72 can be present on dendritic cells (DC) also and that T cell CD100 binds ► CD72, the effects of anti-► CD72 on SRBC response may be more complex, since the early studies did not test for the presence of DCs in the purified cell populations (Yakura et al. 1982; Kumanogoh and Kikutani 2004; Kikutani and Kumanogoh 2003). Hence the ability of anti-► CD72 antibodies to modulate antibody responses to T-independent antigens, in particular TNP-Ficoll (which is a well-established activator of B cells without T cell help) was tested. Consistent with the findings

on synergy between anti-▶ CD72 and anti-BCR antibodies, ▶ CD72 ligation enhanced antigen-specific responses to TNP-Ficoll (Wu 2002). These later studies showed for the first time that ▶ CD72 may also affect B cell differentiation into antibody secreting cells.

Despite the extensive data showing positive effects of ▶ CD72 ligation in B cells, three observations suggested that ▶ CD72 may actually be a negative regulator of B cells. Thus Wu et al. showed that in a cell line model ▶ CD72 may have a role in B cell apoptosis (Wu et al. 1998). The B cells from ▶ CD72-deficient mice exhibited a small increase in B cell proliferation in response to BCR ligation (Pan et al. 1999). The crucial finding that ▶ CD72 associated with an SH2 domain containing protein tyrosine phosphatase, SHP-1 lent strong support to the concept that ▶ CD72 may be a negative regulator of B cells, which will be discussed further in the section on ▶ CD72-mediated signaling (Wu and Bondada 2002, 2009). Despite this ▶ CD72-deficient mice remain relatively healthy although a few aged ▶ CD72-deficient mice develop autoantibodies (Li et al. 2008). Analysis of mice transgenic for hen egg white lysozyme bred to ▶ CD72 knockout mice suggested that ▶ CD72 may have a role in the antigen-induced B cell anergy. Interestingly CD100 (Semaphorin 4D)-deficient mice exhibited an expansion of marginal zone B cells and development of a variety of autoantibodies such as anti-ssDNA, anti-dsDNA, rheumatoid factors, and anti-ribonucleoprotein (Kumanogoh et al. 2005). Somewhat unexpectedly, mice doubly deficient for CD100 and ▶ CD72 did not develop autoimmune disease suggesting that CD100 may be having effects on non-B cells.

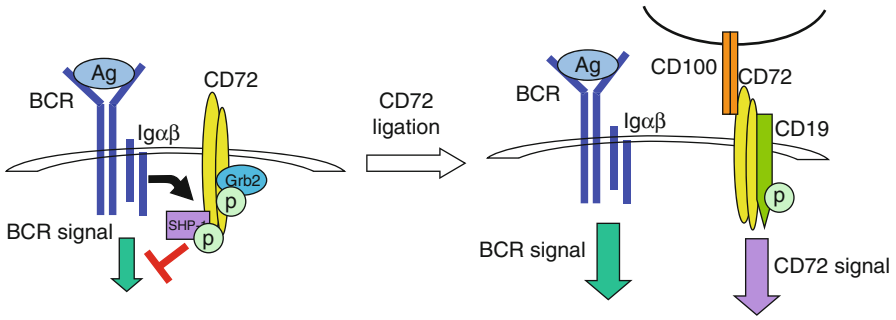
CD72, Table 1 Summary of observations supporting the concept that CD72 ligation can trigger BCR-independent positive signals as well as positively regulate BCR-mediated signals

▶ *CD72 ligation*
–Induced ERK activation in BCR-deficient A20 cell line
–Activated JNK in SHP-1-deficient splenic B cells
–Rescued BCR ligation-induced apoptosis in immature and Xid B cells
–Induced differential signals when compared to BCR ligation
–▶ CD72 induced while BCR reduced Blimp-1 expression
–▶ CD72 activated ERK, JNK, Lyn, Blk, Btk but not Syk while BCR can activate all these kinases
B cell development in ▶ CD72-deficient mice
–Reduced numbers of mature B cells in the bone marrow, spleen, lymph nodes, and Peyer's patches
–Reduced numbers of follicular B cells in the spleen
BCR ligation in CD72-deficient BAL-17 cell line
–Less BCR-induced $Ca2^+$ influx
–Impaired BCR-mediated phosphorylation of CD19, Btk, Vav, and PLCγ2 and association of CD19 with PI-3 kinase
–Reduced ERK, JNK, and p38 MAPK activation

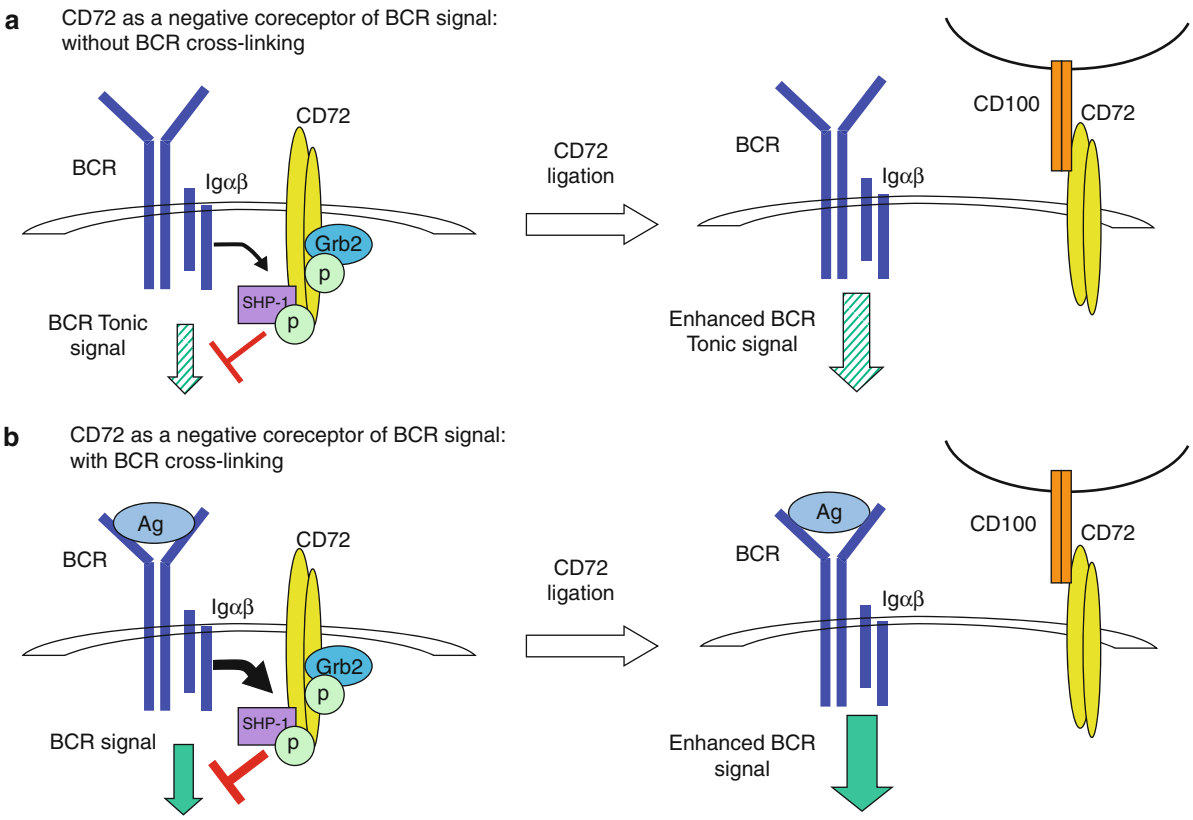
Reproduced from Wu and Bondada (2009)

Signaling Mechanisms Important for the Functional Effects of ▶ CD72 in B Cells

Earlier studies indicated that ▶ CD72 ligation mediated positive signaling events in B cells (Wu and Bondada 2009) (Table 1). However, many later studies focused on ▶ CD72 as a negative regulator of B cell receptor (BCR) signaling pathway (Kumanogoh and Kikutani 2004; Pan et al. 1999; Wu et al. 1998; Adachi et al. 2000) (Table 1). A dual signaling model was proposed to explain both positive and negative roles of ▶ CD72 in B cells based on the published data as summarized below (Fig. 1). The cytoplasmic domain of ▶ CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIM). When antigen encounters BCR, the signals triggered through BCR induce recruitment of ▶ CD72 to the kinase-rich BCR complex which leads to the phosphorylation of ▶ CD72 and recruitment of SHP-1 and Grb2 to ▶ CD72 (Wu et al. 1998; Kumanogoh et al. 2005). The ▶ CD72-associated SHP-1, a tyrosine phosphatase, can dephosphorylate protein tyrosine kinases and negatively regulate BCR signal. Hence, ▶ CD72 is a negative regulator of B cells. In this regard, the positive signals observed after ▶ CD72 ligation are due to ligation-triggered ▶ CD72 dephosphorylation that causes the dissociation of ▶ CD72 and SHP-1 which in turn leads to enhanced tonic BCR signaling (Fig. 2a) (Wu and Bondada 2009; Wu et al. 1998; Adachi et al. 2000). However, BCR-induced CD72 phosphorylation is mostly observed in immature but not mature B cells suggesting that ▶ CD72 may have stronger negative effects on BCR signals in immature cell types but not so much in mature B cells or B cell lines (Fig. 2a). Indeed, in contrast to immature B cells, ▶ CD72 was shown to regulate positive signals in mature B cells. Thus, a reduced BCR ligation induced Ca^{2+}, ERK, and JNK activation was observed in



CD72, Fig. 1 *Dual signaling model of CD72*. Overview of BCR and B-cell coreceptor signaling. CD19 complex enhances while CD22 decreases BCR signals. CD19 associates with BCR transiently upon BCR ligation. ▶ CD72 may increase or decrease BCR signals depending on its association with the CD19 complex. The figure shows only the CD19 molecule to be associated with ▶ CD72 but currently there is no data about its association with CD21 and CD81 partners of the CD19 complex (Reproduced from Wu and Bondada 2009)



CD72, Fig. 2 *Two models for CD72-mediated signals*. ▶ CD72 as a negative coreceptor of BCR signaling. (**a**) Without antigen stimulation, tonic BCR signals can trigger a low level of ▶ CD72 phosphorylation which can recruit SHP-1 and Grb2 that in turn negatively regulate tonic signals derived from BCR. Ligation of ▶ CD72 removes ▶ CD72 away from the kinase-rich BCR signaling complex resulting in dephosphorylation of ▶ CD72. This relieves the negative effect of ▶ CD72 from BCR tonic signals. Therefore, the positive signal seen in ▶ CD72 ligation is simply an enhanced BCR tonic signal. (**b**) Same as A, except that BCR ligation triggers a stronger ▶ CD72 phosphorylation, and presumably, creates a stronger negative effect of ▶ CD72 on BCR signals. ▶ CD72 ligation generates an enhanced BCR signal. (c) Dual signaling model of ▶ CD72. In addition to being a negative coreceptor of BCR-mediated signals (same as panels **a** and **b**), ligation of ▶ CD72 can also trigger positive signals through its association with CD19 (Reproduced from Wu and Bondada 2009)

a ▶ CD72$^{-/-}$ mature B cell line, BAL cells. Importantly, these effects can be mostly reversed by reexpression of ▶ CD72 (Ogimoto et al. 2004). This result suggested that ▶ CD72 can function as a positive regulator of B cells. In addition, just as in the normal B cells, ▶ CD72 ligation induced similar levels of ERK activation in a mature B cell line, A20, and in BCR-deficient A20 cells (Wu 2002). Moreover, the JNK activation of B cells from SHP-1 deficient, viable moth-eaten mice was strongly enhanced by ▶ CD72 ligation. These results suggest that ▶ CD72 can signal on its own in the absence of BCR or SHP-1. Thus, ▶ CD72 ligation can also transmit BCR-independent positive signals. ▶ CD72 might transmit positive signals through its association with CD19, a B cell coreceptor, or Grb2, an adaptor protein required for activation of the Ras/▶ MEK/ERK pathway (Fusaki et al. 2000). Thus, ▶ CD72 ligation not only can trigger BCR-independent positive signals but also can positively regulate BCR-mediated signals. Therefore, it was hypothesized that ▶ CD72 can positively or negatively regulate BCR signaling. The dual role of ▶ CD72 explains why both positive effect of ▶ CD72/CD100 interaction, such as its roles in mature B cell signaling events and B cell development, and negative effect of ▶ CD72/CD100 interaction such as its roles in autoimmune diseases have been both observed in the past studies (Mizui et al. 2009; Pan et al. 1999; Li et al. 2008; Rojas et al. 2003; Besliu et al. 2011). Therefore, the contribution of ▶ CD72 signaling to the outcome of B cell fate is likely to depend on a combination of multiple factors such as the developmental stage of B cells, the strength of BCR signals, and the accessibility and concentration of CD100, the ▶ CD72 ligand, during the interaction of B cells and their milieu.

CD72 Signaling in Other Immune Cell Types

In addition to B cells, ▶ CD72 was also found to be expressed on mast cells and NK cells in recent reports (Kataoka et al. 2010; Alcón et al. 2009). In contrast to the resting B cells, there appears to be a constitutive phosphorylation of ▶ CD72 and association of ▶ CD72 with SHP-1 in resting human mast cells (Kataoka et al. 2010; Wu et al. 1998). Therefore, ▶ CD72 may help maintain the steady state of the mast cells. Simultaneous activation of ▶ CD72 and KIT further enhanced the phosphorylation of ▶ CD72 and the recruitment of SHP-1 to ▶ CD72. Subsequently, the ▶ CD72-SHP-1 complex can reduce the KIT-dependent mast cell activity: growth of mast cells, stem cell factor-induced mast cell chemotaxis, chemokine ligand 2 production, and the IgE-dependent degranulation.

Unlike B cells, not all NK cell express ▶ CD72 on their surface. Activation of NK cells by IL-2 can further increase the ▶ CD72 expression on NK cells. Upon stimulation with IL-12 and IL-18, NK cells that expressed ▶ CD72 produced significantly less IFN-γ than those expressing none or low levels of ▶ CD72 (Alcón et al. 2009). Ectopic expression of ▶ CD72 in the ▶ CD72-deficient murine NK-cell line, KY2 cells, inhibits cytokine-induced ▶ IFN-γ production in NK cells. Both ITIM motifs are required for inhibition of IFN-γ secretion by ▶ CD72. Interestingly, although the extracellular domain of ▶ CD72 was also shown to be required for inhibitory effect of ▶ CD72, ligation of ▶ CD72 on NK cells with anti-▶ CD72 had no effect on ▶ IFN-γ production by NK cells. Thus, it still remains to be determined whether binding of ▶ CD72 to its natural ligand could have any impact on the function of NK cells.

Functional Effects and Disease Associations of Human ▶ CD72

The positive and negative regulatory effects of ▶ CD72 signaling in BCR-dependent and independent activation suggest a potential role for ▶ CD72 in autoimmunity and cancer. The developmental stage of B cells where ▶ CD72 exhibits differential effects upon CD100 interaction in the context of BCR signaling could influence the outcome of B cell responses in health and disease. Differential regulation of ▶ CD72 and BCR-induced response to protein kinase A and ▶ IFN-γ-mediated signaling events suggest potential alternative ▶ CD72-mediated regulatory pathways to overcome signals that downregulate BCR-induced proliferation and differentiation signals (Wu and Bondada 2002, 2009; Wu 2002). Defective ▶ CD72 signaling events are likely to influence the B cell selection, expansion, and immune response thus contributing to autoreactive B cells in the bone marrow and/or abnormal expansion in response to antigenic and microenvironmental stimuli in the periphery.

Polymorphisms in the negative regulatory receptor FcRγIIb (Ile232thr) are associated with systemic lupus erythematosus (SLE) in humans. Because both FcRγIIb and ▶ CD72 contain ITIM motifs, Hitomi and colleagues investigated the human ▶ CD72 polymorphisms for a possible association with SLE (Hitomi et al. 2004). Although no association with susceptibility to SLE was identified, the ▶ CD72 *1 allele, which contains a single 13-bp repeat in intron 8, was significantly associated with nephritis in Japanese patients. An alternatively spliced ▶ CD72 transcript that replaced 42 amino acids of the extracellular domain with 49 amino acids caused by skipping of exon 8 and inclusion of exon 9 was noticed in these patients. The ratio of alternatively spliced/common isoforms was found to be increased in individuals with *2/*2 genotype, which contains two 13-bp repeats in intron 8, when compared with *1/*1 or *1/*2 genotypes. Interestingly, significant association of the inhibitory FcRγIIb receptor 232thr/thr genotype with SLE was observed only within the ▶ CD72 *1/*1 genotype. Thus ▶ CD72 *2 allele may decrease risk for human SLE conferred by FCGR2B 232thr/thr, possibly by increasing the alternative isoform of ▶ CD72 (Hitomi et al. 2004).

Abnormalities in CD100 or ▶ CD72 counter receptors involved in T and B cell interaction were associated with autoimmunity. This was exemplified by the report from Besliu et al. (2011) who showed increases in $CD100^{+}$ T cells in the PBMCs from patients with systemic sclerosis (SSc), a connective tissue disease characterized by immune abnormalities, chronic inflammation, and fibrosis of skin and internal organs with microvascular damage and thrombosis. Both in human and mice CD100 has been shown to influence T cell activation, ▶ CD40 ligand-induced B cell aggregation, and survival (Kumanogoh and Kikutani 2004; Kikutani and Kumanogoh 2003). Triggering CD100 with mAb has shown to result in costimulation of T cells in response to CD3 or CD2. Association of CD100 with CD45 results in increased T cell homotypic adhesion induced by a CD45 mAb and interaction of CD100 cytoplasmic domain with a serine-threonine kinase activity in T and NK cells suggest a potential role of ▶ CD72/CD100 signaling in activation of T cells and NK cells (Kumanogoh and Kikutani 2004; Kikutani and Kumanogoh 2003). Association of CD100 with tyrosine phosphatase activities in B lymphocytes, its promotion of homotypic adhesion as well as increase in survival of B cells provide evidence for the functional role for CD100/▶ CD72 in B cell function (Kumanogoh and Kikutani 2004; Kikutani and Kumanogoh 2003). Aberrations in the expression and/or function of either of these molecules are likely to result in deregulation of the B and T cell homeostasis leading to autoimmune functions (Kumanogoh et al. 2005). A role for ▶ CD72 in CD100-mediated T cell costimulation remains to be explored further.

Aberrant ▶ CD72/CD100 interaction in mature B cell signaling events could potentially contribute to hitherto unidentified lympho-proliferative disorders. Deaglio et al. have described a potential role for CD100/▶ CD72 network in chronic lymphocytic leukemia (CLL) (Deaglio et al. 2005). ▶ CD38/CD31 interaction triggers relocalization of BCR/CD19 to the ▶ CD38/CD31 contact areas, increased cell survival and proliferation of CLL B cells. This is associated with upregulation of CD100, mainly in proliferating cells, and a concomitant decrease in ▶ CD72, low-affinity CD100 ligand (Deaglio et al. 2005). Nurse-like cells from B-CLL patients express CD31 and plexin-B1, a high-affinity ligand for CD100, which deliver growth and survival signals to ▶ CD38+/CD100+ B-CLL as well as downregulation of ▶ CD72 (Deaglio et al. 2005). Simultaneous cross-linking of ▶ CD72 has been shown to inhibit BCR-mediated apoptosis in Burkitt's lymphoma cells, but enhanced CD20-mediated apoptosis suggesting receptor-dependent positive and negative regulatory roles for ▶ CD72 in leukemia and lymphoma (Mimori et al. 2003). Consistent with a role for ▶ CD72 in B cell malignancies, CLL and mantle cell lymphoma (MCL) cells have been shown to proliferate in response to ▶ CD72 activation exhibiting ▶ CD40-like costimulatory effects. CD5 and/or ▶ CD72 engagement also has been shown to deliver critical costimulatory signals in B-1a, B-2, and B cells from patients with chronic lymphocytic leukemia, but with different requirements and patterns (Planken et al. 1998). Interestingly ▶ CD72 is reported to be present at high levels in B-lineage acute lymphoblastic leukemias (ALL) and has also been shown to serve as an effective target for therapy with anti-▶ CD72 immunotoxin for refractory ALL in vitro and in vivo animal models (Myers and Uckun 1995). High levels of expression of ▶ CD72 in a wide range of B cell malignancies including precursor B-cell

lymphomas, Burkitt's lymphomas, germinal center lymphomas, chronic lymphocytic leukemias, and hairy cell leukemia (Schwarting et al. 1992) warrants further exploration and development of ▶ CD72 and CD100 targeted therapeutic agents.

Summary

▶ CD72 is a B cell surface molecule with ability to modulate B cell receptor-derived signals. CD100, a member of semaphorin family, is the physiological ligand for ▶ CD72. ▶ CD72 is type II transmembrane protein with two immunoreceptor inhibitory motifs in the cytoplasmic domain. ▶ CD72 has been shown to associate with SHP-1 and Grb2 which play a role in its positive and negative regulatory effects. In lupus patients certain polymorphisms in the FcγRIIb are associated with polymorphisms in ▶ CD72. ▶ CD72 is also expressed in a variety of B cell malignancies and could be a therapeutic target. Recent studies suggest that mast cells and natural killer cells also express ▶ CD72 where it may negatively regulate their ability to degranulate and secrete γ-interferon respectively.

References

Adachi T, Wakabayashi C, Nakayama T, Yakura H, Tsubata T. CD72 negatively regulates signaling through the antigen receptor of B cells. J Immunol. 2000;164:1223–9.

Alcón VL, Luther C, Balce D, Takei F. B-cell co-receptor CD72 is expressed on NK cells and inhibits IFN-γ production but not cytotoxicity. Eur J Immunol. 2009;39:826–32.

Besliu A, Banica L, Predeteanu D, Vlad V, Ionescu R, et al. Peripheral blood lymphocytes analysis detects CD100/SEMA4D alteration in systemic sclerosis patients. Autoimmunity. 2011;44:427–36.

Bikah G, Lynd FM, Aruffo AA, Ledbetter JA, Bondada S. A role for CD5 in cognate interactions between T cells and B cells, and identification of a novel ligand for CD5. Int Immunol. 1998;10:1185–96.

Deaglio S, Vaisitti T, Bergui L, Bonello L, Horenstein AL, et al. CD38 and CD100 lead a network of surface receptors relaying positive signals for B-CLL growth and survival. Blood. 2005;105:3042–50.

Fusaki N, Tomita S, Wu Y, Okamoto N, Goitsuka R, et al. BLNK is associated with the CD72/SHP-1/grb2 complex in the WEHI231 cell line after membrane IgM cross-linking. Eur J Immunol. 2000;30:1326–30.

Hitomi Y, Tsuchiya N, Kawasaki A, Kyogoku C, Ohashi J, et al. CD72 polymorphisms associated with alternative splicing modify susceptibility to human systemic lupus erythematosus through epistatic interaction with FCGR2B. Hum Mol Genet. 2004;13:2907–17.

Kataoka TR, Kumanogoh A, Bandara G, Metcalfe DD, Gilfillan AM. CD72 negatively regulates KIT-mediated responses in human mast cells. J Immunol. 2010;184:2468–75.

Kikutani H, Kumanogoh A. Semaphorins in interactions between T cells and antigen-presenting cells. Nat Rev Immunol. 2003;3:159–67.

Kumanogoh A, Kikutani H. Biological functions and signaling of a transmembrane semaphorin, CD100/Sema4D. Cell Mol Life Sci. 2004;61:292–300.

Kumanogoh A, Shikina T, Watanabe C, Takegahara N, Suzuki K, et al. Requirement for CD100-CD72 interactions in fine-tuning of B-cell antigen receptor signaling and homeostatic maintenance of the B-cell compartment. Int Immunol. 2005;17:1277–82.

Li DH, Winslow MM, Cao TM, Chen AH, Davis CR, et al. Modulation of peripheral B cell tolerance by CD72 in a murine model. Arthritis Rheum. 2008;58:3192–204.

Mimori K, Kiyokawa N, Taguchi T, Suzuki T, Sekino T, et al. Costimulatory signals distinctively affect CD20- and B-cell-antigen-receptor-mediated apoptosis in Burkitt's lymphoma/leukemia cells. Leukemia. 2003;17:1164–74.

Mizui M, Kumanogoh A, Kikutani H. Immune semaphorins: novel features of neural guidance molecules. J Clin Immunol. 2009;29:1–11.

Myers DE, Uckun FM. An anti-CD72 immunotoxin against therapy-refractory B-lineage acute lymphoblastic leukemia. Leuk Lymphoma. 1995;18:119–22.

Nakayama E, von Hoegen I, Parnes JR. Sequence of the Lyb-2 B-cell differentiation antigen defines a gene superfamily of receptors with inverted membrane orientation. Proc Natl Acad Sci. 1989;86:1352–6.

Ogimoto M, Ichinowatari G, Watanabe N, Tada N, Mizuno K, et al. Impairment of B cell receptor-mediated Ca2+ influx, activation of mitogen-activated protein kinases and growth inhibition in CD72-deficient BAL-17 cells. Int Immunol. 2004;16:971–82.

Pan C, Baumgarth N, Parnes JR. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. Immunity. 1999;11:495–506.

Planken EV, Van de Velde H, Falkenburg JH, Thielemans K, Willemze R, et al. Selective response of CD5+ B cell malignancies to activation of the CD72 antigen. Clin Immunol Immunopathol. 1998;87:42–9.

Robinson WH, Landolfi MMT, Schafer H, Parnes JR. Biochemical identity of the mouse Ly-19.2 and 32.2 alloantigens with the B cell differentiation antigen Lyb-2/CD72. J Immunol. 1993;151:4764–72.

Rojas A, Xu F, Rojas M, Thomas JW. Structure and function of CD72 in the non-obese diabetic (NOD) mouse. Autoimmunity. 2003;36:233–9.

Sato H, Boyse EA. A new alloantigen expressed selectively on B cells: the Lyb-2 system. Immunogenetics. 1976;3:565–72.

Schwarting R, Castello R, Moldenhauer G, Pezzutto A, von Hoegen I, et al. Human Lyb-2 homolog CD72 is a marker for progenitor B-cell leukemias. Am J Hematol. 1992;41:151–8.

Subbarao B, Mosier DE. Induction of B lymphocyte proliferation without antibody secretion by monoclonal anti-Lyb2 antibody. J Immunol. 1983;130:2033–7.

Wu H-J. The positive signaling role of CD72 in B lymphocyte activation and function (doctoral thesis). Lexington: University of Kentucky; 2002.
Wu H-J, Bondada S. Positive and negative roles of CD72 in B cell function. Immunol Res. 2002;25:155–66.
Wu HJ, Bondada S. CD72, a coreceptor with both positive and negative effects on B lymphocyte development and function. J Clin Immunol. 2009;29:12–21.
Wu Y, Nadler MJ, Brennan LA, Gish GD, Timms JF, et al. The B-cell transmembrane protein CD72 binds to and is an in vivo substrate of the protein tyrosine phosphatase SHP-1. Curr Biol. 1998;8:1009–17.
Wu HJ, Venkataraman C, Estus S, Dong C, Davis RJ, et al. Positive signaling through CD72 induces mitogen-activated protein kinase activation and synergizes with B cell receptor signals to induce X-linked immunodeficiency B cell proliferation. J Immunol. 2001;167:1263–73.
Yakura H, Shen F-W, Bourcet E, Boyse EA. Evidence that Lyb-2 is critical to specific activation of B cells before they become responsive to T and other signals. J Exp Med. 1982;155:1309.

CD72 Antigen

▶ CD72

CD81

Shoshana Levy
School of Medicine – Division of Oncology Center for Clinical Sciences Research, Stanford University, Stanford, CA, USA

Synonyms

TAPA-1; TSPAN 28

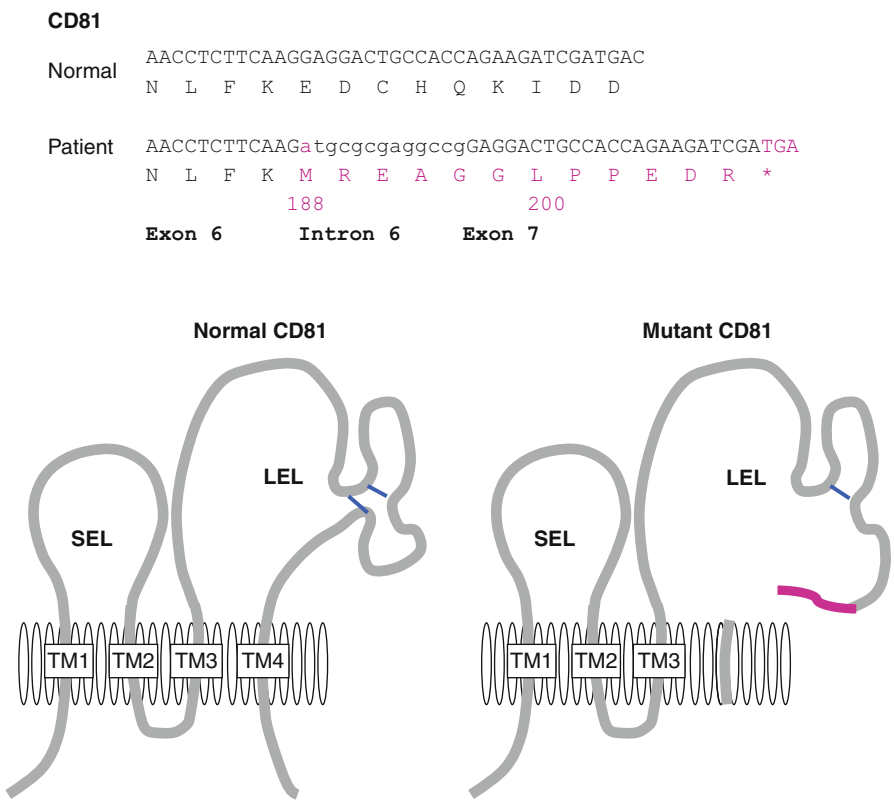
Historical Background

CD81 was originally identified as a Target of an Anti-Proliferative Antibody (TAPA-1) in a study that also defined a new family of transmembrane proteins, later named tetraspanins (Oren et al. 1990). CD81 is expressed on most human cell types; however, Oren et al. have shown that the sensitivity of diverse cell lineages to the anti-proliferative effect of the anti-CD81 antibody differed (Oren et al. 1990). Therefore, subsequent immune-co-precipitation studies were performed to reveal that CD81 associates with different partner proteins in the various cells types. For example, in B cells CD81 associates with CD19, a B cell specific signaling molecule. Similar studies on additional family members confirmed that tetraspanins tend to associate in the membrane with each other and with additional partner proteins. These tetraspanin-enriched microdomains (TEMs) are dynamic membrane entities, which act as signaling platforms (reviewed in (Levy and Shoham 2005)). Indeed, CD81 functions as an organizer and a facilitator of signaling for its associated partners, it is also required for cell fusion and cell–cell interactions, functions that have been subverted by human pathogens.

The discovery that CD81 is a receptor for the hepatitis C virus (HCV) was made more than a decade ago (Pileri et al. 1998), it demonstrated that the viral envelope protein E2 binds CD81. Multiple subsequent studies confirmed the key role of CD81 in the life cycle of HCV (reviewed in (Dubuisson et al. 2008)). Remarkably, HCV is the only known natural ligand for human CD81.

Subsequent studies have shown that CD81 is also required for the life cycle of another major human pathogen, *Plasmodium,* the malaria-causing pathogen. A study by Silvie et al. has shown that $Cd81^{-/-}$ mice cannot be infected by *P. yoelii* sporozoites, the first stage of the parasite's life cycle (Silvie et al. 2003). Follow-up studies demonstrated that anti-human CD81 mAbs blocked the infection of hepatocytes by *P. falciparum* sporozoites, the human malarial pathogen (Silvie et al. 2003). The mechanism by which CD81 enables the productive invasion of sporozoites into liver cells and their subsequent development into merozoites is not known; however, CD81 is not a receptor for this pathogen, as it does not bind the sporozoites directly.

Additional studies have suggested that CD81 has a role in the life cycle of HIV, especially it has been found to co-localize with this viral proteins in TEMs. For example, CD81 and additional tetraspanins have been shown to associate with the viral Gag and Env proteins and to modulate the release of HIV particles from a chronically infected T cell line. Interestingly, anti-CD81 mAb reduced HIV release and infectivity, whereas silencing of CD81 reduced viral release but increased infectivity (Grigorov et al. 2009).



CD81, Fig. 1 A common variable immunodeficiency disorder (CVID) diagnosed in a human patient with a homozygous splice site mutation in CD81. *Upper panel*: location of a homozygous splice site CD81 mutation in the recently diagnosed CVID patient. The G → A mutation, frameshift, and stop codon are shown (*magenta*). *Lower panel*: Normal CD81 contains two disulfide bonds in the large extracellular loop (LEL) (*blue lines*), whereas the mutant CD81 protein does not form the second disulfide bond in LEL. It contains a frameshift peptide (*magenta*) and is not anchored in the membrane by TM4

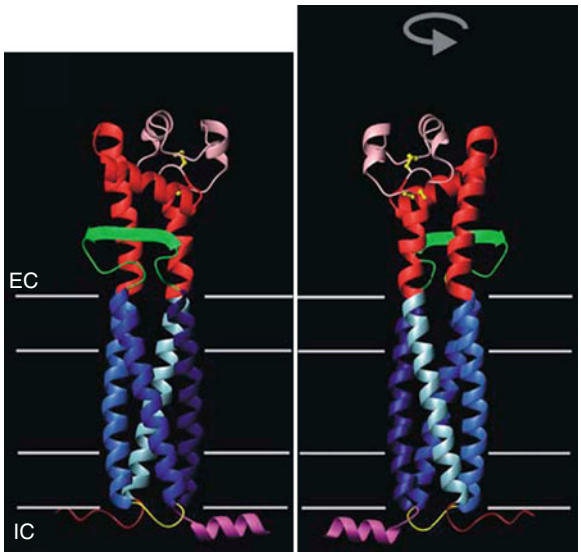
CD81 also plays a role in fusion of egg with sperm – *Cd81*$^{-/-}$ female mice are infertile (Rubinstein et al. 2006). Interestingly, this defect became evident only after several backcrosses of the original outbred knockout mice onto inbred mice strains, because of this impairment *Cd81*$^{-/-}$ mice need to be bred as heterozygotes.

CD81 also plays a role in cell surface expression of its associated partner proteins. The best-studied example is in B cells, where CD81 is required for normal expression levels of CD19, an important B cell signaling molecule. In human, an immunodeficient girl was recently diagnosed. Further characterization has shown that her B cells lacked CD19 surface expression; however, no mutations were detected in her CD19 locus. Because CD19 was shown to associate with CD81 subsequent studies focused on CD81, which was also absent in the patient. The deficiency was due to a homozygous splice site mutation downstream of exon 6 in the CD81 gene (van Zelm et al. 2010), as illustrated in Fig. 1. By contrast, surface expression of CD19 is not completely abolished (just reduced) in three independently derived lines of *Cd81*$^{-/-}$ mice.

Structure

CD81 is embedded in the plasma membrane by four transmembrane domains that flank short amino and carboxyl cytoplasmic termini and a small and large extracellular loop, SEL and LEL, respectively (Seigneuret 2006). This overall structure is shared by tetraspanins, a large evolutionarily conserved family of proteins (Garcia-Espana et al. 2008). In addition to a similar overall topology, tetraspanins share certain conserved amino acid motifs that distinguish them from other four transmembrane domain molecules. To date, human CD81 is the only tetraspanin molecule whose three-dimensional (3D) structure of the LEL domain has been determined (Kitadokoro et al. 2001). CD81-LEL is composed of a stalk of two longer α-helices and a novel mushroom-like head structure folded with the help of two disulfide bridges. Seigneuret (2006) proposed a structural model, based on the LEL 3D structure and on additional molecular modeling, as shown in Fig. 2.

Unlike most other tetraspanins, CD81 is not glycosylated. The molecule contains six juxtamembrane cysteines, which have been shown to be palmitoylated,



CD81, Fig. 2 Architecture and polarity of the modeled CD81 3D structure. Ribbon representation of the CD81 tertiary structure and topology. TM1-TM4, the conserved and variable subdomains of the large extracellular loop, the small extracellular loop, the intracellular loop, and the N-terminal and C-terminal regions are, respectively, represented in *marine blue, blue, royal blue, light blue, red, pink, green, yellow, magenta, and brown.* Disulfide bridges are in *yellow* (Reprinted from (Seigneuret 2006). Figure 6A with permission from the Biophysical Society and Elsevier)

a modification that is common to tetraspanins. No other modifications have been reported for CD81.

The short cytoplasmic domains of CD81 do not contain known signaling motifs.

CD81-Associated Membrane Partner Proteins

CD81 associates with partner proteins, the latter differ in various cell types. A large number of studies have documented these interactions; few examples highlighting the functional consequences of such interactions are detailed below.

CD19

Early biochemical studies aimed at understanding the preferential sensitivity of B cell lines to engagement by an anti-CD81 mAb revealed an association with CD19, a signaling molecule belonging to the immunoglobulin (Ig) superfamily. Subsequently, B cells derived from three independently derived lines of $Cd81^{-/-}$ mice showed reduced CD19 expression. Interestingly, complete lack of CD81 in mice results in a milder effect on CD19 expression than that seen in a human patient diagnosed with a homozygous exon splice mutation in CD81 (Fig. 1), which resulted in complete lack of surface CD19. It is noteworthy that although redundancy in tetraspanins' function has been suggested frequently, CD19 expression is dependent exclusively on CD81 and not on other tetraspanins. Indeed, re-introduction of CD81 into human or mouse B cells that lack CD81 restores CD19 expression (van Zelm et al. 2010). The absence of normal CD81 expression, both in human and in mouse, results in aberrant glycosylation of CD19. Thus, the absence of CD81 results in impaired trafficking of CD19, as evident by high mannose glycans that are sensitive to digestion by endoglycosidase-H, indicating residence in the endoplasmic reticulum (ER) without further exit through the Golgi to the cell surface.

CD4

In T cells CD81 was shown to be associated with CD4, a T cell specific molecule expressed on T helper cells. The cytoplasmic region of CD4 was sufficient for the association with CD81. Interestingly, these molecules associated with each other after removal of the Lck-binding site from the cytoplasmic domain of CD4, and the binding of Lck to CD4 inhibited the association with CD81. These findings led the authors' suggestion that CD4 exist in two states, one associated with Lck, the other with tetraspanins (Imai et al. 1995).

EWI-2

Another Ig family member that associates with CD81 is EWI-2, an association that is maintained in harsh lysis conditions. This cellular partner of CD81 has been shown to modulate susceptibility to HCV infection. Hepatocytes express the complete EWI-2 and are susceptible to viral infection. However, EWI-2 exists in a form that lacks an Ig domain "without its N-terminus (EWI-2wint)" in some cell types. Cells that express EWI-2wint are not susceptible to HCV infection. For example, EWI-2wint, which is expressed in lymphocytes, blocks HCV entry by inhibiting the interaction of the viral envelope proteins with CD81 (Rocha-Perugini et al. 2008).

Integrins

Biochemical studies have repeatedly shown that tetraspanins tend to associate with specific integrins in various cell types. This specificity of interaction was

demonstrated in cells where only one specific integrin associated with an individual tetraspanin molecule, although the cell contained several integrin molecules. A study that analyzed the functional consequence of the association of CD81 with α4β1 integrin in B cells has shown that CD81 facilitated integrin-dependent adhesion strengthening (Feigelson et al. 2003).

G-Protein-Coupled Receptors (GPCR)

A search for CD81-associated proteins in a teratocarcinoma cell line identified GPR56, an orphan G-protein-coupled receptor. The association with CD81 was also seen in transfected cells where an additional tetraspanin molecule, CD9, was associated with GPR56 (Little et al. 2004). Further studies demonstrated that CD81 was required for the association of GPR56 with $G\alpha_{q/11}$ and that engagement of CD81 by an antibody led to the dissociation of the G-protein from the GPCR (Little et al. 2004).

Role of CD81 in Cell Signaling

The presence or absence of CD81 can greatly affect downstream signaling events in a cell-of-origin-dependent manner. For example, while B cell signaling is impaired in the CVID patient shown in Fig. 1, her T cells responded normally to mitogens as measured by proliferation and interferon γ (IFNγ) production. Correspondingly, responses to CD81 engagement are highly dependent on the cell type. The examples below summarize studies that have delineated signaling pathways triggered in response to CD81 engagement either by antibodies or by the HCV envelope proteins. It is noteworthy that although these signaling cascades differ in the various cell types, they all ultimately provide a connection to the actin cytoskeleton.

B Cells

The engagement of CD81 on human B cells induces tyrosine phosphorylation of a large number of proteins. Analysis of these phosphorylated proteins by mass spectrometry identified ezrin, an actin-binding protein, as the major tyrosine-phosphorylated band (Coffey et al. 2009). Coffey et al. also demonstrated that engagement of CD81 induced the phosphorylation of spleen tyrosine kinase (Syk) and that phosphorylation of Syk was required for the tyrosine phosphorylation of ezrin. Finally, the study demonstrated that the cytoplasmic C-terminal domain of CD81 was required for the induction of activated ezrin. The model emerging from this study is that CD81 interfaces between the cell membrane and the cytoskeleton where its engagement leads to activation of Syk, which in turn activates ezrin, which then recruits filamentous actin (F-actin) to facilitate cytoskeletal organization and cell signaling.

T Cells

CD81 was demonstrated to be a costimulatory molecule both in human and in mouse T cells, namely, the coengagement of CD81 potentiated the stimulation induced in response to engagement of ▶ CD3 on these cells. Interestingly, these studies have also shown that the costimulatory effect induced by engaging CD81 is similar in magnitude to that induced in response to coengagement of CD3 and CD28, the latter being a classical T cell costimulatory molecule. However, while the cytoplasmic domain of CD28 contains tyrosine activation motifs, CD81 lacks such motifs. The detailed mechanism of T cell costimulation by CD81 is yet to be delineated. However, a recent study demonstrated that costimulation of CD3 and CD81 induced phosphorylation of Erk1/2 and activated the actin cytoskeleton (Crotta et al. 2006). An earlier study by the same group has shown that costimulation of T cells by CD81 was mediated by Lck. These and additional studies, which were aimed at understanding the interaction of HCV with human T cells have shown that the costimulatory potential of the envelope protein E2 of HCV (HCV-E2) was comparable to that of the anti-CD81 mAbs.

Hepatocytes

The hepatocyte cell line, Huh-7, which is susceptible to infection by HCV in cell culture (HCVcc) was used in the following study. Engagement of CD81 in this cell line by a mAb or by HCV-E2 induced the phosphorylation of Erk; moreover, an inhibitor of the Raf/Mek/Erk signaling pathway inhibited viral infection (Brazzoli et al. 2008). The same study also demonstrated that CD81 provided a linkage to the actin cytoskeleton. Briefly, engagement of CD81 induced its movement to the zone of cell–cell contact; in addition, treatment by drugs that inhibit F-actin formation blocked the relocation of CD81 to the cell-to-cell contact areas. Further analysis revealed that CD81 mediated the activation of the actin cytoskeleton by increasing the level of the active GTP-bound Rho

family GTPases (Rho-A, Rac1, and Cdc42). Moreover, use of knockdown and of inhibitors of these GTPases reduced viral infectivity (Brazzoli et al. 2008). Thus, HCV subverts key cellular functions of CD81.

Summary

The tetraspanin molecule CD81 is a membrane-embedded protein. It functions as an organizer and a facilitator of signaling for its associated protein partners. CD81 associates with different partners in the various cell types. CD81, its partner proteins, and additional tetraspanins form TEMs, which act as signaling platforms connecting the cell membrane to the actin cytoskeleton. The role of CD81 has been subverted by major human pathogens.

References

Brazzoli M, Bianchi A, Filippini S, Weiner A, Zhu Q, Pizza M, Crotta S. CD81 is a central regulator of cellular events required for hepatitis C virus infection of human hepatocytes. J Virol. 2008;82:8316–29.

Coffey GP, Rajapaksa R, Liu R, Sharpe O, Kuo CC, Krauss SW, Sagi Y, Davis RE, Staudt LM, Sharman JP, Robinson WH, Levy S. Engagement of CD81 induces ezrin tyrosine phosphorylation and its cellular redistribution with filamentous actin. J Cell Sci. 2009;122:3137–44.

Crotta S, Ronconi V, Ulivieri C, Baldari CT, Valiante NM, Abrignani S, Wack A. Cytoskeleton rearrangement induced by tetraspanin engagement modulates the activation of T and NK cells. Eur J Immunol. 2006;36:919–29.

Dubuisson J, Helle F, Cocquerel L. Early steps of the hepatitis C virus life cycle. Cell Microbiol. 2008;10:821–7.

Feigelson SW, Grabovsky V, Shamri R, Levy S, Alon R. The CD81 tetraspanin facilitates instantaneous leukocyte VLA-4 adhesion strengthening to vascular cell adhesion molecule 1 (VCAM-1) under shear flow. J Biol Chem. 2003;278:51203–12.

Garcia-Espana A, Chung PJ, Sarkar IN, Stiner E, Sun TT, Desalle R. Appearance of new tetraspanin genes during vertebrate evolution. Genomics. 2008;91:326–34.

Grigorov B, Attuil-Audenis V, Perugi F, Nedelec M, Watson S, Pique C, Darlix JL, Conjeaud H, Muriaux D. A role for CD81 on the late steps of HIV-1 replication in a chronically infected T cell line. Retrovirology. 2009;6:28.

Imai T, Kakizaki M, Nishimura M, Yoshie O. Molecular analyses of the association of CD4 with two members of the transmembrane 4 superfamily, CD81 and CD82. J Immunol. 1995;155:1229–39.

Kitadokoro K, Bordo D, Galli G, Petracca R, Falugi F, Abrignani S, Grandi G, Bolognesi M. CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs. EMBO J. 2001;20:12–8.

Levy S, Shoham T. The tetraspanin web modulates immune-signalling complexes. Nat Rev Immunol. 2005;5:136–48.

Little KD, Hemler ME, Stipp CS. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-Galpha q/11 association. Mol Biol Cell. 2004;15:2375–87.

Oren R, Takahashi S, Doss C, Levy R, Levy S. TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. Mol Cell Biol. 1990;10:4007–15.

Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. Science. 1998;282:938–41.

Rocha-Perugini V, Montpellier C, Delgrange D, Wychowski C, Helle F, Pillez A, Drobecq H, Le Naour F, Charrin S, Levy S, Rubinstein E, Dubuisson J, Cocquerel L. The CD81 partner EWI-2wint inhibits hepatitis C virus entry. PLoS One. 2008;3:e1866.

Rubinstein E, Ziyyat A, Prenant M, Wrobel E, Wolf JP, Levy S, Le Naour F, Boucheix C. Reduced fertility of female mice lacking CD81. Dev Biol. 2006;290:351–8.

Seigneuret M. Complete predicted three-dimensional structure of the facilitator transmembrane protein and hepatitis C virus receptor CD81: conserved and variable structural domains in the tetraspanin superfamily. Biophys J. 2006;90:212–27.

Silvie O, Rubinstein E, Franetich JF, Prenant M, Belnoue E, Renia L, Hannoun L, Eling W, Levy S, Boucheix C, Mazier D. Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. Nat Med. 2003;9:93–6.

van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, Ferster A, Kuo CC, Levy S, van Dongen JJ, van der Burg M. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest. 2010;120:1265–74.

CD87

► Structure and Functions of the Urokinase Receptor

CD91

Sudesh Pawaria and Robert J. Binder
Department of Immunology, University of Pittsburgh, Pittsburgh, PA, USA

Synonyms

Alpha-2-macroglobulin receptor (A2MR); Apolipoprotein receptor (APR); CED1; Low density lipoprotein receptor-related protein 1 (LRP1)

Historical Background

CD91, also known as the low density lipoprotein receptor-related protein 1 (LRP1), is a receptor on the plasma membrane involved in receptor-mediated endocytosis and in various signaling events. CD91 is a member of a gene family found in diverse species, including *C. elegans*, *Drosophila*, *Xenopus*, and mammals (Gaultier et al. 2008). Seven structurally closely related cell surface receptors constitute the core of the low density lipoprotein (LDL) receptor gene family. They include the LDL receptor, the LDL receptor-related protein (CD91), LRP1b, megalin, very low density lipoprotein (VLDL) receptor, apolipoprotein E receptor 2 (apo-ER2), and MEGF7 (multiple epidermal growth factor-like domains containing protein 7) (Herz and Strickland 2001). This review focuses on CD91 and its role as a signaling receptor for several ligands and in various physiological processes.

CD91 was originally identified by virtue of its structural similarity and sequence homology to the LDL receptor (Herz et al. 1988). As expected of a lipoprotein receptor, CD91 was found to play an important role in lipoprotein metabolism and cholesterol homeostasis by the discovery of apolipoprotein E as its ligand. The function of CD91 in vivo as a chylomicron remnant receptor is now well established. The sequencing of the receptor soon revealed that it was identical to the receptor proposed for removal of α_2M-proteinase complexes (Lillis et al. 2008).

Protein Structure

CD91 is synthesized as a 600-kDa type I transmembrane protein which during transit to the cell surface is processed by furin within a β-propeller domain into a large 515-kDa α- and a smaller 85-kDa β-subunit in the *trans*-Golgi network. An ER-resident chaperone, termed the receptor associated protein (RAP), binds tightly to CD91 on multiple sites and prevents premature association of ligands to the newly synthesized CD91 in the ER enabling it to be successfully delivered to the plasma membrane (Lillis et al. 2008).

CD91 is composed of modular structures that include cysteine-rich complement-type repeats, EGF repeats, and β-propeller domains, all on the α-chain, and a transmembrane and cytoplasmic domain on the β-chain. The cysteine-rich complement-type repeats, also commonly referred to as ligand-binding repeats, are localized into regions as clusters and are termed clusters I–IV, each containing variable numbers of complement-type repeats. Most CD91 ligands have been shown to bind to clusters II and IV (Lillis et al. 2008).

The β-chain contains a small extracellular domain, a single-pass transmembrane domain, and an intracellular domain of 100 amino acid residues. After furin cleavage in the ER, the β-chain stays non-covalently connected to the larger α-subunit through its small extracellular domain. The intracellular domain of CD91 contains many potential motifs which are postulated to have a role in basolateral sorting, internalization, recycling of the receptor, and binding of several different adaptor proteins for signal transduction. These motifs on the β-chain include two NPXY motifs, the distal one overlapping with an YXXL internalization motif; two di-leucine internalization motifs; and several serine, threonine, and tyrosine phosphorylation sites. Unlike the other LDL receptors, the YXXL motif rather than the NPXY and di-leucine motifs was shown to be the dominant endocytosis signal in CD91 mini-receptor constructs (Li et al. 2000).

The receptor is expressed in a variety of cell types, including macrophages (Misra et al. 1995), dendritic cells (Basu et al. 2001), T helper cells (Banerjee et al. 2002), adrenal cortical cells, hepatocytes, neurons and neuroblastoma, follicular cells of the ovary, fibroblasts, measengial cells (Zheng et al. 1994), and adipocytes (Corvera et al. 1989).

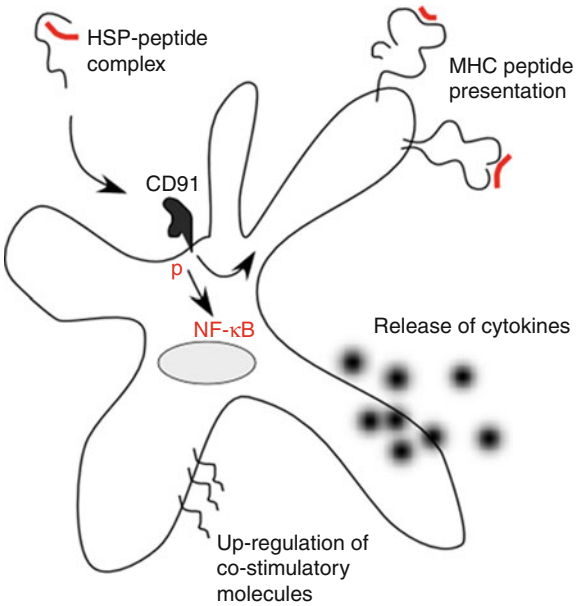
The Role of CD91 in Cell Signaling

A significant number of biological functions have been attributed to CD91 due to its ability to bind to a large number of ligands. Mice deficient in CD91 expression die early during embryonic development demonstrating a critical function for CD91 in prenatal development (Herz and Strickland 2001). Mice engineered to lack expression of CD91 in vascular smooth muscle cells display overexpression of, and abnormal signaling through, the platelet-derived growth factor (PDGF) receptor which causes proliferation of smooth muscle cells, aneurysm formation, and marked susceptibility to cholesterol-induced atherosclerosis (Boucher et al. 2003). Generally the functions of CD91 are mediated

through two major physiological processes; endocytosis and regulation of signaling pathways. During endocytosis, CD91 is capable of binding approximately 30 ligands with high affinity and delivering them to endo-lysosomal compartments. The receptor itself is usually recycled. The details of CD91-mediated endocytosis are outside the scope of this review.

In addition to the numerous ligands that are recognized and bound by the CD91 α-chain in the extracellular space, it is becoming apparent that several cytoplasmic proteins also associate with the CD91 β-chain. CD91 promotes intracellular signaling, which downstream mediates proliferation, migration, maturation and differentiation of different types of cells, including macrophages, dendritic cells, vascular smooth muscle cells, and neurons (Herz and Strickland 2001; Lillis et al. 2008; Pawaria and Binder 2011). Phosphorylation of the CD91 intracellular domain regulates association with adaptor molecules. While phosphotyrosine binding PTB domains were originally thought to recognize phosphorylated tyrosine residues within the NPXY sequence, in an increasing number of cases it has become apparent that phosphorylation of the tyrosine residue in these motifs is not required for binding to PTB domains. Both the proximal NPXY and distal NPXYXXL motifs are capable of interacting with cytoplasmic adaptors and scaffold proteins, like Disabled-1 (DAB-1), FE65, JNK-interacting protein 1 (JIP1), Postsynaptic density protein 95 (PSD-95), Src homology 2 domain containing protein (SHC), Sorting nexin 17 (Snx17), and CED-6/GULP (May et al. 2007). Although most of these proteins have been shown to bind only the distal NPXYXXL motif in the CD91 intracellular domain, FE65 can bind both motifs, and Snx17 is the only interacting protein so far identified to bind exclusively to the proximal NPXY motif. Phosphorylation at the Tyr can be inhibitory also as in case of Snx17 where binding is inhibited after Tyr 4473 phosphorylation (Betts et al. 2008). Recently Guttmen et al. used each NPXY motif microdomain separately in both phosphorylated and unphosphorylated forms and reported a large number of proteins that selectively interact with either the phosphorylated or the unphosphorylated form (Guttman et al. 2009). Very few binding partners were detected for the membrane proximal Tyr 4473 including Shc3 and Grb2. The proximal NPXY 4473 motif has been shown to be involved in receptor recycling and demonstrated a strong phenotype in knock-in mice (Roebroek et al. 2006). The proteins that bound to the Tyr 4507 included signaling molecules like PLC-γ, ▶ PI3K, Shp-2, ▶ Src, CSK, Shc-3, and Grb-2, which get phosphorylated on the tyrosine in response to extracellular signals (Guttman et al. 2009). Other studies have implicated the NPXY4507 motif in CD91 in binding intracellular signaling proteins such as ShcA, Fe65, and Disabled (Dab1) which have a phosphotyrosine binding (PTB) domain (Barnes et al. 2001). The association of CD91 with specific (set of) adaptor molecules is dictated by the binding of different ligands and is essential for the differentiation of CD91 function in cargo transport or in signaling pathways. The diversity of extracellular and intracellular CD91 binding partners highlights its multiple signaling functions in physiological and pathophysiological processes.

1. *The role of CD91 signaling in apoptosis:* CD91 is one of the numerous cell surface receptors that have been shown to engage apoptotic cells and promote phagocytosis (Kinchen and Ravichandran 2007). CD91 was first shown to be a receptor for calreticulin, a Ca^{2+}-binding protein normally found in the endoplasmic reticulum, in 2001 (Basu et al. 2001). Those experiments were performed with soluble calreticulin in the extracellular space. In an extension of those studies, CD91 has been shown to bind calreticulin on the surface of apoptotic cells which in turn directly interacts with phosphatidylserine on the apoptotic cell surface. These interactions are important for clearance of apoptotic cells by signaling an "eat me" signal within phagoctytic cells such as macrophages (Gardai et al. 2006). The nature of the signaling pathway with respect to CD91 and apoptosis is currently unknown.
2. *The role of CD91 signaling in immune cells:* The HSPs, gp96 (▶ Grp94), hsp70, hsp90, and calreticulin are immunogenic. Upon immunization with HSPs, frogs, mice, rats, and humans elicit immune responses to peptides that are chaperoned by the HSP (Binder 2008). Upon examination of the mechanism of action, CD91 was first identified by Binder et al. as the receptor for gp96 (Binder et al. 2000), and then by Basu et al. as a common receptor for other immunogenic HSPs such as hsp70, hsp90, and calreticulin (Basu et al. 2001). Utilizing antigen-presenting cells in the form of dendritic cells or macrophages, CD91 has been demonstrated in



CD91, Fig. 1 Dual effects of HSPs are mediated through CD91. Peptides chaperoned by HSPs are cross-presented on MHC I and II after CD91-dependent endocytosis. HSPs signal through CD91 by phosphorylating CD91 leading to activation of NF-kB. As a result of this signaling pathway, antigen-presenting cells release cytokines and upregulate expression of co-stimulatory molecules

numerous laboratories to be essential for endocytosis of HSP-peptide complexes and cross-presentation of the chaperoned peptide. While the role of CD91-dependent endocytosis in HSP-mediated immunity is well established, its potential role in signaling is just beginning to be evaluated. Recent data shows that in response to binding any one of the immunogenic HSPs, gp96, hsp70, or calreticulin, (a) CD91 is phosphorylated; (b) CD91 associates with the Shc adaptor protein, a process that involves the Src family kinases; and (c) ▶ NF-κB is activated in a CD91-dependent manner (Pawaria and Binder 2011). This signaling pathway which includes various kinases is important for the maturation of antigen-presenting cells and the resulting elaboration of co-stimulation. CD91 thus appears to play a dual role in HSP-mediated immunity; provision of signal 1 by way of cross-presentation of antigens and signal 2 via co-stimulation (Fig. 1). These events play key roles in initiation of antitumor immune responses in tumor-bearing mice and for therapeutic purposes in the clinic.

3. *The intracellular domain of CD91 as a transcriptional regulator:* It has been recognized for some time that a soluble form of CD91 circulates in the plasma, indicating that the receptor is subject to proteolysis, thereby releasing portions of receptor as a soluble polypeptide (Quinn et al. 1999). Similar to other type I integral membrane proteins, the CD91 cytoplasmic domain can also be cleaved by an enzyme with γ-secretase-like activity (May et al. 2002) releasing CD91 polypeptides of about 12kDa into the cytosol. This polypeptide has been shown to efficiently translocate to the nucleus where it limits the transcription of the inflammatory genes ▶ TNF-α and IL-6 in response to LPS activation of cells (Zurhove et al. 2008). Briefly, the nuclear CD91 polypeptide co-localizes with histone acetyltransferase Tip60, a transcriptional modulator with a role in linking proteolytic cleavage of β-amyloid precursor protein (APP) to transcriptional activation (Cao and Sudhof 2001; Baek et al. 2002). These studies suggest that a portion of the β-chain of CD91 can function directly as a transcriptional regulator. The identification of other genes regulated in a similar mechanism by this polypeptide is expected.
4. *The role of CD91 signaling in the nervous system:* Bu et al. found that the cytoplasmic tail of CD91 is phosphorylated in neuronal-derived cell lines and that nerve growth factor rapidly increases the amount of phosphorylation (Bu et al. 1998). A result of this is the rapid increase of cell surface CD91. CD91 serves as a receptor for α_2M with or without the amyloid peptide apolipoprotein E, tissue factor pathway inhibitor, and APP among others. These proteins are associated with the onset or pathology of Alzheimer's disease. An increase in CD91 expression on neurons suggests a mechanism for increased clearance of these proteins in the brain. In addition, signaling through phosphorylation of CD91 has been suggested to be crucial for the redistribution of CD91 from a silent pool (in the endosomes) to an active endocytic pool (on the cell surface). Redistribution of CD91 in neurons by this mechanism serves as a rapid way to regulate concentration of its ligands in the local environment of the brain.
5. *Other signaling events that have not been associated with a physiological outcome*: These studies

have largely been performed on cells in an in vitro setting and thus their role in physiological events is not yet readily apparent. Barnes et al. identified the CD91 β-chain bound to Shc PTB domain and showed that CD91 is tyrosine-phosphorylated in v-Src transformed cells. The tyrosine-phosphorylated CD91 binds to Shc which might provide a link to the RAS-ERK/MAP kinase pathway and alternative pathways downstream of Shc (Barnes et al. 2001). Those results are consistent with our own observations on the role of CD91 in immune cells described above. Tyrosine phosphorylation of CD91 suggested that a member of the receptor tyrosine kinase family might be responsible for this effect. Loukinova et al. found that PDGF BB induces a transient tyrosine phosphorylation of the CD91 cytoplasmic domain in a process dependent on PDGFβ receptor activation and ▶ c-Src family kinase activity (Loukinova et al. 2002). Lastly, CD91 is phosphorylated on cytoplasmic tyrosines in response to platelet-derived growth factor (Boucher et al. 2002) and Tyr 4507 was identified as the principle v-Src phosphorylation site (Barnes et al. 2001).

Summary

The endocytic functions of CD91 have been avidly examined and described. With respect to over 30 ligands described to bind to the α-chain of CD91, most have been demonstrated to be endocytosed into endo-lysosomes. There are an increasing number of examples of important roles for signaling via CD91 in various physiological events as described here. While an association of CD91 and its ligands in Alzheimer's disease has been studied for a number of years, the role of CD91 in immune responses is only beginning to emerge. In other physiological events, the crucial role of CD91 in clearing apoptotic cells signifies the far reaching consequences a deficiency of CD91 function would have. Indeed no such deficiencies have been found. In our own laboratory signaling of CD91 in response to immunogenic HSPs is a critical pathway for initiation of immune responses by dendritic cells and also for directing the type of immune response that is primed. This ancient receptor that has a major role in endocytosis appears to have been hijacked for evolutionarily newer functions of higher organisms. A major challenge for the study of CD91 is its sheer size of 600kDa and the intricate conformation of its domains which makes expression of recombinant protein with the right protein conformation challenging. In addition mice that are created to be deficient in CD91 expression die early during embryonic development due to its role in prenatal development (Herz and Strickland 2001). Recent advances in artificial chromosomes and conditional knock-out mice technology using the Cre-lox system will provide invaluable reagents for further investigation into the role in pathology and pathogenesis of disease and the role of CD91 signaling in physiological pathways.

References

Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, Rosenfeld MG. Exchange of N-CoR corepressor and Tip60 activator complexes links gene expression by NFkappaB and beta-amyloid precursor protein. Cell. 2002;110:55–67.

Banerjee PP, Vinay DS, Mathew A, Raje M, Parekh V, Prasad DV, Kumar A, Mitra D, Mishra GC. Evidence that glycoprotein 96 (B2), a stress protein, functions as a Th2-specific costimulatory molecule. J Immunol. 2002;169:3507–18.

Barnes H, Larsen B, Tyers M, Van Der GP. Tyrosine phosphorylated LDL receptor-related protein 1 (LRP1) associates with the adaptor protein Shc in Src-transformed cells. J Biol Chem. 2001;22:19119–25.

Basu S, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity. 2001;14:303–13.

Betts GN, van der Geer P, Komives EA. Structural and functional consequences of tyrosine phosphorylation in the LRP1 cytoplasmic domain. J Biol Chem. 2008;283:15656–64.

Binder RJ. Heat-shock protein-based vaccines for cancer and infectious disease. Expert Rev Vaccines. 2008;7:383–93.

Binder RJ, Han DK, Srivastava PK. CD91: a receptor for heat shock protein gp96. Nat Immunol. 2000;1:151–5.

Boucher P, Liu P, Gotthardt M, Hiesberger T, Anderson RG, Herz J. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. J Biol Chem. 2002;277:15507–13.

Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science. 2003;300:329–32.

Bu G, Sun Y, Schwartz AL, Holtzman DM. Nerve growth factor induces rapid increases in functional cell surface low density lipoprotein receptor-related protein. J Biol Chem. 1998;273:13359–65.

Cao X, Sudhof TC. A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science. 2001;293:115–20.

Corvera S, Graver DF, Smith RM. Insulin increases the cell surface concentration of alpha 2-macroglobulin receptors in 3T3–L1 adipocytes. Altered transit of the receptor among intracellular endocytic compartments. J Biol Chem. 1989;264:10133–8.
Gardai SJ, Bratton DL, Ogden CA, Henson PM. Recognition ligands on apoptotic cells: a perspective. J Leukoc Biol. 2006;79:896–903.
Gaultier A, Arandjelovic S, Li X, Janes J, Dragojlovic N, Zhou GP, Dolkas J, Myers RR, Gonias SL, Campana WM. A shed form of LDL receptor-related protein-1 regulates peripheral nerve injury and neuropathic pain in rodents. J Clin Invest. 2008;118:161–72.
Guttman M, Betts GN, Barnes H, Ghassemian M, van der Geer P, Komives EA. Interactions of the NPXY microdomains of the low density lipoprotein receptor-related protein 1. Proteomics. 2009;22:5016–28.
Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J Clin Invest. 2001;108:779–84.
Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK. Surface location and high affinity for calcium of a 500kDa liver membrane protein closely related to the LDL-receptor suggest a physiolocical role as lipoprotein receptor. EMBO J. 1988;7:4119–27.
Kinchen JM, Ravichandran KS. Journey to the grave: signaling events regulating removal of apoptotic cells. J Cell Sci. 2007;120:2143–9.
Li Y, Marzolo MP, van Kerkhof P, Strous GJ, Bu G. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. J Biol Chem. 2000;275:17187–94.
Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. Physiol Rev. 2008;88:887–918.
Loukinova E, Ranganathan S, Kuznetsov S, Gorlatova N, Migliorini MM, Loukinov D, Ulery PG, Mikhailenko I, Lawrence DA, Strickland DK. Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function betwenn LRP and the PDGF. J Biol Chem. 2002;277:15499–506.
May P, Reddy YK, Herz J. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. J Biol Chem. 2002;277:18736–43.
May P, Woldt E, Matz RL, Boucher P. The LDL receptor-related protein (LRP) family: an old family of proteins with new physiological functions. Ann Med. 2007;39:219–28.
Misra UK, Gawdi G, Pizzo SV. Ligation of the alpha 2-macroglobulin signalling receptor on macrophages induces protein phosphorylation and an increase in cytosolic pH. Biochem J. 1995;309:151–8.
Pawaria S, Binder RJ. CD91-dependent programming of T helper cell responses following heat shock protein immunization. Nat Commun. 2011;2:521. doi:10.1038/ncomms1524.
Quinn KA, Pye VJ, Dai YP, Chesterman CN, Owensby DA. Characterization of the soluble form of the low density lipoprotein receptor-related protein (LRP). Exp Cell Res. 1999;251:433–41.
Roebroek AJ, Reekmans S, Lauwers A, Feyaerts N, Smeijers L, Hartmann D. Mutant Lrp1 knock-in mice generated by recombinase-mediated cassette exchange reveal differential importance of the NPXY motifs in the intracellular domain of LRP1 for normal fetal development. Mol Cell Biol. 2006;26:605–16.
Zheng G, Bachinsky DR, Stamenkovic I, Strickland DK, Brown D, Andres G, McCluskey RT. Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/alpa 2MR, and the receptor-associated protein (RAP). J Histochem Cytochem. 1994;42:531–42.
Zurhove K, Nakajima C, Herz J, Bock HH, May P. Gamma-secretase limits the inflammatory response through the processing of LRP1. Sci Signal. 2008;1:ra15.

CDC25

► RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor)

CDC25L

► RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor)

CDC25Mm

► RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor)

Cdc2l

► CDK11

CDC2L1

► CDK11

CDC2L2

► CDK11

CDC2L3

▶ CDK11

Cdc7

Hisao Masai
Genome Dynamics Project, Department of Genome Medicine, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Synonyms

Cdc7 (*S. cerevisiae*); Cdc7L1; Hsk1 (homologue of Cdc7 [seven] kinase, *S. pombe*); Spo4 (second Cdc7 homologue in *S. pombe*)

Historical Background

Cdc7 was originally discovered as a temperature-sensitive mutant of budding yeast defective in progression of cell cycle (Hartwell 1973). The growth of *cdc7* (ts) is arrested immediately before the onset of the S phase at a nonpermissive temperature. Upon return to a permissive temperature, cells resume growth and can complete S phase in the absence of ongoing protein synthesis. This led to the notion that Cdc7 is required for DNA replication at the stage where all other proteins required for DNA synthesis are prepared. Cdc7 was later cloned and was identified as encoding a serine/threonine kinase (Hollingsworth and Sclafani 1990). Dbf4, another cell cycle regulator required at the onset of the S phase, was then shown to encode an activation subunit for Cdc7. An ortholog of Cdc7 was first identified in fission yeast (Hsk1; (Masai et al. 1995)) and was later shown to be conserved in higher eukaryotes as well (Sato et al. 1997).

Structure of Cdc7 Kinase and Activation by Dbf4 Subunit

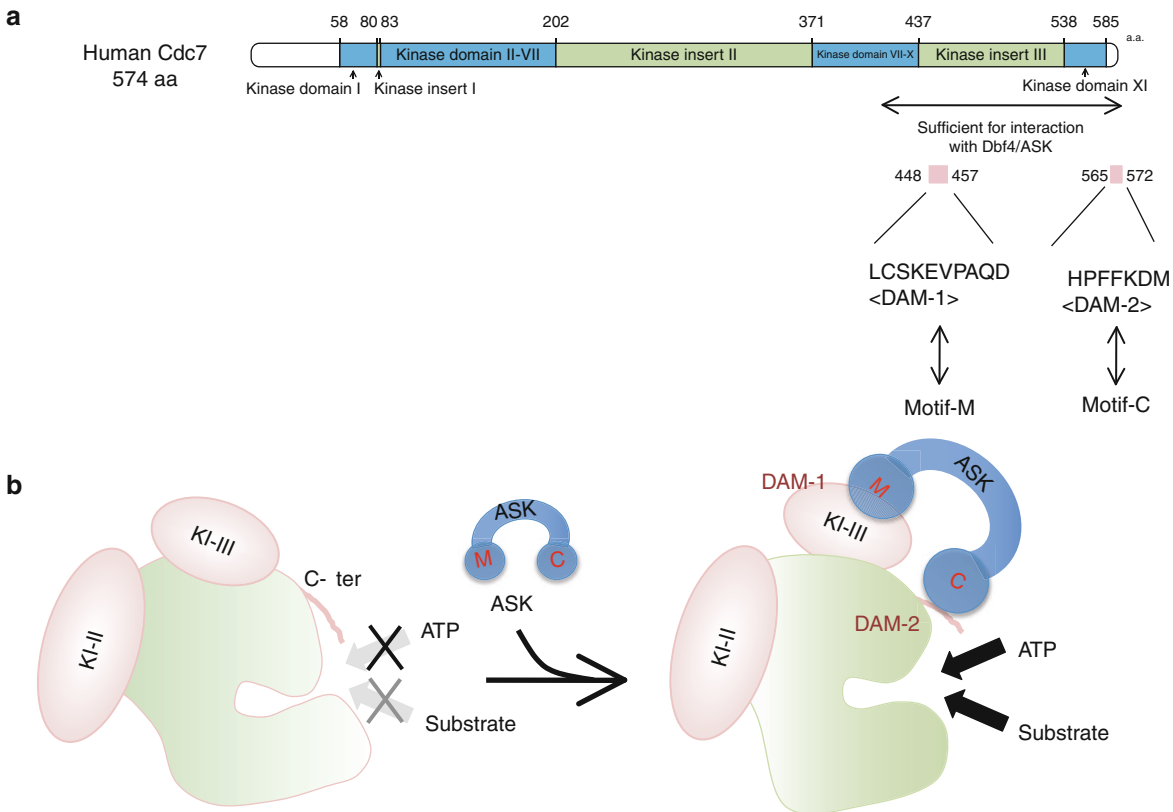
Cdc7 belongs to a rather unique branch of the kinase phylogeny tree and the closest member may be the casein kinase. Cdc7 kinase is unique in that it carries two or three so-called kinase insert sequences at the conserved locations within the kinase-conserved domains (Kim et al. 1998). The sequences and lengths of the kinase insert are not generally conserved. Analyses in human Cdc7 kinase indicated that the kinase insert II between the kinase domains VII and VIII is required for efficient nuclear localization as well as for chromatin association of Cdc7 (Kim et al. 1998; Masai and Arai 2002), whereas a small segment (DAM-1, Dbf4/ASK Association Motif-1) in the kinase insert III between the domains X and XI is required for interaction with Dbf4/ASK activation subunit. A conserved sequence present at the C-terminus of Cdc7 (DAM-2) also plays an essential role in interaction with Dbf4/ASK. Furthermore, DAM-1 and DAM-2 were shown to interact with motif-M and motif-C of Dbf4/ASK (Kim et al. 2007), respectively. Dbf4/ASK stimulates binding of ATP to the Cdc7 catalytic subunit as well as recognition of specific substrates ((Ogino et al. 2001); Fig. 1).

Identification of Orthologs of Cdc7 in Other Species

An ortholog of budding yeast Cdc7 was first identified in fission yeast (*hsk1*$^+$; (Masai et al. 1995)). Hsk1 and Cdc7 shares about 40% identity in the kinase-conserved domains (Kim et al. 1998). Hsk1 contains characteristic insert sequences at the conserved locations among the kinase-conserved domains. Cdc7 homologues were then identified in humans, mice, and *Xenopus* (Jiang and Hunter 1997; Sato et al. 1997; Kim et al. 1998). It is now known to be conserved over a wide range of species (Masai and Arai 2002). In fission yeast, another kinase, *spo4*$^+$, related to Cdc7 was identified (Nakamura et al. 2002). Spo4 is expressed specifically during late meiosis and forms a complex with Spo6, a meiosis-specific Dbf4-like molecule. Spo4-Spo6 is required for second meiotic cell division and sporulation.

Regulation of Expression

In normal proliferating yeast cells, Cdc7 is expressed constitutively during cell cycle. In fission yeast, the transcription of *hsk1*$^+$ slightly oscillates during cell



Cdc7, Fig. 1 Structure of Cdc7 kinase and interaction with Dbf4/ASK subunit. (**a**) Schematic drawing of amino acid sequences of human Cdc7. Two small segments of Cdc7 (*pink boxes*) termed DAM-1 and DAM-2 interact with motif-M and motif-C, respectively, of Dbf4/ASK protein (Ogino et al. 2001; Kitamura et al. 2011). (**b**) Schematic drawing showing interaction between Cdc7 and Dbf4/ASK. The interaction induces conformational change in Cdc7, thus permitting access to ATP and facilitating the recognition of substrates

cycle, peaking at the G1/S boundary. However, the protein level of Hsk1 is more or less constant during cell cycle (Takeda et al. 1999). In mammalian cells, the transcription of Cdc7 is under regulation of E2F transcription factor (Kim et al. 1998). Its transcription is repressed in quiescent cells, and induced after addition of mitogenic stimuli. The human Cdc7 transcription is regulated also during the cell cycle, increasing at late G1 through early S phase. The Cdc7 protein level also increases after growth stimulation of quiescent cells. In cycling cells, human Cdc7 protein level decreases during G1 phase, probably due to proteolytic degradation, since the Cdc7 protein expressed from a constitutive promoter similarly oscillates during cell cycle (Masai et al. 2006).

Cdc7 protein associates with chromatin during S phase and dissociates from it during G2/M phase (Masai et al. 2006). It is expressed also during M phase, and is present in cytoplasm. It is phosphorylated by Cdk (cyclin-dependent kinase) during M phase, and the phosphorylated Cdc7 is dissociated from chromatin, although the significance of this phosphorylation is not clear at the moment.

Substrate Specificity of Cdc7 Kinase

Cdc7 is a serine-threnine kinase, the primary structure of which shares some similarity with casein kinase. Like casein kinase, Cdc7 is an acidophylic kinase, favoring the acidic environment surrounding the target site. It has been reported that prior phosphorylation by another kinase such as Cdk or a checkpoint kinase can stimulate Cdc7-mediated phosphorylation by creating acidic environment surrounding the target sites (Masai et al. 2000; Montagnoli et al. 2006; Wan et al. 2008; Randell et al. 2010). SSP or STP may be one of the typical Cdc7 target sequences in which second serine

or threonine is first phosphoryalated by Cdk and the first serine is subsequently phosphorylated by Cdc7 (Cho et al. 2006; Masai et al. 2000; Montagnoli et al. 2006; Wan et al. 2008).

Functions in Initiation of DNA Replication

Cdc7 is essential for cell viability in yeasts under normal growth condition. Conditional knockout of Cdc7 gene in ES cells resulted in inhibition of DNA synthesis and eventual cell death due to accumulation of DNA damages (Kim et al. 2002), indicating that Cdc7 is also essential for mammalian cell growth. Early characterization of *cdc7*(ts) in budding yeast suggested its essential role in initiation of S phase. Later, it was shown that Cdc7 is required for initiation of DNA replication at each replication origin throughout S phase (Donaldson et al. 1998; Bousset and Diffley 1998). One-hybrid assays showed association of Dbf4 with replication origins in budding yeast (Dowell et al. 1994). These findings led to the notion that the pre-RC generated at each origin may be the target of Cdc7. Indeed, human Cdc7 was shown to phosphorylate MCM (minichromosome maintenance) in vitro (Sato et al. 1997). In budding yeast, MCM2 was shown to be phosphorylated in a manner dependent on Cdc7 (Lei et al. 1997). Accumulating evidence pointed to MCM complex as a major conserved substrate of Cdc7 kinase. Phosphorylation of MCM2 and MCM4 at the N-terminal segments promotes association of Cdc45 ((Masai et al. 2006; Sheu and Stillman 2006); Fig. 2), facilitating generation of the replication fork complex containing an active DNA helicase. In fission yeast, Hsk1 was shown to be essential for loading of Sld3 which is needed for assembly of an active replication fork. Hsk1 was also shown to regulate the efficiency of origin firing in fission yeast.

A bypass mutation (*bob1*) of *cdc7* or *dbf4* function was reported (Hardy et al. 1997). *bob1* was mapped in mcm5 (P83L), supporting the importance of phosphorylation of MCM in Cdc7-mediated regulation of initiation. The mutation may cause conformational changes in the MCM complex which may be permissive for origin activation in the absence of Cdc7 (Hoang et al. 2007). More recently, deletion of an N-terminal segment of MCM4 was shown to bypass Cdc7 function. It was proposed that the sole essential function of Cdc7 in budding yeast is to relieve an inhibitory activity residing within this N-terminal domain of MCM4 (Sheu and Stillman 2010). It remains to be seen whether this is conserved in other species.

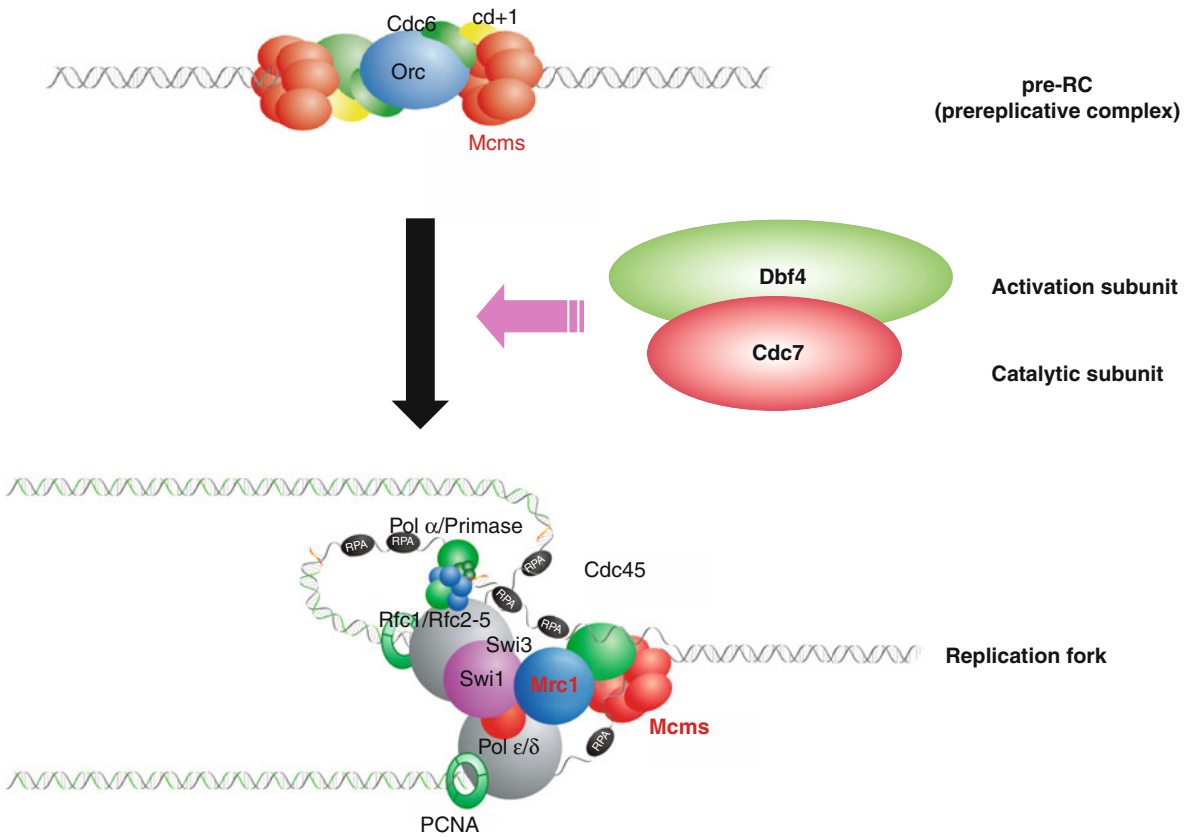
Functions During Mitotic Cell Cycle

Although the major and conserved role of Cdc7 kinase is in regulation of initiation of DNA replication, recent investigation has pointed to important roles of Cdc7 kinase in regulation of other mitotic events (see Fig. 3 and Table 1). Centrometric sister chromatid cohesion is partially impaired in *hsk1*(ts) (Takeda et al. 2001; Bailis et al. 2003). Consistent with this, the mutants are synthetic lethal with Rad21, a cohesin component, and is sensitive to the anti-microtubule drug thiabendazole (TBZ, (Takeda et al. 2001)). These results suggest a role of Hsk1 kinase in regulation of sister chromatid cohesion. In *Xenopus* egg extracts, Cdc7 was shown to form a complex with Scc2-Scc4 which is required for loading of cohesin onto chromatin (Takahashi et al. 2008). The kinase activity of Cdc7 is required for this process, although the exact target of Cdc7 is not known. It is also not known whether Cdc7 behaves similarly in other species including mammals.

In budding yeast, Cdc7-Dbf4 interacts with Polo kinase (*cdc5*) through N-terminal nonessential segment of Dbf4, and appears to regulate mitotic exit. In fission yeast as well, *hsk1*(ts) is synthetic lethal with a *plo1* (fission yeast homologue of Polo kinase) mutant (Matsumoto et al.). In human cancer cells, depletion of Cdc7 inhibited the cell-cycle progression from G2 phase arrest induced by nocodazole. These results suggest conserved roles of Cdc7 kinase during mitosis.

Functions During Meiotic Cell Cycle

Important roles of Cdc7 in meiotic cell cycle were originally reported in *cdc7*(ts) mutants of budding yeast (see Fig. 3 and Table 1). Defect in meiotic recombination and in synaptonemal complex formation were initially observed, while premeiotic DNA replication was reported not to be significantly affected. More recently, using fission yeast *hsk1*(ts) mutant, it was demonstrated that Hsk1 is required for formation of DSBs (double-stranded DNA breaks) which is prerequisite for meiotic recombination



Cdc7, Fig. 2 Roles of Cdc7 kinase in initiation of DNA replication. Cdc7-Dbf4 kinase recognizes pre-replicative complex (pre-RC) on the chromosome and phosphorylates MCM complex. This will facilitate the recruitment of replisome factors including Cdc45 required for generation of an active replication fork

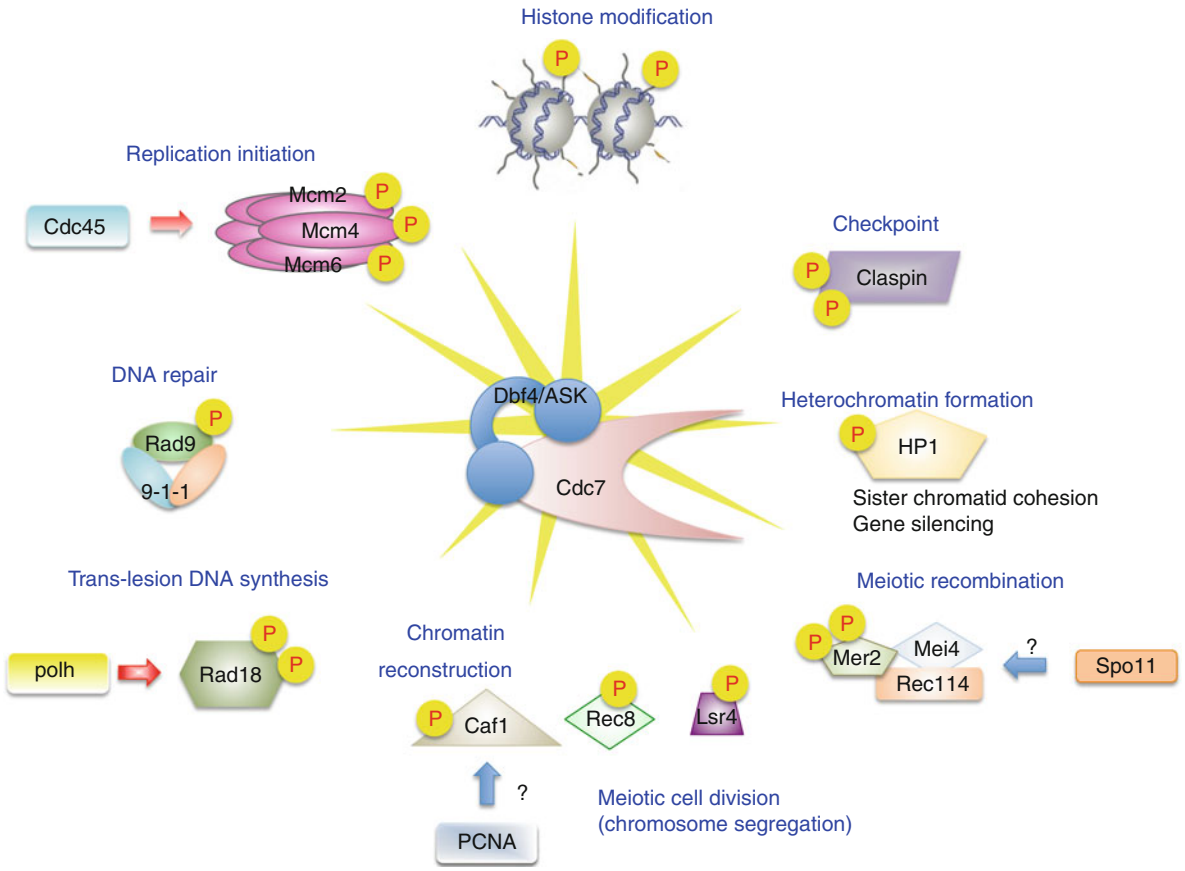
(Ogino et al. 2006). Premeiotic DNA replication could continue in *hsk1*(ts) mutant, albeit at a slightly slower rate. Similar conclusion was reached in budding yeast. However, using a tet-repressible transcription system, premeiotic DNA replication was shown to require Dbf4 function in budding yeast. Thus, it appears that Cdc7 may facilitate the initiation of premeiotic DNA replication but its requirement may be less stringent than for mitotic DNA replication. Phosphorylation of Mer2 protein, a component of the Mer2-Mei4-Rec114 complex required for initiation of meiotic recombination, by combined actions of Cdk and Cdc7 was shown to play essential roles in generation of meiotic DSB (Sasanuma et al. 2008; Wan et al. 2008).

Cells arrest with one nucleus state in a *hsk1*(ts) mutant in meiosis, suggesting that Hsk1 is required also for meiotic cell division. Consistent with this prediction, Cdc7 in budding yeast, in conjunction with casein kinase 1, was shown to phosphorylate Rec8 and to regulate cohesion cleavage by separase. Cdc7 was also shown to play essential roles in separation of the homologous chromosomes during meiosis I by phosphorylating Lsr2 protein and also by stimulating NDT80 transcription (Lo et al. 2008; Matos et al. 2008).

Cdc7 during meiosis is likely to be conserved in mammalian cells. Cdc7 hypomorphic mutant mice were sterile and germ cell development was severely disrupted (Kim et al. 2003). Sperm development was arrested at an early prophase, consistent with completion of premeiotic S phase but arrest before meiotic cell division.

Roles of Cdc7 in Other Chromosome Transactions

Cdc7 regulates chromosome processes other than DNA replication (see Fig. 3 and Table 1). It regulates



Cdc7, Fig. 3 Chromosome dynamics regulated by Cdc7 kinase. Various chromosome transactions, as indicated in the figure, are regulated by Cdc7 kinase. The potential target proteins in these events are shown. The red arrows indicate the recruitment by the phosphorylation which has been proven, and blue arrows indicate that which has been speculated but not been proven

DNA replication checkpoint, since *hsk1*(ts) is sensitive to HU and checkpoint kinase Cds1 is not fully activated in this mutant (Takeda et al. 2001) as well as in *cdc7* deletion and hsk1deletion cells made viable by a suppressor. Activation of Chk1 in response to replication stress in human cells also requires Cdc7 function and claspin may be a target of Cdc7 kinase during this process as well as during the normal course of DNA replication (Kim et al. 2008).

Roles of Cdc7 in UV-induced mutagenesis were suggested in the initial characterization of budding yeast *cdc7*(ts) mutants. More recently, budding yeast cdc7 was shown to be required for trans-lesion synthesis branch of Rad6 epistasis group (Pessoa-Brandão et al. 2004). In humans, Cdc7 phosphorylates a C-terminal segment of Rad18 and this phosphorylation facilitates the recruitment of a lesion-bypass polymerase, Polη (Rad30 counterpart), at the site of damage though its interaction with the phoshophorylated Rad18 (Day et al. 2010).

In fission yeast, Hsk1 phoshporylates Rad9 clamp loader and induces its dissociation from the damaged sites, facilitating the repair process (Furuya et al. 2010). Replication-associated phosphorylation of threonine 45 of Histone H3 is mediated by Cdc7-Dbf4 in budding yeast and may play a role in maintenance of genomic integrity during DNA replication and repair (Baker et al. 2010). Human Cdc7 interacts with chromatin assembly factor 1 (CAF1), and phosphorylates p150 subunit. This phosphorylation facilitates interaction of Caf1 with PCNA (proliferating cell nuclear antigen, known as a clamp protein) (Gérard et al. 2006). Thus, Cdc7 may play a role in the coordination between DNA replication and chromatin assembly by Caf1.

Cdc7, Table 1 List of chromosome events regulated by Cdc7 kinase, potential target proteins, and known phosphorylation sites mediated by Cdc7

Chromosome transactions	Target	Phosphorylation sites	Collaboration with Cdk?	Protein recruited	References
DNA replication	MCM2 MCM4	S26, S40, and other SSP	Yes (S27, S41/T7)	Cdc45 (Sld3)	(Masai et al. 2000, 2006; Montagnoli et al. 2006; Sheu and Stillman 2006)
Meiotic recombination	Mer2/Rec7	S29 and S11,15,19,22 (Mer2)	Yes (S30)	Spo11/ Rec12	(Ballabeni et al. 2009; Landis and Tower 1999; Takahashi and Walter 2005)
Trans-lesion DNA synthesis	Rad18	S434 and S432,433,436, 438,441,442,443,444	?	Polh	(Day et al. 2010)
DNA repair	Rad9	S319,320,T321	?	?	(Furuya et al. 2010)
Checkpoint	Mrc1/ Claspin	?	?	?	(Masai et al. 2010)
Chromatin formation	Cac1	?	?	?	(Gérard et al. 2006)
Meiosis I	Monopolin Lrs4	?	?	?	(Tudzarova et al. 2010; Montagnoli et al. 2004)
Meiosis I	Rec8	?	?	?	(Brott and Sokol 2005)
Heterochromatin formation	HP1	?	?	?	(Bailis et al. 2003)
Histone mod.	Histone H3	T46	?	?	(Baker et al. 2010)
Sister chromatid cohesion	Rad21?, Scc2?	?	?	?	(Takeda et al. 2001; Takahashi et al. 2008)

Proteins Interacting with Cdc7

Cdc7 is an enzyme which may interact with the substrates only transiently. Thus, interaction with Cdc7-Dbf4 kinase is generally weak. Physical interaction with MCM subunits have been reported, as expected from the fact that MCM is one of the most robust substrates of Cdc7. Yeast two-hybrid assays with mouse Cdc7 showed its interaction with Orc1 and 6 and with MCM2, 4, 5, and 7 (Kneissl et al. 2003).

Interaction of Cdc7 with other replication factors has been reported. In human cells and fission yeast, Cdc7 (Hsk1) interacted with Claspin (Mrc1) protein (Kim et al. 2008; Uno and Masai 2011). In human cells, Cdc7 interacts with Cdt1 and this interaction may facilitate the loading of Cdc45 onto chromatin (Ballabeni et al. 2009).

Developmental Role of Cdc7

The *Drosophila* chiffon mutant exhibits thin, fragile chorions and female sterility, and is deficient in chorion gene amplification (Landis and Tower 1999). The mutant also shows rough eyes and thin thoracic bristles. Chiffon encodes a Dbf4-like molecule which is required for normal development of Drosophila.

In *Xenopus* egg extracts, Cdc7-Drf1/ASKL1 is a major Cdc7 complex, but the level of Drf1/ASKL1 decreases and that of Dbf4 increases after gastrulation, when the cell cycle acquires somatic characteristics (Takahashi and Walter 2005). It is not known whether similar developmental regulation operates in human cells.

Xenopus Dbf4 (XDbf4) is required for heart and eye development. XDbf4 inhibits Wnt signaling through interaction with Frodo. This role of XDbf4 is independent of its function as an activator of Cdc7 kinase, since XDbf4 that cannot activate Cdc7 can still rescue the cardiac marker expression induced by XDbf4 depletion (Brott and Sokol 2005).

Cdc7 as a Target for Novel Cancer Therapy

Inhibition of Cdc7 expression in normal somatic cells generally causes stable G1 arrest (Tudzarova et al. 2010; Montagnoli et al. 2004). On the other hand, inhibition of Cdc7 in cancer cell lines causes robust cell death regardless of the p53 status. Generally in p53-negative background, cells tend to get arrested at G2 and then proceed into aberrant mitosis, whereas p53-positive cancer cells have tendency to proceed into aberrant S phase, before they die. These incidents

happen because of deficiency in various checkpoint systems found in cancer cells. Efforts are being made to develop specific chemical inhibitors of Cdc7 kinase and use them as potent agents that may induce cancer cell-specific cell death. Indeed, panels of low molecular weight chemical compounds have been identified that inhibit Cdc7 kinase in vitro and can suppress tumor growth in xenograft cancer model mice (Montagnoli et al. 2008). Those who are interested in learning more about eukaryotic DNA replication are recommended to read the reference (Masai et al. 2010).

Summary

Cdc7 is a serine-threonine kinase conserved from yeasts to human and regulates initiation of DNA replication by phosphorylating MCM, essential components of pre-replicative complexes generated at prospective replication origins. This phosphorylation triggers recruitment of Cdc45 and other replisome proteins to generate active replication forks. Cdc7 forms a complex with an activation subunit Dbf4/ASK to generate an active kinase. In addition to DNA replication, Cdc7 regulates varieties of chromosome dynamics including meiotic DNA recombination, meiotic cell division, DNA damage repair through trans-lesion DNA synthesis, DNA replication checkpoint and chromatin structures. Cdc7 emerges as a novel cancer therapy target since inhibition of Cdc7 in cancer cells causes efficient cell death by inducing abortive DNA synthesis or aberrant mitosis while that in normal cells generally arrests the cell growth in G1.

References

Bailis JM, Bernard P, Antonelli R, Allshire RC, Forsburg SL. Hsk1-Dfp1 is required for heterochromatin-mediated cohesion at centromeres. Nat Cell Biol. 2003;5:1111–6.

Baker SP, Phillips J, Anderson S, Qiu Q, Shabanowitz J, Smith MM, Yates JR. 3rd, Hunt DF, Grant PA. Histone H3 Thr 45 phosphorylation is a replication-associated post-translational modification in *S. cerevisiae*. Nat Cell Biol. 2010;12:294–8.

Ballabeni A, Zamponi R, Caprara G, Melixetian M, Bossi S, Masiero L, Helin K. Human CDT1 associates with CDC7 and recruits CDC45 to chromatin during S phase. J Biol Chem. 2009;284:3028–36.

Bousset K, Diffley JF. The Cdc7 protein kinase is required for origin firing during S phase. Genes Dev. 1998;12:480–90.

Brott BK, Sokol SY. A vertebrate homolog of the cell cycle regulator Dbf4 is an inhibitor of Wnt signaling required for heart development. Dev Cell. 2005;8:703–15.

Cho WH, Lee YJ, Kong SI, Hurwitz J, Lee JK. CDC7 kinase phosphorylates serine residues adjacent to acidic amino acids in the minichromosome maintenance 2 protein. Proc Natl Acad Sci USA. 2006;103:11521–6.

Day TA, Palle K, Barkley LR, Kakusho N, Zou Y, Tateishi S, Verreault A, Masai H, Vaziri C. Phosphorylated Rad 18 directs DNA Polymerase {eta} to sites of stalled replication. J Cell Biol. 2010;191:953–66.

Donaldson AD, Fangman WL, Brewer BJ. Cdc7 is required throughout the yeast S phase to activate replication origins. Genes Dev. 1998;12:491–501.

Dowell SJ, Romanowski P, Diffley JF. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science. 1994;265:1243–6.

Furuya K, Miyabe I, Tsutsui Y, Paderi F, Kakusho N, Masai H, Niki H, Carr AM. DDK phosphorylates checkpoint clamp component Rad9 and promotes its release from damaged chromatin. Mol Cell. 2010;40:606–18.

Gérard A, Koundrioukoff S, Ramillon V, Sergère JC, Mailand N, Quivy JP, Almouzni G. The replication kinase Cdc7-Dbf4 promotes the interaction of the p150 subunit of chromatin assembly factor 1 with proliferating cell nuclear antigen. EMBO Rep. 2006;7:817–23.

Hardy CF, Dryga O, Seematter S, Pahl PM, Sclafani RA. mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci USA. 1997;94:3151–5.

Hartwell LH. Three additional genes required for deoxyribonucleic acid synthesis in *Saccharomyces cerevisiae*. J Bacteriol. 1973;115:966–74.

Hoang ML, Leon RP, Pessoa-Brandao L, Hunt S, Raghuraman MK, Fangman WL, Brewer BJ, Sclafani RA. Structural changes in Mcm5 protein bypass Cdc7-Dbf4 function and reduce replication origin efficiency in *Saccharomyces cerevisiae*. Mol Cell Biol. 2007;27:7594–602.

Hollingsworth Jr RE, Sclafani RA. DNA metabolism gene CDC7 from yeast encodes a serine (threonine) protein kinase. Proc Natl Acad Sci USA. 1990;87:6272–6.

Jiang W, Hunter T. Identification and characterization of a human protein kinase related to budding yeast Cdc7p. Proc Natl Acad Sci USA. 1997;94:14320–5.

Kim JM, Sato N, Yamada M, Arai K, Masai H. Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to *Saccharomyces cerevisiae* CDC7 essential for G1/S transition. Structure, chromosomal localization, and expression of mouse gene for *S. cerevisiae* Cdc7-related kinase. J Biol Chem. 1998;273:23248–57.

Kim JM, Nakao K, Nakamura K, Saito I, Katsuki M, Arai K, Masai H. Inactivation of Cdc7 kinase in mouse ES cells results in S-phase arrest and p53-dependent cell death. EMBO J. 2002;21:2168–79.

Kim JM, Takemoto N, Arai K, Masai H. Hypomorphic mutation in an essential cell-cycle kinase causes growth retardation and impaired spermatogenesis. EMBO J. 2003;22:5260–72.

Kim BJ, Kim SY, Lee H. Identification and characterization of human cdc7 nuclear retention and export sequences in the context of chromatin binding. J Biol Chem. 2007;282:30029–38.

Kim JM, Kakusho N, Yamada M, Kanoh Y, Takemoto N, Masai H. Cdc7 kinase mediates Claspin phosphorylation in DNA replication checkpoint. Oncogene. 2008;27:3475–82.

Kitamura R, Fukatsu R, Kakusho N, Cho YS, Taniyama C, Yamazaki S, Toh GT, Yanagi K, Arai N, Chang HJ, Masai H. Molecular mechanism of activation of human Cdc7 kinase: bipartite interaction with Dbf4/ASK stimulates ATP binding and substrate recognition. J Biol Chem. 2011;286:23031–43.

Kneissl M, Pütter V, Szalay AA, Grummt F. Interaction and assembly of murine pre-replicative complex proteins in yeast and mouse cells. J Mol Biol. 2003;327:111–28.

Landis G, Tower J. The Drosophila chiffon gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. Development. 1999;126:4281–93.

Lei M, Kawasaki Y, Young MR, Kihara M, Sugino A, Tye BK. Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev. 1997;11:3365–74.

Lo HC, Wan L, Rosebrock A, Futcher B, Hollingsworth NM. Cdc7-Dbf4 regulates NDT80 transcription as well as reductional segregation during budding yeast meiosis. Mol Biol Cell. 2008;19:4956–67.

Masai H, Arai K. Cdc7 kinase complex: a key regulator in the initiation of DNA replication. J Cell Physiol. 2002;190:287–926. Review.

Masai H, Miyake T, Arai K. hsk1+, a Schizosaccharomyces pombe gene related to Saccharomyces cerevisiae CDC7, is required for chromosomal replication. EMBO J. 1995;14:3094–104.

Masai H, Matsui E, You Z, Ishimi Y, Tamai K, Arai K. Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 by Cdks. J Biol Chem. 2000;275:29042–52.

Masai H, Taniyama C, Ogino K, Matsui E, Kakusho N, Matsumoto S, Kim JM, Ishii A, Tanaka T, Kobayashi T, Tamai K, Ohtani K, Arai K. Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem. 2006;281:39249–61.

Masai H, Matsumoto S, You Z, Yoshizawa-Sugata N, Oda M. Eukaryotic chromosome DNA replication: where, when, and how? Annu Rev Biochem. 2010;79:89–130.

Matos J, Lipp JJ, Bogdanova A, Guillot S, Okaz E, Junqueira M, Shevchenko A, Zachariae W. Dbf4-dependent CDC7 kinase links DNA replication to the segregation of homologous chromosomes in meiosis I. Cell. 2008;135:662–78.

Montagnoli A, Tenca P, Sola F, Carpani D, Brotherton D, Albanese C, Santocanale C. Cdc7 inhibition reveals a p53-dependent replication checkpoint that is defective in cancer cells. Cancer Res. 2004;64:7110–6.

Montagnoli A, Valsasina B, Brotherton D, Troiani S, Rainoldi S, Tenca P, Molinari A, Santocanale C. Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J Biol Chem. 2006;281:10281–90.

Montagnoli A, Valsasina B, Croci V, Menichincheri M, Rainoldi S, Marchesi V, Tibolla M, Tenca P, Brotherton D, Albanese C, Patton V, Alzani R, Ciavolella A, Sola F, Molinari A, Volpi D, Avanzi N, Fiorentini F, Cattoni M, Healy S, Ballinari D, Pesenti E, Isacchi A, Moll J, Bensimon A, Vanotti E, Santocanale C. A Cdc7 kinase inhibitor restricts initiation of DNA replication and has antitumor activity. Nat Chem Biol. 2008;4:357–65.

Nakamura T, Nakamura-Kubo M, Nakamura T, Shimoda C. Novel fission yeast Cdc7-Dbf4-like kinase complex required for the initiation and progression of meiotic second division. Mol Cell Biol. 2002;22:309–20.

Ogino K, Takeda T, Matsui E, Iiyama H, Taniyama C, Arai K, Masai H. Bipartite binding of a kinase activator activates Cdc7-related kinase essential for S phase. J Biol Chem. 2001;276:31376–87.

Ogino K, Hirota K, Matsumoto S, Takeda T, Ohta K, Arai K, Masai H. Hsk1 kinase is required for induction of meiotic dsDNA breaks without involving checkpoint kinases in fission yeast. Proc Natl Acad Sci USA. 2006;103:8131–6.

Pessoa-Brandão L, Sclafani RA. CDC7/DBF4 functions in the translesion synthesis branch of the RAD6 epistasis group in Saccharomyces cerevisiae. Genetics. 2004;167:1597–610.

Randell JC, Fan A, Chan C, Francis LI, Heller RC, Galani K, Bell SP. Mec1 is one of multiple kinases that prime the Mcm2-7 helicase for phosphorylation by Cdc7. Mol Cell. 2010;40:353–63.

Sasanuma H, Hirota K, Fukuda T, Kakusho N, Kugou K, Kawasaki Y, Shibata T, Masai H, Ohta K. Cdc7-dependent phosphorylation of Mer2 facilitates initiation of yeast meiotic recombination. Genes Dev. 2008;22:398–410.

Sato N, Arai K, Masai H. Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. EMBO J. 1997;16:4340–51.

Sheu YJ, Stillman B. Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell. 2006;24:101–13.

Sheu YJ, Stillman B. The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4. Nature. 2010;463:113–7.

Takahashi TS, Walter JC. Cdc7-Drf1 is a developmentally regulated protein kinase required for the initiation of vertebrate DNA replication. Genes Dev. 2005;19:2295–300.

Takahashi TS, Basu A, Bermudez V, Hurwitz J, Walter JC. Cdc7-Drf1 kinase links chromosome cohesion to the initiation of DNA replication in Xenopus egg extracts. Genes Dev. 2008;22:1894–905.

Takeda T, Ogino K, Matsui E, Cho MK, Kumagai H, Miyake T, Arai K, Masai H. A fission yeast gene, him1(+)/dfp1(+), encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. Mol Cell Biol. 1999;19:5535–47.

Takeda T, Ogino K, Tatebayashi K, Ikeda H, Ki A, Masai H. Regulation of initiation of S phase, replication checkpoint signaling, and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. Mol Biol Cell. 2001;12:1257–74.

Tudzarova S, Trotter MW, Wollenschlaeger A, Mulvey C, Godovac-Zimmermann J, Williams GH, Stoeber K. Molecular architecture of the DNA replication origin activation checkpoint. EMBO J. 2010;29:3381–94.

Uno S, Masai H. Efficient expression and purification of human replication fork-stabilizing factor, Claspin, from mammalian cells: DNA-binding activity and novel protein interactions. Genes Cells. 2011;16:842–56.
Wan L, Niu H, Futcher B, Zhang C, Shokat KM, Boulton SJ, Hollingsworth NM. Cdc28-Clb5 (CDK-S) and Cdc7-Dbf4 (DDK) collaborate to initiate meiotic recombination in yeast. Genes Dev. 2008;22:386–97.

Cdc7 (*S. cerevisiae*)

▶ Cdc7

Cdc7L1

▶ Cdc7

CDK11

Dongli Hu and Jill M. Lahti
Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, USA

Synonyms

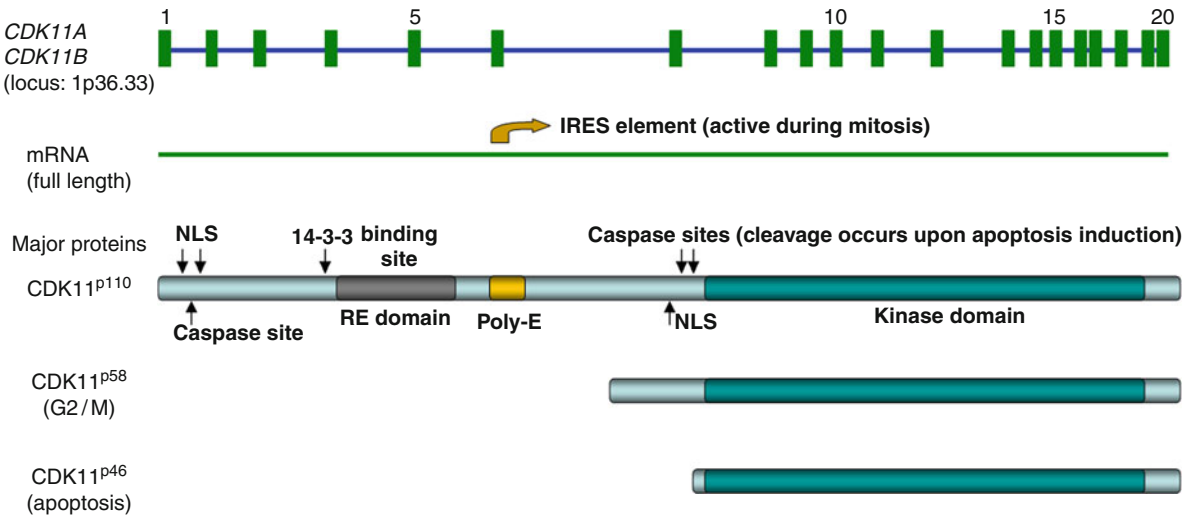
Cdc2l; CDC2L1; CDC2L2; CDC2L3; CDK11A^{p110}; CDK11A^{p46}; CDK11A^{p58}; CDK11B^{p110}; CDK11B^{p46}; CDK11B^{p58}; CDK11^{p110}; CDK11^{p46}; CDK11^{p58}; FLJ59152; MGC131975; p58; p58CDC2L1; p58CLK-1; p58GTA; PITSLRE; PK58

Historical Background

The CDK11A and CDK11B kinases (cyclin-dependent kinase 11A and B), formerly known as PITSLRE protein kinases, are serine/threonine protein kinases that belong to p34CDC2-related protein kinase superfamily. p34CDC2-related kinases play essential roles in many aspects of the cellular processes, especially cell cycle control and the regulation of transcription. There are two *CDK11* genes in humans (*CDK11A* and *CDK11B* formerly known as *CDC2L2* and *CDC2L1*), and one *CDK11* gene in mouse (*Cdk11*, previously known as *cdc2*l) (Malumbres et al. 2009). *CDK11* genes are evolutionarily conserved. *CDK11* homologs are found in human, mouse, rat, frog, cattle, fruit fly, yeast, and even in protozoa amoeba (Trembley et al. 2004; Goldberg et al. 2006). The almost identical human *CDK11A* and *CDK11B* genes are tandemly located within ~140 kb on chromosome 1p36.33. Each gene contains 20 exons and 19 introns and the full-length CDK11A^{p110} and CDK11B^{p110} proteins differ by only 16 amino acids (Gururajan et al. 1998; Lahti et al. 1994; Trembley et al. 2004). All data to date suggest that these two genes are functional equivalent; therefore, to simplify the nomenclature we will use the term "CDK11" to represent the human genes and proteins as well as the single mouse gene and protein in the following text.

CDK11 mRNA and protein are expressed in all cell types examined to date, although the mRNA and proteins are most abundant in testes, bone marrow, spleen, brain, lymph nodes, thymus, and hematopoietic cell lines (Xiang et al.1994). The three major CDK11 isoforms are CDK11^{p110}, CDK11^{p58}, and CDK11^{p46} (Fig. 1). There are many human transcript variants encoding the most abundant CDK11 isoform, CDK11^{p110}. These transcripts are generated by alternate promoter usage and alternative splicing of exons 1–11 which are located upstream of the Ser/Thr protein kinase domain of the CDK11 genes. CDK11^{p110} is expressed throughout the cell cycle. In contrast, CDK11^{p58} is specifically expressed during G2/M (Gap 2 and mitosis) phase of the cell cycle. This protein is generated via an IRES (internal ribosome entry site) located within the coding region of the CDK11 full-length transcript (Cornelis et al. 2000; Wilker et al. 2007). Finally, CDK11^{p46} is produced by caspase-mediated cleavage of both CDK11^{p110} and CDK11^{p58} during apoptosis (Lahti et al. 1995; Beyaert et al. 1997; Tang et al. 1998). Like other CDKs, CDK11 protein kinases are associated with and regulated by the cyclin partners, cyclin L1 and L2 (Dickinson et al. 2002; Loyer et al. 2008).

CDK11 is an essential gene. Genetic deletion of the *Cdk11* gene in mouse leads to early embryonic lethality at E3.5 (embryonic day 3.5), while the heterozygous mice (CDK11$^{+/-}$mice) appear to develop



CDK11, Fig. 1 The schematic diagram of the human CDK11 gene, transcript, and the major protein isoforms. The human *CDK11A* and *CDK11B* genes are located on chromosome 1p36.33. The exons are represented by the *green bars*. Several known functioning motifs are indicated on the full-length CDK11^{p110} diagram, and the relative location of the internal ribosomal entry site (IRES) element facilitating the mitotic expression of CDK11^{p58} is indicated on the transcript. The caspase cleavage sites used for generating apoptotic CDK11^{p46} are also indicated by *arrows* on the full-length protein. Additional CDK11 isoforms are generated by alternative splicing of exons 1–11 of the pre-mRNA

normally (Li et al. 2004). Likewise, decreasing CDK11 expression using siRNA leads to mitosis abnormalities, apoptosis, and cell death (Hu et al. 2007; Petretti et al. 2006). Interestingly, heterozygous deletion or translocations of the human *CDK11* genes are frequently observed implying its role in tumorigenesis (Trembley et al. 2004). In addition to potential roles in tumorigenesis and mitosis, data published to date have shown that CDK11 is involved in transcription, RNA processing, neuronal signaling, apoptosis, and cell cycle regulation.

CDK11 and Transcription

The first clue that CDK11 might be involved in transcriptional regulation came from yeast two hybrid studies using CDK11^{p110} as a bait protein. The RNA polymerase II transcription elongation factor ELL2 was identified as a CDK11^{p110} interacting protein in these studies (Trembley et al. 2002). Characterization of purified CDK11 complexes from Hela nuclear extracts revealed that the largest subunit of RNA polymerase II (RNAP II), the SSRP1 (structure-specific recognition protein 1), and SPT16 (chromatin-specific transcription elongation factor 140 kDa subunit) subunits of the transcription elongation factor FACT (facilitates chromatin transcription, a histone chaperone) and ▶ Casein kinase II (CKII) and ELL2, all proteins involved in transcription, are present in CDK11^{p110} complexes. Furthermore, co-immunoprecipitation analyses indicated that CDK11^{p110} associates with the TFIIF (transcription initiation factor IIF), TFIIS (transcription elongation factor IIS), and both hypo- and hyperphosphorylated forms of the largest subunit of RNAP II. Importantly, addition of CDK11 antibody, which bind to the carboxyl terminal domain of CDK11, and presumably disrupts interactions between CDK11 and transcriptional complex proteins, substantially reduced the production of RNA transcript in an in vitro transcription assay (Trembley et al. 2002, 2003).

CDK1, CDK7, CDK8, and CDK9 are also involved transcription regulators and phosphorylate the carboxyl-terminal domain (CTD) of RNAP II complexes to positively or negatively regulate transcription (reviewed in Hirose and Ohkuma 2007). In contrast, CDK11^{p110} does not directly phosphorylate the RNAPII CTD; however, CDK11^{p110} interacts with casein kinase II (CKII) which phosphorylates both CDK11 and the RNAP II CTD (Trembley et al. 2002, 2003, 2004). Despite data clearly indicating that CDK11^{p110} interacts with transcriptional proteins and is required for high level in vitro transcription, the

exact role of CDK11^{p110} in transcription in vivo and the biological substrates of the kinase remain undetermined.

CDK11 and RNA Processing

The role of CDK11^{p110} is consistent with the fact that CDK11^{p110} has several putative nuclear localization signal sequences. Indirect immunofluorescence analysis of CDK11 localization revealed that a portion of the cellular CDK11^{p110} is diffusely localized in the nucleoplasm, while the majority of the protein is found in nuclear speckles. These nuclear speckles also contain general splicing factors suggesting that CDK11^{p110} may also play a role in splicing. Consistent with this hypothesis, the splicing factors RNPS1 and 9G8 were identified as CDK11^{p110} interacting proteins by yeast two-hybrid analyses and 9G8 is phosphorylated by CDK11^{p110} (Loyer et al. 1998; Hu et al. 2003). Additionally, the CDK11 regulatory proteins, cyclin L1 and L2, have roles in RNA splicing (Loyer et al. 1998, 2008; Dickinson et al. 2002; Hu et al. 2003).

Further evidence for the role of CDK11^{p110} in splicing was demonstrated both in vitro and in vivo. Immunodepletion of CDK11^{p110} from nuclear extract severely reduced splicing activity in an in vitro splicing assay using a β-globin minigene as a substrate, while re-addition of the CDK11^{p110} complexes restored splicing activity. Additionally, disrupting the interaction between CDK11^{p110} and other splicing component using a recombinant N-terminal 50 amino acids fragment of the CDK11^{p110} protein kinase greatly reduced splicing activity (Hu et al. 2003). Ectopic expression of cyclins L1α, L1β, L2α, L2β, or catalytically active CDK11^{p110} proteins individually enhances intron splicing activity, while expression of catalytically inactive form of CDK11, CDK11^{p110DN}, CDK11^{p58}, or CDK11^{p46} represses splicing activity when using the pTN24 transcription/splicing dual in vivo reporting system. More importantly, co-expression of cyclin Lα and Lβ and CDK11^{p110} alters constitutive splicing of the TN24 plasmid and alternative slicing of the E1A minigene reporter in vivo (Loyer et al. 2008). CDK11^{p110} and 9G8 also appear to be involved in mRNA 3′ end processing based on a recent study which showed that CDK11 and 9G8 are involved in regulating HIV-1 mRNA 3′ end processing. These investigators also found that the eukaryotic initiation factor 3 subunit f (eIF3F) can block the 3′ mRNA processing by altering the 3′ end pre-mRNA sequence recognition by 9G8 coupled with CDK11 (Valente et al. 2009). Taken together, these data indicates CDK11^{p110} protein kinase activity plays crucial role in pre-mRNA splicing processing possibly by coupling mRNA processing with transcription.

CDK11 and Mitosis

CDK11^{p58} is specifically expressed during the G2/M phase of the cell cycle from the same mRNA that produces CDK11^{p110} via an internal ribosome entry site (IRES) sequence in the coding region of the transcript (Cornelis et al. 2000). This uniquely regulated expression strongly suggested that CDK11^{p58}, most likely in association with cyclin L proteins, functions during mitosis. Defects in IRES directed cap-independent translation during mitosis (e.g., lack of translation of the IRES regulated protein 14-3-3σ) leads to reduced CDK11^{p58} expression and mitotic defects, such as the absence of polo-like kinase 1 (Plk1) at the midbody, the impairment of cytokinesis, and the accumulation of binucleated cells (Wilker et al. 2007). Further evidence that CDK11^{p58} expression is tightly regulated during G2/M comes from studies showing that minimal overexpression of CDK11^{p58} in CHO (Chinese hamster ovary) cells resulted in aneuploidy, a delay in cytokinesis exemplified by the present of a large number of cells with tubulin midbodies, telophase defects, and apoptosis (Lahti et al. 1995; Bunnell et al. 1990). More recent studies using siRNAs targeting CDK11 have shown that CDK11^{p58} is required for proper mitosis (Petretti et al. 2006; Hu et al. 2007). Moderate depletion of CDK11 expression in Hela cells using siRNAs targeting CDK11 causes misaligned chromosomes but does not prevent mitotic progression. Further diminution of CDK11 expression leads to centrosome morphological defects, centrosome number abnormalities, abnormal mitotic spindle, defective chromosome congression as well as premature sister chromatid separation, permanent mitotic arrest, and cell death. Importantly, the mitotic defects caused by CDK11 depletion can be rescued, at least in part, by ectopic expression of GFP-CDK11^{p58} (Hu et al. 2007; Petretti et al. 2006).

These studies and immunofluorescence experiments also revealed that CDK11 associates with

centrosomes throughout the cell cycle but that the association intensity is increased as the mitotic spindles form and the chromosomes congress. Staining is then reduced as the cells enter anaphase (Petretti et al. 2006). Studies in *Xenopus* system have also shown that CDK11 localizes on spindle poles and microtubules, stabilizes microtubules in RanGTP-dependent manner, and that CDK11 kinase activity is important for spindle assembly and function (Yokoyama et al. 2008).

CDK11 depletion also causes spindle checkpoint activation and mitotic arrest. The spindle checkpoint machinery does not function properly when CDK11 is depleted since sister chromatids are able to partially separate and BubR1 (a mitotic checkpoint regulatory serine/threonine-protein kinase) is retained on the separated kinetochores. Moreover, immunofluorescence studies on CDK11 RNAi-treated cells showed that the cohesion subunit Scc1 prematurely dissociates from the kinetochores and the localization of the cohesion guardian Sgo1 at the kinetochore region is altered (Hu et al. 2007). These data support the conclusion that CDK11 plays a crucial role in the regulation of sister chromatid cohesion.

Finally, *CDK11* gene knockout studies demonstrate a role for CDK11 in mitosis during development. Deletion of the mouse *Cdk11* gene resulted in embryonic lethality at E3.5. The CDK11-deficient blastocyst cells exhibited cell proliferation defects (as judged by the low level of BrdU incorporation) and mitotic arrest (illustrated by staining with Histone 3 phosphor-Ser10 antibody) (Li et al. 2004), indicating at least one isoform of the CDK11 protein kinase is involved in mitotic regulation.

CDK11 and Apoptosis

The early hypothesis that CDK11 might function in apoptosis was based on the discovery of the $CDK11^{p46}$ isoform. This 46kD isoform is generated by caspase-dependent cleavage of the preexisting pools of $CDK11^{p110}$ and $CDK11^{p58}$ upon the induction of apoptosis. $CDK11^{p46}$ contains the intact kinase catalytic domain but lacks the N-terminal region of p110 and the N-terminal 52 amino acids that are present in the $CDK11^{p58}$ isoform (Lahti et al. 1995; Beyaert et al. 1997). The production of this isoforms may be regulated by phosphorylation since phosphorylation of $CDK11^{p110}$ is coupled with caspase cleavage during Fas-mediated cell death (Tang et al. 1998). Ectopic expression of the kinase active form of $CDK11^{p46}$, not the inactive form, in CHO cells induces apoptosis (Lahti et al. 1995). It has also been reported that $CDK11^{p46}$ induces anoikis, a special form of apoptosis caused by disruption of cell–matrix interactions, and associates with PAK1 (p21-activated kinase 1) leading to the inhibition of PAK1 kinase activity (Chen et al. 2003). Other studies demonstrated that $CDK11^{p46}$ interacts with and phosphorylates elF3f, a subunit of eukaryotic elongation factor 3. The phosphorylation of elF3f enhances its association with the core elF3 complex during apoptosis, suggesting that phosphorylation of this protein by $CDK11^{p46}$ may inhibit protein translation by enhancing the binding of elF3f to the elF3 core during apoptosis (Shi et al. 2009). Like the other larger CDK11 isoforms, $CDK11^{p46}$ associates with cyclin L1α, L2α, and L2β, although it is not clear whether this association regulates $CDK11^{p46}$ function. Taken together the data mentioned above suggest $CDK11^{p46}$ is involved in apoptotic signaling pathway, like p34cdc2 (Shi et al. 1994).

CDK11 and Tumorigenesis

Given the many important biological functions observed thus far for CDK11, it is very logical to speculate that any genetic abnormalities for *CDK11* gene and its regulation defects could lead to severe human disease. Indeed, deletion of one allele of 1p36.3 chromosome region, which contains the two human *CDK11* genes, is often observed in human tumors, including neuroblastoma, melanoma, colon cancer, ovarian cancer, breast cancer, and pheochromocytoma (reviewed in Trembley et al. 2004). Although the $Cdk11^{+/-}$ heterozygous mice have normal development (Li et al. 2004) indicating that loss of *Cdk11* alone is not sufficient for tumorigenesis, when *Cdk11* heterozygous and control mice were challenged with skin cancer inducing carcinogens there was an threefold increase in the number of tumors per mouse and an increased frequency of larger papillomas in *Cdk11* heterozygous mice as compared to wild-type mice (Chandramouli et al. 2007). Furthermore, a 2.5-fold downregulation of *Cdk11* gene expression was found in the invasive mouse skin carcinomas in association with mutant ▶ *p53* and mutant H-*ras* status (Zhang et al. 2005). Another line of evidence implicating

CDK11 in tumorigenesis comes from the discovery that CDK11 regulates the Hh (hedgehog) signaling pathway. Disruption or improper activation of this pathway is associated with developmental abnormalities and tumorigenesis (Evangelista et al. 2008). CDK11 was also identified as a kinase regulator for Wnt/β-catenin signaling pathway in a high-throughput siRNA screening (Naik et al. 2009). These studies showed that decreasing CDK11 expression resulted in reduced β-catenin-dependent transcription suggesting that CDK11 could be a potential therapeutic target in tumors with dysregulation of Wnt/β-catenin signaling, for example, colorectal cancer, breast cancer, and hepatocellular carcinoma (Clevers 2006).

Loss of CDK11 also causes defects in cell cycle, especially during mitosis. Cell cycle misregulation is one of the hallmarks of cancer cells. As mentioned above, the G2/M-specific CDK11^{p58} isoform regulates mitotic progression (Petretti et al. 2006; Hu et al. 2007). Defects in IRES directed cap-independent translation machinery, which is responsible for generating most of the proteins that are produced during late G2 and mitosis, causes reduced CDK11^{p58} expression and which results in mitotic abnormalities and in turn contributes to aneuploidy and tumorigenesis (Wilker et al. 2007). Expression of the ▶ Myc oncogene in Eμ-Myc/+ mice disrupts IRES-mediated translation of proteins during mitosis and leads to tumor formation (Barna et al. 2008). Further analysis of these tumors revealed decreased CDK11^{p58} expression and increased aneuploidy (Barna et al. 2008). Importantly, this defect can be suppressed by restoring CDK11^{p58} expression to normal levels (Barna et al. 2008). These observations strongly suggest that *CDK11* may be a tumor suppressor gene.

CDK11, Table 1 The major functions of different CDK11 protein isoforms

CDK11 proteins	Major functions
CDK11^{p110}	Transcription
	RNA processing/splicing
	Hh signaling
	Wnt/β-catenin signaling
	Other functions?
CDK11^{p58}	Mitosis progression
	Centrosome function
	Spindle formation and functioning
	Sister chromatid cohesion
	Spindle checkpoint control
	Chromosome congression
	Other functions?
CDK11^{p46}	Apoptosis
	Other functions?

Summary

Since the discovery of *CDK11* gene roughly two decades ago, studies from many labs have advanced our understanding of *CDK11* gene function and regulation. Based on these studies it is now clear that CDK11 is essential for cell growth, and that the different CDK11 isoforms function in distinct cellular processes such as transcription, mRNA processing/splicing (CDK11^{p110}), mitotic progression (CDK11^{p58}), apoptosis (CDK11^{p46}), and tumorigenesis (Table 1). More detailed analysis is needed to establish the roles of the various CDK11 isoform in vivo and the roles in tumorigenesis. Likewise, although CDK11 protein kinases associate with cyclin L1 and L2, and co-expression of L cyclins and CDK11^{p110} strongly affect alternative splicing and results in differences in the number and type of transcripts that are produced by alternate splicing in comparison with expressing those proteins individually (Loyer et al. 2008), the detailed regulatory mechanisms on their cooperative and synergistic activities remain to be clarified. Current studies are also highly focused on identifying CDK11 kinase substrates. Since CDK11 protein kinases have multiple functions in different cellular processes, the identification of more bona fide substrates is a key step in improving our understanding of the function and regulation of the various CDK11 isoforms. To date, 9G8 and eIF3f are the only substrates that have been studied extensively. However, the exact CDK11^{p110} phosphorylation site (s) on 9G8 and the functional relevance of this phosphorylation are yet to be elucidated. The other challenge in understanding CDK11 function is generating appropriate molecular tools to define or discriminate between the many different CDK11 protein isoforms and splice variants and to determine their functions in various signaling pathways. Additionally, determining how CDK11 influences tumorigenesis remains an important question for further investigation. Generating CDK11 mouse models that allow modulation of CDK11 expression in specific tissues is necessary to

understand the role of CDK11 in tumorigenesis and to address the possibility of developing CDK11-specific protein kinase inhibitors for the therapeutic purposes.

References

Barna M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, Rao PH, Ruggero D. Suppression of Myc oncogenic activity by ribosomal protein haploinsufficiency. Nature. 2008;456:971–5.

Beyaert R, Kidd VJ, Cornelis S, Van de Craen M, Denecker G, Lahti JM, Gururajan R, Vandenabeele P, Fiers W. Cleavage of PITSLRE kinases by ICE/CASP-1 and CPP32/CASP-3 during apoptosis induced by tumor necrosis factor. J Biol Chem. 1997;272:11694–7.

Bunnell BA, Heath LS, Adams DE, Lahti JM, Kidd VJ. Increased expression of a 58-kDa protein kinase leads to changes in the CHO cell cycle. Proc Natl Acad Sci USA. 1990;87:7467–71.

Chen S, Yin X, Zhu X, Yan J, Ji S, Chen C, Cai M, Zhang S, Zong H, Hu Y, Yuan Z, Shen Z, Gu J. The C-terminal kinase domain of the p34cdc2-related PITSLRE protein kinase (p110C) associates with p21-activated kinase 1 and inhibits its activity during anoikis. J Biol Chem. 2003; 278:20029–36.

Clevers H. Wnt/β-catenin signaling in development and disease. Cell. 2006;127:469–80.

Cornelis S, Bruynooghe Y, Denecker G, Van Huffel S, Tinton S, Beyaert R. Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. Mol Cell. 2000;5:597–605.

Chandramouli A, Shi J, Feng Y, Holubec H, Shanas RM, Bhattacharyya AK, Zheng W, Nelson MA. Haploinsufficiency of the cdc2l gene contributes to skin cancer development in mice. Carcinogenesis. 2007;28:2028–35.

Dickinson LA, Edgar AJ, Ehley J, Gottesfeld JM. Cyclin L is an RS domain protein involved in pre-mRNA splicing. J Biol Chem. 2002;277:25465–73.

Evangelista M, Lim TY, Lee J, Parker L, Ashique A, Peterson AS, Ye W, Davis DP, de Sauvage FJ. Kinome siRNA screen identifies regulators of ciliogenesis and hedgehog signal transduction. Sci Signal. 2008;1(39):ra7.

Goldberg JM, Manning G, Liu A, Fey P, Pilcher KE, Xu Y, Smith JL. The dictyostelium kinome–analysis of the protein kinases from a simple model organism. PLoS Genet. 2006;2(3):e38.

Gururajan R, Lahti JM, Grenet J, Easton J, Gruber I, Ambros PF, Kidd VJ. Duplication of a genomic region containing the Cdc2L1-2 and MMP21-22 genes on human chromosome 1p36.3 and their linkage to D1Z2. Genome Res. 1998;8:929–39.

Hirose Y, Ohkuma Y. Phosphorylation of the C-terminal domain of RNA polymerase II plays central roles in the integrated events of eucaryotic gene expression. J Biochem. 2007;141:601–8.

Hu D, Mayeda A, Trembley JH, Lahti JM, Kidd VJ. CDK11 complexes promote pre-mRNA splicing. J Biol Chem. 2003;278:8623–9.

Hu D, Valentine M, Kidd VJ, Lahti JM. CDK11(p58) is required for the maintenance of sister chromatid cohesion. J Cell Sci. 2007;120:2424–34.

Lahti JM, Valentine M, Xiang J, Jones B, Amann J, Grenet J, Richmond G, Look AT, Kidd VJ. Alterations in the PITSLRE protein kinase gene complex on chromosome 1p36 in childhood neuroblastoma. Nat Genet. 1994;7:370–5.

Lahti JM, Xiang J, Heath LS, Campana D, Kidd VJ. PITSLRE protein kinase activity is associated with apoptosis. Mol Cell Biol. 1995;15:1–11.

Li T, Inoue A, Lahti JM, Kidd VJ. Failure to proliferate and mitotic arrest of CDK11(p110/p58)-null mutant mice at the blastocyst stage of embryonic cell development. Mol Cell Biol. 2004;24:3188–97.

Loyer P, Trembley JH, Lahti JM, Kidd VJ. The RNP protein, RNPS1, associates with specific isoforms of the p34cdc2-related PITSLRE protein kinase in vivo. J Cell Sci. 1998;111:1495–506.

Loyer P, Trembley JH, Grenet JA, Busson A, Corlu A, Zhao W, Kocak M, Kidd VJ, Lahti JM. Characterization of cyclin L1 and L2 interactions with CDK11 and splicing factors: influence of cyclin L isoforms on splice site selection. J Biol Chem. 2008;283:7721–32.

Malumbres M, Harlow E, Hunt T, Hunter T, Lahti JM, Manning G, Morgan DO, Tsai LH, Wolgemuth DJ. Cyclin-dependent kinases: a family portrait. Nat Cell Biol. 2009;11:1275–6.

Naik S, Dothager RS, Marasa J, Lewis CL, Piwnica-Worms D. Vascular endothelial growth factor receptor-1 is synthetic lethal to aberrant β-catenin activation in colon cancer. Clin Cancer Res. 2009;15:7529–37.

Petretti C, Savoian M, Montembault E, Glover DM, Prigent C, Giet R. The PITSLRE/CDK11p58 protein kinase promotes centrosome maturation and bipolar spindle formation. EMBO Rep. 2006;7:418–24.

Shi J, Hershey JW, Nelson MA. Phosphorylation of the eukaryotic initiation factor 3f by cyclin-dependent kinase 11 during apoptosis. FEBS Lett. 2009;583:971–7.

Shi L, Nishioka WK, Th'ng J, Bradbury EM, Litchfield DW, Greenberg AH. Premature p34cdc2 activation required for apoptosis. Science. 1994;263:1143–5.

Tang D, Gururajan R, Kidd VJ. Phosphorylation of PITSLRE p110 isoforms accompanies their processing by caspases during Fas-mediated cell death. J Biol Chem. 1998;273:16601–7.

Trembley JH, Hu D, Hsu LC, Yeung CY, Slaughter C, Lahti JM, Kidd VJ. PITSLRE p110 protein kinases associate with transcription complexes and affect their activity. J Biol Chem. 2002;277:2589–96.

Trembley JH, Hu D, Slaughter CA, Lahti JM, Kidd VJ. Casein kinase 2 interacts with cyclin-dependent kinase 11 (CDK11) in vivo and phosphorylates both the RNA polymerase II carboxyl-terminal domain and CDK11 in vitro. J Biol Chem. 2003;278:2265–70.

Trembley JH, Loyer P, Hu D, Li T, Grenet J, Lahti JM, Kidd VJ. Cyclin dependent kinase 11 in RNA transcription and splicing. Prog Nucleic Acid Res Mol Biol. 2004; 77:263–88.

Valente ST, Gilmartin GM, Venkatarama K, Arriagada G, Goff SP. HIV-1 mRNA 3′ end processing is distinctively regulated by eIF3f, CDK11, and splice factor 9G8. Mol Cell. 2009;36:279–89.

Wilker EW, van Vugt MA, Artim SA, Huang PH, Petersen CP, Reinhardt HC, Feng Y, Sharp PA, Sonenberg N, White FM, Yaffe MB. 14-3-3sigma controls mitotic translation to facilitate cytokinesis. Nature. 2007;446:329–32.
Xiang J, Lahti JM, Grenet J, Easton J, Kidd VJ. Molecular cloning and expression of alternatively spliced PITSLRE protein kinase isoforms. J Biol Chem. 1994;269:15786–94.
Yokoyama H, Gruss OJ, Rybina S, Caudron M, Schelder M, Wilm M, Mattaj IW, Karsenti E. Cdk11 is a RanGTP-dependent microtubule stabilization factor that regulates spindle assembly rate. J Cell Biol. 2008;180:867–75.
Zhang Z, Yao R, Li J, Wang Y, Boone CW, Lubet RA, You M. Induction of invasive mouse skin carcinomas in transgenic mice with mutations in both H-ras and p53. Mol Cancer Res. 2005;3:563–74.

CDK11A^{p110}

▶ CDK11

CDK11A^{p46}

▶ CDK11

CDK11A^{p58}

▶ CDK11

CDK11B^{p110}

▶ CDK11

CDK11B^{p46}

▶ CDK11

CDK11B^{p58}

▶ CDK11

CDK11^{p110}

▶ CDK11

CDK11^{p46}

▶ CDK11

CDK11^{p58}

▶ CDK11

CDW40

▶ CD40

CEACAMs

Bernhard B. Singer
Department of Anatomy, University Hospital Essen, University Duisburg-Essen, Essen, NRW, Germany

Synonyms

Bgp; C-CAM; CD66

Alternative Names

CEACAM1: Bb-1; Bgp; Bgp1; Bgpa (human); Bgpd (mouse); BGPI; Biliary glycoprotein 1; C-CAM; C-CAM105; NCA-160; nonspecific cross-reacting antigen 160; CD66; CD66a; Cea-1; Cea-7; CEA-related cell adhesion molecule 1; Cea1; Cea7; Carcinoembryonic antigen-related cell adhesion molecule 1; CEACAM1; Ceacam1; mCEA1; MHVR; mmCGM1; mmCGM1a; mmCGM2.

CEACAM3: CD66d; CGM1; MGC119875; W264; W282.

CEACAM4: CGM7.

CEACAM5: CEA; CD66e.

CEACAM6: CD66c; NCA; NCA-90; NCA-50/90; CEAL.

CEACAM7: CGM2.

CEACAM8: CD66b; CD67; CGM6; NCA-95.

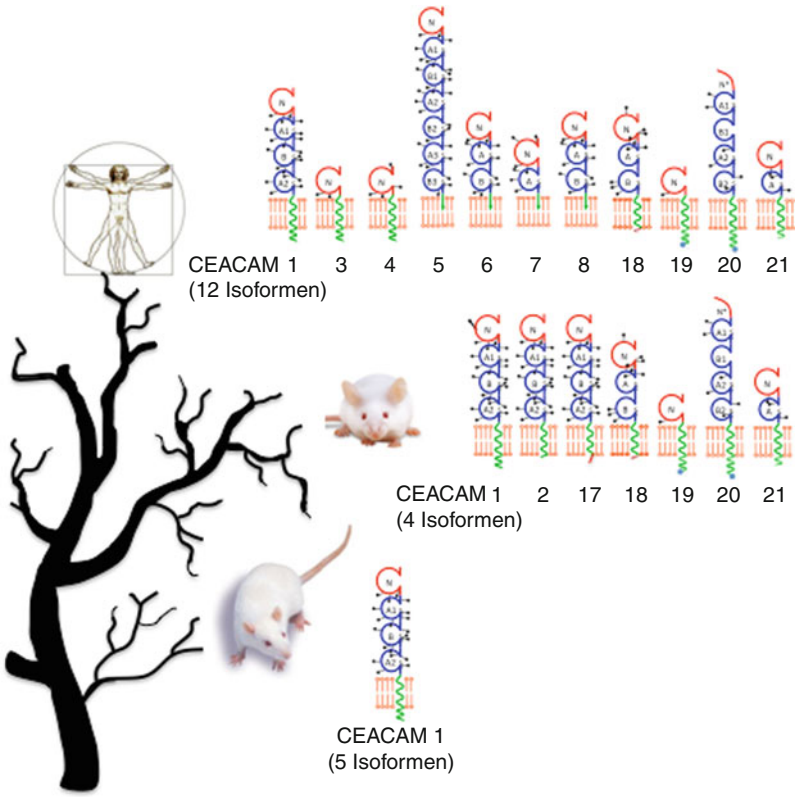
CEACAM9, CEACAM10, CEACAM11, CEACAM12, CEACAM13, CEACAM14, CEACAM15, CEACAM16, CEACAM17, CEACAM18, CEACAM19, CEACAM20, and CEACAM21: no synonyms.

Historical Background

CEA-related cell adhesion molecules (CEACAMs) are members of the carcinoembryonic antigen (*CEA*) gene family, which belongs to the immunoglobulin superfamily. Two subgroups of the *CEA* gene family, the CEA-related cell adhesion molecules and the pregnancy-specific glycoproteins (PSGs), are located within a 1.2 Mb cluster on the long arm of human chromosome 19 (19q13.2), the mouse chromosome 7, or the rat chromosome 1 (Kammerer and Zimmermann 2010). All CEACAMs are heavily glycosylated. The CEACAM subgroup in humans consists of 12 members composed of a single immunoglobulin variable (IgV)-like N-terminal (N) domain followed by zero to six Ig constant (IgC)-like domains of A and B subtypes and one member which consists of two IgC-like domains and two IgV-like domains, one at each end of the molecule. Due to its orthologs found in numerous species, CEACAM1 was identified as the ancestral founder molecule of the CEACAM-family (Beauchemin et al. 1999). However, as a result of its early recognition to be a potent tumor marker CEA/CEACAM5 represents the best-known CEACAM family member (Hammarström 1999; Horst and Wagener 2004). In mice, rats, and cattle, but not in humans, at least two CEACAM1 alleles each have been determined (Kammerer and Zimmermann 2010). Species-specific alternative splicing of several family members further enhances the diversity of the CEACAM family (Singer and Lucka 2005). For example, CEACAM1 mostly appears in at least two co-expressed isoforms one with a long (CEACAM1-L; 73 amino acid), one with a short (CEACAM1-S; 10 amino acid) cytoplasmic tail. The cytoplasmic domain of CEACAM1-L contains tyrosine residues within an immunoreceptor tyrosine inhibition motif (ITIM) that interacts with protein tyrosine kinases of the Src family. CEACAM1, CEACAM3, CEACAM4, CEACAM18, CEACAM19, CEACAM20, and CEACAM21 are transmembrane-anchored molecules while CEA/CEACAM5, CEACAM6, CEACAM7, and CEACAM8 are linked to the cell membrane via glycosylphosphatidylinositol (GPI) anchors, a type of semipenetrating membrane anchorage that exists only in human *CEA* gene family members. Thus, the variety of CEACAMs expressed in rat and mouse is less (Fig. 1). CEACAM16 represents most likely a secreted molecule. Interestingly, also CEACAM1, CEA/CEACAM5, CEACAM6, and CEACAM8 can appear as secreted variants. The transmembrane-anchored CEACAM17 and the secreted CEACAM9, CEACAM10, CEACAM11, CEACAM12, CEACAM13, CEACAM14, and CEACAM15 exist in rat and mouse but not in human. The transmembrane-bound CEACAM2 is solely found in mice (Beauchemin et al. 1999). Because very little is known about the relatively new discovered CEACAM9-CEACAM21 proteins, this entry will concentrate on CEACAM1-CEACAM8.

Expression Patterns of CEACAMs

CEACAMs show a very heterogeneous expression pattern. Thus, CEACAM1 is expressed in several leukocyte-subtypes, most epithelia and endothelia of newly formed small blood vessels (Singer and Lucka 2005). Notably, angiogenically activated microvascular endothelial cells express significant less CEACAM1 than angiogenically activated lymphendothelial cells. The CEACAM1 expression is up-regulated in activated granulocytes, B- and T-lymphocytes as well as in confluent, contact-inhibited epithelial cells (Singer and Lucka 2005; Gray-Owen and Blumberg 2006; Singer et al. 2010). Beside the altered CEACAM expression in contact inhibited versus proliferating epithelia cells, the CEACAM1-L to CEACAM1-S ratio can also differ (Singer et al. 2010). The CEACAM3- and CEACAM8-expression is restricted to human granulocytes whereas CEACAM5 and CEACAM7 can solely be found on epithelia. CEACAM6 is expressed in granulocytes and some epithelia. Until now, the expression pattern of CEACAM4 is not finally understood but it is believed to be present in granulocytes, in T- and B-cell lymphoblastic leukemia.



CEACAMs, Fig. 1 Domain structure of membrane-anchored members of the *CEA* gene family expressed in rat, mouse, and human. Each receptor has an amino-terminal IgV-like domain (N domain) followed by a variable number of A or B subsets of IgC2-like domains, a transmembrane and a cytoplasmic domain. Additionally, GPI-anchored CEACAMs appear in human. Alternative spliced variants can be found in of some of the CEACAMs and further enhance the complexity of the *CEA* gene family. CEACAM1 is believed to be the ancestral founder gene of the *CEA* gene family. The most common isoforms of CEACAM1 carry either a long (CEACAM1-4L) or a short (CEACAM1-4S) cytoplasmic tail. The number of CEACAM1 isoforms varies significantly between the different species. Further information about the *CEA* gene family can be found at http://www.carcinoembryonic-antigen.de

The overexpression of CEA/CEACAM5 in tumors of epithelial origin is the basis of its widespread use as a tumor marker. As a result, the CEA serum levels from individuals with colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, lung carcinoma, breast carcinoma, as well as medullary thyroid carcinoma, and under some non-neoplastic conditions like in ulcerative colitis, pancreatitis, cirrhosis, COPD, Crohn's disease, and in smokers are usually increased (Hammarström 1999; Horst and Wagener 2004). Also the CEACAM6 expression is significantly up-regulated in colon, lung, gastrointestinal, and pancreatic carcinomas. On the contrary, the CEACAM7 expression appears to be decreased in rectal cancer (Horst and Wagener 2004). CEACAM1 can be up-regulated or down-regulated both in the cancerous tumors. For example, in epithelial tumors of colorectal, liver, breast, prostate, bladder, and renal cancer the CEACAM1 expression level is decreased, whereas in thyroid, gastric, lung cancer as well as in malignant melanoma the CEACAM1 expression is significantly higher (Öbrink 2008). Thus, it is believed that the deregulated expression of CEA/CEACAM5, CEACAM6, CEACAM7, and CEACAM1 is able to provide important tumorigenic contributions to carcinogenesis. However, malignant tumors seem to produce so far unidentified factors that induce microvascular endothelial and lymphendothelial cells to express significant amounts of CEACAM1 (Öbrink 2008).

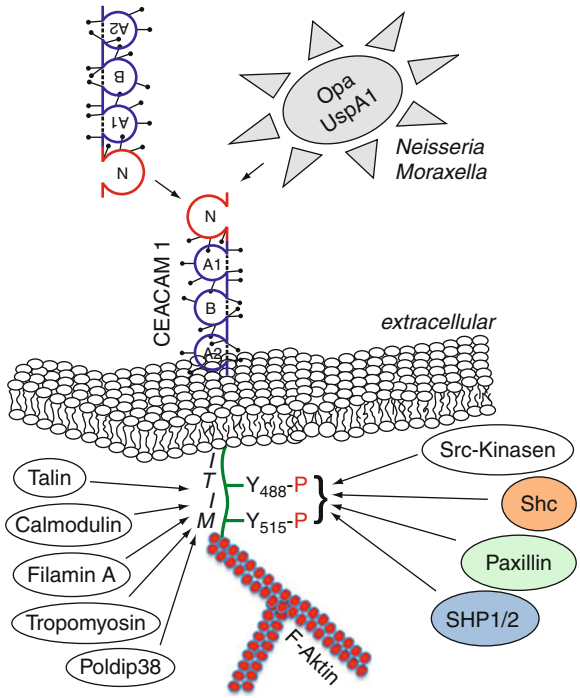
The fact that at least two distinct isoforms of CEACAM1, namely, CEACAM1-L and CEACAM1-S, are co-expressed in most if not all cell types enhances the complexity of the CEACAM1 expression

pattern. In addition, human CEACAM1 appears with cell type specific glycosylation levels altering its molecular weight from approximately 120 to 160 kDa in epithelial cells and granulocytes, respectively (Singer and Lucka 2005).

Functions Mediated by CEACAMs

Members of the CEA family trigger a broad range of diverse functions. It is well established that CEACAM1, CEA/CEACAM5, and CEACAM6 act as cell-cell adhesion molecules (Fig. 2). They mediate either homophilic adhesion (e.g., CEACAM1 to CEACAM1) or they interact heterophilically by binding to other CEACAMs (e.g., CEACAM1 binds to CEA/CEACAM5 and CEACAM6) (Öbrink 2008). CEACAM1 can bind to membrane-anchored as well as soluble CEACAMs (Öbrink. 1997). However, these intercellular adhesive bonds are rather weak and thus CEACAMs most likely represent sensor molecules at the cell surface that regulate cellular signaling. Importantly, the N-domains of CEACAMs are crucial for the homophilic and heterophilic interaction. Furthermore, the N-domains of CEACAM1, CEACAM3, CEACAM5, and CEACAM6 emerged to be pathogen receptors (Fig. 2). It was found that opacity-associated (Opa) proteins of various *Neisseria* strains, UspA1 molecules of *Moraxella catarrhalis*, and OmpP5 proteins of *Haemophilus influenzae* specifically ligate to human CEACAMs whereas the Mouse Hepatatis Virus (MHV) solely binds to murine CEACAM1 (Gray-Owen and Blumberg 2006; Slevogt et al. 2008). Furthermore, in human, CEACAM1 is a receptor for *Salmonella* and *Escherichia coli*, binds to galectin-3 and to E-selectin via its LewisX and sialyl-LewisX-epitopes latter specifically expressed in CEACAM1 of human granulocytes (Singer and Lucka 2005). It is worth noting that CEACAM ligands like microorganisms, CEACAMs, and CEACAM specific antibodies of one species are not cross-reactive with CEACAMs of any other species.

In answer to ligand binding, membrane-anchored CEACAM1 controls apoptosis, cell migration, cell invasion, morphogenesis, insulin metabolism, endocytosis, angiogenesis, lymphangiogenesis, and cell proliferation (Öbrink 2008; Singer and Lucka 2005). Hereby, CEACAM1-L, but not CEACAM1-S, appears to be decisive in mediating these cellular functions.

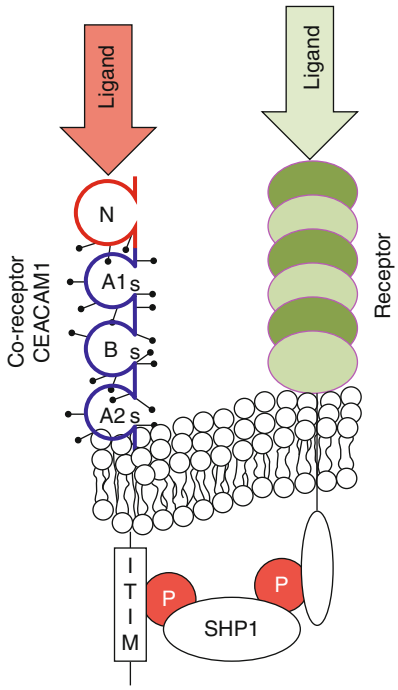


CEACAMs, Fig. 2 Overview of the extracellular ligands and intracellular interaction partners of CEACAM1-L. Precious few extracellular binding partners exist for CEACAM1. Intracellularly, many different molecules can interact with CEACAM1. Thus the evolutionary pressure toward the extracellular CEACAM1 domains should be less than toward its cytoplasmic tail. That notion could explain why CEACAMs expressed in different species share common intracellular binding partners but never extracellular ones

Thus, in epithelia, CEACAM1-L negatively triggers proliferation via its ITIM domain and maintains the post-confluent contact inhibition, whereas the disturbances of the CEACAM1-L signaling by CEACAM1-4S, CEACAM6 or CEA/CEACAM5 leads to undifferentiated cell growth and malignant transformation (Singer et al. 2010). In agreement with this finding, CEACAM6-overexpressing pancreatic adenocarcinoma cells were more proliferative, more invasive and more chemoresistant to gemcitabine compared to cells with normal CEACAM6 level (Duxbury et al. 2004). CEACAM6 and CEA/CEACAM5 also were shown to inhibit apoptosis/anoikis further emphasizing their role in promoting aberrant growth (Kuespert et al. 2006).

In immunity, CEACAM1 regulates natural killer cell inhibition, dendritic cell maturation, granulocyte survival, and T and B lymphocyte proliferation and

activation (Gray-Owen and Blumberg 2006). Also CEACAM3, CEACAM6, and CEACAM8 are crucial for the regulation of granulocyte activation. Notably, CEACAM8 (CD66b) is a widely utilized differentiation and activation marker for human granulocytes. Recently, it has been shown that several pathogens can diminish the immune response by binding to CEACAM1 on CD4+ T cells as well as in pulmonary epithelial cells via CEACAM1-L(ITIM) mediated signaling (Sadarangani et al. 2010; Slevogt et al. 2008). However, antibodies to CEACAM1 have been reported to increase or decrease T-cell activation in response to T-cell receptor cross-linkage in vitro (Singer and Lucka 2005; Gray-Owen and Blumberg 2006). This apparent discrepancy seems to reflect the different signaling capabilities of CEACAM1 to generate both stimulatory and inhibitory signals via the complex cooperation between the ratio of CEACAM1-L to -S, the equilibrium between monomers, *cis*- and *trans*-dimers, levels of phosphorylation and the interactions with intracellular and cytoskeletal molecules. In contrast, CEACAM3 harbors an immunoreceptor tyrosine-based activation motif (ITAM) within its cytoplasmic tail and is believed to function as a phagocytic receptor involved in the clearance of CEACAM-binding bacteria by human granulocytes (Sadarangani et al. 2010).



CEACAMs, Fig. 3 Initial steps of the CEACAM1-L mediated signaling. Ligation of CEACAM1-L triggers the tyrosine phosphorylation within its cytoplasmic ITIM and the recruitment of SHP-1. Subsequently, CEACAM1 can interact via SHP-1 with other receptors and modulate their signaling capacity and thus influence the functional outcome

Regulation of the Activity of CEACAMs

CEACAMs represent multifunctional adhesion receptor proteins influencing pleiotropic effects in epithelia, endothelia, and hematopoietic cells. The long isoform of CEACAM1 and some other transmembrane-anchored CEA family members can transduce signals directly via their cytoplasmic domains into the cytoplasma. Hereby, CEACAM1-L forms homodimers in a cis-configuration (Öbrink 1997). However, the GPI-linked CEACAMs have a higher lateral mobility in the plasma membrane but need to utilize transmembrane partner molecules like CEACAM1 for signaling. As an example, GPI-linked CEACAMs are able to alter the CEACAM1-L maintained post-confluent contact inhibition (Singer et al. 2010). In addition, CEACAM1-S can interfere with the CEACAM1-L mediated functions most likely via *cis*-dimerization (Öbrink 1997). Thus, the ratio of CEACAM1-L to CEACAM1-S possibly together with additionally co-expressed CEA family members seems to be decisive for the functional outcome mediated by CEACAM1 (Singer et al. 2010).

The homophilic and heterophilic adhesion of CEACAM1 and other CEACAMs as well as pathogen binding is mediated by N-terminal domain interactions (Öbrink 1997). The ligand binding induces signal cascades starting with the phosphorylation of distinct tyrosine residues within the cytoplasmic tail of the transmembrane-anchored CEACAMs (Fig. 3). Human CEACAM1 contains two ITIM in its cytoplasmic domain, whereas in rodents one CEACAM1 ITIM is replaced by an immunoreceptor tyrosine-based switch motif (ITSM) (Kammerer and Zimmermann 2010). Human CEACAM3 and probably CEACAM4, CEACAM19, and CEACAM20 carry ITAM in their cytoplasmic tails. In contrast, except for CEACAM19 and CEACAM20 no such ITAM-containing CEA family members exist in rodents. It is general accepted that the phosphorylation of the tyrosine residues within the ITIM represents the initial step of CEACAM1 mediated

signaling. CEACAM1-L can be phosphorylated by protein tyrosine kinases of the ▶ Src family, by the insulin receptor kinase (IR) and by the epithelial growth factor receptor (EGFR) (Öbrink 1997; Singer and Lucka 2005). Upon phosphorylation, CEACAM1-L can bind and activate both protein tyrosine kinases (such as c-src, lyn, and hck) and protein tyrosine phosphatases such as SHP-1 and SHP-2 (Singer and Lucka 2005).

Besides CEACAM1-L, CEACAM1-S has the potential to directly induce signal transduction. Thus, protein kinase C (PKC) can phosphorylate the serine and threonine residues of CEACAM1-S and -L (Singer and Lucka 2005). Additionally, both splice variants interact with the DNA polymerase delta-interacting protein 38 (Poldip 38) (Klaile et al. 2007) as well as with actin, talin, paxillin, and filamin A (Fig. 2) (Singer and Lucka 2005). The PKC mediated phosphorylation of CEACAM1 induces calmodulin binding to the cytoplasmic domain of CEACAM1 and as a result regulates its cis-dimerization. CEACAM1 associates also in cis with integrin αvβ3 probably to promote cellular invasion (Singer and Lucka 2005). Moreover, CEACAM3 is phosphorylated within its ITAM by Src-kinases, in particular Hck and Fgr (Kuespert et al. 2006). This phosphorylation is triggered by bacterial binding stimulating in the following the small GTPases Rac. Consequently, the pathogen becomes phagocytozed and killed. In addition, there are reports showing that GPI-anchored members of the CEA family are able to induce signal transduction. Thus, ligation of CEACAM8 leads to an activation of the extracellular signal-regulated kinase 1/2 (Erk1/2) and the overexpression of CEACAM6 leads to an increased activity of the tyrosine kinase c-Src and the serine/threonine kinase akt-1 (Singer et al. 2002; Duxbury et al. 2004). Furthermore, CEACAM6 and CEACAM8 (as well as CEACAM1 and CEACAM3) can initiate adhesion of neutrophilic granulocyte to endothelial cells most likely by activating integrins (Singer and Lucka 2005). Although it is not finally understood how GPI-anchored CEACAMs manage to kick off their signaling cascades, it is likely that they employ CEACAM1 or other transmembrane molecules for signaling.

In recent times, several groups have demonstrated that CEACAM1 acts as a co-receptor of numerous cell receptors (Fig. 3). In lymphendothelial cells membrane-bound CEACAM1 mediates pro-angiogenic functions by interfering with the vascular endothelial growth factor receptor-3 (VEGFR-3) (Kilic et al. 2007). Hereby, CEACAM1-L(ITIM) is tyrosine-phosphorylated upon VEGF treatment in a SHP-1- and Src-dependent manner. Lately, CEACAM1 was shown to be co-receptor of the VEGFR-2 regulating the VEGFR2/Akt/eNOS-mediated vascular permeability pathway and angiogenesis (Nouvion et al. 2010). Furthermore, under certain circumstances IR and EGFR are able to phosphorylate Tyr-488 of the CEACAM1-L (ITIM). Subsequently, the SH2 domain of the Src homology 2 domain-containing transforming protein 1 (Shc) can associate leading to decreased Ras/MAPK Erk1/2 pathway induction (Kuespert et al. 2006). As a result, CEACAM1 negatively regulates cell proliferation in response to insulin and EGF, respectively. Moreover, various antibodies to CEACAM1 have been reported to enhance or diminish T-cell activation in response to ligation of the T-cell receptor (TCR) (Singer and Lucka 2005). However, in activated $CD4^+$ T-lymphocytes, CEACAM1 appeared to be an inhibitory co-receptor of the TCR/▶ CD3 complex. CEACAM1-L (ITIM) phosphorylation and subsequent association with SHP-1 were identified as the initiating signaling steps. Afterward, CEACAM1 interacts via SHP1 with the TCR/CD3 complex which leads to decreased CD3-zeta, ▶ ZAP-70, and IL2-R signaling (Gray-Owen and Blumberg 2006; Sadarangani et al. 2010). Similar to its function as co-receptor for the TCR, CEACAM1 was described to function as an inhibitory co-modulator of the human B cell receptor (BCR), likely through the recruitment of SHP-1 and inhibition of the phosphatidylinositol 3-OH kinase–Akt kinase (PI3K)-promoted pathway (Lobo et al. 2009). In contrast, other studies showed that certain CEACAM1-specific monoclonal antibodies or the homophilic CEACAM1-CEACAM1 interaction strongly triggered proliferation of mouse B cells when combined with the ligation of the BCR (Greicius et al. 2003). CEACAM1 acts also as co-inhibiting receptor of the Toll-like receptor 2 (TLR2). The inhibitory effects were mediated by tyrosine phosphorylation of CEACAM1-L(ITIM) and recruitment of SHP-1, which negatively regulates the TLR2-dependent activation of the PI3K activation pathway and the initiation of the NF-kappa B–dependent inflammatory responses (Slevogt et al. 2008). Consequently, the generation and release of pro-inflammatory cytokines such as GM-CSF and IL8 were reduced. Last but not least, CEACAM1 was described to be a co-inhibitory receptor molecule for the granulocyte

colony-stimulating factor receptor (G-CSFR) regulating granulopoiesis through CEACAM1-L(ITIM) phosphorylation and SHP-1 interaction leading to decreased Stat3 activation (Pan and Shively 2010).

Summary

CEACAMs are members of the *CEA*-gene family. Nowadays, the CEACAM1 to CEACAM8 are well analyzed whereas very little is known about CEACAM9 to CEACAM21. The CEACAM1 to CEACAM8 expression pattern varies considerably in accordance with the species, the cell type, the cell growth, and activation state. Notably, the CEACAM expression is frequently deregulated in many types of cancer indicating their putative role in tumor-growth regulation. However, most membrane-bound CEACAMs function as cell-adhesion receptor molecules mediating a broad range of functions including apoptosis, cell migration, cell invasion, contact-inhibition, morphogenesis, insulin metabolism, endocytosis, angiogenesis, lymphangiogenesis, and cell proliferation. In addition, CEACAM1 was found to be a crucial co-receptor for TCR, BCR, TLR-2, IR, EGFR, G-CSFR, VEGFR-2, and VEGFR-3. CEACAM1 interacts with diverse signal transducing proteins depending on its phosphorylation stage. Some CEACAMs were also identified to bind species specific certain pathogens like *Moraxella catarrhalis* or *Neisseria gonorrhoeae*. Thus, CEACAMs seem to play a central role in controlling pathogens by the innate immune system. Furthermore, a putatively pathogen driven evolution of the *CEA* gene family could be responsible for the high variability of the number of diverse CEACAMs expressed in the different species. However, more knowledge is needed about the abundance of CEACAM1, the relative proportion of CEACAM1-L to CEACAM1-S isoforms and the appearance of further co-expressed CEACAMs to fully understand the signaling and functional potential of members of the CEA family.

References

Beauchemin N, Draber P, Dveksler G, Gold P, Gray-Owen S, Grunert F, Hammarström S, Holmes KV, Karlsson A, Kuroki M, Lin SH, Lucka L, Najjar SM, Neumaier M, Öbrink B, Shively JE, Skubitz KM, Stanners CP, Thomas P, Thompson JA, Virji M, von Kleist S, Wagener C, Watt S, Zimmermann W. Redefined nomenclature for members of the carcinoembryonic antigen family. Exp Cell Res. 1999; 252(2):243–9.

Duxbury MS, Ito H, Ashley SW, Whang EE. CEACAM6 cross-linking induces caveolin-1-dependent, Src-mediated focal adhesion kinase phosphorylation in BxPC3 pancreatic adenocarcinoma cells. J Biol Chem. 2004;279(22):23176–82.

Gray-Owen SD, Blumberg RS. CEACAM1: contact-dependent control of immunity. Nat Rev Immunol. 2006;6(6):433–46.

Greicius G, Severinson E, Beauchemin N, Öbrink B, Singer BB. CEACAM1 is a potent regulator of B cell receptor complex-induced activation. J Leukoc Biol. 2003;74(1):126–34.

Hammarström S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Semin Cancer Biol. 1999;9(2):67–81.

Horst AK, Wagener C. CEA-related CAMs. In Handbook of experimental pharmacology. Springer, New York. 2004; pp. 283–341.

Kammerer R, Zimmermann W. Coevolution of activating and inhibitory receptors within mammalian carcinoembryonic antigen families. BMC Biol. 2010;8:12.

Kilic N, Oliveira-Ferrer L, Neshat-Vahid S, Irmak S, Obst-Pernberg K, Wurmbach JH, Loges S, Kilic E, Weil J, Lauke H, Tilki D, Singer BB, Ergün S. Lymphatic reprogramming of microvascular endothelial cells by CEA-related cell adhesion molecule-1 via interaction with VEGFR-3 and Prox1. Blood. 2007;110(13):4223–33.

Klaile E, Müller MM, Kannicht C, Otto W, Singer BB, Reutter W, Öbrink B, Lucka L. The cell adhesion receptor carcinoembryonic antigen-related cell adhesion molecule 1 regulates nucleocytoplasmic trafficking of DNA polymerase delta-interacting protein 38. J Biol Chem. 2007;282 (36):26629–40.

Kuespert K, Pils S, Hauck CR. CEACAMs: their role in physiology and pathophysiology. Curr Opin Cell Biol. 2006; 18(5):565–71.

Lobo EO, Zhang Z, Shively JE. Pivotal advance: CEACAM1 is a negative coreceptor for the B cell receptor and promotes CD19-mediated adhesion of B cells in a PI3K-dependent manner. J Leukoc Biol. 2009;86(2):205–18.

Nouvion A-L, Oubaha M, LeBlanc S, Davis EC, Jastrow H, Kammerer R, Breton V, Turbide C, Ergun S, Gratton J-P, Beauchemin N. CEACAM1: a key regulator of vascular permeability. J Cell Sci. 2010;123:4221–30.

Öbrink B. CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. Curr Opin Cell Biol. 1997; 9(5):616–26.

Öbrink B. On the role of CEACAM1 in cancer. Lung Cancer. 2008; 60(3):309–12.

Pan H, Shively JE. Carcinoembryonic antigen-related cell adhesion molecule-1 regulates granulopoiesis by inhibition of granulocyte colony-stimulating factor receptor. Immunity. 2010; 33(4):620–31.

Sadarangani M, Pollard AJ, Gray-Owen SD. Opa proteins & CEACAMs: pathways of immune engagement for pathogenic *Neisseria*. FEMS Microbiol Rev. 2010;35(3):498–514. doi:10.1111/j.1574-6976.2010.00260.x.

Singer BB, Lucka L. CEACAM1. UCSD-Nature Molecule Pages. 2005. doi:10 1038/mp a003597 01.

Singer BB, Scheffrahn I, Heymann R, Sigmundsson K, Kammerer R, Öbrink B. Carcinoembryonic antigen-related cell adhesion molecule 1 expression and signaling in human, mouse, and rat leukocytes: evidence for replacement of the short cytoplasmic domain isoform by glycosylphosphatidylinositol-linked proteins in human leukocytes. J Immunol. 2002;168(10):5139–46.
Singer BB, Scheffrahn I, Kammerer R, Suttorp N, Ergun S, Slevogt H. Deregulation of the CEACAM expression pattern causes undifferentiated cell growth in human lung adenocarcinoma cells. PLoS ONE. 2010;5(1):e8747.
Slevogt H, Zabel S, Opitz B, Hocke A, Eitel J, N'guessan PD, Lucka L, Riesbeck K, Zimmermann W, Zweigner J, Temmesfeld-Wollbrueck B, Suttorp N, Singer BB. CEACAM1 inhibits Toll-like receptor 2-triggered antibacterial responses of human pulmonary epithelial cells. Nat Immunol. 2008;9(11):1270–8.

CED1

► CD91

Cek4 (Chicken)

► EphA3, Erythropoietin-Producing Hepatocellular Carcinoma Cell Receptor A3

Cell Proliferation-Inducing Gene 17

► TMEM85 (Transmembrane Protein 85)

Cell Surface Glycoprotein CD53

► CD53

CELSR

► Cadherins

CENTA1

► ADAP1

Centaurin Alpha

► ADAP1

Centaurin Delta 3

► ARAP3

Centd3

► ARAP3

c-FGR

► FGR (Gene Name)

Chemokine (C-X-C Motif) Ligand 10

► CXCL10

Chemokine Receptor CCR1

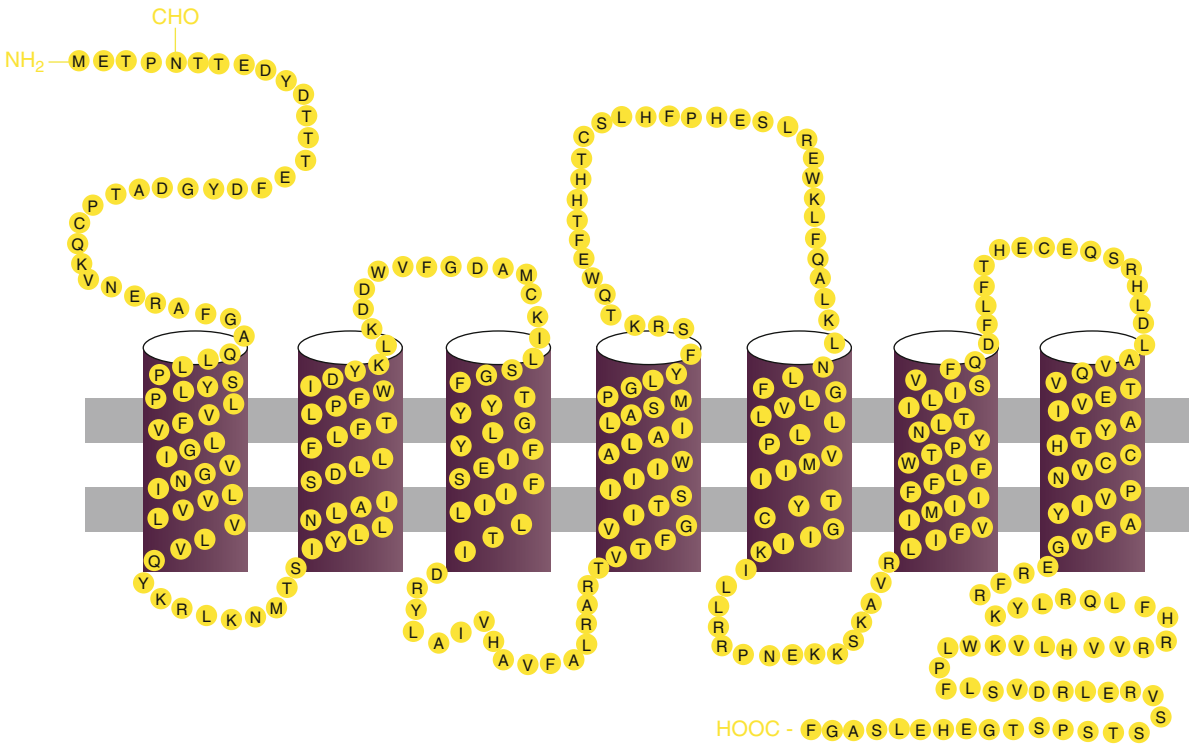
Richard Horuk
Department of Pharmacology, UC Davis, Davis, CA, USA

Synonyms

CC CKR1; CD191; CKR1; CMKBR1; HM145; LD78 receptor; MIP-1α/R; MIP-1α/RANTES

Historical Background

Although numerous reports had described specific effects of the chemokines RANTES (CCL5) and MIP-1α (CCL3) on T lymphocytes and monocytes

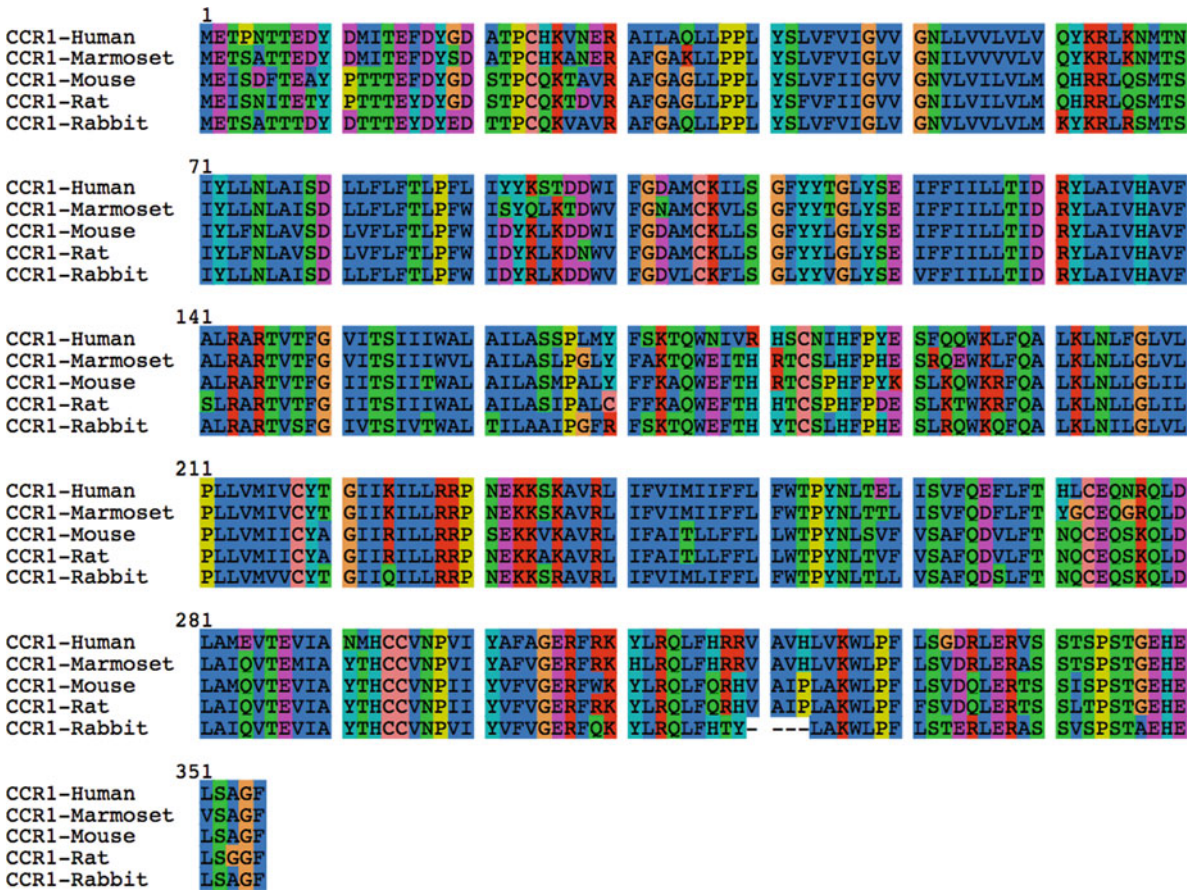


Chemokine Receptor CCR1, Fig. 1 Proposed membrane topography of CCR1. Membrane spanning alpha helices are defined based on hydropathy analysis. CHO, potential N-Linked glycosylation sites

the identity of the putative receptor for these ligands was unknown. However, cloning of this receptor was aided by the fact that the primary sequences of the C5a, fMLP, and IL-8 receptors revealed domains which were conserved in receptors associated with cell motility, but not in other seven-transmembrane-spanning receptors (Holmes et al. 1991). These similarities were exploited using polymerase chain reaction (PCR) technology to obtain several orphan receptor cDNA clones which were then expressed and screened by receptor binding and functional assays. Using this homology hybridization cloning approach, the molecular cloning and functional expression of CCR1 was reported by two separate groups (Gao et al. 1993; Neote et al. 1993). The open reading frame for human CCR1 is on a single exon and predicts a protein of 355 amino acids (Fig. 1). The gene, CCR1, is located on human chromosome 3p21. The expressed human CCR1 was able to bind CCL3 and CCL5 with high affinity and physiological concentrations of both ligands induced an increase in intracellular Ca^{2+}. CCR1 was specific for these ligands and showed a poor response to MIP-1ß (CCL4) and MCP-1 (CCL2). In addition, to these ligands CCR1 has been shown to respond with high affinity and to signal in response to a variety of other C-C chemokines including MCP-3 (CCL7), MPIF-1 (CCL23), leukotactin-1(CCL15), and HCC-1 (CCL14).

CCR1 was the first human CC chemokine receptor to be identified at the cDNA level. It has a functional viral homolog, US28, which is a human cytomegalovirus (Gao et al. 1993; Neote et al. 1993). Chemokine receptors like CCR1 are all members of the rhodopsin-like subfamily of G-protein-coupled receptors (GPCRs) (Gao et al. 1993; Neote et al. 1993). Although CCR1 was initially described as a receptor for the chemokines CCL3 and CCL5, a number of other chemokine ligands have since been identified, including CCL6, CCL7, CCL9 (MIP-1 gamma), CCL14, CCL15, CCL16 (LEC), and CCL23. Interestingly CCL6, CCL9, CCL15, and CCL23 (CKbeta-8) are initially weak CCR1 agonists but appear to be made more potent by proteolytic modification at the N-terminus during inflammatory responses in vivo.

Amino-terminal processing of chemokines can change the specificity of the ligand for its receptor.



Chemokine Receptor CCR1, Fig. 2 Alignment of the primary structures of the cloned human, marmoset, rat, mouse, and rabbitt CCR1

For example, activated human peripheral lymphocytes secrete an amino-terminal truncated form of CCL4 (3–69) with novel functional specificity for CCR1, 2, and 5 (Guan et al. 2004). This chemokine is generated by proteolytic cleavage of the full length CCL4, which has poor affinity for CCR1, by CD26 a membrane-bound ectopeptidase with dipeptidyl peptidase IV activity. The kinetics of conversion of CCL4 from intact to CCL4(3–69) in activated peripheral lymphocytes correlates with cell surface expression of CD26.

The protein sequences of CCR1 receptors from 14 different species including human, rat mouse, marmoset and rabbit have been cloned (Fig. 2). The sequences of CCR1 are highly homologous and the human receptor is 81% and 80% identical to the mouse and rat sequences, respectively. Interestingly, human CCR1 has also been proposed to have a consensus site for tyrosine sulfation, which affects the affinity and binding of some chemokine receptors to their ligand (s) and has been hypothesized to play an important role in regulating their activity (Liu et al. 2008).

CCRI plays an important role in host defense and is involved in regulating the chemotaxis of immune cells, a feature it shares in common with all chemokine receptors. Dysregulation of this response leads to autoimmunity and CCR1 has been associated with the pathophysiology of a number of diseases including rheumatoid arthritis, multiple sclerosis, transplant rejection, and allergic inflammation (Murphy et al. 2000). CCR1 activates the Gi/o class of guanine nucleotide binding regulatory proteins (Murphy et al. 2000). This activation, can in turn, regulate several effectors, including adenylyl cyclase (inhibition), phospholipase C (activation), protein kinase C (activation), calcium flux (stimulation), and phospholipase A2 (PLA2) (activation).

Not all CCR1 ligands appear to mediate the same functions. For example, although CCL3, CCL5, and CCL16 are all able to transduce signals through CCR1 in a human osteosarcoma line via Gi/Go, phospholipase C, and protein kinase Cdelta mediated pathways, CCL16 can also signal via p38 MAP kinase and in contrast to the other two ligands does not induce calcium transients. The CCR1 ligands CCL3, CCL7, CCL5, and CCL15 can all inhibit adenylyl cyclase activity in cells transiently transfected with CCR1. Of these CCR1 ligands, however, only CCL15 was unable to signal via Ga14 or Ga16 coupled pathways. In addition, most of the CCR1 ligands are promiscuous and can activate other chemokine receptors (Murphy et al. 2000).

The cytomegalovirus, which causes acute, latent, and chronic infections in humans, has been shown to encode a CCR1 homolog, US28, which strongly activates classical G-protein signal transduction networks within the cell (Gao et al. 1993; Neote et al. 1993). It can bind a number of chemokines including CCL3 and CCL5 with high affinity (Gao et al. 1993; Neote et al. 1993). In addition, US28 can signal in a ligand-independent, constitutive manner both through the Gq/phospholipase C pathway and through activation of the transcription factor NF-kappaB as well as through the cyclic AMP response element binding protein.

Regulation of Activity

In common with most GPCRs CCR1 undergoes receptor desensitization upon ligand binding (Neote et al. 1993). The mechanism involves phosphorylation of specific serines in the serine-rich carboxy-terminal tail which is mediated by G-protein receptor kinases and results in the uncoupling of the receptor from its G-protein and the recruitment of arrestin. These processes induce receptor internalization and terminate CCR1 signaling.

A series of CCR1 cytoplasmic tail mutants indicate that the phosphorylation sites for CCR1 are located in a cluster of serine and threonine residues between amino acids 340–346 (Richardson et al. 2000). Phosphorylation of these residues leads to receptor desensitization and inhibits CCL3 and CCL5-induced calcium transients and GTPase activity. Interestingly the activity of CCR1 can be independently regulated by unrelated chemokine receptors. For example in a cell line coexpressing CCR1 and CXCR2 the CXCR2 ligand CXCL8 was able to cross-phosphorylate CCR1 and desensitize its ability to stimulate GTPase activity and Ca^{2+} mobilization. Conversely CCR1 ligands were able to induce the phosphorylation and desensitization of CXCR2.

Retinoic acid has been shown to increase the expression of CCR1 in human monocytes (Ko et al. 2007). It increased calcium influx and chemotactic activity in response to the CCR1 ligands CCL15, CCL3, and CCL5 and appears to be mediated through a p38 MAPK and ERK pathway since pretreatment with inhibitors of these kinases blocked the retinoic acid-induced effects.

In an animal model of myocarditis a CCR1 antagonist, BX 471 was able to inhibit disease and improve cardiac function (Futamatsu et al. 2006). The beneficial effects of the antagonist were likely associated with its ability to block T-cell proliferation and suppress ERK1/2 and JNK activities in T cells stimulated with myosin.

Interactions with Ligands and Other Proteins

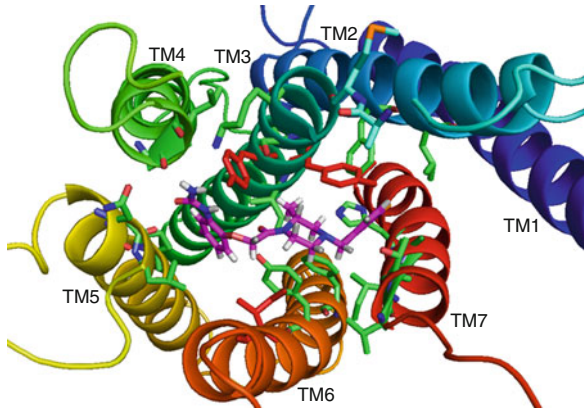
CCR1 responds to a number of protein ligands and to small molecule antagonists. The interaction of the receptor with these distinct ligands has been described in a number of studies and is very different. Structure-function studies have revealed that chemokines such as CCL3 bind to CCR1 via residues in the N-terminus and in the extracellular loops (Pease et al. 1998; Zoffmann et al. 2002). For example, a photoactivatable derivative of CCL3 labeled with a benzophenone group at the N-terminal end was used to probe contact points on CCR1 (Zoffmann et al. 2002). These studies revealed that the N terminus of CCL3 interacts in a specific manner with the second extracellular loop of CCR1, within a segment comprising amino acids 178 – 194. The chemokine receptors CCR1 and CCR3 are 54% identical in amino acid sequence and chimeras of these receptors have been used to map the CCL3 binding determinants for CCR1 (Pease et al. 1998). Using this approach, chimeras in which the N-terminal extracellular segments of the two receptors were switched were constructed. CCL3 was able to bind with high affinity to both chimeras; however, it was not able to function as an agonist with the chimeric receptors in either calcium flux or chemotaxis assays. These data are consistent with the idea that chemokines bind to their receptors via multiple extracellular binding sites which determine their selectivity and their ability to activate their receptors.

CCR1 Antagonists

In contrast, potent CCR1 antagonists like BX 471, in common with other chemokine receptor antagonists binds to CCR1 in the transmembrane domain (Vaidehi et al. 2006), similar to the transmembrane binding domain of 11-cis-retinal in rhodopsin. Through its interaction with residues in the transmembrane domain, Tyr113 and Tyr114 on transmembrane domain 3 and Ile259 on transmembrane domain 6, BX 471 induces a conformational change in the receptor that leads to displacement of bound chemokine from its extracellular binding site (Fig. 3). In contrast, when radiolabeled BX 471 is bound, CCR1 agonists are unable to competitively displace the antagonist (Vaidehi et al. 2006).

Several companies have disclosed small molecule CCR1 antagonists and a standard feature of many of these compounds is their lack of crossreactivity with non-human CCR1. This is a common problem in drug discovery for GPCRs and numerous examples abound in the literature. Drug substances that are limited in specificity to human target proteins can be problematic during drug development because they are difficult to test in surrogate animal efficacy models. Without efficacy data, it can become very difficult to justify further development of the drug given the considerable risks and costs involved.

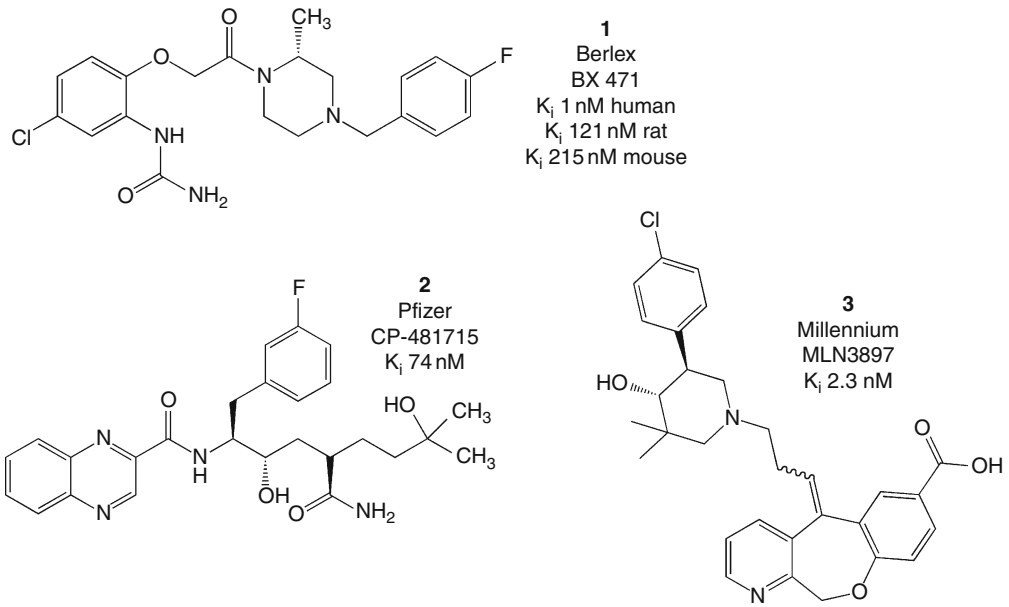
Berlex has disclosed a number of CCR1 compounds and the most advanced BX 471 is a potent diacyl piperazine more than 1000-fold selective for CCR1 (Liang et al. 2000). The antagonist has a reported K_D of 1.0 nM for human CCR1 calculated from radiolabeled binding studies. The compounds from this series also had issues with crossreactivity with rodent CCR1 (Fig. 4 compound 1) but they had sufficient affinity that they could be tested in animal models. BX 471 was efficacious in a number of mouse and rat models of disease including an acute rat experimental allergic encephalomyelitis (EAE) model of multiple sclerosis (Liang et al. 2000) a rat heart transplant model in the rat (Horuk et al. 2001) and mouse models of renal fibrosis (Anders et al. 2002) and multiple myeloma (Menu et al. 2006). Based on these data and positive phase I safety studies the CCR1 antagonist entered phase II clinical trials for multiple sclerosis in early 2004. Although the drug was well tolerated and showed no safety concerns its development was stopped after the clinical Phase II study failed to show a reduction in the number of new inflammatory central nervous system (CNS) lesions (detected by Magnetic Resonance Imaging, MRI) .



Chemokine Receptor CCR1, Fig. 3 The structure of human CCR1 predicted using MembStruk showing the BX 471 antagonist binding site predicted using HierDock. A top view of the predicted structure of BX 471 in the CCR1 binding pocket. The residues shown in red (Tyr113, Tyr114 and Ileu259) are responsible for anchoring the ligand in this cavity

Pfizer has been very active in the CCR1 field and has disclosed a number of CCR1 compounds including CP-481,715, a quinoxaline-2-carboxylic acid derivative (Gladue et al. 2003) (Fig. 4 compound 2). The antagonist has a reported K_i of 74 nM for human CCR1 calculated from displacement of radiolabeled CCL3 binding studies, and 71 nM from inhibition of Ca^{2+} transients (Gladue et al. 2003). The CCR1 antagonist is a competitive and reversible antagonist and is more than 100-fold selective for CCR1 as compared to a panel of G protein-coupled receptors including related chemokine receptors (Gladue et al. 2003). The compound successfully completed phase I safety studies and was successful in a 16 patient phase Ib clinical trial (Haringman et al. 2003). In this trial patients with active rheumatoid arthritis responded to the antagonist as demonstrated by a significant reduction in the number of macrophages and CCR1 positive cells in the synovium compared with a placebo group. A trend but no significant clinical improvements were seen in treated patients (Haringman et al. 2003). Based on these data CP-481715 entered phase II studies for rheumatoid arthritis in February 2004, however the trial was stopped after 6 weeks since although the compound was well tolerated it did not demonstrate any efficacy.

Millennium has disclosed a number of CCR1 antagonists the most advanced of which is MLN3897 (Fig. 4



Chemokine Receptor CCR1, Fig. 4 Structures of Berlex, Pfizer, and Millenium CCR1 receptor antagonists

compound 3) a substituted pyridylbenzoxepine. It demonstrated high affinity binding for CCR1 (K_i 1.2 nM) and had an IC_{50} of 3.4 nM for the inhibition of chemotaxis induced by CCL3. The compound was effective *in vivo* and demonstrated an EC_{50} of 0.03 mg/kg in inhibiting CCL3 induced immune cell recruitment in a guinea pig skin sensitization model (Pharmacokinetic studies revealed that it had a half-life of 3 h in rat and oral bioavailability of 35% in rat and 100% in dog. In 2004, Millennium announced that they were in phase I clinical trials with MLN3897 and the major indications appeared to be rheumatoid arthritis, multiple sclerosis and psoriasis. In November 2007, Millennium announced that they were terminating the development of MLN3897 for rheumatoid arthritis because it failed to reach its clinical endpoint in a phase II trial. Millennium, along with partner Sanofi-Aventis is assessing the next steps for the program.

Regulation of Concentration

CCR1 has been reported to be regulated by a number of factors including cytokines, lipopolysaccharide, statins, substance P, and estrogen amongst others.

CCR1 activity is coordinately regulated by cytokines like GM-CSF and IL-2. For example, although CCR1 is constitutively expressed on murine neutrophils, almost no expression is seen on human cells. However the mRNA for CCR1 is rapidly upregulated in cells that have been treated with the cytokine GM-CSF, and these cells, but not untreated neutrophils, respond to the CCR1 ligands CCL3 and CCL5 in chemotaxis and intracellular calcium mobilization assays . Freshly isolated CD45RO + lymphocytes cultured for 10 days with IL-2 migrated in response to the CCR1 ligand CCL5 while control cells cultured in the absence of the cytokine did not. This functional response of these cells correlated to an induction of mRNA for CCR1 in the cytokine-treated cells.

Interestingly recent work shows that proinflammatory signals can also deactivate normally functional chemokine receptors and turn them into decoy receptors. Treatment of dendritic cells with LPS + IL-10 showed high expression of CCR1 and other CC chemokine receptors. However, these receptors were unable to elicit migration in response to their ligands. Similar results were obtained for monocytes exposed to activating signals and IL-10. Thus, in an inflammatory environment, IL-10 generates functional decoy receptors on DC and monocytes, which act as molecular sinks and scavengers for inflammatory chemokines.

In addition to their lipid-lowering effects statins have potent anti-inflammatory properties and part of this can be ascribed to their ability to modulate

chemokine and chemokine receptor activity. *In vitro* experiments on human vascular endothelial cells and human primary macrophages. revealed that simvastatin significantly reduced CCL2 in endothelial cells and macrophages stimulated with TNF-alpha or IFN-gamma, respectively. Messenger RNA analysis revealed that expression of the chemokines CCL2, CCL3, and CCL4, as well as the chemokine receptors CCR1, CCR2, CCR4, and CCR5, was decreased by simvastatin, both in endothelial cells and macrophages. The statin effects were reversed by mevalonate and mimicked by the geranylgeranyl transferase inhibitor, whereas the farnesyl transeferase inhibitor had no effect. These results suggest that statins act via inhibition of the geranylgeranylation of proteins.

Neuropeptides like substance P play an important role in the active communication between the nervous and immune systems. A recent study revealed the modulatory effect of substance P on chemokine production and chemokine receptor expression in primary mouse neutrophils. Substance P induced both the mRNA and protein expression of the chemokines CCL3 and CXCL2 in neutrophils and upregulated their receptors CCR1 and CXCR2. This stimulatory effect on chemokine and chemokine receptor expression in neutrophils was further found to be neurokinin-1 receptor specific.

It is well established that estrogens appear to be beneficial in reducing relapses in patients with multiple sclerosis. This protective effect of the hormone may be partly due to its effects on the expression of chemokines and chemokine receptors as revealed by a recent study{Matejuk, 2001 #7003}. In EAE protected mice, 17 beta-estradiol strongly inhibited the mRNA expression of a number of chemokines including CCL3 and CCL5, and of the chemokine receptors CCR1, CCR2, and CCR5. Conversely, ovariectomy, which abrogated basal 17 beta-estradiol levels and increased the severity of EAE, enhanced the expression of CCR3 that were over-expressed by inflammatory mononuclear cells. Elevated expression of CCR1 and CCR5 by lymph node cells was also inhibited in 17 beta-estradiol treated mice with EAE. These results suggest that the beneficial effects of 17 beta-estradiol are mediated in part by strong inhibition of recruited inflammatory cells, resulting in reduced production of inflammatory chemokines and cytokines in CNS.

Major Sites of Expression

CCR1 is widely expressed in both immune and nonimmune cells (Murphy et al. 2000). Expression of this receptor has been observed in airway smooth muscle cells in the lung suggesting a possible role in asthma, in both normal neurons (Hesselgesser and Horuk 1999) and in dystrophic neurons from patients with Alzheimer's dementia (Halks-Miller et al. 2003), in astrocytes (Tanabe et al. 1997), which suggests a role in inflammation in the CNS, in endothelial cells which can be induced to chemotax in response to CCL23 and may play a role in angiogenesis, and in multiple myeloma cells and osteoclasts (Moller et al. 2003; Oba et al. 2005) hinting at a role for CCR1 in bone cancer. CCR1 is also abundantly expressed on immune cells, including monocytes, memory T cells, basophils, dendritic cells (Gao et al. 1993; Murphy et al. 2000; Neote et al. 1993) and is induced by GM-CSF in neutrophils.

Phenotypes

CCR1-deficient mice generated by targeted gene disruption have revealed that CCR1 has a number of non-redundant functions in host defense and inflammation. The role of CCR1 in host defense has been examined in a number of studies. In the first study when CCR1 knockout mice were challenged with Aspergillus fumigatus, a fungus controlled mainly by neutrophils, they showed an increase in mortality. Interestingly, knockout animals in the same study showed a 40% reduction, compared to their wild-type littermates, in the size of lung granulomas induced by intravenous injection of Schistosoma mansoni eggs. In another study CCR1 knockout mice were challenged with the obligate intracellular protozoan parasite Toxoplasma gondii. In comparison with parental wild-type mice, knockout mice showed an increased mortality to T. gondii in association with an increased tissue parasite load. The increased mortality appeared to be correlated to a reduction in the trafficking of neutrophils to inflamed areas in the knockout mice during early infection.

The role of CCR1 in host defense was further examined in a model of viral-induced neurologic disease. Intracerebral infection of mice with mouse hepatitis virus results in an acute encephalitis followed by a chronic demyelinating disease similar in pathology to that observed in multiple sclerosis. Although no increase in mortality was observed during the acute phase of disease following infection by 21 days

post-infection, 74% of CCR1 knockout mice had died compared to only 32% mortality of wild-type mice. The CCR1 knockout mice appeared to have a reduction in T-cell accumulation within the CNS during acute, but not chronic, disease. However, despite this other components of the immune response appeared to be unaltered in the knockout mice Despite the reduction in T-cell trafficking into the CNS of CCR1 knockout mice during acute disease, components of host defense such as T-cell effector functions including cytolytic activity and proliferation and the expression of IFN-gamma remained unaltered. These findings suggest that T-cell and macrophage trafficking are not dependent on CCR1 and suggest a non-redundant role for CCR1 in promoting survival during chronic mouse hepatitis virus infection.

Both CCR1 and one of its ligands, CCL3, appear to be important for protection against infection with paramyxovirus pneumonia virus in mice. Infection of wild-type mice with the virus results in pulmonary neutrophilia and eosinophilia accompanied by local production of CCL3. Mice deficient in CCL3 or its receptor, CCR1 showed an attenuated inflammatory response to the virus with limited neutrophil trafficking and no eosinophils detected in bronchoalveolar lavage fluid. This was accompanied by a higher rate of mortalitry compared to wild-type mice. These results suggest that the CCL3/CCR1-mediated acute inflammatory response protects mice by delaying the lethal events of infection.

CCR1 also appears to play an important role in the pathopysiology of disease. In an animal model of inflammation CCR1 knockout mice were protected from pulmonary inflammation secondary to acute pancreatitis. The protection from lung injury was associated with decreased levels of cytokines such as TNF-alpha suggesting that the activation of the CCR1 receptor is an early event in the systemic inflammatory response syndrome. CCR1 also appears to be involved in remodeling after myocardial infarction. Studies with CCR1 deficient mice revealed that when myocardial infarction was induced, although initial infarct areas and areas at risk did not differ between groups, the infarct size increased in wild-type mice compared to CCR1 deficient animals. This attenuation in infarct expansion was associated with preserved left ventricular function and was accompanied by an altered post-infarct inflammatory pattern characterized by diminished neutrophil infiltration, accelerated monocyte/lymphocyte infiltration, decreased apoptosis, increased cell proliferation and earlier myofibroblast population in the infarcted tissue. This reduction in functional impairment and structural remodeling after myocardial infarction in CCR1 knockout animals suggests a role for this receptor in cardiac disease.

Since CCR1 appears to be expressed in demyelinating lesions in patients with multiple sclerosis and neutralization of one of its ligands, CCL3, is protective in a relapsing remitting model of multiple sclerosis in mice the role of the receptor was examined in a myelin oligodendrocyte glycoprotein (MOG) induced model of disease in mice. After immunization with MOG nearly all of the wild-type animals developed disease with a clinical score of 2.5. In contrast CCR1 deficient mice showed a decreased incidence to disease, less than 55%, and the severity of those with disease was reduced by around 50% with a clinical score of 1.2. In addition there were elevated levels of mRNA for the chemokines CCL2 and CCL5 in the CNS of wild-type animals compared to CCR1 deficient animals.

While there is no question that CCR1 gene deletion studies in mice have been extremely useful in illuminating the role of this receptor both in normal and in pathophysiology we need to exercise some caution in extrapolating these studies for human CCR1. It is known that the immune system in rodents can be very different from that in humans. For example CCR1 is expressed constitutively in mouse neutrophils whereas it is inducible in human neutrophils (Murphy et al. 2000). Thus it is likely that the role of this receptor will be somewhat different in rodents than in humans when responding to an attack by pathogenic organisms. In addition, although CCR1 knockouts hint at a role for CCR1 in diseases like multiple sclerosis the fact that there is not a total abrogation of the disease in CCR1 deficient animals suggests that there is some redundancy in the system and this might partly account for the failure of a number of CCR1 antagonists in clinical trials.

Summary

CCR1 was the first human CC chemokine receptor to be identified at the cDNA level. It has a functional viral homolog, US28, which is a human cytomegalovirus (Gao et al. 1993; Neote et al. 1993). Although CCR1 was initially described as a receptor for the

chemokines CCL3 and CCL5 a number of other chemokine ligands have been described, some of which are generated from weak agonists by proteases during inflammatory responses in vivo. The ligands of many of the chemokine receptors, including those of CCR1, can also act as agonists on other chemokine receptors and this ligand redundancy has made it difficult to successfully therapeutically target these proteins. In humans, CCR1 is expressed in a variety of cell types including monocytes, memory T cells, basophils, dendritic cells, platelets, neurons, and astrocytes (Gao et al. 1993; Neote et al. 1993; Weber et al. 2001) and is induced by GM-CSF in neutrophils. Since CCR1 appears to play role in host defense and inflammation it represents a potential target for therapeutic intervention in a variety of clinically important diseases including rheumatoid arthritis, multiple sclerosis, transplant rejection, and allergic inflammation. Several companies have disclosed small molecule CCR1 antagonists and a number of these have been tested in clinical trials, for multiple sclerosis, rheumatoid arthritis, psoriasis, and endometriosis. Disappointingly, however, none of these approaches reached their clinical endpoints and the programs were all halted.

References

Anders HJ, Vielhauer V, Frink M, Linde Y, Cohen CD, Blattner SM, et al. A chemokine receptor CCR-1 antagonist reduces renal fibrosis after unilateral ureter ligation. J Clin Invest. 2002;109(2):251–9.

Futamatsu H, Suzuki J, Koga N, Adachi S, Kosuge H, Maejima Y, et al. A CCR1 antagonist prevents the development of experimental autoimmune myocarditis in association with T cell inactivation. J Mol Cell Cardiol. 2006;40(6):853–61.

Gao JL, Kuhns DB, Tiffany HL, McDermott D, Li X, Francke U, et al. Structure and functional expression of the human macrophage inflammatory protein 1 alpha/RANTES receptor. J Exp Med. 1993;177(5):1421–7.

Gladue RP, Tylaska LA, Brissette WH, Lira PD, Kath JC, Poss CS, et al. CP-481,715: A potent and selective CCR1 antagonist with potential therapeutic implications for inflammatory diseases. J Biol Chem. 2003;278:40473–80.

Guan E, Wang J, Norcross MA. Amino-terminal processing of MIP-1beta/CCL4 by CD26/dipeptidyl-peptidase IV. J Cell Biochem. 2004;92(1):53–64.

Halks-Miller M, Schroeder ML, Haroutunian V, Moenning U, Rossi M, Achim C, et al. CCR1 is an early and specific marker of Alzheimer's disease. Ann Neurol. 2003;54(5):638–46.

Haringman JJ, Kraan MC, Smeets TJ, Zwinderman KH, Tak PP. Chemokine blockade and chronic inflammatory disease: proof of concept in patients with rheumatoid arthritis. Ann Rheum Dis. 2003;62(8):715–21.

Hesselgesser J, Horuk R. Chemokine and chemokine receptor expression in the central nervous system. J Neurovirol. 1999;5(1):13–26.

Holmes WE, Lee J, Kuang WJ, Rice GC, Wood WI. Structure and functional expression of a human interleukin-8 receptor. Science. 1991;253(5025):1278–80.

Horuk R, Clayberger C, Krensky AM, Wang Z, Grone HJ, Weber C, et al. A non-peptide functional antagonist of the CCR1 chemokine receptor is effective in rat heart transplant rejection. J Biol Chem. 2001;276(6):4199–204.

Ko J, Yun CY, Lee JS, Kim JH, Kim IS. p38 MAPK and ERK activation by 9-cis-retinoic acid induces chemokine receptors CCR1 and CCR2 expression in human monocytic THP-1 cells. Exp Mol Med. 2007;39(2):129–38.

Liang M, Mallari C, Rosser M, Ng HP, May K, Monahan S, et al. Identification and characterization of a potent, selective, and orally active antagonist of the CC chemokine receptor-1. J Biol Chem. 2000;275(25):19000–8.

Liu J, Louie S, Hsu W, Yu KM, Nicholas Jr HB, Rosenquist GL. Tyrosine sulfation is prevalent in human chemokine receptors important in lung disease. Am J Respir Cell Mol Biol. 2008;38(6):738–43.

Menu E, De Leenheer E, De Raeve H, Coulton L, Imanishi T, Miyashita K, et al. Role of CCR1 and CCR5 in homing and growth of multiple myeloma and in the development of osteolytic lesions: a study in the 5TMM model. Clin Exp Metastasis. 2006;23(5–6):291–300.

Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G. Expression and function of chemokine receptors in human multiple myeloma. Leukemia. 2003;17(1):203–10.

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev. 2000;52(1):145–76.

Neote K, DiGregorio D, Mak JY, Horuk R, Schall TJ. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. Cell. 1993;72(3):415–25.

Oba Y, Lee JW, Ehrlich LA, Chung HY, Jelinek DF, Callander NS, et al. MIP-1alpha utilizes both CCR1 and CCR5 to induce osteoclast formation and increase adhesion of myeloma cells to marrow stromal cells. Exp Hematol. 2005;33(3):272–8.

Pease JE, Wang J, Ponath PD, Murphy PM. The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1alpha and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. J Biol Chem. 1998;273(32):19972–6.

Richardson RM, Pridgen BC, Haribabu B, Snyderman R. Regulation of the human chemokine receptor CCR1. Cross-regulation by cxcr1 and cxcr2. J Biol Chem. 2000;275(13):9201–8.

Tanabe S, Heesen M, Berman MA, Fischer MB, Yoshizawa I, Luo Y, et al. Murine astrocytes express a functional chemokine receptor. J Neurosci. 1997;17(17):6522–8.

Vaidehi N, Schlyer S, Trabanino RJ, Floriano WB, Abrol R, Sharma S, et al. Predictions of CCR1 chemokine receptor structure and BX 471 antagonist binding followed by experimental validation. J Biol Chem. 2006;281(37):27613–20.

Weber C, Weber KS, Klier C, Gu S, Wank R, Horuk R, et al. Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-like/CD45RO(+) T cells. Blood. 2001;97(4):1144–6.
Zoffmann S, Chollet A, Galzi JL. Identification of the extracellular loop 2 as the point of interaction between the N Terminus of the chemokine MIP-1alpha and Its CCR1 receptor. Mol Pharmacol. 2002;62(3):729–36.

Chiffon (*Drosophila*)

▶ Dbf4

CHK

▶ CSK-Homologous Kinase

Cholecystokinin Receptor 1

▶ Cholecystokinin-1 Receptor

Cholecystokinin Type A Receptor

▶ Cholecystokinin-1 Receptor

Cholecystokinin-1 Receptor

Laurence J. Miller
Department of Molecular Pharmacology and Experimental Therapeutics and Department of Internal Medicine, Division of Gastroenterology, Mayo Clinic, Scottsdale, AZ, USA

Synonyms

CCK(A) receptor; CCK1; CCK-A receptor; CCKA; CCKAR; CCK-AR; Cholecystokinin receptor 1; Cholecystokinin type A receptor; Cholecystokinin-1 receptor: CCK-1 receptor; Peripheral type CCK receptor

Historical Background

The hormone cholecystokinin (CCK) was discovered in 1928 by Ivy and Oldberg, based on its ability to stimulate gallbladder contraction. Fifteen years later, Harper and Raper described a factor capable of stimulating pancreatic exocrine secretion (pancreozymin). When Jorpes and Mutt finally isolated the CCK peptide from porcine duodenum in 1966, it became clear that this single hormone was responsible for both of these classical physiological gastrointestinal regulatory activities. Many years later, in 1992, the receptor mediating these effects was finally identified and characterized as a family A guanine nucleotide-binding protein (G protein)-coupled receptor, the CCK-1 receptor (Liddle 1994). Of interest, the cDNA encoding this receptor was initially cloned and described in the same issue of the *Proceedings of the National Academy of Sciences, USA,* as the structurally closely related CCK-2 receptor (see the following chapter on this receptor). The CCK-1 receptor has subsequently been recognized as having multiple effects on tissues including gallbladder, pancreas, gut, and neurons in the periphery and central nervous system, with most of these effects related to maintenance of nutritional homeostasis. This includes optimization of the digestive milieu, regulation of the rate of transit of intestinal chyme for optimal nutrient absorption, and the control of appetite. Indeed, this receptor has become the target of substantial efforts by pharmaceutical companies to develop an agonist drug that might be useful in the treatment of obesity as a noncaloric satiety agent.

Tissue Distribution and Physiological Actions of CCK-1 Receptors

The distribution and functions of CCK receptors differ among species, with much of the fundamental physiologic studies having been performed in rats, mice, guinea pigs, and dogs in the period of time before the CCK receptor genes had been cloned. Now that the molecular nature of the two types of CCK receptors have been established, and highly specific and sensitive assays for their presence and activity are now in place, the role of the CCK-1 receptor in human physiology can be better established. Human tissues with established physiological functions for the CCK-1 receptor include gallbladder smooth muscle,

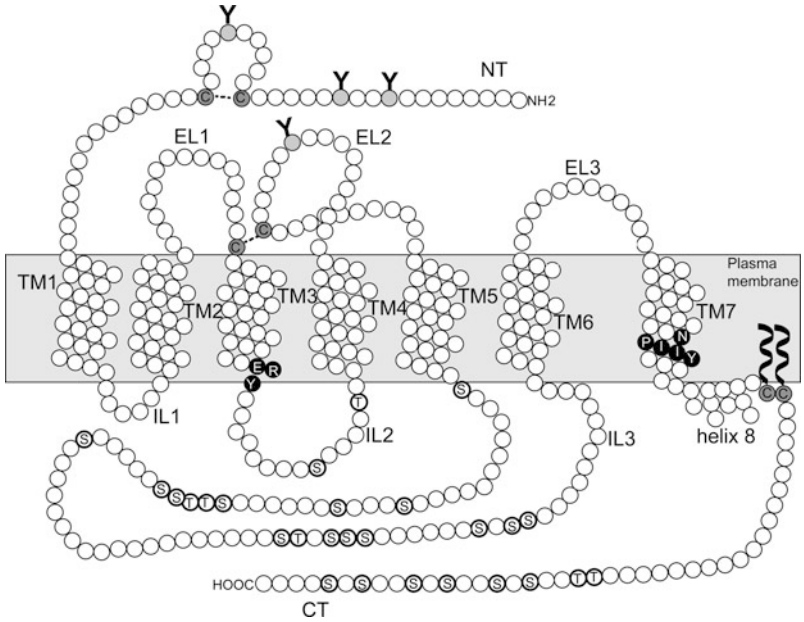
pancreatic neurons (and low levels of expression on acinar cells), gastric and intestinal smooth muscle, vagal afferent neurons, and distinct central nervous system nuclei (Noble et al. 1999). These targets are involved in the normal physiologic stimulation of gallbladder emptying, stimulation of pancreatic exocrine secretion, regulation of gastric emptying and bowel transit, and induction of postcibal satiety. These functions naturally follow from the distribution and secretory pattern of the hormone that activates them. CCK is produced in I cells scattered within the mucosa of the proximal small intestine, and is secreted into the bloodstream in response to luminal nutrients, such as protein and fat. These complex nutrients require micelle formation (fat), digestion (protein and fat), and regulated enteric transit of chyme for optimal absorption. CCK is also produced in some neurons where it can have neurocrine effects. Other possible effects of this hormone on CCK-1 receptors expressed on healthy cells, with less clearly established physiological roles, include stimulation of the secretion of somatostatin from gastric D cells and secretion of pepsinogen and leptin from chief cells, trophic effects on the exocrine pancreas, and the regulation of secretion of insulin, pancreatic polypeptide, and somatostatin from the pancreatic islets.

Structure of CCK-1 Receptors and Molecular Basis of Ligand Binding

The CCK-1 receptor is a typical peptide-binding receptor in the rhodopsin-β adrenergic family (family A) of G protein-coupled receptors (Miller and Gao 2008). It contains seven transmembrane helical segments that form a helical bundle within the plasma membrane, glycosylated extracellular loop and amino-terminal tail domains, a conserved disulfide bond linking cysteine residues at the top of transmembrane segment 3 with a cysteine in the second extracellular loop, an intradomain disulfide bond linking two cysteine residues within the amino-terminal tail region, phosphorylated intracellular loop and carboxyl-terminal tail domains, and palmitoylated cysteine residues beyond the seventh transmembrane segment that help to form a membrane-associated intracellular eighth helical segment region (Fig. 1). This receptor includes the signature sequences typical of this receptor family, including the D/ERY motif at the cytosolic face of transmembrane segment 3 and the NPxxY motif in transmembrane segment 7. The glycosylation of this receptor appears to help with its normal folding during biosynthesis and to protect the receptor against proteolytic cleavage. The cytosolic sites of phosphorylation are on serine and threonine residues within the second and third intracellular loop and carboxyl-terminal tail domains, representing targets of protein kinase C, a signaling kinase activated by CCK action, as well as by action of a G protein-coupled receptor kinase (Klueppelberg et al. 1991).

The natural peptide agonist ligand for the CCK-1 receptor binds at the extracellular surface of this receptor, with key determinants within loop and amino-terminal tail epitopes (Miller and Gao 2008). This has been well established in receptor truncation and mutagenesis studies, chimeric receptor studies, ligand structure-activity studies, and photoaffinity labeling studies. The latter represent the most direct evidence for mode of docking, and have been performed to establish spatial approximation constraints involving six of the seven residues within the focused pharmacophoric domain of this hormone, as well as the amino-terminal region of the hormone just outside this domain. Of note, analogous studies with the CCK-2 receptor have suggested a distinct mechanism for the binding of the same peptide to that receptor, with the possibility that the carboxyl terminus of CCK dips down within the helical bundle of the CCK-2 receptor. It is noteworthy that the structure-activity characteristics for peptide binding and activation of the two types of CCK receptors are quite distinct, with the CCK-1 receptor requiring the carboxyl-terminal heptapeptide-amide of CCK for high-affinity binding and activation, while only the carboxyl-terminal tetrapeptide-amide is required for the CCK-2 receptor. With gastrin sharing the carboxyl-terminal pentapeptide-amide with all CCK peptide species, it is clear that both gastrin and CCK bind and activate the CCK-2 receptor with high affinity and potency, while only CCK peptides have these actions at the CCK-1 receptor.

For the CCK-1 receptor, in addition to the orthosteric natural peptide hormone-binding site, there is an allosteric ligand-binding pocket within the intramembranous helical bundle that is the site of docking a series of small molecule benzodiazepine ligands. This pocket has been shown to be totally distinct from the CCK-binding site using classical



Cholecystokinin-1 Receptor, Fig. 1 Shown is a schematic diagram of the primary structure of the human CCK-1 receptor, with the seven transmembrane helices (TM) shown within the gray area representing the plasma membrane and the extracellular amino-terminal tail (NT) and loop (EL) regions above the membrane and the intracellular loop (IL) regions, helix 8 and carboxyl-terminal tail (CT) regions below the membrane. Also identified are sites of glycosylation ("Y" structures), disulfide bonds (S-S), and sites of palmitoylation (*curved lines*). Sites of phosphorylation (S and T) are present in the intracellular loop and tail regions. Conserved D/ERY and NPxxY motifs are also shown in their positions in the cytosolic face of TM3 and the end of TM7

manipulations, such as the ability of natural ligand to accelerate the dissociation of receptor-bound benzodiazepine (Gao et al. 2008). Molecular models also support the separate and distinct nature of the peptide and non-peptidyl small molecule binding sites within the CCK-1 receptor.

Signaling at the CCK-1 Receptor

The classical pathway of signaling stimulated by natural agonist action at the CCK-1 receptor is mediated via coupling with heterotrimeric G proteins in the G_q family (G_q, G_{11}, and G_{14}), involving the rapid hydrolysis of phosphatidylinositol bisphosphate by phospholipase C enzymes (principally β isoforms) to generate inositol trisphosphate and diacylglycerol. This results in calcium mobilization from intracellular stores and the subsequent activation of various isoforms of protein kinase C (Williams 2001). High concentrations of CCK can also stimulate coupling of this receptor with G_s, with subsequent activation of adenylate cyclase, increase in cAMP, and activation of protein kinase A, although the physiologic nature of this signaling pathway is not clear. There have also been reports of CCK acting through this receptor to stimulate mitogen-activated protein kinases (MAPKs). These include extracellular signal-regulated kinases 1 and 2 (ERK 1 and ERK 2), c-jun kinases (JNKs), and ERK 5 and p38 MAPK. These signaling events are downstream of the G protein coupling events. Phosphatidylinositol 3-kinases (PI 3-kinases), p125-focal adhesion kinase (FAK), and Janus kinases (JAKs) are also activated by CCK action on this receptor. Current understanding of the linkage and interrelationship of these signaling molecules has been reviewed elsewhere (Williams 2001; Cawston and Miller 2010).

In addition to association with the classical proximal mediator of signaling, representing coupling of the heterotrimeric G proteins with the CCK-1 receptor, this receptor is also known to associate with arrestin molecules. Arrestin binding to a G protein-coupled receptor can occur as a result of agonist binding with signaling kinase action leading to phosphorylation of the receptor, as well as direct interaction of arrestin

with non-phosphorylated portions of the receptor. Arrestin is a known multifunctional adapter protein that can contribute to receptor endocytosis and to signaling events in the cell, including some of those described above (Whalen et al. 2011).

Biochemical and Cellular Regulation of the CCK-1 Receptor

Like most G protein-coupled receptors, the CCK-1 receptor is regulated by both biochemical and cellular mechanisms to protect the cell from overstimulation (Miller and Gao 2008). Indeed, hyperstimulation through this receptor is the basis for one of the most frequently used animal models of pancreatitis, in which very high doses of the CCK analogue caerulein is administered and results in fusion of the zymogen granules with the lateral (rather than the apical) plasmalemma and release of these enzymes into the pancreatic parenchyma where they become activated and initiate autodigestion of this organ and surrounding tissues. The most common biochemical mechanism of regulation of this receptor involves phosphorylation. This occurs exclusively on serine and threonine residues within the intracellular loops and carboxyl-terminal tail domains. The earliest and most sensitive phosphorylation events result from activation of protein kinase C. Of interest the phosphorylation of key protein kinase C substrates within the third intracellular loop has been shown to produce a conformational change that makes the second loop accessible for its phosphorylation and for its interaction with cellular regulatory molecules. Higher concentrations of CCK also stimulate the slower action of a G protein-coupled receptor kinase that adds to the phosphorylation of this receptor. These phosphorylation events have been shown to interfere with the normal coupling of this receptor with its G proteins. Presumably, this also contributes to arrestin association, known to effect such disruption of coupling.

Because the natural agonists for the CCK-1 receptor are hydrophilic peptides that are unable to cross the lipid bilayer, internalization of the receptor is another very effective mechanism for desensitization in which the hormone is unable to bind to and stimulate the receptor. Indeed, the agonist occupation of this receptor has been shown to result in the internalization of this receptor, with clathrin-dependent endocytosis representing the major pathway (Roettger et al. 1993). In CHO cell lines, potocytosis into caveolae has also been described. Another interesting cellular mechanism for desensitization that has been described was identified as insulation in which the receptor is moved into a highly specialized plasmalemmal domain that is devoid of G proteins for coupling with the receptor. These cellular regulatory mechanisms are typically slower of onset and slower to reverse than the biochemical mechanisms described above.

Clinical Relevance of the CCK-1 Receptor

The CCK-1 receptor has been implicated in mediating some of the intestinal dysmotility responsible for abdominal crampy pain and abnormal bowel habits in irritable bowel syndrome, as well as the gallbladder inertia and incomplete gallbladder emptying found in cholesterol gallstone disease. It has also been proposed as playing a role in the reduced satiety and increased food consumption present in some patients with obesity and obesity-related medical disorders. Indeed, it is the latter that has stimulated most of the work by pharmaceutical companies to develop noncaloric agonists of this receptor as a means for inducing satiety and reducing food intake.

There has also been substantial work focused on CCK receptors in neoplastic diseases (Reubi et al. 1997). This follows the established trophic effects of this hormone on target tissues that are mediated by these receptors. Indeed, many epithelial cancer cell lines have been described to express CCK-1 and/or CCK-2 receptors, or misspliced variants of these receptors (Korner and Miller 2009). The major cancers that can express the CCK-1 receptor include pancreatic ductal adenocarcinoma, colorectal carcinoma, meningiomas, and some neuroblastomas. Antagonists of this receptor are being studied to examine their potential therapeutic role in pancreatic cancer. Additionally, reagents directed to this receptor may have an imaging, staging, or stratification role for these tumors.

Summary

The CCK-1 receptor is physiologically quite important for normal digestion, bowel transit, and appetite

control, all roles key for normal nutritional homeostasis. Additionally, this receptor plays various roles in common gastrointestinal disorders, including irritable bowel syndrome and gallstone disease, as well as likely contributing to the development of obesity and potentially playing a therapeutic role in this disorder and in obesity-related medical diseases. It also can be expressed on various cancers, where agents targeting this receptor can be used in diagnosis, staging, stratification, and possibly also treatment.

References

Cawston EE, Miller LJ. Therapeutic potential for novel drugs targeting the type 1 cholecystokinin receptor. Br J Pharmacol. 2010;159:1009–21.

Gao F, Sexton PM, Christopoulos A, Miller LJ. Benzodiazepine ligands can act as allosteric modulators of the Type 1 cholecystokinin receptor. Bioorg Med Chem Lett. 2008;18:4401–4.

Klueppelberg UG, Gates LK, Gorelick FS, Miller LJ. Agonist-regulated phosphorylation of the pancreatic cholecystokinin receptor. J Biol Chem. 1991;266:2403–8.

Korner M, Miller LJ. Alternative splicing of pre-mRNA in cancer: focus on G protein-coupled peptide hormone receptors. Am J Pathol. 2009;175:461–72.

Liddle RA. Cholecystokinin. In: Walsh JH, Dockray GJ, editors. Gut peptides: biochemistry and physiology. New York: Raven; 1994. p. 175–216.

Miller LJ, Gao F. Structural basis of cholecystokinin receptor binding and regulation. Pharmacol Ther. 2008;119:83–95.

Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, et al. International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol Rev. 1999;51:745–81.

Reubi JC, Schaer JC, Waser B. Cholecystokinin(CCK)-A and CCK-B/gastrin receptors in human tumors. Cancer Res. 1997;57:1377–86.

Roettger BF, Rentsch RU, Pinon DI, Holicky E, Hadac E, Larkin JM, et al. Dual pathwasy of internalization of the cholecystokinin receptor. J Cell Biol. 1993;128:1029–41.

Whalen EJ, Rajagopal S, Lefkowitz RJ. Therapeutic potential of beta-arrestin- and G protein-biased agonists. Trends Mol Med. 2011;17:126–39.

Williams JA. Intracellular signaling mechanisms activated by cholecystokinin-regulating synthesis and secretion of digestive enzymes in pancreatic acinar cells. Annu Rev Physiol. 2001;63:77–97.

Cholecystokinin-1 Receptor: CCK-1 Receptor

► Cholecystokinin-1 Receptor

Cholecystokinin-2 Receptor

Jean Morisset
Service de gastroentérologie, Département de médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada

Synonyms

The cholecystokinin-B receptor

Historical Background

With the availability of modern biochemical and molecular techniques developed in the 1980s and 1990s, it was possible to characterize and clone the two cholecystokinin receptors known today. They are now identified as the cholecystokinin-1 (CCK-1) and the cholecystokinin-2 (CCK-2) receptor subtypes.

In this review entry, we will summarize what we now know about the CCK-2 receptor. More specifically, we will discuss its biochemical characterization, its organ localization, its functions in normal organs, its role in cancer along with the intracellular signaling routes used to relay its messages. References to the other cholecystokinin receptor are given in the preceding chapter.

Biochemical Characterization

Initially, the CCK-1 receptor was known as the CCK_A receptor (A for alimentary) and the CCK-2 receptor as the CCK_B receptor (B for brain). This reclassification of the CCK receptor subtypes occurred recently based on recommendations from the International Union of Pharmacology Committee regarding receptor nomenclature and drug classification.

These two receptors can be differentiated by their respective affinity for CCK and gastrin, their respective natural agonist. Indeed, the CCK-1R binds and responds to sulfated CCK with a 500–1,000-fold higher affinity or potency than sulfated gastrin or nonsulfated CCK. On the contrary, the CCK-2R binds and responds to gastrin and CCK with almost the same affinity or potency, and discriminates poorly between sulfated and nonsulfated agonists (Dufresne et al. 2006). The CCK-2R gene has been cloned in five

different species (human, rat, mouse, dog, and rabbit). According to published data, the human CCK-2R has 90% identity to the rat and canine receptor. The predicted mouse CCK-2R shares 87% and 92% amino acid identity with the human and rat receptor, respectively. The rabbit CCK-2R protein exhibits 93–97% amino acid similarly with corresponding cDNA identified in human, canine and rat brain or stomach. In general, the CCK-2R cloned from different species exhibits a comparable high affinity for the agonists CCK-8 and gastrin-17-1 with IC50 in the nM range. On the contrary, it has a higher affinity for its specific antagonist, L-365,260 (IC_{50} around 10 nM) than for the CCK-1R antagonist L-364,718 (IC_{50} around 1 μM). Finally, as opposed to the CCK-1R, the CCK-2R binds to a single class of high affinity sites with a K_D in the range of 0.2–2.0 nM (Morisset 2005).

Localization

The presence and cellular localization of a receptor in a specific organ can be detected and determined by at least five different techniques: agonist- or antagonist-binding studies, RT-PCR, Western blot, immunological staining, and autoradiography. Of these five, the Western blot and the immunological staining techniques are the most reliable and informative on the presence of the receptor and its specific localization if the specificity of the antibodies used has been carefully evaluated. However, so far, the nonquantitative RT-PCR technique has been the major method of analysis. This technique is indeed very sensitive but there is always this possibility that the message levels detected could be extremely low and therefore, may not translate into the expression of functional levels of receptors. The autoradiography technique with a radioactive ligand seems the least sensitive while the binding technique mostly used cell membrane preparations. All five techniques can be used for receptor detection but cellular localization can be most efficiently and accurately revealed by immunological staining and confirmation with Western blot using preferably the same antibody. Therefore, to relate a physiological response to a specific receptor's occupation, we need to know that the receptor is there and that it is really localized on the cell where the physiological response originates from in the target organ.

The CCK-2R was mainly found throughout the central nervous system in areas that parallel the distribution of CCK and gastrin immunoreactivity and mRNAs. Samples from rat brain tissue allowed the detection of CCK-2R mRNA. Mature forms of the receptor mRNA were identified in cerebral cortex, hypothalamus, and hippocampus. Moreover, a truncated form of the receptor was identified in all regions of the brain studied (Jagerschmidt et al. 1994). Using the PCR amplification technique, the CCK-2R was also identified in the brain, but also in the stomach, pancreas, small intestine, liver, colon, spleen, lung, thymus, ovary, breast, prostate, testes, adrenals, and kidneys, as opposed to what was observed previously (Morisset 2005).

CCK binding sites were also identified on rat adipocyte membranes and their occupation lead to the control of leptin release (Attoub et al. 1999). In the adrenal glands, CCK-stimulated aldosterone release was related to occupation of both CCK subtypes in rats, but exclusively through CCK-2R occupation in humans (Mazzocchi et al. 2004). In normal esophagus, the CCK-2R has been revealed by RT-PCR and localized to columnar epithelia within the glandular and lower crypt regions of the mucosa by microautoradiography (Haigh et al. 2003).

Even though the CCK-2R was first identified in the brain, historically it was originally cloned by Kopin through the expression of a canine parietal cell cDNA library and isolation of a cDNA clone encoding a 453-amino-acid protein.

Although there is no doubt that the CCK-2R is present in the stomach, its precise location at the cellular level still presents some controversies depending on the technique used for detection. By RT-PCR, the receptor was identified in rat and mice antrum mucosae, and in the fundus by Northern blot analysis. By in situ hybridization, the CCK-2R was present only on parietal cells during the perinatal period in rats and mainly in ECL cells in adult rodents (Waki et al. 1998). This absence of CCK-2Rs on rat adult parietal cells was confirmed by Fluo-CCK-8 binding studies which located the receptor only on ECL cells co-localized with histidine decarboxylase (Bakke et al. 2001). These data contradict the cloning data of Kopin performed on a 95% pure isolated canine parietal cell preparation, but contamination with only a few ECL cells could be enough to give one CCK-2R clone.

Adding to the confusion about CCK-2R localization in the stomach, immunohistochemistry and electron

microscopy studies performed with polyclonal antibodies to human gastrin indicate, on one hand, that gastrin binding sites were found in the guinea pig stomach on parietal and chief cells but much less on ECL and somatostatin cells (Tarasova et al. 1996). On the other hand, using an antibody raised against the C-terminal decapeptide of the CCK-2R, it was found that undifferentiated rat gastric epithelial cells express CCK-2R. Furthermore, other epithelial cells in the progenitor zone of the adult gastric gland also expressed the receptor and among them were the parietal, chief, and ECL cells (Tommeras et al. 2002).

In the pancreas, we encountered the same location problems as in the other organs with the usual techniques used. Indeed, from whole human pancreas and isolated acini, RT-PCR amplified both receptor subtypes mRNA but the receptor protein could not be identified. On the contrary, this CCK-2R has been detected in the pig pancreas by Northern blot analysis as well as in the whole human and rat fetal and adult pancreas and isolated islets; this finding was confirmed by Western blot analysis and by immunohistochemistry with a precise location on islet somatostatin cells (Julien et al. 2004). Binding studies with labeled G-17 confirmed the presence of high affinity sites on dog pancreatic acini (Fourmy et al. 1984). A similar situation was found in guinea pig pancreas with the same technique (Yu et al. 1987) and on rat acini by immunofluorescence (Morisset 2005). Such a location on pancreatic delta cells was also confirmed in six different species, the calf, pig, horse, rat, human, and dog. However, with a different antibody, the receptor was also localized on human islet glucagon cells. The only other location observed for the CCK-2R in the pancreas by immunofluorescence besides the somatostatin cells was on the ductal cells in the young calf and adult cow (Morisset 2005).

By comparing and analyzing all these data using different techniques, we can easily ascertain that technology has not yet been successful in helping us localize this CCK-2R in any tissues at the cellular level. We therefore need to produce more specific receptor antibodies which will have to be certified for their specificity by different laboratories using the same technique of immunohistochemistry for cellular localization and Western blot for protein detection. When this is elucidated, it will be much easier to definitively associate this receptor occupation with a cellular physiological response.

Physiological Response to the CCK-2 Receptor Occupation

The stomach remains one of the main targets of gastrin, and the CCK-2R expressed in this organ is an important part of the system that regulates functions of the gastric epithelium. Two main pathways of activation of stomach secretion by gastrin have been proposed: direct and indirect. Direct implies the gastrin action on parietal and chief cells while indirect means that the gastrin effect is mediated by histamine stored and released by the enterochromaffin-like (ECL) cells of the mucosa. According to Bakke (2001), the indirect effect of gastrin via the ECL cells would prevail for the control of acid secretion since they could not locate the CCK-2R on parietal cells. Since these data were obtained by fluorescence CCK-8 binding, they will have to be confirmed by immunofluorescence or immunocytochemistry. Studies performed in gastrin-CCK double KO mice to study control of acid secretion via the CCK-2R are of limited use since acid secretion in such a case becomes only controlled by cholinergic vagal stimulation (Tarasova et al. 1996).

Besides being involved in the control of acid secretion, the occupation of the CCK-2R was also associated with proliferation of the gastric mucosa, especially during lactation in rats (Takeuchi and Johnson 1987). Gastrin was also described as a growth factor for the small intestine and colon. However, an elaborate study performed in fed rats using relatively high doses of pentagastrin for 5 days did not have any major effect on DNA synthesis in the stomach, duodenum, and colon, nor on the total organ weight of these three organs and their total DNA contents (Solomon et al. 1987). Furthermore, more recently, it was shown that gastrin elicited increased DNA synthesis in ECL cells but failed to do so in parietal cells. This increased DNA synthesis was preceded by phosphorylation and activation of MAP kinase as well as *c-fos* and *c-jun* gene expression only in the ECL cells (Kinoshita et al. 1998). These data clearly indicate that occupation of the CCK-2R can induce growth in this specific subpopulation of gastric cells and suggest that gastrin could act to promote commitment or differentiation of stomach precursor cells. The presence of the CCK-2R on the proliferating progenitor cell population remains to be confirmed.

As indicated earlier in this review, the location of the CCK-2R in the pancreas of mammals as well as humans remains an unsolved situation which should be

addressed soon. Indeed, in recent literature, we can observe that the human pancreatic acinar cells lack functional responses to CCK and gastrin (Ji et al. 2001), while a more recent study (Murphy et al. 2008) clearly claimed a secretory response of purified human pancreatic acinar cells to CCK. However, when the human CCK-2R receptor is either transfected in purified human acinar cells or present in the murine pancreas via transfection using the elastase promoter, both models exhibited amylase release from their respective acini in response to CCK and gastrin (Ji et al. 2001; Saillan-Barreau et al. 1998). This indicates that the acini of these preparations can respond to CCK-2R activation when the receptor is present and that it can use the cell's intracellular machinery leading to enzyme release.

The implication of the CCK-2R in the control of pancreas growth and regeneration via its agonist gastrin is far from being established. However, pentagastrin, given to pregnant rats, was found to be the most potent factor, with hydrocortisone, responsible for fetal pancreas growth as its effect was blocked by the specific CCK-2R antagonist L-365,260. The fetal rat pancreas also expressed gastrin whose expression disappeared early after birth (Morisset et al. 2004). Furthermore, after pancreatitis induction in rats, significant overexpression of the CCK-2R mRNA was observed quite early during the destruction period as well as during early regeneration; the presence of caerulein was however constantly needed for prolonged expression of the receptor (Morisset and Calvo 1998). Recent data obtained in transgenic CCK-2R mice with CCK-2R overexpression in pancreatic acinar cells supported the possibility of this receptor being involved in pancreas growth as significant increases were observed in pancreas weight and areas occupied by the exocrine cells. Further increases in pancreatic weight were observed when these rats were bred with insulin-gastrin transgenic mice to achieve continuous stimulation of the acinar cells overexpressing the CCK-2R (Morisset et al. 2004). With regard to the human pancreas, even though Tang claimed that the CCK-2R are distributed all over the exocrine pancreas as established by phosphorimaging detection, it remains that no one has yet established first, that the CCK-2Rs are present at any other location than on the endocrine delta cells and second, that the human pancreas did not show any sign of regeneration after partial pancreatectomy. Since we and others have shown that the pig pancreas can regenerate after partial pancreatectomy, perhaps we should use this species as a model to study pancreas regeneration (Morisset et al. 2004). However, prior to initiating such studies, it is mandatory that we clearly establish the location of this CCK-2R in human and pig pancreas to secure the pig model.

Role of CCK-2R in Cancer

The expression of both CCK receptor subtypes in human gastrointestinal cancers remains poorly documented and is still of controversial nature. From a potential therapeutic point of view, it is very important to ascertain which CCK receptors are present on gastrointestinal tumors. Indeed, these receptors might act in concert with oncogenes to promote neoplastic transformation or contribute to tumor invasiveness. The presence of a known receptor may offer the opportunity to use receptor antagonists or a cytotoxic toxin linked to a stable ligand of this receptor as an adjunct in the treatment of a target cancer.

As an example of the confusion existing on the presence of CCK-2R on specific tumors, one study indicated that this receptor, evaluated by RT-PCR, was not detected in esophageal cancers (Clerc et al. 1997), while its presence was confirmed with gastrin in 100% of patients with Barrett's metaplasia and in 70% of patients with esophageal adenocarcinomas. This suggests that an autocrine signal could be involved in the pathogenesis of Barrett's metaplasia before development of dysplasia and cancer. Does this mean that this receptor disappears during cancer development?

Controversy also exists on the presence of the CCK-2R on gastric cancer cells. The receptor and gastrin were detected in some human gastric adenocarcinoma by immunohistochemistry while detected in only 7% of gastric cancer samples by RT-PCR or by receptor autoradiography. To amplify the doubt, another study, using the RT-PCR technique, claimed that seven out of eight specimens of gastric adenocarcinomas express the receptor (Clerc et al. 1997).

Data on the presence of the CCK-2R in colon cancer are less controversial. Indeed, by autoradiography the CCK-2R was either rarely or not at all expressed in colorectal cancer in one study, while found in only two out of 12 colon adenocarcinoma samples by RT-PCR in another study. In this organ, however, isoforms of the CCK-2R were detected, the short and longer forms. The short form, also called CCK-C, was detected in

100% of the tumors tested along with gastrin mRNA. This truncated form does not discriminate between amidated and glycine-extended forms of gastrin, and since the colonic adenocarcinomas synthesize progastrin but fail to process the prohormone, this prohormone could be the specific agonist of this receptor and thus be the intracrine growth factor in human colorectal cancer (Biagini et al. 1997). It thus becomes important to search for this receptor type in any other type of gastrointestinal cancers, and the presence of this new subtype may be the reason why so few CCK-2Rs were detected in colon cancer, we were not looking for the appropriate one.

The pancreatic tumors are no different than the others as some authors have failed to find transcripts of the CCK-2R at the mRNA level whereas others reported expression. A CCK-C receptor was also characterized in human pancreatic cancer (Smith et al. 2002). However, it is not yet clear if this newly reported pancreatic CCK-CR is comparable to the one reported in the colon. Although some authors claimed that the CCK-2Rs were present in all pancreatic carcinomas, others reported their occasional presence in gastroenteropancreatic tumors with rare expression in ductal pancreatic carcinoma (Morisset et al. 2004). However, when the CCK-2R is transfected in pancreatic acinar cells of the murine pancreas along with the already present CCK1R, its new presence seems to play a key role in the development of pre- and neoplastic lesions in the pancreas of these mice (Clerc et al. 2002). The new feature of this experimental approach is the simultaneous expression of CCK-1 and CCK-2Rs in the same cell. Could this result in novel signaling secondary to receptor cross talk and/or heterodimerization of these receptors? We could expect potentiation of signal transduction, and heterodimerization may represent a new mechanism for modulation of these receptors which may lead to cancer development.

The presence of both CCK receptor subtypes in cancer cell lines is not a negligible phenomenon, as we will see. Furthermore, the expression of CCK receptors in cancer cell lines has mostly given rise to controversial reports with regard to their type and density.

In SEG-1 cells, a human esophageal adenocarcinoma cell line, RT-PCR has established the presence of the CCK-2R receptors responsive to gastrin for their growth with growth inhibition by L-365,260, the specific CCK-2R antagonist. However, one study on esophageal cancers reported expression of low levels of CCK-2R mRNA with the CCK-1Rs being overexpressed (Morisset 2005).

In AGS-B cells, a human gastric carcinoma cell line expressing the CCK-2R, amidated gastrin G-17 was associated with increased DNA synthesis which was inhibited by the L-365,260 antagonist. This cell line could be derived from the 7% of the gastric cancer samples expressing the CCK-2R (Morisset 2005).

Contrary to the rare incidence of CCK receptors in colon cancer as seen above, it seems that some colorectal carcinoma cell lines Colo 320, HCT 116, LoVo, and the immortalized mouse colon cell line YAMC express the CCK-2R as they respond to gastrin for their growth and tumorigenicity. However, such cell lines are believed to be the exception because, as for tumors, most colorectal carcinoma cell lines do not express gastrin/CCK-2 receptors or the CCK-1Rs (Baldwin and Shulkes 1998). In general, however, expression of the CCK-2R isoform is always coupled with co-expression of the gastrin gene in GI tumor cell lines, implying the presence of a gastrin/CCK-2R autocrine loop. If there is any involvement of peptides of the CCK and gastrin family in the development of colon cancer, it could be through glycine-extended gastrin and progastrin as high concentrations of these two peptides have been observed in colon tumors and in blood of patients with colorectal cancer. The receptor for these peptides was described but never cloned (Dufresne et al. 2006).

In non-GI tract tumor cell lines, co-expression of gastrin and CCK-2R was not found, except in the lymphoblastic leukemia cell line Molt 4 which also expresses the receptor's isoform. High CCK-2R density and incidence was reported in medullary thyroid carcinomas, small cell lung cancers, and stromal ovarian cancers; leiomyo-carcomas express both CCK-R subtypes (Dufresne et al. 2006).

In some pancreatic cell lines of ductal origin, a gastrin autocrine loop involving the CCK-2R exists as in the MIA PaCa-2 cells but such is not the case in the PANC-1 cells possessing also the CCK-2R (Morisset et al. 2004). In these same two cell lines, transfection of both CCK-R subtypes led to inhibition of their growth by CCK-8, a result which contradicts what was previously observed in these same cells. Would it be possible that overexpression of these CCK-Rs leads to inhibition of their growth? In the

rat AR4-2J cells, an acinar cell line which expresses both CCK-R subtypes, a trophic response to gastrin has been shown which is coupled to CCK-2R occupation (Scemama et al. 1989). In BxPC-3 human cancer cells, the expression of the recently characterized CCK-CR was confirmed as its specific antibody resulted in growth inhibition (Smith et al. 2002). In the RIN-14B cells, a pancreatic somatostatin endocrine cell line, the presence of both CCK-Rs was revealed by Western blot and immunofluorescence; in these cells, occupation of both receptors by caerulein and gastrin led to somatostatin secretion, while cell growth was inhibited during stimulation of the CCK-1R (El-Kouhen and Morisset 2010). The message which can be drawn from these studies on cancer cell lines driven from any type of cancer is that it is very important to establish what kind of CCK-Rs are present and if there is any type of autocrine loop existing involving both specific agonists, CCK and gastrin. The other major observation remains that cancers in situ or cancer cell lines are unique in their growth control and that each typical cancer cell could respond to a specific chimiotherapeutic treatment depending on the intracellular signaling systems activated by the agonist.

Intracellular Signaling Pathways

Besides mutations of specific genes often causing cancer development, activation of intracellular signaling pathways by occupation of hormones and growth factor receptors in an unregulated way can be part of the tumor growth and invasiveness processes.

Occupation of the CCK-2R by gastrin is no exception and results in activation of a variety of cell-type–specific transduction pathways involved in proliferation. Among them are phospholipase C, c-src-like tyrosine kinases, p125 FAK, phosphatidylinositol 3-kinase and the MAPK, ERK and p38 kinases (Rozengurt and Walsh 2001).

Besides proliferation, malignant transformation also results in loss of epithelial differentiation, acquirement of mesenchymal characteristics, and increasing invasive and metastatic potential. Gastrin and the CCK-2R seem to be involved in epithelial-mesenchymal transition, and this process could involve p60-src. PI-3-kinase and ERK1/2 activation for periods of up to 4 h, leading to reduced association between E-cadherin, $p120^{CTN}$, and β-catenin resulting in loss of cell adhesion and scattering (Bierkamp et al. 2002).

How can we now reconcile the growth-promoting effect of gastrin via occupation of its CCK-2R on different cancer cells with recent observations indicating that gastrin through this same CCK-2R can prevent rather than promote colorectal carcinogenesis via activation of the MAPK/ERK/AP-1 pathway and inhibition of NF-κB (Muerkoster et al. 2005)? Furthermore, how to explain this other finding that occupation of the CCK-2R in CHO and Swiss 3T3 cells activated similar intracellular signals with opposite growth effects. The reason given was that it depends on the cell model (Detjen et al. 1997).

With so many conflicting data on the effects of gastrin through occupation of its CCK-2R regarding growth and intracellular signaling pathways activated, it seems that all of these controversies come from the choice of the cell line selected to perform these studies and whether they possess the CCK-2 receptor or that it has been transiently or stably transfected. Indeed, we can list these cell lines: the MDCK cells, the CHO cells, the Swiss 3T3 cells, the COS-7 cells, the AR42J cells, the Colo 320 cells, the Lovo cells, the SW707 cells, and the HCT-115 cells, among others. It is believed that in order to have a clear image of the effects of gastrin on cancer cell growth and transformation through CCK-2R occupation, all the different signaling pathways involved in these processes should be studied in one selected cell line preferably expressing the CCK-2R. In doing so, it will then be possible to assign pathways to different responses happening in the cell leading to cancer development, growth control, and invasiveness.

Summary

The message to be stretched out of this review is that a lot of uncertainty still exists on the location and roles played by this CCK-2R on normal and tumoral cells. In order to clarify the existing dilemmas, we need, as scientists, to set up standard protocols to be followed using well-accepted techniques and tools which everybody agrees on. In this way, it will then be possible to clearly establish which organ or tumor expresses the receptor and on which specific cell it is operating. With all this new knowledge, we shall be able to clearly establish the physiological roles of this receptor on normal cells and possibly target or use the receptor for chemotherapy on tumor cells, if applicable.

References

Attoub S, Levasseur S, Buyse M, Goiot H, Laigneau JP, Moizo L, et al. Physiological role of cholecystokinin B/gastrin receptor in leptin secretion. Endocrinology. 1999;140:4406–10.

Bakke I, Qvigstad G, Sandvik AK, Waldum HL. The CCK-2 receptor is located on the ECL cell, but not on the parietal cell. Scand J Gastroenterol. 2001;36:1128–33.

Baldwin GS, Shulkes A. Gastrin, gastrin receptors and colorectal carcinoma. Gut. 1998;42:581–4.

Biagini P, Monges G, Vuarogueaux V, Parriaux D, Cantaloube JF, De Micco P. The human gastrin/cholecystokinin receptors: type B and type C expression in colonic tumors and cell lines. Life Sci. 1997;61:1009–18.

Bierkamp C, Kowalski-Chauvel A, Dehez S, Fourmy D, Pradayrol L, Seva C. Gastrin mediated cholecystokinin-2 receptor activation induces loss of cell adhesion and scattering in epithelial MDCK cells. Oncogene. 2002;21:7656–70.

Clerc P, Dufresne M, Saillan C, Chastre E, André T, Escrieut C, et al. Differential expression of the CCK-A and CCK-B/gastrin receptor genes in human cancers of the esophagus, stomach and colon. Int J Cancer. 1997;72:931–6.

Clerc P, Leung-Theung-Long S, Wang TC, Dockray GJ, Bonisson M, Delisle MB, Vaysse N, Pradayrol L, Fourmy D, Dufresne M. Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferentiation of acinar cells and neoplasia. Gastroenterology. 2002;122:428–37.

Detjen K, Yule D, Tseng M-J, Williams JA, Logsdon CD. CCK-B receptors produce similar signals but have opposite growth effects in CHO and Swiss 3T3 cells. Am J Physiol. 1997;273:G1449–57.

Dufresne M, Seva C, Fourmy D. Cholecystokinin and gastrin receptors. Physiol Rev. 2006;86:805–47.

El-Kouhen K, Morisset J. Control of somatostatin (SS) secretion by CCK-1 and CCK-2 receptors' occupation in RIN-14B cells, a rat pancreatic islet cell line. Pancreas. 2010;39:127–34.

Fourmy D, Zahidi A, Pradayrol L, Vayssette J, Ribet A. Relationship of CCK/gastrin receptor binding to amylase release in dog pancreatic acini. Regul Pept. 1984;10:57–68.

Haigh CR, Attwood SEA, Thompson DG, Jankowski JA, Kirton CM, Pritchard DM, et al. Gastrin induces proliferation in Barrett's metaplasia through activation of the CCK_2 receptor. Gastroenterology. 2003;124:615–25.

Jagerschmidt A, Popovici T, O'Donohue M, Roques BP. Identification and characterization of various cholecystokinin B receptor mRNA forms in rat brain tissue and partial determination of the cholecystokinin B receptor gene structure. J Neurochem. 1994;63:1199–206.

Ji B, Bi Y, Simeone D, Mortensen RM, Logsdon CD. Human pancreatic acinar cells lack functional responses to cholecystokinin and gastrin. Gastroenterology. 2001;121:1380–90.

Julien S, Lainé J, Morisset J. The rat pancreatic islets: a reliable tool to study islet responses to cholecystokinin receptor occupation. Regul Pept. 2004;121:73–81.

Kinoshita Y, Nakata H, Kishi K, Kawanami C, Sawada M, Chiba T. Comparison of the signal transduction pathways activated by gastrin in enterochromaffin-like and parietal cells. Gastorenterology. 1998;115:93–100.

Mazzocchi G, Malendowicz LK, Aragona F, Spinazzi R, Nussdorfer GG. Cholecystokinin (CCK) stimulates aldosterone secretion from human adrenocortical cells via CCK2 receptors coupled to the adenylate cyclase/protein kinase A signaling cascade. J Clin Endocrinol Metab. 2004;89:1277–84.

Morisset J. The gastrointestinal cholecystokinin receptors in health and diseases. Ann Acad Med Bialostocensis. 2005;50:21–36.

Morisset J, Calvo E. Trophicity of the pancreas: from rodents to man. J Physiol Pharmacol. 1998;49(Suppl 2):25–37.

Morisset J, Lainé J, Biernat M, Julien S. What are the pancreatic target cells for gastrin and its CCK_B receptor? Is this a couple for cancerous cells? Med Sci Monit. 2004;10:RA 242–6.

Muerkoster S, Isberner A, Arlt A, Witt M, Reimann B, Blaszezuk E, et al. Gastrin suppresses growth of CCK2 receptor expressing in colon cancer cells by inducing apoptosis in vitro and in vivo. Gastroenterology. 2005;129:952–68.

Murphy JA, Criddle DN, Sherwood M, Chvanov M, Mukherjee R, McLaughlin E, et al. Direct activation of cytosolic Ca^{2+} signalling and enzyme secretion by cholecystokinin in human pancreatic acinar cells. Gastroenterology. 2008;135:632–41.

Rozengurt E, Walsh JH. Gastrin, CCK, signaling, and cancer. Annu Rev Physiol. 2001;63:49–76.

Saillan-Barreau C, Clerc P, Adato M, Escrieut C, Vaysse N, Fourmy D, Dufresne M. Transgenic CCK-B/gastrin receptor mediates murine exocrine pancreatic secretion. Gastroenterology. 1998;115:988–96.

Scemama J, DeVries L, Pradayrol L, Seva C, Tronchere H, Vaysse N. Cholecystokinin and gastrin peptides stimulate ODC activity in a rat pancreatic cell line. Am J Physiol. 1989;256:G846–50.

Smith JP, Verderame MF, McLaughlin P, Martenis M, Ballard E, Zagon IS. Characterization of the CCK-C (cancer) receptor in human pancreactic cancer. Int J Mol Med. 2002;10:689–94.

Solomon TE, Morisset J, Wood JG, Bussjaeger LJ. Additive interaction of pentagastrin and secretin on pancreatic growth in rats. Gastroenterology. 1987;92:429–35.

Takeuchi K, Johnson LR. The role of food intake on gastric mucosal growth and gastrin receptors during pregnancy and lactation. Proc Soc Exptl Biol Med. 1987;186:198–204.

Tarasova NI, Romanov VI, Da Pinto SP, Michejda CJ. Numerous cell targets for gastrin in the guinea pig stomach revealed by gastrin/CCK_B receptor localization. Cell Tissue Res. 1996;283:1–6.

Tommeras K, Hammer P, Sundler F, Borch K, Mardh S, Cabero JL. Immunolocalization of cholecystokinin-2 receptors in rat gastric mucosa. Scand J Gastroenterol. 2002;37:1017–24.

Waki S, Kinoshita Y, Wang H-Y, Asahara M, Matsushima Y, Sazzad Hassan MD, et al. Effect of aging on gastrin receptor gene expression in rat stomach. Peptides. 1998;19:225–9.

Yu DH, Noguchi M, Zhou ZC, Villanueva ML, Gardner JD, Jensen RT. Characterization of gastrin receptors on guinea pig pancreatic acini. Am J Physiol. 1987;253:G793–801.

c-H-Ras

► RAS (H-, K-, N-RAS)

CIS (Cytokine-Inducible SH2-Containing Protein)

▶ SOCS

CK2

▶ Casein Kinase II

CK-I

▶ Glycogen Synthase Kinase-3

CKII

▶ Casein Kinase II

CKIP-1

David W. Litchfield
Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada
Department of Oncology, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

Synonyms

CKIP-1 (CK2 interacting protein-1); JBP (c-Jun-binding protein); OC120 (osteoclast maturation-associated gene 120 protein); PLEKHO1 (pleckstrin-homology domain containing, family O member 1)

Historical Background

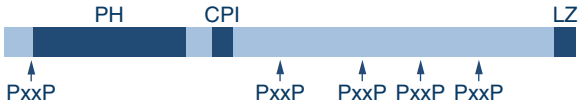
CKIP-1 (CK2-interacting protein-1) was initially described on the basis of its interactions with protein kinase ▶ CK2 (Bosc et al. 2000). In this respect, the first publication on CKIP-1 described isolation of a cDNA encoding this novel protein from a human B-cell cDNA library using a yeast two-hybrid screen to identify interaction partners of protein kinase CK2. Interactions between CK2 and CKIP-1 were confirmed by co-immunoprecipitation experiments and in vitro interaction assays using recombinant and in vitro translated proteins. Prior to that report, a partial cDNA encoding its C-terminal 72 amino acids had been isolated from a mouse embryo cDNA library, using a yeast two-hybrid screen to identify interaction partners for the leucine-zipper region of the c-Jun transcription factor (Chevray and Nathans 1992). Collectively, these reports suggested that the primary functions of CKIP-1 were mediated by interactions with other proteins within cells. Since these initial reports, subsequent studies have reinforced that suggestion by demonstrating that CKIP-1 interacts with a number of other cellular proteins.

Domain Structure and Architecture

The human CKIP-1 cDNA encodes a protein of 409 amino acids with an N-terminal pleckstrin-homology (PH) domain and a C-terminal domain containing a putative leucine zipper (Fig. 1). The central region that separates the PH domain and leucine zipper contains a significant number of proline residues including P-X-X-P motifs that may be involved in protein interactions and S-P or T-P motifs that could be sites of phosphorylation for proline-directed protein kinases such as ▶ MAP kinases or cyclin-dependent kinases (CDKs). Homologs of CKIP-1 in other species, including mouse, rats, chickens, and zebrafish contain the same general domain structure and a reasonably high level of conservation. For example, the deduced protein sequences of mouse, chicken, or zebrafish CKIP-1 display approximately 90%, 80%, and 60% identity to human CKIP-1, respectively. Independent reports have demonstrated that CKIP-1 has the capacity to form leucine-zipper-mediated dimers, an event that may regulate interactions with other cellular partners (Zhang et al. 2007; Tokuda et al. 2007)

Regulation of CKIP-1

While a detailed understanding of its regulation remains incomplete, a number of potential mechanisms for



CKIP-1, Fig. 1 Schematic illustration of the domain structure of CKIP-1. The pleckstrin homology (*PH*) domain, capping protein interaction (*CPI*) motif, and putative leucine zipper (*LZ*) are indicated. *P-X-X-P* motifs that may also be involved in protein: protein interactions are also marked

controlling the activity of CKIP-1 have been reported. These mechanisms include both changes in its level of expression and posttranslational mechanisms. In terms of changes in its level of expression, CKIP-1 has been shown to be up-regulated during muscle differentiation (Safi et al. 2004) and to be induced by cytokines (Zhang et al. 2007). Posttranslational mechanisms include ▶ PI 3-kinase-dependent interactions between its PH domain and phosphatidylinositol 3-phosphate in the plasma membrane (Safi et al. 2004). By comparison, independent studies have demonstrated that CKIP-1 is constitutively localized to the plasma membrane and exhibits relatively nonspecific interactions with membrane phospholipids raising questions about its precise interactions with phosphatidylinositols (Olsten et al. 2004). It does not appear that the PH domain of CKIP-1 is exclusively involved in interactions with membrane phospholipids. In this respect, the PH domain of CKIP-1 has also been shown to be important for interactions with some of its protein partners, including ▶ CK2 and Akt (Olsten et al. 2004; Tokuda et al. 2007). In addition to its localization to the plasma membrane, it has also been reported that CKIP-1 can be localized within the cytoplasm and the nucleus of cultured cells (Bosc et al. 2000; Litchfield et al. 2001; Xi et al. 2010). In one instance, CKIP-1 has been identified as a substrate for caspase 3 following the induction of apoptosis (Zhang et al. 2005). In this situation, a C-terminal fragment of CKIP-1 is translocated to the nucleus where it represses AP-1 activity, presumably through interactions with c-Jun. An additional mechanism that may contribute to its regulation in cells is phosphorylation (Zhang et al. 2005; Zhang et al. 2006).

Biological Functions of CKIP-1

Based on its domain structure and absence of obvious sequence similarities to known catalytic domains, it has been proposed that CKIP-1 could be an adaptor protein or targeting protein that participates in cellular events through interactions with other cellular proteins (Litchfield et al. 2001; Canton et al. 2005; Canton and Litchfield 2006). Following its identification as a ▶ CK2-interacting protein (Bosc et al. 2000) and putative partner for the leucine zipper of the c-Jun transcription factor (Chevray and Nathans 1992), additional partners for CKIP-1 were identified (Fig. 2). CKIP-1 interacting proteins included other protein kinases such as ATM (Zhang et al. 2006) and Akt (Tokuda et al. 2007). As was the case with CK2, CKIP-1 was shown to recruit a proportion of ATM to the plasma membrane. Other interaction partners for CKIP-1 include the heterodimeric actin capping protein comprised of CPα and CPβ (Canton et al. 2005; Canton et al. 2006; Hernandez-Valladares et al. 2010; Takeda et al. 2010), interferon-induced proteins IFP35 and Nmi (Zhang et al. 2007), and Smurf1 that is a HECT-type ubiquitin ligase involved in SMAD protein regulation in the bone morphogenetic protein (BMP) pathway (Barrios-Rodiles et al. 2005; Lu et al. 2008).

In view of its relatively diverse collection of binding partners, it is perhaps not surprising that CKIP-1 appears to participate in a variety of biological events (Fig. 2). In this respect, when it is expressed in cells under the control of an inducible promoter, increased expression of CKIP-1 promotes changes in cell morphology and the actin cytoskeleton, presumably through its interactions with the actin capping protein (Canton et al. 2005). Through its induction by cytokines and interactions with IFP35 and Nmi, CKIP-1 has also been implicated in cytokine signaling (Zhang et al. 2007). Since interfering with activation of the ▶ PI 3-kinase pathway or the expression of CKIP-1 in C2C12 cells blocks differentiation, it appears that CKIP-1 is a component of ▶ PI 3-kinase-regulated muscle differentiation (Safi et al. 2004). Additional involvement with the ▶ PI 3-kinase pathway comes from the demonstration that increased CKIP-1 expression results in impaired growth of tumor cells in vitro and in mouse Xenografts that is accompanied by inactivation of Akt (Tokuda et al. 2007). In addition to its involvement in these events, there is mounting evidence demonstrating that CKIP-1 has an important role in bone homeostasis (Lu et al. 2008), a function that may relate to its designation as an Osteoclast Maturation-Associated Gene (i.e., OC120). Through its interactions with Smurf1, CKIP-1 promotes an activation of the ubiquitin ligase

CKIP-1, Fig. 2 Interaction partners for CKIP-1 and consequences of interaction. Additional detail is presented in the text

CKIP-1 PARTNER	FUNCTION	CONSEQUENCES OF INTERACTION
Akt	protein kinase	inhibition of Akt/PI3 kinase pathway
ATM	protein kinase	membrane recruitment
CK2	protein kinase	membrane recruitment
CPα/CPβ	F-actin capping	regulation of actin cytoskeleton/morphology
IFP35/Nmi	interferon-inducible	modulation of Akt/PI3 kinase pathway
Smurf1	ubiquitin ligase	increased activity & Smad 1/5 degradation

activity of Smurf1. Furthermore, in CKIP-1 deficient mice, a decrease in Smurf1 activity is accompanied by an age-dependent increase in bone mass. Additional confirmation of its role in bone formation comes from studies where bone mass was enhanced in rats in response to targeted delivery of CKIP-1-specific siRNA to osteogenic cells in rats (Zhang et al. 2012).

Summary

Since its original characterization as a CK2-interacting protein and as a putative partner for the leucine zipper of the c-Jun transcription factor, additional partners for CKIP-1 have been characterized. In conjunction with the identification of its partners, CKIP-1 has been implicated in a number of distinct biological events. Although it would appear that a comprehensive understanding of CKIP-1 and its regulation and functions is far from complete, its involvement in processes such as muscle differentiation, tumor growth, and bone homeostasis suggests that a detailed understanding of CKIP-1 could ultimately yield novel approaches for preventing or treating disease. It can also be envisaged that the involvement of CKIP-1 in processes that underlie human health and disease will spur more attention on its regulation and functional properties. Challenges for the future include efforts to elucidate its high-resolution structure, comprehensive identification of its cellular partners, and thorough evaluation of its expression levels at both gene and protein levels during development and in response to different stimuli. Collectively, this information will yield a more precise understanding of CKIP-1 and its role in biological processes that could ultimately instruct efforts to target its activity for the prevention or treatment of diseases such as cancer or musculoskeletal disorders.

References

Barrios-Rodiles M, Brown KR, Ozdamar B, Bose R, Liu Z, Donovan RS, Shinjo F, Liu Y, Dembowy J, Taylor IW, Luga V, Przulj N, Robinson M, Suzuki H, Hayashizaki Y, Jurisica I, Wrana JL. High-throughput mapping of a dynamic signaling network in mammalian cells. Science. 2005;307:1621–5.

Bosc DG, Graham KC, Saulnier RB, Zhang C, Prober D, Gietz RD, Litchfield DW. Identification and characterization of CKIP-1, a novel pleckstrin homology-domain containing protein that interacts with protein kinase CK2. J Biol Chem. 2000;275:14295–306.

Canton DA, Litchfield DW. The shape of things to come: an emerging role for protein kinase CK2 in the regulation of cell morphology and the cytoskeleton. Cell Signal. 2006;18:267–75.

Canton DA, Olsten ME, Kim K, Doherty-Kirby A, Lajoie G, Cooper JA, Litchfield DW. The pleckstrin homology domain-containing protein CKIP-1 is involved in regulation of cell morphology and the actin cytoskeleton and interaction with actin capping protein. Mol Cell Biol. 2005;25:3519–34.

Canton DA, Olsten ME, Niederstrasser H, Cooper JA, Litchfield DW. The role of CKIP-1 in cell morphology depends on its interaction with actin-capping protein. J Biol Chem. 2006;281:36347–59.

Chevray PM, Nathans D. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc Natl Acad Sci USA. 1992;89:5789–93.

Hernandez-Valladares M, Kim T, Kannan B, Tung A, Aguda AH, Larsson M, Cooper JA, Robinson RC. Structural characterization of a capping protein interaction motif defines a family of actin filament regulators. Nat Struct Mol Biol. 2010;17:497–503.

Litchfield DW, Bosc DG, Canton DA, Saulnier RB, Vilk G, Zhang C. Functional specialization of CK2 isoforms and characterization of isoform-specific binding partners. Mol Cell Biochem. 2001;227:21–9.

Lu K, Yin X, Weng T, Xi S, Li L, Xing G, Cheng X, Yang X, Zhang L, He F. Targeting WW domains linker of HECT-type ubiquitin ligase Smurf1 for activation by CKIP-1. Nat Cell Biol. 2008;10:994–1002.

Olsten ME, Canton DA, Zhang C, Walton PA, Litchfield DW. The Pleckstrin homology domain of CK2 interacting protein-1 is required for interactions and recruitment of protein kinase CK2 to the plasma membrane. J Biol Chem. 2004;279:42114–27.

Safi A, Vandromme M, Caussanel S, Valdacci L, Baas D, Vidal M, Brun G, Schaeffer L, Goillot E. Role for the pleckstrin homology domain-containing protein CKIP-1 in phosphatidylinositol 3-kinase-regulated muscle differentiation. Mol Cell Biol. 2004;24:1245–55.

Takeda S, Minakata S, Koike R, Kawahata I, Narita A, Kitazawa M, Ota M, Yamakuni T, Maéda Y, Nitanai Y. Two distinct mechanisms for actin capping protein regulation– steric and allosteric inhibition. PLoS Biol. 2010;8:e1000416.

Tokuda E, Fujita N, Oh-hara T, Sato S, Kurata A, Katayama R, Itoh T, Takenawa T, Miyazono K, Tsuruo T. Casein kinase 2-interacting protein-1, a novel Akt pleckstrin homology domain-interacting protein, down-regulates PI3K/Akt signaling and suppresses tumor growth in vivo. Cancer Res. 2007;67:9666–76.

Xi S, Tie Y, Lu K, Zhang M, Yin X, Chen J, Xing G, Tian C, Zheng X, He F, Zhang L. N-terminal PH domain and C-terminal auto-inhibitory region of CKIP-1 coordinate to determine its nucleus-plasma membrane shuttling. FEBS Lett. 2010;584:1223–30.

Zhang L, Xing G, Tie Y, Tang Y, Tian C, Li L, Sun L, Wei H, Zhu Y, He F. Role for the pleckstrin homology domain-containing protein CKIP-1 in AP-1 regulation and apoptosis. EMBO J. 2005;24:766–78.

Zhang L, Tie Y, Tian C, Xing G, Song Y, Zhu Y, Sun Z, He F. CKIP-1 recruits nuclear ATM partially to the plasma membrane through interaction with ATM. Cell Signal. 2006;18:1386–95.

Zhang L, Tang Y, Tie Y, Tian C, Wang J, Dong Y, Sun Z, He F. The PH domain containing protein CKIP-1 binds to IFP35 and Nmi and is involved in cytokine signaling. Cell Signal. 2007;19:932–44.

Zhang G, Guo B, Wu H, Tang T, Zhang BT, Zheng L, He Y, Yang Z, Pan X, Chow H, To K, Li Y, Li D, Wang X, Wang Y, Lee K, Hou Z, Dong N, Li G, Leung K, Hung L, He F, Zhang L, Qin L. A delivery system targeting bone formation surfaces to facilitate RNAi-based anabolic therapy. Nat Med. 2012;18:307–14.

CKIP-1 (CK2 Interacting Protein-1)

► CKIP-1

CKR1

► Chemokine Receptor CCR1

c-K-Ras

► RAS (H-, K-, N-RAS)

Classic Cadherins

► Cadherins

Classic TRP Channels, TRPC1-7

► TRP (Transient Receptor Potential Cation Channel)

CLEC1

► CLEC-1

CLEC-1

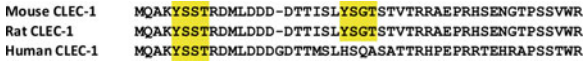
Anita R. Mistry and Christopher A. O'Callaghan
Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Synonyms

CLEC1; Clec1a; C-type lectin-domain family 1 member A; C-type lectin-domain family 1, member a; C-type lectin-like receptor 1

Historical Background

CLEC-1 is a C-type lectin-like molecule. Based on its protein sequence, it is predicted to be a type 2 transmembrane protein with an N-terminal cytoplasmic tail, a single transmembrane domain and a C-terminal extracellular C-type lectin-like domain, that is separated from the transmembrane domain by a short stalk region. The cytoplasmic domain contains a tyrosine residue within the sequence YSST, which may represent a novel signaling motif (Fig. 1). CLEC-1 is encoded within the natural killer gene complex on human chromosome 12 and mouse chromosome 6 in a cluster of genes termed the Dectin-1 cluster, which also includes MICL, CLEC-2, CLEC12B, CLEC9A,



CLEC-1, Fig. 1 Alignment of the cytoplasmic domains of mouse, rat, and human CLEC-1. The human CLEC-1 cytoplasmic domain contains a single tyrosine residue in the sequence YxxT. The mouse and rat CLEC-1 cytoplasmic domains each contain two tyrosine residues in the sequence YxxT x_{13} YxxT. Several serine and threonine residues are also present and could be phosphorylation sites

Dectin-1, and LOX-1. Like other Dectin-1 family members, CLEC-1 is predominantly expressed by dendritic cells, endothelial cells, monocytes, and macrophages. Expression of CLEC-1 is downregulated by inflammatory stimuli and increased by immunoregulatory mediators. Recently, a role for CLEC-1 in immune tolerance has been proposed. CLEC-1 lacks the conserved residues that mediate calcium coordination and sugar binding in classical carbohydrate-binding C-type lectins, suggesting that CLEC-1 may not have a carbohydrate ligand. Currently, there are no known ligands for CLEC-1.

CLEC-1 Expression

Human *CLEC-1* is transcribed in placenta, lung, and bone marrow (Colonna et al. 2000). Lower expression levels are found in thymus and heart, and very low levels are also detected in pancreas, kidney, bladder, prostate, testis, ovary, small intestine, and colon (Colonna et al. 2000). The wide tissue distribution of human *CLEC-1* transcription is also found in the rat, with high levels of expression in spleen, kidney, lung, lymph node and aorta, moderate expression in liver, heart, muscle and brain, and low expression levels in bone marrow, thymus, and testis (Thebault et al. 2009; Flornes et al. 2010). At the protein level, rat CLEC-1 is strongly expressed in lung, moderately expressed in spleen and heart, and weakly expressed in thymus, consistent with the transcript expression distribution (Thebault et al. 2009).

At the cellular level, human *CLEC-1* is transcribed in unstimulated dendritic cells (DC) and in DC stimulated with lipopolysaccharides (LPS), tumor necrosis factor alpha (TNF-α), or by CD40-CD40 ligand interaction (Colonna et al. 2000; Sobanov et al. 2001). High levels of human *CLEC-1* transcripts are also detected in endothelial cells (unstimulated and LPS-stimulated human umbilical vein endothelial cells (HUVEC)) (Sobanov et al. 2001). *CLEC-1* is weakly expressed in primary human monocytes (Colonna et al. 2000) and in the U937 human monocytic lymphoma cell line following LPS stimulation (Sobanov et al. 2001). No expression was detected in peripheral blood mononuclear cells, granulocytes, B, T, or NK cells (Colonna et al. 2000).

In the rat, *CLEC-1* is expressed in concavalin-A stimulated blasts, DC, peritoneal macrophages, B cells, granulocytes, and LAK (IL2-stimulated NK cells or lymphokine-activated killers) (Thebault et al. 2009; Flornes et al. 2010). Low-level rat-*CLEC-1* transcription is detected in $CD4^+$ and $CD8^+$ T cells, RNK16 (NK cell line), and an endothelial cell line (Thebault et al. 2009; Flornes et al. 2010). Rat CLEC-1 is observed at the cell surface of bone marrow-derived DC, macrophages, and endothelial cells (Thebault et al. 2009). However, in transfected COS cells (Colonna et al. 2000) and transfected HUVEC (Sobanov et al. 2001), CLEC-1 is located intracellularly. Immunofluorescence studies demonstrate that CLEC-1 accumulates in perinuclear regions of the transfected cells (Sobanov et al. 2001). These observations raise the possibility that CLEC-1 may need to associate with a second molecule to be expressed as a transmembrane protein at the cell surface. Certain C-type lectin molecules such as CLEC4E interact with adaptor proteins in the plasma membrane, and this interaction is required for efficient cell-surface expression (Yamasaki et al. 2008). It is unclear whether CLEC-1 has a similar requirement, but it does not have a charged residue in its predicted transmembrane region, and such associations are typically mediated by charge-charge interactions within the plasma membrane.

Regulation of CLEC-1 Expression

As discussed above, *CLEC-1* expression is induced in human monocytic U937 cells following LPS stimulation (Sobanov et al. 2001). However, in rat bone marrow–derived DC, *CLEC-1* expression is reduced by LPS, interferon (IFN) gamma, or poly (I:C) treatment after 48 h (Thebault et al. 2009). IFN-γ and LPS treatment also significantly reduce *CLEC-1* expression in human aortic endothelial cells and human monocyte–derived DC, respectively. In contrast, treatment with IL10 or TGF-beta increases *CLEC-1* expression

in rat bone marrow–derived DC and human monocyte-derived DC. Furthermore, IL10 and TGF-beta are able to inhibit the LPS-induced downregulation of *CLEC-1* expression in human monocyte–derived DC (Thebault et al. 2009).

In a model of rat allograft tolerance, CLEC-1 has been shown to be overexpressed on PECAM-1^+ endothelial cells of tolerated allografts compared with allografts that are chronically rejected or syngeneic grafts (Thebault et al. 2009). In vitro and in vivo induction of CLEC-1 expression in the endothelial cells of tolerated allografts is dependent upon alloantigen-specific regulatory $CD4^+CD25^+$ T cells from tolerant recipients, involving a cell contact-dependent mechanism. $CD4^+CD25^+$ T cells from naive rats are not able to upregulate *CLEC-1* in allograft endothelial cells in vitro, and neither regulatory $CD4^+CD25^+$ T cells from tolerated recipients nor T cells from naive rats can induce CLEC-1 expression on syngeneic grafts in vivo (Thebault et al. 2009). Therefore, regulatory $CD4^+CD25^+$ T cells from tolerant recipients that accumulate in allografts may be able to regulate the expression of CLEC-1 in donor-specific endothelial cells.

CLEC-1 is an Orphan Receptor

There are no known endogenous or exogenous ligands for CLEC-1. Unlike classical C-type lectins which contain conserved residues that mediate the coordination of calcium, which is involved in sugar binding, CLEC-1 is a C-type lectin-like receptor that lacks these conserved residues and so is unlikely to have a carbohydrate ligand. CLEC-1 may bind to protein ligands, as has been shown for other C-type lectin-like molecules, including CLEC-2 (Watson and O'Callaghan 2005; Suzuki-Inoue et al. 2006; Suzuki-Inoue et al. 2007; Christou et al. 2008), NKG2D (Bauer et al. 1999; Cerwenka et al. 2000; Diefenbach et al. 2000) and CD94/NKG2A/B (Braud et al. 1998). CLEC-1 belongs to the Dectin-1 family of C-type lectin-like receptors, which are encoded in the natural killer gene complex on human chromosome 12 and mouse chromosome 6 ((Sobanov et al. 2001); reviewed in (Kanazawa 2007; Huysamen and Brown 2009)), and Dectin-1 is able to bind to fungal β-glucans (Brown and Gordon 2001) in a calcium-independent manner and so has a different mode of sugar binding to the classical C-type lectins (Adachi et al. 2004, Brown et al. 2007). A carbohydrate ligand for CLEC-1 cannot be ruled out.

CLEC-1 is highly conserved; rat and mouse CLEC-1 have 95.5% identity, human and rat CLEC-1 have 71.3% identity, and human and mouse CLEC-1 have 69.4% identity (Flornes et al. 2010).

CLEC-1 Function and Regulation of its Activity

CLEC-1 contains a tyrosine residue within the sequence YSST in its cytoplasmic domain, which may represent a novel signaling motif. Mouse and rat CLEC-1 contain an additional tyrosine residue in the intracellular domain (YxxT x_{13} YxxT) (Flornes et al. 2010) (Fig. 1). The cytoplasmic domain of CLEC-1 also contains several serine and threonine residues that are putative phosphorylation sites. Putative CLEC-1 signaling and downstream effector functions have not been deciphered, but it is possible that ligand binding to CLEC-1 may lead to phosphorylation of the intracellular tyrosine(s), triggering effector functions. A knockout mouse model of CLEC-1 has not yet been generated.

Inhibition of CLEC-1 expression in rat LPS-stimulated bone marrow–derived DC using RNAi does not affect DC generation or maturation as assessed by cytokine production and expression of MHC class II, CD86, and CD80 cell-surface markers (Thebault et al. 2009). However, in a mixed leukocyte reaction, inhibition of CLEC-1 in DC increases the differentiation of allogeneic Th17 T cells and decreases the number of regulatory $Foxp3^+$ T cells, but has no overall effect on T cell proliferation (Thebault et al. 2009). These effects could not be accounted for by levels of the cytokines IL6 and TGF-β, which were unaffected by CLEC-1 inhibition. The increase in $CD4^+$ Th17 differentiation is not due to a direct effect on Th17 differentiation of naïve T cells or to the plasticity of regulatory $CD4^+CD25^+$ T cells. It is suggested that the Th17 differentiation may be due to an effect on the suppression mediated by regulatory $CD4^+CD25^+$ T cells (Thebault et al. 2009). Therefore, CLEC-1 expressed on DC can inhibit Th17 differentiation and increase the numbers of regulatory $Foxp3^+$ $CD4^+CD25^+$ T cells. Inhibition of CLEC-1 expression also downregulates IL13 secretion by $CD4^+CD25^-$ T cells (Thebault et al. 2009). This suggests a role for CLEC-1 in Th2 differentiation.

In a rat model of long-term tolerated allografts, in which CLEC-1 expression on graft endothelial cells is upregulated, the level of IL17 transcript is reduced, and the level of Foxp3 transcript is increased compared to the levels in chronically rejected allografts, which do not significantly express CLEC-1 (Thebault et al. 2009). This is consistent with the idea that CLEC-1 regulates the balance between effector and regulatory T cells and, hence, modulates T-cell activation. CLEC-1 may therefore play a role in allograft tolerance.

Summary

CLEC-1 is a C-type lectin-like receptor belonging to the Dectin-1 family of immune receptors. It is predominantly expressed by DC, endothelial cells, monocytes, and macrophages. Expression of CLEC-1 is downregulated by inflammatory stimuli and increased by the immunoregulatory mediators IL10 or transforming growth factor beta (TGF-β). CLEC-1 is overexpressed on endothelial cells of tolerated allografts compared with allografts that are chronically rejected or syngeneic grafts in a rat model, and CLEC-1 is believed to play a role in allograft tolerance by regulating the balance between Th17 effector and $Foxp3^+$ regulatory T cells. A ligand for CLEC-1 has not yet been identified. Ligand binding to CLEC-1 might lead to phosphorylation of the intracellular tyrosine(s) present in the cytoplasmic domain, and this may trigger effector functions. Future studies to identify endogenous and exogenous ligands for CLEC-1 are required to better understand CLEC-1 signaling and function.

References

Adachi Y, Ishii T, Ikeda Y, Hoshino A, Tamura H, Aketagawa J, et al. Characterization of beta-glucan recognition site on C-type lectin, dectin 1. Infect Immun. 2004;72(7):4159–71.

Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999; 285(5428):727–9.

Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature. 1998;391(6669):795–9.

Brown GD, Gordon S. Immune recognition. A new receptor for beta-glucans. Nature. 2001;413(6851):36–7.

Brown J, O'Callaghan CA, Marshall AS, Gilbert RJ, Siebold C, Gordon S, et al. Structure of the fungal beta-glucan-binding immune receptor dectin-1: implications for function. Protein Sci. 2007;16(6):1042–52.

Cerwenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH, et al. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity. 2000;12(6):721–7.

Christou CM, Pearce AC, Watson AA, Mistry AR, Pollitt AY, Fenton-May AE, et al. Renal cells activate the platelet receptor CLEC-2 through podoplanin. Biochem J. 2008;411(1):133–40.

Colonna M, Samaridis J, Angman L. Molecular characterization of two novel C-type lectin-like receptors, one of which is selectively expressed in human dendritic cells. Eur J Immunol. 2000;30(2):697–704.

Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol. 2000;1(2):119–26.

Flornes LM, Nylenna O, Saether PC, Daws MR, Dissen E, Fossum S. The complete inventory of receptors encoded by the rat natural killer cell gene complex. Immunogenetics. 2010;62(8):521–30.

Huysamen C, Brown GD. The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors. FEMS Microbiol Lett. 2009;290(2):121–8.

Kanazawa N. Dendritic cell immunoreceptors: C-type lectin receptors for pattern-recognition and signaling on antigen-presenting cells. J Dermatol Sci. 2007;45(2):77–86.

Sobanov Y, Bernreiter A, Derdak S, Mechtcheriakova D, Schweighofer B, Duchler M, et al. A novel cluster of lectin-like receptor genes expressed in monocytic, dendritic and endothelial cells maps close to the NK receptor genes in the human NK gene complex. Eur J Immunol. 2001;31(12):3493–503.

Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood. 2006;107(2):542–9.

Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, et al. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. J Biol Chem. 2007;282(36):25993–6001.

Thebault P, Lhermite N, Tilly G, Le Texier L, Quillard T, Heslan M, et al. The C-type lectin-like receptor CLEC-1, expressed by myeloid cells and endothelial cells, is up-regulated by immunoregulatory mediators and moderates T cell activation. J Immunol. 2009;183(5):3099–108.

Watson AA, O'Callaghan CA. Crystallization and X-ray diffraction analysis of human CLEC-2. Acta Crystallogr F Struct Biol Cryst Commun. 2005;61(Pt 12):1094–6.

Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol. 2008;9(10):1179–88.

Clec1a

► CLEC-1

Clec1b

▶ CLEC-2

CLEC1B, C-type Lectin-Domain Family 1, Member B

▶ CLEC-2

Clec2

▶ CLEC-2

CLEC-2

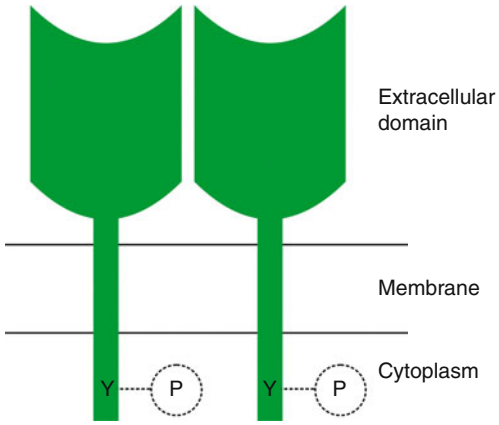
Angharad E. Fenton-May and
Christopher A. O'Callaghan
Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Synonyms

Clec1b; CLEC1B, C-type lectin-domain family 1, member B; Clec2; CLEC2B; C-type lectin-domain family 1, member b; C-type lectin-like receptor 2; mCLEC-2

Historical Background

CLEC-2 is a 32 kDa C-type lectin-like immune receptor (Colonna et al. 2000; O'Callaghan 2009). CLEC-2 (gene name CLEC1B) is part of the NK (natural killer) gene cluster found on human chromosome 12 and mouse chromosome 6. Within this cluster, CLEC-2 is part of the Dectin-1 subfamily which consists of all type 2 transmembrane proteins with extracellular C-type lectin-like domains (CTLDs) and immune or homeostatic roles. Other members of this cluster include: ▶ CLEC-1, ▶ Dectin-1, CLEC8A (Lox-1), CLEC9A, CLEC12A, and CLEC12B.



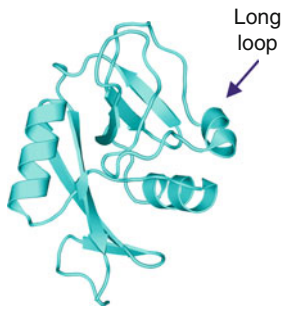
CLEC-2, Fig. 1 Schematic representation of CLEC-2. Upon ligand engagement, the cytoplasmic tyrosine residue in the YxxL motif is phosphorylated, initiating a signalling cascade

CLEC-2 is a type 2 transmembrane signalling protein with its N-terminal region within the cell and its C-terminal region outside the cell. CLEC-2 has a short cytoplasmic region containing a single YxxL motif followed by a single pass transmembrane domain and then an extracellular C-type lectin-like domain (Colonna et al. 2000) (Fig. 1).

CLEC-2 is expressed on the surface of platelets and ligand binding results in platelet activation and aggregation (Suzuki-Inoue et al. 2006). CLEC-2 is also expressed on megakaryocytes and liver sinusoidal endothelial cells (Chaipan et al. 2006), and transcripts have been reported in peripheral blood cells, including NK cells, monocytes, granulocytes, and dendritic cells (Colonna et al. 2000; Suzuki-Inoue et al. 2006; Fuller et al. 2007; Christou et al. 2008). CLEC-2 has also been shown to be a phagocytic receptor on mouse neutrophils. There are two currently identified ligands for CLEC-2: the snake venom protein rhodocytin and the endogenous protein podoplanin (Suzuki-Inoue et al. 2006; Suzuki-Inoue et al. 2007; Christou et al. 2008).

Structure

The protein sequence of CLEC-2 shares similarity with other C-type lectin-like proteins, such as ▶ NKG2D. The structure of the extracellular portion of CLEC-2 has been solved by crystallization and X-ray diffraction analysis and reveals a C-type lectin-like structure consisting of two antiparallel β-sheets flanked by two α-helices with a short 3–10 helix in the long loop



CLEC-2, Fig. 2 The structure of CLEC-2. This view is based upon the crystal structure of the extracellular domain of CLEC-2 (Watson et al. 2007). The molecule is oriented as if the membrane were at the bottom of the figure. The long loop region which contributes to ligand binding is indicated

region (Watson and O'Callaghan 2005; Watson et al. 2007) (Fig. 2). Site-directed mutagenesis studies demonstrate that this flexible long loop region is important for ligand binding (Watson et al. 2007).

Sugar-binding C-type lectins typically coordinate calcium ions as part of their interaction with a sugar. However, CLEC-2 does not contain the residues required for calcium coordination, and no Ca^{2+} ions were seen in the CLEC-2 crystal structure, (Watson et al. 2007) indicating that CLEC-2 is unlikely to bind a carbohydrate ligand. Furthermore, the CLEC-2 ligand rhodocytin is unglycosylated, indicating that this interaction is not mediated by sugars. CLEC-2 has been shown to be found at the cell surface as a non-disulphide-linked homodimer (Watson et al. 2009).

Signalling

CLEC-2 has a single conserved YxxL motif in its cytoplasmic domain (Colonna et al. 2000). This YxxL motif is preceded by three acidic residues and has been termed a *hemiTAM*. As CLEC-2 is dimeric at the cell surface, two such motifs will be in close proximity (Watson et al. 2009) (Fig. 1).

Binding of ligand or antibody to CLEC-2 triggers phosphorylation of the tyrosine residue in the YxxL motif (Suzuki-Inoue et al. 2006) and mutation of this tyrosine prevents signalling (Fuller et al. 2007). Upon ligand engagement, CLEC-2 translocates to lipid rafts, and this translocation appears essential for phosphorylation and, therefore, signalling (Pollitt et al. 2010). Tyrosine phosphorylation is mediated by ▶ Src kinases, and Src kinase inhibition prevent signalling (Fuller et al. 2007). CLEC-2 phosphorylation results in binding to, and phosphorylation of, Syk (spleen tyrosine kinase) (Suzuki-Inoue et al. 2006; Fuller et al. 2007). This initiates a signalling cascade culminating in phosphorylation of PLCγ2 (phospholipase-Cγ2) (Suzuki-Inoue et al. 2006; Fuller et al. 2007). Both of the SH2 (Src-homology domain 2) domains of Syk are required for productive binding to phosphorylated CLEC-2 (Fuller et al. 2007). Since CLEC-2 only has a single YxxL motif, these two SH2 domains must therefore bind to two chains of CLEC-2 (Hughes et al. 2010b), although whether this is achieved through binding to two YxxLs within a single CLEC-2 dimer or to one from each of two dimers is not currently understood. Downstream phosphorylation has additionally been shown of Vav3, LAT, Btk, and ▶ SLP-76 (Src-homology 2 domain-containing leukocyte protein of 76 kDa), although activation through CLEC-2 is only partially dependent upon the SLP-76/Blnk family of adapter molecules (Suzuki-Inoue et al. 2006; Fuller et al. 2007).

Ligands

Two ligands, rhodocytin and podoplanin, have been identified for CLEC-2 (Suzuki-Inoue et al. 2006; Suzuki-Inoue et al. 2007; Christou et al. 2008). Rhodocytin (also known as aggretin) is a venom protein from the Malayan pit viper *Calloselasma rhodostoma* that was known to activate platelets; CLEC-2 was identified as its ligand by affinity purification and mass spectrometry (Suzuki-Inoue et al. 2006). The crystal structure of rhodocytin shows it to be a tetramer, consisting of two heterodimers each composed of an α and a β chain (Watson et al. 2007; Hooley et al. 2008). These α and β chains each contain C-type lectin-like domains. Rhodocytin binds directly to CLEC-2 and is not glycosylated (Watson et al. 2007).

Podoplanin (also known as aggrus) is an endogenous ligand for CLEC-2 and binds directly to CLEC-2 (Suzuki-Inoue et al. 2007; Christou et al. 2008). Podoplanin was identified as a ligand for CLEC-2 when similarities were noted between the signalling profiles of platelets stimulated with rhodocytin and podoplanin (Watanabe et al. 1990; Suzuki-Inoue et al. 2006). Podoplanin is a heavily O-glycosylated type 1 membrane protein with a short cytoplasmic region. However, it seems unlikely that CLEC-2 could recognize a carbohydrate ligand in addition to the non-glycosylated protein rhodocytin. A recent

study indicates that the state of glycosylation of podoplanin does not have a significant effect on the interaction with CLEC-2 (Cueni et al. 2010).

Podoplanin is expressed on podocytes in the kidney (from where it takes its name) and on lymphatic endothelial cells, but not upon vascular endothelial cells. Podoplanin expression has also been reported in skeletal tissue, muscle, type I alveolar cells in the lung, heart, myofibroblasts in breast and salivary glands, osteoblasts, certain mesothelial cells, and follicular dendritic cells. Podoplanin is expressed by several tumor types, and expression at the leading edge of some tumors has been observed. CLEC-2 has been identified as an attachment factor for HIV-1, facilitating capture of the virus by platelets (Chaipan et al. 2006). CLEC-2 appears to bind to podoplanin incorporated into the virion from the virus-producing cells (Chaipan et al. 2010).

Knockout Studies

A number of groups have generated murine CLEC-2 knockout models which are embryonic/neonatal lethal (Bertozzi et al. 2010; Hughes et al. 2010a; Suzuki-Inoue et al. 2010; Tang et al. 2010). Knockout results in severe defects in lymphatic development with blood-filled lymphatics indicating a failure of blood/lymphatic separation. Similarly, mice deficient in podoplanin die at birth from respiratory failure and also have a failure of blood/lymphatic separation (Schacht et al. 2003). Indeed, the phenotypes of CLEC-2, podoplanin, Syk, SLP76, PLCγ2, and platelet-defective mice all share similar characteristics of a failure in blood/lymphatic separation (Abtahian et al. 2003; Schacht et al. 2003; Ichise et al. 2009; Bertozzi et al. 2010; Hughes et al. 2010a; Suzuki-Inoue et al. 2010; Tang et al. 2010). This suggests that the developmental interaction between CLEC-2 on platelets and podoplanin on the future lymphatic endothelial cells may induce separation of the vascular and lymphatic systems by triggering platelet aggregation, so sealing the future lymphatic vessels.

Antibody-mediated depletion of CLEC-2 from circulating platelets results in normal adhesion, but reduced aggregation, of platelets under flow conditions and is associated with increased bleeding times and inhibition of occlusive arterial thrombus formation (May et al. 2009). One of the knockout studies in which irradiated wild type mice were reconstituted with CLEC-2 deficient fetal liver cells and so have CLEC-2 deficient platelets also showed a decrease in aggregation under flow and observed defects in thrombosis formation following injury, supporting a role for CLEC-2 in stabilizing thrombus formation (Suzuki-Inoue et al. 2010). However, a second, independent, knockout study observed no decrease in platelet aggregation under shear conditions (Hughes et al. 2010a). Neither of these mouse knockout studies reported statistically significant decreases in tail bleeding times (Hughes et al. 2010a; Suzuki-Inoue et al. 2010), in contrast to the study in which CLEC-2 expression was reduced by antibody administration (May et al. 2009), and the role of CLEC-2 in hemostasis and thrombosis remains unclear.

Summary

CLEC-2 is an important platelet receptor, which may also have an immune role. Ligand engagement of CLEC-2 results in tyrosine phosphorylation in its cytoplasmic YXXL motif and triggers platelet activation and aggregation. Knockout models reveal a key role for CLEC-2 in blood/lymphatic separation during development. For the function of CLEC-2 beyond platelets to be understood, its cellular expression pattern and the regulation of this expression need further study, especially in immune cells.

The involvement of CLEC-2 in platelet aggregation makes it a key medical target in a variety of contexts, including myocardial infarction and ischaemic stroke (O'Callaghan 2009). The structure of podoplanin should accelerate the development of reagents to inhibit the CLEC-2 axis.

References

Abtahian F, Guerriero A, Sebzda E, Lu MM, Zhou R, Mocsai A, et al. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science. 2003;299(5604):247–51.

Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, et al. Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. Blood. 2010;116(4):661–70.

Chaipan C, Soilleux EJ, Simpson P, Hofmann H, Gramberg T, Marzi A, et al. DC-SIGN and CLEC-2 mediate human immunodeficiency virus type 1 capture by platelets. J Virol. 2006;80(18):8951–60.

Chaipan C, Steffen I, Tsegaye TS, Bertram S, Glowacka I, Kato Y, et al. Incorporation of podoplanin into HIV released from HEK-293T cells, but not PBMC, is required for efficient binding to the attachment factor CLEC-2. Retrovirology. 2010;7:47.
Christou CM, Pearce AC, Watson AA, Mistry AR, Pollitt AY, Fenton-May AE, et al. Renal cells activate the platelet receptor CLEC-2 through podoplanin. Biochem J. 2008;411(1):133–40.
Colonna M, Samaridis J, Angman L. Molecular characterization of two novel C-type lectin-like receptors, one of which is selectively expressed in human dendritic cells. Eur J Immunol. 2000;30(2):697–704.
Cueni LN, Chen L, Zhang H, Marino D, Huggenberger R, Alitalo A, et al. Podoplanin-Fc reduces lymphatic vessel formation in vitro and in vivo and causes disseminated intravascular coagulation when transgenically expressed in the skin. Blood. 2010;116(20):4376–84.
Fuller GL, Williams JA, Tomlinson MG, Eble JA, Hanna SL, Pohlmann S, et al. The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. J Biol Chem. 2007;282(17):12397–409.
Hooley E, Papagrigoriou E, Navdaev A, Pandey AV, Clemetson JM, Clemetson KJ, et al. The crystal structure of the platelet activator aggretin reveals a novel (alphabeta)2 dimeric structure. Biochemistry. 2008;47(30):7831–7.
Hughes CE, Navarro-Nunez L, Finney BA, Mourao-Sa D, Pollitt AY, Watson SP. CLEC-2 is not required for platelet aggregation at arteriolar shear. J Thromb Haemost. 2010a;8(10):2328–32.
Hughes CE, Pollitt AY, Mori J, Eble JA, Tomlinson MG, Hartwig JH, et al. CLEC-2 activates Syk through dimerization. Blood. 2010b;115(14):2947–55.
Ichise H, Ichise T, Ohtani O, Yoshida N. Phospholipase Cgamma2 is necessary for separation of blood and lymphatic vasculature in mice. Development. 2009;136(2):191–5.
May F, Hagedorn I, Pleines I, Bender M, Vogtle T, Eble J, et al. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. Blood. 2009;114(16):3464–72.
O'Callaghan CA. Thrombomodulation via CLEC-2 targeting. Curr Opin Pharmacol. 2009;9(2):90–5.
Pollitt AY, Grygielska B, Leblond B, Desire L, Eble JA, Watson SP. Phosphorylation of CLEC-2 is dependent on lipid rafts, actin polymerization, secondary mediators, and Rac. Blood. 2010;115(14):2938–46.
Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, et al. T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J. 2003;22(14):3546–56.
Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood. 2006;107(2):542–9.
Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, et al. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. J Biol Chem. 2007;282(36):25993–6001.
Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, et al. Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem. 2010;285(32):24494–507.
Tang T, Li L, Tang J, Li Y, Lin WY, Martin F, et al. A mouse knockout library for secreted and transmembrane proteins. Nat Biotechnol. 2010;28(7):749–55.
Watanabe M, Sugimoto Y, Tsuruo T. Expression of a Mr 41,000 glycoprotein associated with thrombin-independent platelet aggregation in high metastatic variants of murine B16 melanoma. Cancer Res. 1990;50(20):6657–62.
Watson AA, O'Callaghan CA. Crystallization and X-ray diffraction analysis of human CLEC-2. Acta Crystallogr F Struct Biol Cryst Commun. 2005;61(Pt 12):1094–6.
Watson AA, Brown J, Harlos K, Eble JA, Walter TS, O'Callaghan CA. The crystal structure and mutational binding analysis of the extracellular domain of the platelet-activating receptor CLEC-2. J Biol Chem. 2007;282(5):3165–72.
Watson AA, Christou CM, James JR, Fenton-May AE, Moncayo GE, Mistry AR, et al. The platelet receptor CLEC-2 is active as a dimer. Biochemistry. 2009;48(46):10988–96.

CLEC2B

▶ CLEC-2

CLEC4E

Anita R. Mistry and Christopher A. O'Callaghan
Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Synonyms

CLECSF9; C-type (calcium-dependent, carbohydrate recognition domain) lectin superfamily member 9; C-type lectin domain family 4 member E; Immunoreceptor; Macrophage-inducible C-type lectin; MINCLE

Historical Background

CLEC4E is a member of the C-type lectin family of immune receptors. It is expressed on the cell surface of activated macrophages, and its expression is

strongly upregulated by inflammatory stimuli. CLEC4E is encoded in the natural killer gene complex on human chromosome 12 and mouse chromosome 6 within a cluster of genes, which also includes BDCA-2, DCAR, DCIR, Dectin-2, and Clecsf8. *CLEC4E* encodes a type 2 transmembrane protein with a short N-terminal cytoplasmic domain, a single transmembrane domain and a C-terminal extracellular C-type lectin carbohydrate recognition domain. The transmembrane domain contains a positively charged arginine residue, which mediates association with the Fc receptor gamma chain (FcεRIγ) signaling adaptor which carries an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. CLEC4E is a classical C-type lectin that contains conserved residues for calcium coordination and sugar binding within its extracellular domain. This includes a predicted mannose-binding motif, suggesting that CLEC4E might have mannose-containing sugar ligands. CLEC4E is a fungal receptor on macrophages and has a role in the innate immune response to *Candida albicans* and *Malassezia* species. However, the ligand on fungi has not yet been identified. Recently, CLEC4E has been identified as a mycobacterial receptor, and it recognizes the mycobacterial glycolipid trehalose-6,6′-dimycolate. Recognition of *Malassezia*, and mycobacteria has been shown to be dependent upon the predicted mannose-binding motif of CLEC4E. However, CLEC4E also binds an endogenous nuclear protein ligand released from necrotic cells, spliceosome-associated protein 130 (SAP130), in a carbohydrate-independent manner. Ligand binding to CLEC4E leads to cytokine and chemokine production through a pathway involving FcεRIγ, spleen tyrosine kinase (Syk) and caspase recruitment domain family, member 9 (CARD9). CLEC4E is also essential for granuloma formation, which is an important characteristic of *Mycobacterium tuberculosis* infection.

CLEC4E Expression Pattern and Regulation of Expression

At the transcript level, mouse *CLEC4E* is expressed in bone marrow, lymph node, spleen, and lung (Comelli et al. 2006), and has recently been found to be expressed in healing corneas (Saravanan et al. 2010). *CLEC4E* is transcribed in a wide range of leukocytes, including macrophages, neutrophils, dendritic cells, B cells, $CD4^+$ T cells, $CD8^+$T cells, concanavalin A blasts, and M1 myeloblastic leukemia cells (Matsumoto et al. 1999; Flornes et al. 2004; Nakamura et al. 2006), as well as in microglia in the brain (McKimmie et al. 2006). At the protein level, mouse CLEC4E is predominantly expressed on activated macrophages (Matsumoto et al. 1999; Wells et al. 2008; Yamasaki et al. 2008; Yamasaki et al. 2009; Schoenen et al. 2010) and also on the cell surface of $CD11b^+Gr1^+$ neutrophils, present in the thymus of irradiated mice (Yamasaki et al. 2008). Human *CLEC4E* is transcribed in LPS-stimulated THP1 monocytic leukemia cells (Matsumoto et al. 1999) and in bone marrow–derived mononuclear cells of rheumatoid arthritis patients (Nakamura et al. 2006). Human CLEC4E protein was detected in in vitro $CD34^+$ progenitor-derived mast cells upon IgE receptor cross-linking (Ribbing et al. 2010).

CLEC4E expression is induced on macrophages by a variety of inflammatory stimuli, including lipopolysaccharide (LPS), ▶ TNF-α, interleukin (IL)-6, ▶ interferon-gamma (IFNγ), zymosan, mannan, β-glucan, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) (a bacterial peptidoglycan-derived small peptide), CpG oligodeoxyribonucleotides, curdlan, and mycobacterial glycolipids (Matsumoto et al. 1999; Guo et al. 2009; Schoenen et al. 2010). Expression of *CLEC4E* is also induced by various infections, including *Streptococcus pneumoniae*, influenza A virus (Rosseau et al. 2007); *Mycobacterium tuberculosis* bacillus Calmette-Guérin (Khajoee et al. 2006), Semliki forest virus (McKimmie et al. 2006), and *C. albicans* (Wells et al. 2008). At the protein level, CLEC4E is upregulated by *Malassezia* fungal stimulation of thioglycollate-elicited peritoneal mouse macrophages (Yamasaki et al. 2009). Human *CLEC4E* is upregulated in bone marrow-derived mononuclear cells from rheumatoid arthritis patients compared with those from osteoarthritis patients (Nakamura et al. 2006).

CLEC4E is a transcriptional target of the CCAAT/enhancer binding protein, beta (C/EBP-β) and interferon regulatory factor-8 (IRF-8) transcription factors (Matsumoto et al. 1999; Tamura et al. 2005). C/EBP-β activity is induced by inflammatory stimuli, and it plays a role in the regulation of genes involved in immune and inflammatory responses in activated macrophages. IRF-8 is a transcription factor that controls myeloid differentiation.

Interaction with Endogenous Ligands and Pathogens

CLEC4E is a classical C-type lectin with a carbohydrate recognition domain that has conserved residues forming what has been termed "Ca^{2+}-binding site 2," which plays a role in calcium-dependent carbohydrate binding (Matsumoto et al. 1999; Flornes et al. 2004). CLEC4E is, therefore, predicted to bind carbohydrate ligands, and in particular mannose-containing carbohydrates, since it contains an EPN (glutamic acid-proline-asparagine) motif, which is a well-characterized mannose-binding motif present in a number of other C-type lectins, including DC-SIGN (CD209), Dectin-2, and Langerin. Indeed, recombinant CLEC4E protein binds to a highly multivalent form of α-mannose in a Ca^{2+}-dependent manner, but notably not to mannan (Yamasaki et al. 2009).

CLEC4E is a receptor for *Candida albicans* and *Malassezia* fungal species, although the precise ligand on fungi has yet to be identified. Mouse CLEC4E binds to a soluble component of heat-killed *Candida albicans* and *Saccharomyces cerevisiae* (Bugarcic et al. 2008; Wells et al. 2008). Recognition of *Malassezia* is dependent upon the EPN motif of CLEC4E (Yamasaki et al. 2009). CLEC4E is also a receptor for mycobacteria, and the mycobacterial glycolipid, trehalose-6,6′-dimycolate (TDM) has been identified as the CLEC4E ligand on mycobacteria (Ishikawa et al. 2009; Schoenen et al. 2010). TDM is a potent immunostimulant causing IL1 and TNF release and granuloma formation in vivo. TDM is also an effective adjuvant. TDM is composed of a trehalose moiety (a disaccharide made up of two glucose units) and two long-chain α-branched, β-hydoxyl fatty acids, known as mycolic acids. Proteins with EPN mannose motifs are also able to bind structurally related sugars such as glucose, and indeed CLEC4E recognition of *M.bovis* and *M.tuberculosis* is dependent upon the EPN motif of CLEC4E. However, neither trehalose nor purified mycolate alone stimulates CLEC4E-expressing cells. In addition, the long, branched structure of mycolic acids is also not essential for stimulatory activity since a synthetic analogue of TDM, trehalose dibehenate (TDB), which contains shorter fatty acid chains, is also a potent stimulator of CLEC4E-expressing cells. CLEC4E may, therefore, be specific for the ester linkage between the fatty acid and trehalose.

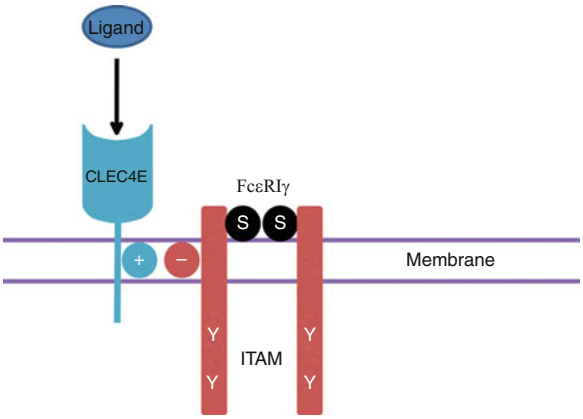
An endogenous ligand for CLEC4E has also been identified (Yamasaki et al. 2008). CLEC4E binds to spliceosome-associated protein 130 (SAP130) in a carbohydrate-independent manner. SAP130 is normally located in the nucleus as a component of the SF3b complex, which forms part of the U2 snRNP. U2 snRNP is involved in the assembly of spliceosomes. It is proposed that during cell death, SAP130 is released into the extracellular environment.

CLEC4E Signaling and Regulation of Activity

The transmembrane domain of CLEC4E contains a positively charged arginine residue, which mediates association with the ITAM-bearing FcεRIγ signaling adaptor (Yamasaki et al. 2008) (Fig. 1). The negatively charged aspartic acid residue in the transmembrane domain of FcεRIγ associates with the positively charged amino acid in the transmembrane domains of other immune receptor molecules (reviewed in (Humphrey et al. 2005)). Ligand binding to FcεRIγ-associated receptors results in phosphorylation of FcεRIγ on the two tyrosine residues at positions 65 and 76 that form the ITAM motif. A number of related C-type lectin and C-type lectin-like proteins also interact with signaling molecules in the plasma membrane, including ▶ CLEC5A which interacts with Dap12, ▶ NKG2D which associates with Dap10 and Dap12 and DCAR (CLEC4B) which associates with FcεRIγ.

Efficient cell-surface expression of CLEC4E on bone marrow–derived macrophages in response to LPS stimulation requires the expression of FcεRIγ (Yamasaki et al. 2008). FcεRIγ is essential for CLEC4E-mediated signaling. Stimulation of thioglycollate-elicited peritoneal macrophages or bone marrow–derived macrophages with monoclonal antibodies to CLEC4E (Yamasaki et al. 2008), or with fungal species (Wells et al. 2008; Yamasaki et al. 2009), induces the production of proinflammatory cytokines and chemokines in an FcεRIγ-dependent manner (Yamasaki et al. 2008). Furthermore, inflammatory cytokine production, lung swelling, and the formation of granulomas in response to TDM stimulation in vivo are dependent upon FcεRIγ (Ishikawa et al. 2009). FcεRIγ is also essential for the adjuvant properties of TDB (Schoenen et al. 2010).

Cross-linking of CLEC4E induces phosphorylation of the kinases Syk and extracellular signal-regulated kinase (Erk) in peritoneal macrophages (Yamasaki et al. 2008). The adaptor molecule CARD9 has an important role in the downstream signaling, resulting in chemokine production (Yamasaki et al. 2008).



CLEC4E, Fig. 1 MINCLE interactions. MINCLE interacts with the FcεRIγ signaling adaptor within the membrane. The interaction employs a charge–charge interaction between polar residues within the membrane

The CLEC4E cytoplasmic domain contains two putative phosphorylation sites, Ser 3 and Thr 12 (Matsumoto et al. 1999).

Functional Significance of CLEC4E Signaling

CLEC4E is a macrophage receptor for a number of pathogens, including *Candida albicans*, several species of *Malassezia*, and mycobacteria (Bugarcic et al. 2008; Wells et al. 2008; Ishikawa et al. 2009; Yamasaki et al. 2009; Schoenen et al. 2010). An endogenous ligand, SAP130, has also been identified (Yamasaki et al. 2008). Ligand binding and triggering of CLEC4E on macrophages stimulates a signaling cascade through FcεRIγ, Syk, and CARD9, which induces the production of cytokines and chemokines, including TNF-α, ▶ IL6, IL10, CXCL1 (KC), and CXCL2 (MIP-2) (Wells et al. 2008; Yamasaki et al. 2008; Ishikawa et al. 2009). Absence or inhibition of CLEC4E reduces cytokine and chemokine production in response to challenge with *Candida albicans* (Wells et al. 2008) and *Malassezia* species (Yamasaki et al. 2009). Partial inhibition of the transcription of G-CSF and IL6 and MIP-2 production in CLEC4E-deficient bone marrow macrophages were observed following stimulation with *M.tuberculosis and M.bovis* BCG (Bacille Calmette-Guerin) (Ishikawa et al. 2009; Schoenen et al. 2010). However, no inhibition of TNF production was observed in these cells. Since whole mycobacteria contain many immunostimulatory components, it is suggested that the lack of significant impairment in cytokine and chemokine production is due to signaling through other receptors, including toll-like receptors (TLR) and the NOD-like receptor family. Indeed, cytokine production is substantially inhibited in MyD88$^{-/-}$CLEC4E$^{-/-}$ double deficient macrophages compared with macrophages deficient in CLEC4E or MyD88 alone following stimulation with *M.tuberculosis* (Ishikawa et al. 2009).

In response to TDM or TDB, mouse bone marrow–derived macrophages produce nitric oxide, IL6, and G-CSF in a CLEC4E-dependent manner (Ishikawa et al. 2009; Schoenen et al. 2010). Production of nitric oxide is important for the direct killing of mycobacteria. In vivo administration of TDM induces the production of IL6 and TNF in sera, inflammatory lung swelling, and importantly granuloma formation in a CLEC4E- and FcεRIγ-dependent manner (Ishikawa et al. 2009). Transcription of TNF, IL1β, and MIP-1α in the lungs is induced by TDM in a CLEC4E-dependent manner, and TDM induces thymic atrophy through CLEC4E stimulation. Both TDM and TDB are effective adjuvants controlling Th1 and Th17 responses. CLEC4E plays a key role in the adjuvant effect of TDB in subunit vaccination with *M.tuberculosis* fusion protein Ag85B-ESAT6 (H1); the absence of CLEC4E reduces cellularity in the draining lymph node, and significantly impairs IFN gamma and IL17 production by lymph node cells (Schoenen et al. 2010).

The production of inflammatory cytokines and chemokines plays an important role in the recruitment of neutrophils to sites of inflammation (Yamasaki et al. 2008). In vivo, mouse CLEC4E has a role in neutrophil recruitment to sites of thymocyte necrosis in the thymus caused by whole-body irradiation, or to the peritoneal cavity after injection of dead cells or *Malassezia* fungal species (Yamasaki et al. 2008; Yamasaki et al. 2009). Blockade of CLEC4E-mediated MIP-2 and TNF-α production from macrophages by injection of a monoclonal antibody to CLEC4E significantly inhibits the migration of neutrophils to sites of substantial cell death in vivo (Yamasaki et al. 2008).

CLEC4E localizes to the phagocytic cup that forms around yeast particles after exposure to *C. albicans* (Wells et al. 2008). However, it is not involved in the phagocytosis of *C. albicans* (Wells et al. 2008) or dead cells (Yamasaki et al. 2008). CLEC4E may have a role in autoimmune disease as its expression is considerably upregulated in patients with rheumatoid arthritis (Nakamura et al. 2006).

Phenotype of CLEC4E Knockout Models

To date, two knockout mouse models of *CLEC4E* have been generated (Wells et al. 2008, Yamasaki et al. 2009). In terms of total white blood cell counts and subpopulations of the thymus, spleen, lymph node, and peritoneal cells, no significant phenotype is observed in either of these models. Bone marrow macrophages show normal differentiation, morphology, and number. CLEC4E is therefore not required for the development of the hematopoietic lineage, including the differentiation of macrophages. Immune functions, as measured by delayed-type hypersensitivity responses and immunoglobulin production, are also unaffected by CLEC4E deficiency (Wells et al. 2008).

However, in response to challenge with *C. albicans* (Wells et al. 2008), *Malassezia* species (Yamasaki et al. 2009), and the mycobacterial glycolipid TDM (Ishikawa et al. 2009; Schoenen et al. 2010), macrophages from CLEC4E-deficient mice have significantly impaired production of cytokines (TNF-α, IL10, IL6, and G-CSF), chemokines (MIP-2 and KC), and nitric oxide. In vivo, CLEC4E-deficient mice show increased susceptibility to systemic candidiasis (Wells et al. 2008) and a reduced inflammatory response to intraperitoneal injection of *Malassezia* (Yamasaki et al. 2009) compared with control mice. CLEC4E-mediated production of inflammatory cytokines and chemokines by macrophages is implicated in neutrophil migration into damaged tissue (Yamasaki et al. 2008). In response to challenge with the TDM, CLEC4E-deficient mice display impaired IL6 and TNF-α production in sera and show reduced lung inflammation compared to wild-type mice. Furthermore, TDM-induced granuloma formation is absent in CLEC4E-deficient mice (Ishikawa et al. 2009).

Although *CLEC4E*-knockout mice do not show any gross anatomical phenotype, they seem to show evidence of abnormal heart valves, with accumulation of higher levels of extracellular matrix compared to heart valves from wild-type mice. This suggests a role for CLEC4E in heart valve development (Wells et al. 2008).

Summary

CLEC4E is an important pathogen recognition receptor of the C-type lectin family. It is involved in the recognition of fungal species and mycobacteria in a carbohydrate-dependent manner. There is also evidence indicating that CLEC4E recognizes an endogenous protein ligand, SAP130, in a carbohydrate-independent manner. CLEC4E is predominantly expressed on activated macrophages and associates with the FcεRIγ signaling adaptor. Ligand binding to CLEC4E triggers a signaling cascade through FcεRIγ, Syk, and CARD9, which results in the production of inflammatory cytokines and chemokines. CLEC4E mediates the adjuvant properties of the trehalose glycolipid, TDB. This is an important finding as it provides a target for the design of synthetic adjuvants for effective vaccination strategies against infectious diseases and cancer.

References

Bugarcic A, Hitchens K, Beckhouse AG, Wells CA, Ashman RB, Blanchard H. Human and mouse macrophage-inducible C-type lectin (Mincle) bind Candida albicans. Glycobiology. 2008;18(9):679–85.

Comelli EM, Head SR, Gilmartin T, Whisenant T, Haslam SM, North SJ, et al. A focused microarray approach to functional glycomics: transcriptional regulation of the glycome. Glycobiology. 2006;16(2):117–31.

Flornes LM, Bryceson YT, Spurkland A, Lorentzen JC, Dissen E, Fossum S. Identification of lectin-like receptors expressed by antigen presenting cells and neutrophils and their mapping to a novel gene complex. Immunogenetics. 2004;56(7):506–17.

Guo JP, Verdrengh M, Tarkowski A, Lange S, Jennische E, Lorentzen JC, et al. The rat antigen-presenting lectin-like receptor complex influences innate immunity and development of infectious diseases. Genes Immun. 2009;10(3):227–36.

Humphrey MB, Lanier LL, Nakamura MC. Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. Immunol Rev. 2005;208:50–65.

Ishikawa E, Ishikawa T, Morita YS, Toyonaga K, Yamada H, Takeuchi O, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. J Exp Med. 2009;206(13):2879–88.

Khajoee V, Saito M, Takada H, Nomura A, Kusuhara K, Yoshida SI, et al. Novel roles of osteopontin and CXC chemokine ligand 7 in the defence against mycobacterial infection. Clin Exp Immunol. 2006;143(2):260–8.

Matsumoto M, Tanaka T, Kaisho T, Sanjo H, Copeland NG, Gilbert DJ, et al. A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. J Immunol. 1999;163(9):5039–48.

McKimmie CS, Roy D, Forster T, Fazakerley JK. Innate immune response gene expression profiles of N9 microglia are pathogen-type specific. J Neuroimmunol. 2006;175(1–2):128–41.

Nakamura N, Shimaoka Y, Tougan T, Onda H, Okuzaki D, Zhao H, et al. Isolation and expression profiling of genes

upregulated in bone marrow-derived mononuclear cells of rheumatoid arthritis patients. DNA Res. 2006;13(4): 169–83.
Ribbing C, Engblom C, Lappalainen J, Lindstedt K, Kovanen PT, Karlsson MA, et al. Mast cells generated from patients with atopic eczema have enhanced levels of granule mediators and an impaired Dectin-1 expression. Allergy. 2010;66: 110–119.
Rosseau S, Hocke A, Mollenkopf H, Schmeck B, Suttorp N, Kaufmann SH, et al. Comparative transcriptional profiling of the lung reveals shared and distinct features of Streptococcus pneumoniae and influenza A virus infection. Immunology. 2007;120(3):380–91.
Saravanan C, Cao Z, Head SR, Panjwani N. Analysis of differential expression of glycosyltransferases in healing corneas by glycogene microarrays. Glycobiology. 2010; 20(1):13–23.
Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, et al. Cutting edge: mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol. 2010;184(6):2756–60.
Tamura T, Thotakura P, Tanaka TS, Ko MS, Ozato K. Identification of target genes and a unique cis element regulated by IRF-8 in developing macrophages. Blood. 2005;106(6):1938–47.
Wells CA, Salvage-Jones JA, Li X, Hitchens K, Butcher S, Murray RZ, et al. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans. J Immunol. 2008;180(11):7404–13.
Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol. 2008;9(10): 1179–88.
Yamasaki S, Matsumoto M, Takeuchi O, Matsuzawa T, Ishikawa E, Sakuma M, et al. C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. Proc Natl Acad Sci USA. 2009;106(6):1897–902.

CLEC5A

Michael Reschen and Christopher A. O'Callaghan
Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Synonyms

CLECSF5; C-type (calcium-dependent, carbohydrate-recognition domain) lectin, superfamily member 5; C-type lectin-domain family 5, member A; Ly100; MDL1; MDL-1; Myeloid DAP-12-associating lectin-1

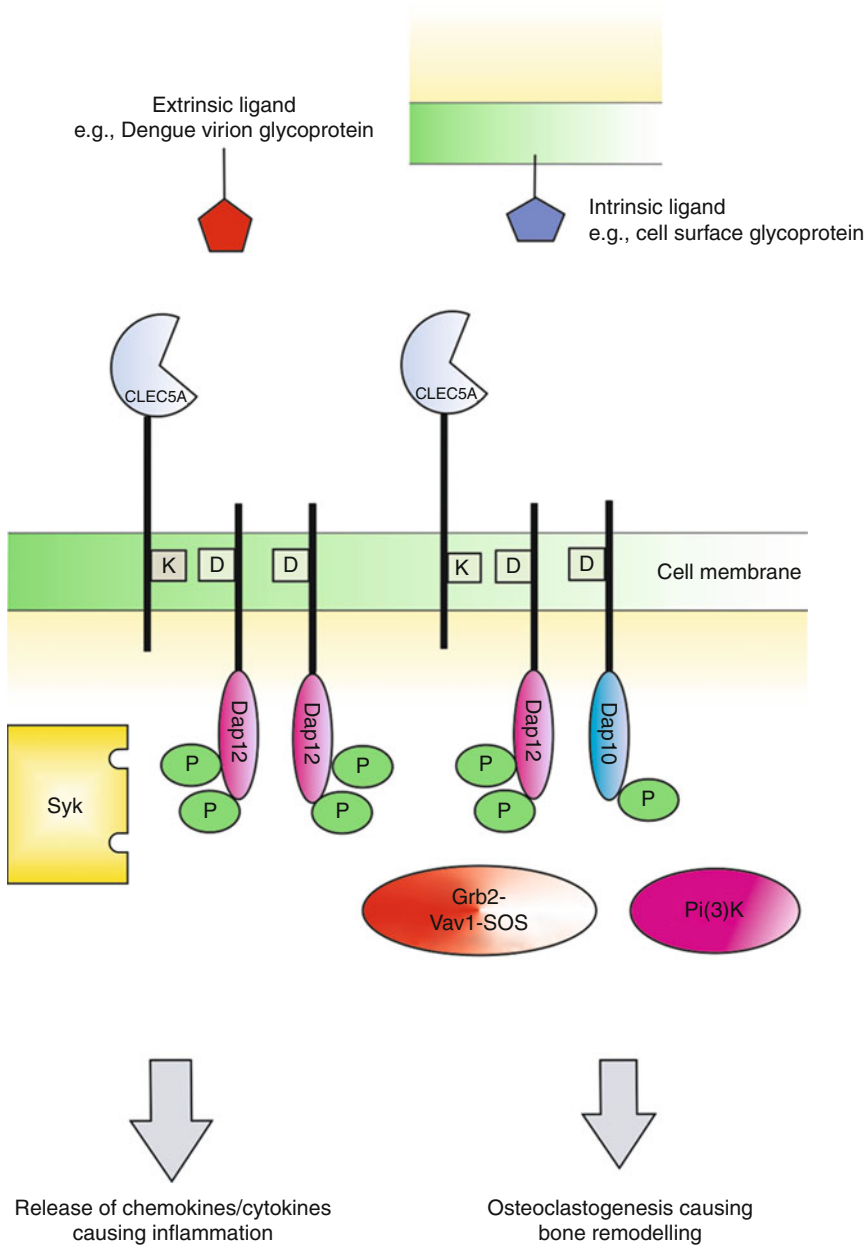
Historical Background

CLEC5A is a type 2 transmembrane receptor originally identified by its ability to stabilize DAP (DNAX associating protein)-12 at the cell surface in myeloid cells (Bakker et al. 1999). It is emerging as a key component of the innate immune system; it activates macrophages, regulates osteoclastogenesis, and plays a role in inflammatory diseases such as dengue virus–induced lethality and autoimmune arthritis (Bakker et al. 1999; Chen et al. 2008; Aoki et al. 2009; Joyce-Shaikh et al. 2010).

Structure

CLEC5A contains a C-type lectin-like domain in its extracellular carboxy-terminal region. However, the N-terminal cytoplasmic tail is predicted to be only four amino acids in length and, thus, lacks a signaling motif. The putative 23 amino acid single pass transmembrane domain contains a conserved lysine residue. CLEC5A associates with DAP-12 by an interaction between this positively charged lysine and a negatively charged aspartate residue which is present in the transmembrane region of DAP-12 (Bakker et al. 1999). DAP-12 transduces activating signals in myeloid and natural killer cells and contains an immunoreceptor tyrosine activation motif (ITAM) in its cytosolic domain. Ligand binding by DAP-12-associated receptors in myeloid cells results in phosphorylation of the two key tyrosine residues within the ITAM motif by ▶ Src kinases; the two SH2 domains of Syk (Spleen tyrosine kinase) can then bind to the phosphorylated ITAM, resulting in Syk activation (Aoki et al. 2003; Lanier 2009). DAP-12 has only a very short extracellular domain and exists as a disulphide linked homodimer in association with CLEC5A or another member of the set of receptors with which it interacts (Lanier et al. 1998; Bakker et al. 1999).

In mouse osteoclasts and bone marrow–derived macrophages, CLEC5A has been shown to associate with both DAP-10 and DAP-12 to form a trimolecular complex (Inui et al. 2009). DAP-10 is a 10 kDa transmembrane protein encoded by a gene which is located very close to the DAP-12 gene. DAP-10 has a cytosolic signaling motif defined by the sequence YINM, that when phosphorylated recruits phosphatidylinositol-3



CLEC5A, Fig. 1 Signaling mechanism of CLEC5A. The positively charged lysine residue (K) in CLEC5A's transmembrane domain interacts with a negatively charged aspartate residue (D) in the DAP-12 or DAP-10 transmembrane domain. Activation of CLEC5A results in phosphorylation of DAP-12 and/or DAP-10 which allows signaling through distinct pathways. Dap12 forms a disulphide-bonded homodimer which contains an ITAM in its intracellular domain. Upon phosphorylation Syk kinases are activated which trigger a signaling cascade that leads to intracellular calcium release and production of chemokines and cytokines. In osteoclasts, DAP-10 can form a heterodimer with DAP-12 and signal through a distinct pathway, ultimately causing osteoclastogenesis and bone remodeling. The intracellular domain of DAP-10 contains a YINM motif which when phosphorylated recruits the p85 subunit of Pi(3)K and a complex of Grb2-Vav1-SOS. CLEC5A interacts directly with the Dengue virion, but other extrinsic or intrinsic ligands have not yet been identified

kinase (► PI3K) and Grb2; Grb2 then interacts with ► Vav1 (Upshaw et al. 2006; Lanier 2008). Thus CLEC5A can access two distinct pathways (see Fig. 1 for a schematic representation of these signaling pathways).

Orthologues of the CLEC5A gene have been identified in several species, including rat, mouse, dog, pig, cow, and chimpanzee (Bakker et al. 1999; Yim et al. 2001). In vitro studies have shown murine and porcine CLEC5A to be glycosylated (Yim et al. 2001; Aoki et al. 2009). Glycosylation is greater in murine neutrophils than macrophages (Aoki et al. 2009), and these differences may influence ligand binding.

Function

Macrophages are phagocytic cells which are derived from monocytes and play a key role in immunity.

CLEC5A can play a role in macrophage activation (Bakker et al. 1999). When the murine macrophage cell line J774 is transfected with CLEC5A, then cross-linking of CLEC5A/DAP-12 complexes results in calcium mobilization (Bakker et al. 1999). Signaling through CLEC5A in murine myeloid 32Dcl3 cells upregulates expression of the leukocyte adhesion molecule CD11b and of cytokines, including RANTES (Regulated upon activation normal T-cell expressed and presumably secreted), IP-10 (Interferon-gamma-induced protein 10), and MDC (macrophage-derived chemokine) (Aoki et al. 2009). Stimulation of ▶ Toll-like receptor 4 by lipopolysaccharide had a synergistic effect with CLEC5A on the expression of RANTES and MDC (Aoki et al. 2009).

CLEC5A interacts with dengue virus causing DAP-12 phosphorylation, which is temporally associated with tumor necrosis factor (TNF)-α production (Chen et al. 2008). Conversely, knockdown of CLEC5A suppresses the release of TNF-α, IL-6, IL-8, macrophage inflammatory protein (MIP), and IP-10. Overall, signaling through CLEC5A increases expression of cell adhesion and chemotactic molecules, contributing to inflammation.

CLEC5A also has a role in bone metabolism. Bone homeostasis depends on the opposing action of osteoblasts of mesencyhmal origin which promote bone-matrix formation and osteoclasts derived from myeloid precursor cells which enhance bone resorption. Stimulation of CLEC5A enhances osteoclastogenesis from murine bone marrow–derived monocytes by signaling through DAP-10 and DAP-12 (Inui et al. 2009; Joyce-Shaikh et al. 2010).

In murine inflammatory arthritis models, CLEC5A stimulation increased mRNA expression of pro-inflammatory cytokines (IL-β, IL-6, IL-17, TNF), bone remodeling genes (receptor activator of NF-κB ligand, Tartrate-resistant acid phosphatase, matrix metallopeptidase 9, ATPV0D2), and myeloid associated genes (CXCL1, CD11b, DAP-12, RANK)(Joyce-Shaikh et al. 2010). These changes are associated with loss of bone and increased inflammation in the murine arthritis model used (see below).

Expression

In humans, CLEC5A is expressed in peripheral blood monocytes and in the monocyte/macrophage cell lines U937 and MonoMac6 but not in cells of nonmyeloid origin (Bakker et al. 1999). Surface expression of CLEC5A was detected on human peripheral blood cells expressing either CD66 or CD14 (Chen et al. 2008). CLEC5A is expressed on porcine monocytes and pulmonary alveolar macrophages but not lymphocytes or polymorphonuclear granulocytes (Yim et al. 2001). In mice, CLEC5A expression has been detected on granulocytes ($CD11b^+$ $Ly6G^{high}$) and monocytes ($CD11b^+$ $Ly6G^{low}$) from bone marrow and peripheral blood, on peritoneal macrophages, thioglycollate-ellicited neutrophils, and osteoclasts (Aoki et al. 2009; Inui et al. 2009; Joyce-Shaikh et al. 2010). In humans and mice, the main tissue sites of expression are in bone marrow, synovium/joint, and lung (Joyce-Shaikh et al. 2010). In inflamed pannus from patients with rheumatoid arthritis CLEC5A was expressed in $CD68^+$ macrophages. (Joyce-Shaikh et al. 2010).

CLEC5A expression is affected by cell ontogeny. CLEC5A expression is higher in peripheral blood derived $CD14^+$ mature monocytes than in undifferentiated $CD34^+$ bone marrow cells. Expression is lower in fetal tissue compared to adult tissue and in malignant cells compared to normal cells (Gingras et al. 2002). Differentiation of myeloid precursor 32Dcl3 cells into neutrophils induces CLEC5A expression (Aoki et al. 2009). Expression is downregulated when monocytes differentiate into dendritic cells (Bakker et al. 1999). Surface expression of murine CLEC5A is upregulated during pulmonary mycobacterial infection (Aoki et al. 2004) and correlates with upregulation of the type 1 cytokines TNF-α and IFN-γ. However, whereas TNF-α is critical for induction of CLEC5A during murine pulmonary mycobacterial infection, IFN-γ suppresses CLEC5A expression (Aoki et al. 2004).

Genetics and Splice Variants

The human CLEC5A gene is on chromosome 7 and encodes a 3,524 basepair mRNA ((Maglott et al. 2007) NM_013252.2). Two isoforms have been identified at the transcript level in humans with isoform 1 (encoding 188 amino acids – Uniprot identifier: Q9NY25-1) being referred to as the canonical sequence. Isoform 2 (Uniprot identifier: Q9NY25-2) differs in that it lacks a lysine residue at position 116. At position 141, there is a single-nucleotide polymorphism (C → T)

encoding a missense mutation altering the amino acid from arginine to histine ((Sherry et al. 2001) rs35942193). Estimates of the frequency of the minor allele (T) range from 0 to 0.027, but this polymorphism is not known to be associated with any diseases. CLEC5A exists as alternatively spliced variants in mice and pigs, with the noncanonical isoforms lacking 25 and 19 amino acids in their extracellular domains, respectively (Bakker et al. 1999; Yim et al. 2001). The significance of these alternatively spliced transcripts is unknown.

CLEC5A −/− mice display a dysregulated bone phenotype with irregular trabecular bone connectivity (Joyce-Shaikh et al. 2010). DAP-10 −/− mice display mild osteopetrosis and a reduction in the number of osteoclasts (Inui et al. 2009). DAP-12 deficient mice also display altered T-cell activity due to impaired antigen priming (Bakker et al. 2000) and have impaired osteoclast development and function (Lanier 2009) and macrophages which are hyperresponsive to in vitro exposure to TLR ligands (Lanier 2008). In humans, lack of DAP-12 results in Nasu-Hakola disease, characterized by presenile dementia and bone cysts (Aoki et al. 2003). However, it is important to note that DAP-12 interacts with a multitude of cell membrane receptors, so this effect is not necessarily mediated by CLEC5A.

Function in Disease

A role for CLEC5A is evident in the immune response and pathogenesis of several infections and in inflammatory arthritis. Human and murine CLEC5A react directly with dengue virus strain 2 in a calcium-independent manner, although the precise ligand on the virus is unknown. This interaction can be inhibited in vitro by the monosaccharide fucose, suggesting that dengue envelope protein glycans are important in mediating this interaction. Infection of macrophages with dengue virus caused phosphorylation of DAP-12, but knockdown of CLEC5A did not reduce viral replication, suggesting that the interaction is important in the inflammatory changes associated with the disease rather than the mechanisms of cellular infection. In vivo blockade of the dengue virus-CLEC5A interaction with an antagonistic anti-CLEC5A antibody reduced lethality of dengue virus infection in STAT−/−mice with decreased plasma leakage and hemorrhage, but no suppression of viral replication. (Chen et al. 2008). CLEC5A may also be involved in the immune response to another flavivirus, Japanese encephalitis virus. Murine infection was associated with increased expression of CLEC5A in brain and splenic tissue and was temporally associated with increased interferon-gamma, TNF-alpha, and IL1-alpha production (Gupta et al. 2010). Murine pulmonary mycobacterial infection induces CLEC5A expression, suggesting a role in mycobacterial immunity (Aoki et al. 2004).

CLEC5A has recently been shown to have an important role in autoimmune-mediated synovial injury and bone erosion in murine models of inflammatory arthritis. Activation of the CLEC5A pathway was associated with a significant increase in the clinical severity, including bone destruction (Joyce-Shaikh et al. 2010). The therapeutic potential of this pathway was demonstrated by improvement in the clinical course brought about by intravenous administration of a CLEC5A-Fc fusion protein. The Fc binding site was mutated to attenuate antibody binding, and the fusion protein presumably bound to and blocked an unknown ligand for CLEC5A. Similar results were achieved by deleting the CLEC5A gene in mice (Joyce-Shaikh et al. 2010).

Summary

CLEC5A is a C-type lectin-like molecule which can activate macrophages and osteoclast production by signaling through DAP-12 and DAP-10. CLEC5A is likely to bind to an endogenous ligand and to viral ligands. It has a role in the pathogenesis of inflammatory arthritis in a mouse model and binds directly to dengue virus contributing to lethality. Future research objectives include: characterization of endogenous and pathogen ligands, understanding the cell surface events that lead to signaling through DAP-12, assessing its role in other TNF-mediated diseases such as inflammatory bowel disease, and exploring the therapeutic potential of CLEC5A blockade.

References

Aoki N, Kimura S, Xing Z. Role of DAP12 in innate and adaptive immune responses. Curr Pharm Des. 2003;9(1):7–10.

Aoki N, Zganiacz A, Margetts P, Xing Z. Differential regulation of DAP12 and molecules associated with DAP12 during host responses to mycobacterial infection. Infect Immun. 2004;72(5):2477–83.
Aoki N, Kimura Y, Kimura S, Nagato T, Azumi M, Kobayashi H, et al. Expression and functional role of MDL-1 (CLEC5A) in mouse myeloid lineage cells. J Leukoc Biol. 2009;85(3):508–17.
Bakker AB, Baker E, Sutherland GR, Phillips JH, Lanier LL. Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells. Proc Natl Acad Sci USA. 1999;96(17):9792–6.
Bakker AB, Hoek RM, Cerwenka A, Blom B, Lucian L, McNeil T, et al. DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming. Immunity. 2000;13(3):345–53.
Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, Lei HY, et al. CLEC5A is critical for dengue-virus-induced lethal disease. Nature. 2008;453(7195):672–6.
Gingras MC, Lapillonne H, Margolin JF. TREM-1, MDL-1, and DAP12 expression is associated with a mature stage of myeloid development. Mol Immunol. 2002;38(11):817–24.
Gupta N, Lomash V, Rao PV. Expression profile of Japanese encephalitis virus induced neuroinflammation and its implication in disease severity. J Clin Virol. 2010;49(1):4–10.
Inui M, Kikuchi Y, Aoki N, Endo S, Maeda T, Sugahara-Tobinai A, et al. Signal adaptor DAP10 associates with MDL-1 and triggers osteoclastogenesis in cooperation with DAP12. Proc Natl Acad Sci USA. 2009;106(12):4816–21.
Joyce-Shaikh B, Bigler ME, Chao CC, Murphy EE, Blumenschein WM, Adamopoulos IE, et al. Myeloid DAP12-associating lectin (MDL)-1 regulates synovial inflammation and bone erosion associated with autoimmune arthritis. J Exp Med. 2010;207(3):579–89.
Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol. 2008;9(5):495–502. doi:10.1038/ni1581.
Lanier LL. DAP10- and DAP12-associated receptors in innate immunity. Immunol Rev. 2009;227(1):150–60.
Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature. 1998;391(6668):703–7.
Maglott D, Ostell J, Pruitt KD, Tatusova T. Entrez Gene: gene-centered information at NCBI. Nucleic Acids Res. 2007;35-(Database issue):D26–31.
Sherry ST, Ward M-H, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res. 2001;29(1):308–11.
Upshaw JL, Arneson LN, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. Nat Immunol. 2006;7(5):524–32. doi:10.1038/ni1325.
Yim D, Jie HB, Sotiriadis J, Kim YS, Kim YB. Molecular cloning and expression pattern of porcine myeloid DAP12-associating lectin-1. Cell Immunol. 2001;209(1):42–8.

CLEC7A

Janet A. Willment[1] and Gordon D. Brown[2]
[1]Institute for Medical Sciences, Aberdeen, UK
[2]Section of Immunology and Infection, Division of Applied Medicine, Institute of Medical Sciences, Aberdeen Fungal Group, Aberdeen, UK

Synonyms

Dectin-1; β-glucan receptor

Historical Background

The existence of a macrophage β-glucan-binding receptor had been recognized for about three decades before the identification of Dectin-1 in 2001 as the receptor for these carbohydrates (Brown and Gordon 2001). Many of the immunomodulatory properties attributed to β-glucans were previously ascribed to the Complement Receptor (CR-3, Mac-1, CD11b/CD18), which was thought to directly recognize β-glucan, among many other ligands. Although additional receptors recognizing β-glucan have been identified, such as lactosylceramide, Langerin, and the scavenger receptors CD5, CL-P1, SCARF1, and CD36, the generation of Dectin-1 knockout (KO) mice has demonstrated that β-glucan recognition by this receptor is central to the immune response to many fungal pathogens. From data generated over the past decade, it is now known that Dectin-1 has a pivotal role in the innate immune protective response to fungi, and that this receptor can induce the adaptive immune system driving the development of T helper (Th-1) and Th-17 responses. Dectin-1 has also been implicated in the recognition of *Mycobacterium tuberculosis* (Mtb), and recognizes an unidentified endogenous ligand. Indeed, Dectin-1 was originally identified in 2000 by Ariizumi et al. as a dendritic cell (DC)-specific molecule which was able to recognize and co-stimulate T cells, via this endogenous ligand. (See Brown 2006; Goodridge et al. 2009; Marakalala et al. 2011b for reviews.)

Structure: Dectin-1 is a type II receptor which contains an extracellular carbohydrate recognition

domain (CRD), a stalk region, a transmembrane domain, and an intracellular tail containing a nonclassical immunoreceptor tyrosine activatory motif (ITAM), known as either an ITAM-like or a hemITAM. Based on CRD sequence similarity, Dectin-1 is part of the Group V C-type lectin-like family with high homology to the natural killer (NK) C-type lectin-like receptors (CLRs), and although it contains the conserved structural characteristics of these proteins, it lacks the residues normally thought to be responsible for carbohydrate binding. In fact, unlike these NK CLRs which have proteinaceous ligands, Dectin-1 binds soluble and insoluble β-(1,3)-linked glucans in a calcium-independent manner. Furthermore, Dectin-1 does not form homo- or heterodimers, and it lacks the cysteine residues within its stalk region; features often found in other NK CLRs (Brown and Gordon 2001; Ariizumi et al. 2000). Dectin-1 does, however, form higher molecular mass structures in the presence of its ligands, such as laminarin (Brown et al. 2007), and two Dectin-1 molecules are thought to be necessary for signaling via spleen tyrosine kinase (Syk) (discussed more in detail below).

Similar to the other closely related C-type lectin-like receptors in the NK gene complex, Dectin-1 is encoded by six exons. Two functional β-glucan-binding isoforms are expressed: one full-length version translated from all six exons (Dectin-1a) and one stalkless version (Dectin-1b) lacking exon 3. The human Dectin-1 has a further six alternatively spliced variants, some lacking intact exons and others with small insertions resulting in premature stop codons. Within the CRD is a shallow surface ligand-binding groove thought to be the ligand-binding site, and Trp221 and His223, which flank this groove, have been shown to be essential for binding to β-glucans. Although the endogenous ligand is still unidentified, binding of Dectin-1 to T cells was not inhibitable by β-glucans suggesting a second ligand-binding site (See Brown 2006, Marakalala et al. 2011b; and Brown 2010 for reviews).

Function and Ligands

The binding efficiency of Dectin-1 depends on both the backbone chain length and degree of branching of the β-glucan polysaccharide. A variety of β-glucan and β-glucan–rich polymers are used in vitro as Dectin-1 ligands, the most frequent being the antagonists, laminarin and glucan-phosphate, the agonist curdlan, and zymosan, a complex *Saccharomyces cerevisiae* – derived β-glucan-rich particle that also contains multiple ligands for receptors other than Dectin-1. The purity, degree of polymerization, branching, structure, and solubility can vary extensively between these ligands and all of these factors influence the cellular responses triggered through Dectin-1. β-glucans are present in the cell wall of most fungal species, but are often only exposed during specific morphological states. Through recognition of these carbohydrates, Dectin-1 binds to a variety of fungal species including *Candida, Aspergillus*, *Coccidioides*, *Pneumocystis*, *Saccharomyces*, *Trichophyton*, *Microsporum*, and *Penicillium*. In addition to fungi, β-glucans are found in plants, and in some bacteria (but not *Mycobacteria*). As β-glucans are not produced by mammals, they act as pathogen-associated molecular patterns (PAMPs) (Brown and Gordon 2001; Adams et al. 2008; Rosas et al. 2008; Brown 2010; Kerrigan and Brown 2010).

Binding of these carbohydrates to Dectin-1 induces the production of various cytokines and chemokines, such as interleukin (IL)-1beta, IL-1alpha, IL-2, IL-6, IL-10, IL-23, macrophage inflammatory protein (MIP)-1alpha and MIP-2, tumor necrosis factor (► TNFalpha), and the production of arachidonate metabolites. Dectin-1 can also induce phagocytosis and the respiratory burst, in both neutrophils and macrophages. Stimulation of Dectin-1 is able to drive maturation of DCs and promote CD4+ and CD8+ T-cell responses to exogenous antigens, leading to the development of Th1 and Th17 adaptive immunity (see Willment and Brown 2010; Kerrigan and Brown 2010 for reviews).

Engagement of Dectin-1 with its unidentified, but non-carbohydrate, endogenous ligand induces the expression of activation markers on T cells, production of ► IFN-γ, and cellular proliferation. Evidence suggests that Dectin-1 may also have a role in phagocytosis of apoptotic cells and cross presentation of cellular antigens. A further endogenous ligand, the intracellular Ran-binding protein RanBPM, has been identified for the cytosolic human Dectin-1E isoform, which lacks both the transmembrane and stalk regions. RanBPM interacts with the GTPase Ran and may act as a scaffolding protein to coordinate signaling from cell surface receptors (Willment and Brown 2010).

Signaling

Upon engagement of Dectin-1, the cytoplasmic ITAM-like motif becomes phosphorylated on the membrane-proximal tyrosine (Tyr 15) by Src family kinases, leading to recruitment of Syk. The exact mechanism of Syk binding is unclear, as binding normally requires two phosphorylated tyrosine residues in traditional ITAMs, although it has been suggested that Syk links two Dectin-1 molecules. Dectin-1 is able to signal through various pathways, the most well characterized to date involves Syk, caspase recruitment domain (CARD)-9, Bcl-10, and ▶ Malt1,which results in the activation of the transcription factor NFkB canonical c-Rel and p65 subunits and the non-canonical RelB subunit. The more recently identified pathway involves the kinase ▶ Raf1 which integrates with the Syk pathway by sequestering the RelB and p65 into inactive dimers and ultimately biases the cytokine production toward a Th1 and Th17 profile. Both Syk and ▶ Raf1 pathways are required for directing Dectin-1-mediated adaptive responses. The following signaling molecules are also involved in Dectin-1-mediated signaling: the Src family kinases (Lck and ▶ Src), ▶ phosphoinositide 3-kinase, mitogen-activated protein kinases (ERK and p38), and protein kinases (Akt and Jnk). The SLP76-BLNK adapter proteins are partially required, and the Tec kinase Btk is differentially used depending on the cell type examined. Dectin-1 signaling also leads to the activation of the transcription factor ▶ NFAT through PLC-γ2, acting downstream of Syk, resulting in the induction of transcription factor early growth response (Egr)2 and Egr3. ▶ NFAT activation by Dectin-1 induces cyclooxygenase (cox)-2 and regulates the production of IL-2, IL-10, and IL-12 (See Goodridge et al. 2009; Kerrigan and Brown 2010; Willment and Brown 2010; Mocsai et al. 2010; den Dunnen et al. 2010; and Kerrigan and Brown 2011 for recent reviews).

Pathogens present a diverse set of ligands or PAMPS to the immune system and therefore it is not surprising that a number of pattern recognition receptors (PPRs) are engaged simultaneously. Depending on the combination of receptors activated, the resultant response can vary significantly from a synergistic enhancement to repression of downstream responses. Triggering via Dectin-1 and MyD88-coupled Toll-like receptors (TLRs), for example, gives rise to a synergistic upregulation of MIP-2, MIP-1α, IL-10, IL-23, IL-6, and ▶ TNFalpha, but simultaneously downregulates the production of IL-12. Both Syk and ▶ Raf1 are required for the collaboration between Dectin-1 and the TLRs, and cross talk between the pathways functions to regulate the balance of Th17 and Th1 responses (Gringhuis et al. 2009).

In addition to collaborating with the TLRs, interactions with other non-TLR PPRs, have been shown to modulate responses to pathogens. For example, triggering the mannose receptor or DC-SIGN, modulates the DC responses to Mtb, and SIGN-R1 and Dectin-1 are both required for the production of reactive oxygen species (ROS) in response to *Candida* in macrophages (Zenaro et al. 2009; Takahara et al. 2011). It was also recently demonstrated that expression of IL-1beta in response to β-glucans in human macrophages occurs via Dectin-1/Syk and required the activation of the intracellular NRLP-3 inflammasome, the production of ROS, and a potassium efflux (Gross et al. 2009; Hise et al. 2009; Kankkunen et al. 2010).

It should be noted that the downstream responses and signaling pathways utilized by Dectin-1 are controlled by the environment within which the receptor is expressed, both in terms of the type of cell and its state of activation, and the nature of the ligand being recognized by the receptor. For example, Dectin-1 does not induce cytokine production in some macrophage populations, but it can induce cytokine production in DCs. This has been linked to differential utilization of CARD9 and effects of various cytokines, such as granulocyte macrophage-colony-stimulating factor (GM-CSF), used to generate the DCs. In the generation of Fms-related tyrosine kinase 3 ligand (Flt3L), matured DCs or bone marrow derived macrophages, both cell types, despite expressing Dectin-1, display Dectin-1–nonresponsive phenotypes. Conversely, use of GM-CSF or IFNgamma in vitro induces a Dectin-1 responsive cell type. Levels of Dectin-1 surface expression are also dependent on posttranslational modifications such as glycosylation, the formation of complexes with other PPRs, such as the TLRs and integral membrane proteins such as CD37 and CD63, and its ability to translocate to lipid rafts where it can co-localize with Syk and PLC-gamma2. The co-localization of Dectin-1 with TLR-2 requires osteopontin, an intracellular protein, which also acts as an adapter molecule facilitating interactions with Syk, increasing Dectin-1-mediated responses, such as cytokine production and facilitating the respiratory burst (Marakalala et al. 2011b; Willment and Brown 2010; Inoue et al. 2011).

Expression and Regulation

Murine Dectin-1 is present in immune cell-rich tissues such as the spleen, lung, thymus, kidney, and liver as well as other organs such as the stomach and small intestine. It is a predominantly myeloid expressed molecule with macrophages, neutrophils, $CD11c^{low}$ and $CR\text{-}3^{+}$ splenocyte, and macrophage subpopulation of splenic red and white pulp all expressing high levels of Dectin-1. Peripheral blood monocytes, inflammatory and alveolar macrophages, Kupffer cells, microglia, lamina propria macrophages, and inflammatory-recruited neutrophils also express this receptor. Dectin-1 positive DC subsets in the peripheral blood, lymph nodes, spleen, dermis, medullary and corticomedullary regions of the thymus, and lamina propria have been identified. Low levels of Dectin-1 expression have been observed on resident peritoneal macrophages, while eosinophils, B cells, and DCs and macrophages of the kidney, heart, eye, and brain are all Dectin-1 negative. The levels of murine Dectin-1 expression can be upregulated by the presence of cytokines that bias the immune response toward a Th-2 profile, such as IL-4 and IL-13, and which gives rise to alternatively activated macrophages. While exogenous GM-CSF will increase Dectin-1 levels, the addition of IL-10, lipopolysaccharide (LPS), dexamethasone, and β-glucans will downregulate surface expression. On T-cell populations, Dectin-1 has been detected on a subset of splenic T cells and on an IL-17–producing subset of γδ T-cell receptor and chemokine receptor 6–positive T cells (See Brown 2006; Willment and Brown 2010; Reid et al. 2009 for reviews).

Human Dectin-1 is expressed on monocytes, immature and mature human monocyte-derived DCs, B cells, eosinophils, neutrophils, $CD1a^{+}$ Langerhans cells, and peripheral blood DCs ($CD1c^{+}CD19^{-}$), but not on plasmacytoid DCs (although there is mRNA present in these cells) or NK cells. However, some unidentified $CD4^{+}$ T-cell subsets do express this receptor. Cell-specific expression of human Dectin-1 mRNA has been reported in monocytes, macrophages, neutrophils, mast cells, eosinophils, and monocytic, B- and T-cell lines. Regulation of the transcription has been observed in vitro with the addition of various cytokines, TLR ligands, and pathogens influencing levels (Willment and Brown 2010).

Dectin-1 is a type II transmembrane receptor, however, when overexpressed in cell lines Dectin-1 localizes to both the perinuclear compartments and the cell surface. There is relatively less Dectin-1b expressed on the cell surface compared with Dectin-1a, possibly due to the lack of glycosylation signals within the former. In human neutrophils, Dectin-1 is located on the plasma membrane (γ-fraction) and within the azurophilic granules (α-fraction) inside the cytoplasm, and in murine macrophages the amounts of intracellular Dectin-1 versus the surface expressed Dectin-1 is affected by the levels of the tetraspanin CD37. The intracellular route of Dectin-1 after binding to soluble ligands, such as laminarin, allows it to be recycled to the surface, whereas larger ligands retain Dectin-1 within the phagosomal compartment leading to de novo–synthesized Dectin-1 expressed on the cell surface. After uptake of ligand, Dectin-1 co-localizes with the lysosomal membrane glycoprotein-1 (LAMP-1) and the tetraspanin CD63. Depending on the nature of the ligand, Dectin-1 can be observed in phagolysosomal compartments, during zymosan uptake, or only in early phagosomes during *Candida* uptake (Brown 2006; Willment and Brown 2010).

Role in Immunity and Homeostatis

The role of Dectin-1 has been extensively examined using murine models but there are now human studies, discussed more in detail below, which highlight the importance of Dectin-1 in antifungal immunity. The Dectin-1 KO mice are phenotypically normal in the absence of infection, however, they show increased susceptibility to infections with *Candida* (with some mouse and *Candida* strain variation), *Aspergillus*, and *Pneumocystis* (Drummond et al. 2011). Macrophages derived from KO animals were still able to recognize *Pneumocystis* and *Candida albicans* (strain 18804, but not strain SC5314), despite the demonstrated in vitro role of Dectin-1 in the recognition of zymosan and live fungi. *Pneumocystis* and *Candida albicans* 18804 elicited similar cytokine profiles in KO and wild-type (WT) macrophages; however, *Candida albicans* SC5314 recognition was impaired by the absence of Dectin-1 and the observed cytokine profile was altered. Both *Pneumocystis* and *Aspergillus* do not induce a ROS response in the KO mice, highlighting Dectin-1's role in killing of fungi (Drummond et al. 2011). In addition to these earlier KO studies, more recent reports have reinforced Dectin-1's role in cell-specific antifungal immunity. Mice with a macrophage-specific

Dectin-1 KO, examined in the context of gastrointestinal models of candidiasis, show a more severe phenotype, and mice with a neutrophil-specific calcineurin deficiency are more susceptible to disseminated candidiasis. Dectin-1, and not the TLRs, was shown to be the activator of calcineurin in neutrophil responses to *Candida* (Greenblatt et al. 2010; Gales et al. 2010).

Classically the induction of a Th-1 immune response was thought to be required for antifungal immunity, but the role of Th-17 and T-regulatory cells has recently become prominent with mice lacking a Th-17 response being more susceptible to *Candida* infection (see Vautier et al. 2010 for review). The induction of the adaptive response, in particular the Th-17 response, is completely dependent on the presence of CARD9, but was not altered in the Dectin-1 KO mice during *Candida* infection (Kerrigan and Brown 2011). This induction was recently demonstrated to be mediated by Dectin-2, signaling via Fc-gamma receptor, Syk, and CARD9, which interacts with the alpha-mannans present in *Candida* (Saijo et al. 2010). In contrast to *Candida* infections, murine Dectin-1 is essential during *Aspergillus* infections for both the innate immune response and the induction of a Th-17 response, modulating the balance between Th-1 and Th-17 responses by inhibiting Th-1 CD4 T-cell differentiation (Werner et al. 2009; Rivera et al. 2011).

There are a few examples where a role for Dectin-1 has been demonstrated in vitro but analysis of the KO response to infectious challenge has not shown any significant role for Dectin-1 in vivo. In vitro experiments had demonstrated roles for Dectin-1 in binding and developing a Th1/Th17 profile for Mtb, whereas the in vivo experiments using the Dectin-1 KO mouse provided evidence that alternative receptors are able to compensate for the lack of Dectin-1 (Kerrigan and Brown 2011; Zenaro et al. 2009; Marakalala et al. 2011a). In the case of *Cryptococcus*, spores were demonstrated to bind Dectin-1 in vitro, but the KO mice are not more susceptible than WT to infection (Giles et al. 2009; Nakamura et al. 2007). Although not yet examined using Dectin-1 KO mice, susceptibility to *Coccidioides* infection was found to be dependent on the strain of mice, with C57BL/6 mice more susceptible than DBA/2. The differences in susceptibility may be due to the relative levels of Dectin-1a and Dectin-1b mRNA transcripts, with some mouse strains either expressing both transcripts equally or predominantly the Dectin-1b transcript. In vitro, these two isoforms have been shown to have slightly different activities, for example, cells expressing Dectin-1b produce more ▶ TNFalpha than Dectin-1a under the same conditions. There are also three murine Dectin-1 single nucleotide polymorphisms (SNPs) (R37Q, S73P, and V165A) differing between C57BL/6 and BALB/c mice that have been identified, but these SNPs have not been linked to any functional effects (Willment and Brown 2010).

A role for Dectin-1 in human antifungal immunity was recently demonstrated with the characterization of two different SNPs, which affect the levels of human Dectin-1 surface expression, and their ability to induce cytokine responses to *Candida*. These data demonstrate that patients with a homozygous Dectin-1 Y238X SNP, resulting in a prematurely truncated protein, are more susceptible to mucocutaneous fungal infections, but not systemic infections. Cells from these patients display defective cytokine production, in particular IL-17, ▶ TNFalpha and IL-6, but are still able to bind and phagocytose live *Candida*. This same SNP also has been implicated in increased susceptibility to both invasive *Aspergillosis* and *Candida* colonization during transplantation, and has clinical relevance if either the donor and/or transplant recipient of hematopoietic stem cells carries the Y238X SNP. The second characterized SNP I223S, which renders cells transduced with I223S Dectin-1 unable to bind fungal particles due to lack of surface expression, may play a role in the immunity to fungi (Marakalala et al. 2011b; Cunha et al. 2010; Plantinga et al. 2010).

The Y238X SNP patient studies have demonstrated a role for human Dectin-1 in mucosal immunity and not in systemic *Candida* fungal infections, due to an impaired Th-17 response, which is now thought to be pivotal during mucosal infections. This is contrary to the role in the mouse where the induction of IL-17 in response to *Candida* is not Dectin-1 dependent (see above). A further difference in host response is observed during *Aspergillus* infections; human Dectin-1 induces a strong Th-1 and a weak Th-17 response, while murine Dectin-1 inhibits the Th-1 response and induces a protective Th-17 response. These differences in immune responses may be due to site-specific immune reactions, such as mucosal versus systemic, and species diversity in the role of Th17 cells. The disparity between the human and

murine immune responses to fungal infections needs further investigation, before significant progress can be made using β-glucan adjuvants targeting Dectin-1 for clinical applications (Vautier et al. 2010; Rivera et al. 2011; Kerrigan and Brown 2011; Chai et al. 2010).

Using a mouse model of zymosan-induced arthritis, a role for Dectin-1 in autoimmune disease has been suggested. The clinical relevance of Dectin-1 in human autoimmune disease is less clear as in studies of chronic inflammation, the Y238X polymorphism did not appear to be a susceptibility factor in irritable bowel diseases or rheumatoid arthritis (RA). This is despite an observed influx of Dectin-1 expressing macrophages in bowel sections obtained from both Crohn's disease and diverticulitis patients, and synovial tissue sections from RA patients, but not those from osteoarthritis patients. It has been suggested, although not proven, that human Dectin-1 may play a role in psoriasis, firstly because Dectin-1 mRNA and those of a number of other PPRs, is upregulated in skin epidermis biopsies obtained from psoriasis patients relative to healthy controls, and secondly, the larger number of infiltrating Langerhans cells in the psoriasis samples are Dectin-1 positive (Willment and Brown 2010; Marakalala et al. 2011b).

Summary

Dectin-1 has a pivotal role in the immune response to fungal pathogens, as evidenced both from human and mouse studies. It is a predominantly myeloid restricted receptor, and its expression on these cell types is crucial for the recognition and killing of fungal pathogens and induction of the subsequent antifungal immune responses. Depending on the cell type and the nature of the ligand, it triggers intracellular signaling via its ITAM-like motif through various pathways, including Syk/CARD9 and Raf1, resulting in the activation of nuclear transcription factors. Collaborating together with multiple other PPRs, a variety of other cellular responses, such as phagocytosis, the respiratory burst, and arachidonate metabolism are initiated and the innate and adaptive immune responses are modulated through the regulation of cytokine and chemokine production. Although Dectin-1 recognizes β-glucans, it also has an uncharacterized ligand on *Mycobacteria* and an endogenous, non-carbohydrate ligand, on T cells and apoptotic cells. In addition to a clearly demonstrated role in antifungal immunity, Dectin-1 has been implicated in autoimmunity and may play a role in other inflammatory diseases. The study of Dectin-1, and its collaboration with other receptors, has given significant new insights into the underlying mechanisms involved in the development of the innate and adaptive immunity to fungi.

References

Adams EL, Rice PJ, Graves B, Ensley HE, Yu H, Brown GD, et al. Differential high affinity interaction of Dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side chain branching. J Pharmacol Exp Ther. 2008;325(1):115–23.

Ariizumi K, Shen GL, Shikano S, Xu S, Ritter 3rd R, Kumamoto T, et al. Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. J Biol Chem. 2000;275:20157–67.

Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol. 2006;6(1):33–43.

Brown GD. Innate antifungal immunity: the key role of phagocytes. Annu Rev Immunol. 2010;29:1–21.

Brown GD, Gordon S. Immune recognition: A new receptor for beta-glucans. Nature. 2001;413(6851):36–7.

Brown J, O'Callaghan CA, Marshall AS, Gilbert RJ, Siebold C, Gordon S, et al. Structure of the fungal beta-glucan-binding immune receptor dectin-1: implications for function. Protein Sci. 2007;16(6):1042–52.

Chai LY, van de Veerdonk F, Marijnissen RJ, Cheng SC, Khoo AL, Hectors M, et al. Anti-Aspergillus human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity. Immunology. 2010;130(1):46–54.

Cunha C, Di Ianni M, Bozza S, Giovannini G, Zagarella S, Zelante T, et al. Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. Blood. 2010;116(24):5394–402.

den Dunnen J, Gringhuis SI, Geijtenbeek TB. Dusting the sugar fingerprint: C-type lectin signaling in adaptive immunity. Immunol Lett. 2010;128(1):12–6.

Drummond RA, Saijo S, Iwakura Y, Brown GD. The role of Syk/CARD9 coupled C-type lectins in antifungal immunity. Eur J Immunol. 2011;41(2):276–81.

Gales A, Conduche A, Bernad J, Lefevre L, Olagnier D, Beraud M, et al. PPARgamma controls dectin-1 expression required for host antifungal defense against *Candida albicans*. PLoS Pathog. 2010;6(1):e1000714.

Giles SS, Dagenais TR, Botts MR, Keller NP, Hull CM. Elucidating the pathogenesis of spores from the human fungal pathogen *Cryptococcus neoformans*. Infect Immun. 2009;77(8):3491–500.

Goodridge HS, Wolf AJ, Underhill DM. Beta-glucan recognition by the innate immune system. Immunol Rev. 2009;230(1):38–50.

Greenblatt MB, Aliprantis A, Hu B, Glimcher LH. Calcineurin regulates innate antifungal immunity in neutrophils. J Exp Med. 2010;207(5):923–31.
Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Wevers B, Bruijns SC, et al. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. Nat Immunol. 2009;10(2):203–13.
Gross O, Poeck H, Bscheider M, Dostert C, Hannesschlager N, Endres S, et al. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature. 2009;459(7245):433–6.
Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, et al. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe. 2009;5(5):487–97.
Inoue M, Moriwaki Y, Arikawa T, Chen YH, Oh YJ, Oliver T, et al. Cutting edge: critical role of intracellular osteopontin in antifungal innate immune responses. J Immunol. 2011;186(1):19–23.
Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, Matikainen S. (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. J Immunol. 2010;184(11):6335–42.
Kerrigan AM, Brown GD. Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. Immunol Rev. 2010;234(1):335–52.
Kerrigan AM, Brown GD. Syk-coupled C-type lectins in immunity. Trends Immunol. 2011;32(4):151–6.
Marakalala MJ, Guler R, Matika L, Murray G, Jacobs M, Brombacher F, et al. The Syk/CARD9-coupled receptor dectin-1 is not required for host resistance to *Mycobacterium tuberculosis* in mice. Microbes Infect. 2011a;13(2):198–201.
Marakalala MJ, Kerrigan AM, Brown GD. Dectin-1: a role in antifungal defense and consequences of genetic polymorphisms in humans. Mamm Genome. 2011b;22(1–2):55–65.
Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol. 2010;10(6):387–402.
Nakamura K, Kinjo T, Saijo S, Miyazato A, Adachi Y, Ohno N, et al. Dectin-1 is not required for the host defense to *Cryptococcus neoformans*. Microbiol Immunol. 2007;51(11):1115–9.
Plantinga TS, Hamza OJ, Willment JA, Ferwerda B, van de Geer NM, Verweij PE, et al. Genetic variation of innate immune genes in HIV-infected African patients with or without oropharyngeal candidiasis. J Acquir Immune Defic Syndr. 2010;55(1):87–94.
Reid DM, Gow NA, Brown GD. Pattern recognition: recent insights from dectin-1. Curr Opin Immunol. 2009;21(1):30–7.
Rivera A, Hohl TM, Collins N, Leiner I, Gallegos A, Saijo S, et al. Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. J Exp Med. 2011;208(2):369–81.
Rosas M, Liddiard K, Kimberg M, Faro-Trindade I, McDonald JU, Williams DL, et al. The induction of inflammation by dectin-1 in vivo is dependent on myeloid cell programming and the progression of phagocytosis. J Immunol. 2008;181(5):3549–57.
Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. Immunity. 2010;32(5):681–91.
Takahara K, Tokieda S, Nagaoka K, Takeda T, Kimura Y, Inaba K. C-type lectin SIGNR1 enhances cellular oxidative burst response against *C. albicans* in cooperation with dectin-1. Eur J Immunol. 2011. doi:10.1002/eji.200940188.
Vautier S, Sousa Mda G, Brown GD. C-type lectins, fungi and Th17 responses. Cytokine Growth Factor Rev. 2010;21(6):405–12.
Werner JL, Metz AE, Horn D, Schoeb TR, Hewitt MM, Schwiebert LM, et al. Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against *Aspergillus fumigatus*. J Immunol. 2009;182(8):4938–46.
Willment JA, Brown GD. Dectin-1. UCSD signaling gateway molecule pages. 2010. http://www.signaling-gateway.org/. doi:10.1038/mp.a004078.01
Zenaro E, Donini M, Dusi S. Induction of Th1/Th17 immune response by *Mycobacterium tuberculosis*: role of dectin-1, mannose receptor, and DC-SIGN. J Leukoc Biol. 2009;86(6):1393–401.

CLECSF5

► CLEC5A

CLECSF9

► CLEC4E

CLK

Leonard Rabinow
Université Paris Sud, CNRS UMR 8195, Orsay, France

Synonyms

CLK kinases; LAMMER kinases; STY (CLK1)

Historical Background

CLK1 (cdc2-like) kinase was initially described in mammalian systems (mouse and human cells), and

was so-named because of amino-acid sequences similar to cdc2 kinase (now cdk1) in specific kinase catalytic subdomains (Stodjl and Bell 1999 and references therein). CLK1 was also referred to as STY, since it was the first kinase which autophosphorylated with dual-specificity, i.e., on Ser/Thr and Tyr (S/T and Y in the single-letter amino-acid code). Subsequent studies identified four CLK kinases in vertebrate genomes, CLK1-4. The cloning and characterization of the *Drosophila* orthologue, DOA, enabled the recognition of family members in many additional species, including *Arabidopsis thaliana* (AFC1-3), *Nicotiana tabacum* (PK12), *Schizosaccharomyces pombe* (Lkh1), and *Saccharomyces cerevisiae* (KNS1) (Yun et al. 1994; Savaldi-Goldstein et al. 2003; Kang et al. 2010 and references therein). These were collectively referred to as "LAMMER" protein kinases, due to a conserved amino-acid motif including this sequence in kinase catalytic domain X, specific to this sub-family. CLK/LAMMER kinases are found throughout eukaryotes and are considered to be among the 347 eukaryotic "signature" proteins (Hartman and Federov 2002). The amino-acid identities leading to the CLK nomenclature ("cdc2-like" kinase) have since been identified in numerous other related kinases, including the cdk and MAP kinases, placing them in the CMGC kinase family. No cyclin-dependence has been described for CLK/LAMMER kinases, and the name CLK has unfortunately been the cause of confusion, since the circadian rhythm genes of the nematode *C. elegant* are CLOCK genes, (clk), and and their human orthologues were named HCLK.

Structure of the LAMMER/CLK Kinases

The catalytic domains of LAMMER/CLK kinases define the C-terminal domain of the protein and comprise roughly two-third of the overall structure in most cases (Yun et al. 1994). The catalytic domains of these kinases are remarkably similar to each other in overall structure and amino-acid sequence, with near 100% amino-acid identity in key residues and motifs throughout catalytic subdomains. Examples are found in kinase catalytic subdomains VIb and VIII where the motifs HTDLKPENIL and STRHYRAPEVIL are respectively either 100% identical or are nearly so in all family members. Both motifs are intimately involved in catalysis or substrate interaction. In particular, the former motif is exclusive to the LAMMER kinases, although the Thr residue, more frequently Arg or Cys in other kinases, is also found in the SRPK kinases. The SRPKs phosphorylate many of the same substrates as the LAMMER kinases, i.e., the SR proteins, albeit not on the same sites. Although the LAMMER motif (EHLAMMERIL) is itself conserved and easily recognizable in all family members, some amino-acid substitutions occurred during evolution, as for example, in *S. cerevisiae* KNS1p (EHMAMMQRIL) and mammalian CLK3 (EHLVMMEKIL, Table 1). Indeed, in *S. cerevisiae* KNS1p an insert of 20 residues relative to other LAMMER kinases occured at the C-terminus of the motif between the terminal I and L residues. The LAMMER motif was hypothesized to lie on the surface of the lower lobe of the kinase domain based upon alignments with other CMGC kinases and thought to perhaps play a role in protein–protein interactions with interacting molecules. However, this idea was not upheld by in vitro studies introducing amino-acid substitutions in the tobacco orthologue PK12 (Savaldi-Goldstein et al. 2003), nor was it validated by recent crystallographic studies performed on the catalytic domains of mammalian CLK1 and CLK3 (Bullock et al. 2009). These latter studies revealed the existence of insertions relative to other kinases placing the LAMMER motif in the interior of the molecule.

Several studies have demonstrated that LAMMER kinases, unlike their cousins in the SRPK family of kinases, do not possess specific docking sites for their substrates, perhaps explaining their broader substrate specificity. Interestingly, the crystal structures for the CLK1 and CLK3 catalytic domains show that they are constitutively active in their non-autophosphorylated state (Bullock et al. 2009). This data is supported by unpublished results showing that autophosphorylation of the DOA catalytic domain represses kinase activity (C. Du and L. Rabinow).

The non-catalytic N-termini of LAMMER kinases are highly diverged from each other and thus were not subjected to stringent constraints during evolution. However, the N-termini are all positively charged, possessing high pI values if considered independently of their catalytic domains (Rabinow unpublished). Most also contain short motifs of Ser-Arg residues in unique or double (but not triple) repeats, somewhat reminiscent of one class of their substrates, the SR proteins. It would nevertheless be erroneous to

CLK, Table 1 Alignment of arbitrarily selected LAMMER kinase catalytic subdomains VII–X, with the "EHLAMMERILG" motif underlined. This motif is limited to this kinase sub-family. DOA: Drosophila (Yun et al. 1994; Kpebe and Rabinow 2008); CLK1, 2, 3: human (Nayler et al. 1997); Afc1, 2: Arabidopsis (Bender and Fink, 1994); PK12: tobacco (Sessa et al. 1996); KNS1: *S. cerevisiae* (Padmanabha et al. 1991). Amino-acid numbering based upon residues in the 55 kD isoform of DOA kinase. Note the insertion in KNS1 that splits the LAMMER motif

481						540	
DOA	VKNTDVRLID	FGSATFDHEH	HSTIVSTRHY	RAPEVILELG		WSQPCDVWSI	GCILFELYLG
CLK1	LINPDIKVVD	FGSATYDDEH	HSTLVSTRHY	RAPEVILALG		WSQPCDVWSI	GCILIEYYLG
CLK2	VKSTAARVVD	FGSATFDHEH	HSTIVSTRHY	RAPEVILELG		WSQPCDVWSI	GCIIFEYYVG
CLK3	VKNTSIRVAD	FGSATFDHEH	HTTIVATRHY	RPPEVILELG		WAQPCDVWSI	GCILFEYYRG
AFC1	PKSSAIKLID	FGSTTFEHQD	HNYIVSTRHY	RAPEVILGVG		WNYPCDLWSI	GCILVELCSG
AFC2	PKSSAIKVID	FGSTTYERQD	QTYIVSTRHY	RAPEVILGLG		WSYPCDVWSV	GCIIVELCTG
PK12	PKSSAIKVID	FGSTAYERPD	HNYIVSTRHY	RAPEVILGLG		WSYPCDLWSV	GCILIELCSG
KNS1	LKNPEIKIID	FGSAIFHYEY	HPPVISTRHY	RAPEIVLGLG		WSFPCDIWSI	ACVLVELVIG
	541						600
DOA	ITLFQTHDNR	EHLAMMERI.	.....	L		GQIPYRMARK	T.KT..K.....
CLK1	FTVFPTHDSK	EHLAMMERI.	.....	L		GPLPKHMIQK	TRKR..K.....
CLK2	FTLFQTHDNR	EHLAMMERI.	.....	L		GPIPSRMIRK	TRKQ..K.....
CLK3	FTLFQTHENR	EHLVMMEKI.	.....	L		GPIPSHMIHR	TRKQ..K.....
AFC1	EALFQTHENL	EHLAMMERV.	.....	L		GPLPPHMVLR	ADRRSEK.....
AFC2	EALFQTHENL	EHLAMMERV.	.....	L		GPFPQQMLKK	VDRHSEK.....
PK12	EALFQTHENL	EHLAMMERV.	.....	L		GPLPSQMLKR	VDRHAEK.....
KNS1	ESLYPIHENL	EHMAMMQRIN	GTPFPTDIID	KMFYKSKHKL		GNSPSDLNST	VIKHFDRKTL

CLK, Table 2 Optimal phosphorylation sites for 3 LAMMER protein kinases. Data from Nikolakaki et al. (2002) obtained via phosphorylation of peptide libraries. Data from Bullock et al. (2009) supports and extends these data with the observation that Pro at the P + 1 position is highly selected by CLK1, and less so for CLK2 and CLK3. " 0 " marks the phosphorylation site, which was fixed as Ser in this approach. The Arg residue fixed at −3 was determined through selection among peptide libraries for those with highest phosphorylation levels by the LAMMER kinases

	−7	−6	−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5
CLK2 (human)	R	R	R	R	R	E	H	S	R	R	D	L	L
DOA	R	R	R	E	R	E	H	S	R	R	D	L	G/D
PK12	H	R	E	E	R	R	R	S	R	R	D	D	D

designate these as "RS" domains such as those found in the SR and SR-like proteins, which contain multiple and extensive Arg-Ser repeats.

LAMMER Kinase Autophosphorylation, Consensus Phosphorylation Sites, and Substrates

All LAMMER kinases tested autophosphorylate on Ser/Thr and Tyr residues, but none has been demonstrated to possess tyrosine kinase activity toward an exogenous substrate. Autophosphorylation of CLK2 affects its intranuclear distribution in cultured cells. The autophosphorylation site is conserved among all mammalian CLKs (Nayler et al. 1998) but not among non-vertebrate LAMMER kinases, although the *A. thaliana* LAMMER kinases AFC1-3 all possess potential autophosphorylation sites at similar locations (Rabinow unpublished). Interestingly, autophosphorylation on different sites of CLK1 differentially alters its substrate specificity (Prasad and Manley 2003).

A consensus phosphorylation sequence for three LAMMER kinases from humans (CLK2), *Drosophila* (DOA), and *N. tabacum* (PK12) revealed substantial substrate similarities among the three kinases (Nikolakaki et al. 2002, Table 2).

The consensus phosphorylation site was defined by in vitro phosphorylation of peptide libraries and contains an Arg residue fixed at −3 to the phosphorylation site. Curiously, both positively and negatively charged

CLK, Table 3 LAMMER protein kinase substrates other than SR proteins and their phosphorylation sites (where known)

Protein substrate	Kinase	Sequence	Reference
Myelin basic protein	DOA, CLK2, KNS1	GRDSRSGSPMAR	Lee et al. 1996
Myelin basic protein	CLK1	QGKGRGLSLSRF	Menegay et al. 2000
PKC-zetazide	CLK1	MPRKRQGSVRRR	Menegay et al. 2000
PTP1B	CLK1, 2	RNRYRDVSPFDH	Moeslein et al. 1999
P1 protamine	DOA, CLK2, PK12	YRCCRSQSRSRY	Nikolakaki et al. 2002
EF1γ	DOA, CLK2, PK12	DDFKRVYSNEDE	Fan et al. 2010
CLK2	CLK2	HRRKRTRSVEDD	Nayler et al. 1998
c-Abl	CLK1, 4	DTEWRSVTLPRD	Nihira et al. 2008
CK1δ	CLK2	MERERKVSMRLH	Giamas et al. 2007
CLASP	CLK4		Katsu et al. 2002
SAF-B	CLK2		Nayler et al. 1998
PRP4	CLK1		Kojima et al. 2001
Histone H1	CLK1		Colwill et al. 1996a
			Colwill et al. 1996b
Tup11, Tup12, Csx1	Lkh1		Kang et al. 2010
			Kang et al. 2007

residues on both sides of the phosphorylation site are favored. To date only Ser residues on exogenous substrates have been found to be phosphorylated with the exception of Thr735 on c-Abl tyrosine kinase, which is phosphorylated by CLK1 and CLK4, creating a potential 14-3-3 protein binding site. The consensus site as defined in the above study did not reveal a preference for a Pro residue at the +1 position, although the familial relationship of LAMMER kinases with "proline-directed" kinases such as cdk's and MAPKs, all in the CMGC group, suggested that Pro should be a favored amino-acid at this position. This hypothesis was borne out by the examination of protein as opposed to peptide substrates, both in vitro and in vivo, as well as by a more recent peptide-based approach using different technology to examine substrate specificity of CLK1 and CLK3 (Bullock et al. 2009). This later study otherwise supports the features of the LAMMER kinase consensus phosphorylation sites for CLK2, DOA, and PK12.

All LAMMER kinase family members tested phosphorylate SR and SR-like proteins in vitro and in vivo (Nayler et al. 1997; Stodjl and Bell 1999; Nikolakaki et al. 2002). The SR proteins regulate multiple steps in mRNA maturation, and their phosphorylation is tightly regulated. Thus LAMMER kinases play important regulatory roles in the alternative and constitutive splicing of pre-mRNAs through phosphorylation of their substrates. However, the presence of the LAMMER orthologue KNS1 in the yeast *S. cerevisiae* suggests that the ancestral function of these kinases must involve a function other than the regulation of splicing, since the genome of this yeast does not encode any SR or SR-like proteins, nor is there alternative splicing per se; and even constitutive splicing is dispensable for vegetative growth of this yeast.

Several publications comparing the substrate specificities of LAMMER and the related SRPK kinases, which also phosphorylate SR proteins, showed that the former possess wider substrate specificity and can conceivably phosphorylate different substrates in addition to the SR proteins (Stodjl and Bell 1999 and references therein). This is in part due to the lack of a substrate-docking site in the LAMMER kinases, as opposed to SRPK kinases (Velazquez-Dones et al. 2005). In vitro substrates used to assay LAMMER kinase activity have included myelin basic protein and histone H1, although the latter is not phosphorylated by all family members, specifically CLK2 and DOA. Aside from SR and SR-like proteins, LAMMER kinase substrates in mammalian cells include the chromatin and RNA-binding protein SAF-B, the protein tyrosine phosphatase PTP1B, prp4 kinase, c-Abl tyrosine kinase, and cyclophilin-RS (Table 3). CK-I was also phosphorylated by CLK2 in vitro. In *Drosophila* several substrates in addition to SR and SR-like proteins have been identified, among which is eEF1βγ (formerly known as EF1γ), a putative translational elongation factor, but which was recently found to participate in the nuclear 3′ end pre-mRNA cleavage complex.

In the yeast *S. pombe*, Lkh kinase phosphorylates RNA-binding protein Csx1 and the TUP11 and TUP12 transcriptional repressors. Global analysis of phosphorylation networks in *S. cerevisiae* has revealed a large number of potential substrates, including the kinases CDC15 and ATG1, although these have not been tested for biological effects.

In short, the substrate specificity of the LAMMER kinases demonstrates that SR and related proteins as well as other regulators of RNA maturation are targeted, although additional processes and substrates including signaling molecules are also clearly phosphorylated.

Genetic, Functional, and Expression Studies on LAMMER Kinases

Mammalian CLKs

CLK1–4 all phosphorylate SR proteins and alter splicing of reporter constructs when over-expressed in cultured cells (Nayler et al. 1997). Alternative splicing of adenovirus is also affected by CLK1. CLK1, 2, and 4 are widely, perhaps ubiquitously, expressed in mammalian tissues, while in contrast CLK3 expression is enriched in testes and spermatozoa (Menegay et al. 1999). It is released from sperm with the acrosome, resulting in the suggestion that it may play a role in fertilization. Mammalian CLK kinases are generally nuclearly localized, but cytoplasmic kinase has also been detected via immunocytochemistry and western blotting when fixation conditions were appropriate (see references Table 3), or on immunoblots when cell fractionations were performed (Prasad and Manley 2003). An antibody directed against the LAMMER motif reveals a large component of nuclear staining in fixed HeLa cells, but cytoplasmic staining as well (K. Lee and L. Rabinow unpublished). Moreover, cell fractionations of HeLa cells followed by immunoblot analysis using the same antibody also detect LAMMER kinases in the cytoplasmic, as well as nuclear fractions (ibid).

Consistent with a role in the phosphorylation of SR proteins, over-expression of CLK1 in cultured cells results in the dispersion of nuclear "speckles," which are thought to be storage sites of SR and other splicing-related proteins (Stodjl and Bell 1999 and references therein). Dispersion of the speckles necessitates transfection with an active form of CLK kinase, since transfections with a catalytically inactive construct of the kinase using a single amino-acid replacement have no effect on speckle number or distribution. All four mammalian CLKs are regulated at the level of splicing, where exclusion of exon 4 (in both mouse and human CLK1) results in use of an in-frame stop codon just upstream of the kinase catalytic domain, resulting in possible production of a truncated peptide. It has been suggested that the mRNA including the premature termination codon is subjected to nonsense-mediated decay (NMD) and thus accumulation of significant levels of truncated CLK peptides might not occur (Hillman et al. 2004). Regulation of CLK1 exon 4 inclusion occurs through autoregulation, but also at least in part through interaction of CLK4 with an SR-like protein, CLASP, the mammalian orthologue of *Drosophila suppressor of white-apricot, su(w^a)* (see reference Table 3). CLASP phosphorylation by CLK4 induces exon 4 exclusion in CLK1 transcripts, thus down-regulating CLK1 levels.

Inclusion or exclusion of exon 4 in CLK1 is regulated in response to stress and also during erythroleukemic cell differentiation. In the first case it was found that cage-restraint and water-immersion of mice reduced accumulation of CLK1 transcripts in the brain, while the balance of the two alternatively spliced transcripts was altered toward the exclusion of exon 4, and hence the production of the non-coding RNA (Murata et al. 2005). Transcripts encoding both the full-length and truncated proteins of all four CLK kinases were up-regulated during HMBA-induced differentiation of MEL erythroleukemia cells (Garcia-Sacristan et al. 2005). In undifferentiated cells, CLK1 transcripts encoding the full-length protein predominated, whereas at later stages of differentiation the transcript including exon 4 became the major form, presumably down-regulating kinase levels and suggesting a role for the kinase in the differentiative process. CLK1 kinase also affects alternative splicing of additional target transcripts which include the choice between pro- and anti-angiogenic forms of VEGF and the splicing of tissue-factor (TF) required for thrombus formation in anucleate human platelets. In addition to its role in the phosphorylation of SR proteins, CLK1 kinase was also reported to negatively regulate expression of Super-Oxide Dismutase (SOD1), since reduction of CLK1 via RNA interference increased SOD1 activity, protein and mRNA in both HeLa and MCF-7 cell lines.

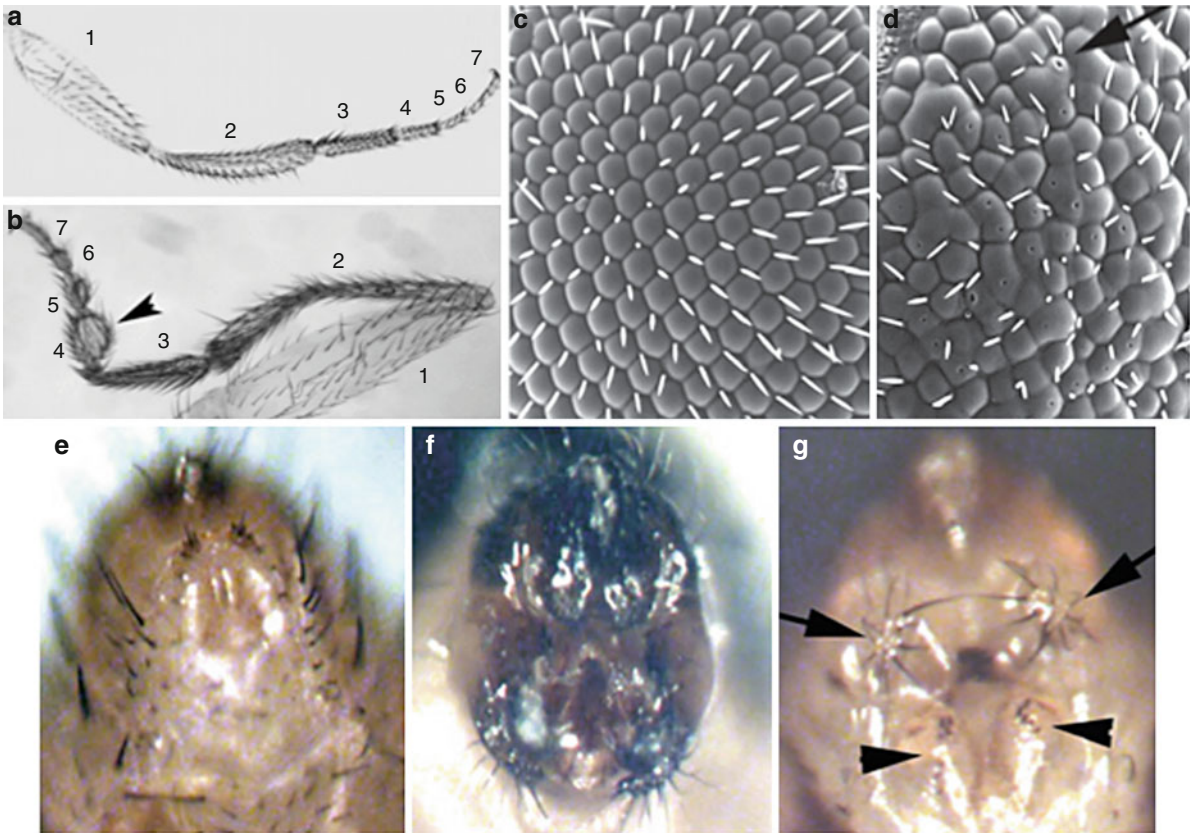
CLK2 was recently described as an insulin-regulated suppressor of gluconeogenesis in primary hepatocytes, repressing activity of the transcriptional co-activator PCG1a through direct phosphorylation of its RS-domain (Rodgers et al. 2010). Repression of PCG1a target genes was also observed in cell cultures when transfected with active CLK2, as well as in the livers of mice infected with an adenovirus-CLK2 transformation construct. Moreover, CLK2 protein levels were induced by feeding, under control of insulin and AKT signaling, while its mRNA levels were largely unaffected. Induction of CLK2 protein was due at least in part to stabilization of the protein and required CLK2 kinase activity and autophosphorylation. Furthermore, diabetic and obese *db*/*db* mice downregulated hepatic CLK2 levels, apparently due to lower protein levels. Reintroduction of active CLK2 restored normal glucose levels to near that of control animals. CLK1 kinase was also directly phosphorylated and activated by AKT kinase (Jiang et al. 2009, see below), leading to effects on alternative splicing of a transcript encoding a PKC isoform.

Interestingly, an earlier report demonstrated the interaction of CLK1, 2, and 4 with an ubiquitin-like molecule (UBL-5, or beacon), whose administration leads to obesity and weight-gain in *Psammomys obesus* (Israeli sand-rats). The beacon molecule was not phosphorylated by the CLKs and so the molecular basis or function of the interaction is not clear. However, these results taken together with those described above suggest that CLK2 is a crucial and newly characterized component of the insulin-signaling pathway. Moreover, these results reveal a specific role for the LAMMER kinases in the regulation of nuclear processes in addition to that of alternative splicing, and their regulation by a crucial evolutionarily conserved signaling pathway.

DOA Kinase of *Drosophila*

The single LAMMER kinase orthologue of *Drosophila* is encoded at the *Darkener of apricot* (*Doa*) locus, so-named because mutants partially suppress the $white^{apricot}$ allele. The locus is the best genetically characterized of all LAMMER kinase-encoding genes due to the facility of genetics in the fly. Mutations are almost invariably recessive lethal at early developmental stages. Homozygous mutant embryos hatch and die as early larvae; this late lethality is allowed by maternal contribution of both RNA and protein to the oocyte (Yun et al. 1994). In contrast, somatic mosaic experiments revealed that null alleles are cell lethal. Clonal analyses also showed that the kinase is essential for early oocyte development. Although homozygous animals are generally inviable, specific allelic combinations (heteroallelic animals) permit recovery of adults, which reveal extensive pleiotropic phenotypes, including roughened eyes, ectopic wing-veins, duplicated bristles (external touch organs of the fly), sterility, transformation of somatic female tissues into male structures, and aberrant development of distal segments of the legs (Fig. 1). Over-expression of specific kinase isoforms can also induce pleiotropic aberrations. Two alleles due to chromosomal rearrangements permit occasional survival of homozygotes which display striking degeneration of retinal photoreceptor cells in the rare surviving adults. Homozygous embryos obtained from heteroallelic mothers (to eliminate maternal contribution) show disrupted development of the central nervous system and cuticular structures. Finally, complete phenotypic suppression of $white^{apricot}$ in several classes of *Doa* heteroallelic mutants is accompanied by reduction in levels of transcripts prematurely truncating within the copia retrotransposon, whose insertion caused this allele. The amount of copia transcript levels in the fly is also doubled. Specific alleles of *Hw* and *Bx* also induced by insertion of copia elements are also phenotypically suppressed by *Doa* mutations. Interestingly, suppression is independent of the orientation of insertion of the element with respect to the affected gene, arguing against a strand-specific (i.e., RNA processing) effect.

DOA is the only LAMMER kinase for which a role in alternative splicing has been documented in whole animals. Female-to-male sex transformations of varying degrees are observed in different classes of *Doa* mutants (Fig. 1), due to aberrant splicing of *doublesex (dsx)* transcripts (Du et al. 1998; Rabinow and Samson 2010). *dsx* encodes a transcription factor whose transcripts are alternatively spliced under the control of several SR and SR-like proteins to yield male or female-specific isoforms. As for other LAMMER kinases, DOA phosphorylates SR and SR-like proteins in vitro. Moreover, the SR and SR-like proteins TRA2 and RBP1 are hypophosphorylated and delocalized from chromatin in *Doa* mutant flies. Finally, genetic epistasis experiments showed that *Doa's* effects on sex-determination were at or parallel to the level of phenotypes induced by mutation of the gene encoding the SR-like protein TRA, which assembles directly



CLK, Fig. 1 Pleiotropic phenotypes of *Doa* mutations Heteroallelism for various combinations of *Doa* alleles or over-expression of specific kinase isoforms yields pleiotropic phenotypes: (**a**) Wild-type leg structure; (**b**) Aberrant leg structure due to failure of the distal segments of the tarsi (tarsi 4–6, *arrow*) to elongate in $Doa^{DEM}/Doa^{l(3)01705}$; (**c**) Wild-type eye at high magnification: (**d**) An eye derived from an animal over-expressing the ubiquitously expressed 55 kD nuclear kinase, showing fused ommatidia and pinpoint holes in the lens covering the photoreceptor cells (*arrow*); (**e**) Normal female genitalia; (**f**) Normal male genitalia; (**g**) Somatic transformation of XX-females (female genitalia, *arrowheads*) to males (male genitalia, *arrows*)

onto the Exonic Splicing Enhancer complex responsible for activation of the female-specific *dsx* splice. It was therefore concluded that DOA directly phosphorylates SR and SR-like proteins responsible for splicing of the *dsx* transcript in vivo.

In contrast to the mammalian LAMMER kinases, no alternative splicing leading to insertion of an exon encoding a premature stop codon has been observed in *Doa* of *Drosophila*. However, the locus encodes six polypeptides of 55, 69, 91, 105, 138, and 227 kD generated by transcripts originating from alternative promoters. These proteins possess virtually identical catalytic domains, but aside from some partial overlaps, differing non-catalytic N-termini. These proteins perform at least three different functions as shown through genetic dissection using isoform-specific mutations, over-expressing alleles, and RNAi constructs. This idea is supported by the observation that the 55 kD protein is primarily nuclearly localized, while another of 105 kD is exclusively cytoplasmic, at least during pupal development. Additionally, the 105 kD protein is not predicted to alter splicing of the transcripts affecting the somatic sex-determination cascade, since over-expression, in vivo RNAi and isoform-specific mutations affecting this isoform do not induce sex transformations. Finally, the 227 kD isoform has recently been implicated in the control of cell trafficking (V. Gelfand, personal communication).

Doa has also been identified in mutageneses, various genomic screens, and systematic RNAi in S2 cells (Table 4). For example, analysis of genes differentially expressed during autophagy and death of the salivary

CLK, Table 4 Identification of DOA kinase in various mutagenic and other screens

Screen/effect	Cells/whole animals	Reference
Suppression of mutagenic effects of copia transposable-element insertions	Whole animal	Rabinow and Birchler 1989
Copia transcript levels elevated in mutants		Rabinow et al. 1993
Oocyte development blocked	Whole animal	Morris et al. 2003
RNAi blocks cell cycle progression, increased% G1	S2 cells	Bettencourt-Dias et al. 2004 and Bjorklund et al. 2006
RNAi blocks protein secretion, DOA localized to ER	S2 cells	Bard et al. 2006
RNAi to DOA inhibits specific splicing events	S2 cells	Park et al. 2004
Doa mRNA induced to high levels during autophagy	Salivary glands (tissue)	Lee et al. 2003 and Gorski et al. 2003
Reduced *Doa* mRNA associated with higher aggression	Whole animal	Edwards et al. 2006
Reduced *Doa* mRNA correlates with reduced "startle" response	Whole animal	Yamamoto et al. 2008
Female-to-male somatic sex transformations; aberrant splicing of *dsx* and *exu* transcripts; hypophosphorylation and aberrant localization of SR and SR-like proteins	Whole animal	Du et al. 1998

glands during pupation demonstrates that *Doa* transcripts are one of the four most induced. *Doa* transcripts were significantly reduced in screens for hyperaggressive flies and in animals showing an altered "startle" response, while screens in S2 cells showed that *Doa* is required for cell cycle progression as well as for protein secretion.

These observations, coupled with the molecular analysis of *Doa* expression and genetic dissection of isoform functions provide further support that LAMMER kinases target multiple processes in addition to alternative splicing through the phosphorylation of SR proteins.

The LAMMER Kinases of Yeast: Lkh1 of *S. pombe* and KNS1p of *S. cerevisiae*

The LAMMER kinase of *Schizosaccharomyces pombe* Lkh1/Kic1 has been well characterized (see references Table 3; Kang et al. 2010). It negatively regulates filamentous growth, and its mutation causes asexual flocculation of the yeast. *lkh* mutants of *S. pombe* are sensitized to oxidative stress due to decreased levels of catalase and super-oxide dismutase. Decreased oxidative stress response is mediated by lower expression of the Atf1 transcription factor in *lkh1* mutants. It was additionally found that the RNA-binding protein Csx1 was directly phosphorylated by Lkh1 in response to oxidative stress and that Csx1 protein binds and stabilizes *atf1* mRNA. This last observation suggests a mechanism by which Lkh1 phosphorylation may reduce accumulation of Atf1 protein. Over-expression of Lkh1/Kic1 produced elongated cells, while others failed to undergo cytokinesis. Finally, it was recently reported that Lkh1 phosphorylates the Tup11 and 12 transcriptional repressors and that this phosphorylation is required for their activity (Table 3 and references therein).

In contrast to the detailed information available concerning the *S. pombe* LAMMER kinase, little is known about the orthologue of budding yeast, KNS1. Mutants undergo normal cell division, vegetative growth, and glucose utilization. Moreover, no authentic SR or SR-like proteins are encoded in the *S. cervevisiae* genome, and no alternative splicing is known for this yeast, strongly suggesting a role for the kinase other than that of splicing regulation. A synthetic lethal screen failed to recover any interacting loci rendering the locus vital (M. Horn, L. Neigeborn and L. Rabinow unpublished). Data from high-throughput proteomic screens revealed interactions between KNS1 and the ATG1 and CDC15 kinases. The former interaction supports the hypothesis of a role for LAMMER kinases in autophagy, as suggested by the observation of strong induction of *Doa* transcripts upon autophagy in the *Drosophila* salivary glands.

Plant LAMMER Kinases

The genome of *Arabidopsis thaliana* encodes three LAMMER kinase paralogues (Table 1 and references therein). AFC1 was identified in transgenic screens

for loci capable of complementing mutations in *S. cerevisiae* FUS3 (a MAP kinase). The AFC2 and AFC3 paralogues were subsequently identified by PCR analysis, but no further work has been performed on them.

The best characterized of the plant LAMMER protein kinases is PK12 of tobacco, whose transcript and protein levels are both induced by the hormone ethylene (Savaldi-Goldstein et al. and references therein). As all other LAMMER kinases tested, PK12 phosphorylates SR proteins and affects alternative splicing in vivo. Analysis of amino-acid replacements in the LAMMER motif showed that this sequence affects subnuclear localization of the kinase, but did not affect substrate selection. Replacement of the motif with random amino-acids instead resulted in loss of catalytic activity. Analysis of transgenic *Arabidopsis* expressing a constitutively active PK12 revealed a subset of developmental effects typical of overexpression of ethylene-pathway response loci.

CLK, Table 5 Modifiers of LAMMER kinase expression/activity

Activation	Modifier	Kinase/Species	Reference
Transcriptional/ Catalytic activity	Ethylene	PK12/ *N. tabacum*	Sessa et al. 1996
Transcriptional	20-0H ecdysone	DOA/ *D. melanogaster*	Kpebe and Rabinow 2008
Phosphorylation, protein stabilization	Insulin/ AKT	CLK 2/ *M. musculus*	Rodgers et al. 2010
Phosphorylation, ionizing radiation	Insulin/ AKT	CLK 2/ *H. sapiens*	Nam et al. 2010
Phosphorylation	AKT	CLK1/ *M. musculus*	Jiang et al. 2009

CLK, Table 6 CLK inhibitors

Inhibitor	Kinase (specificity)	Reference
TG003	CLK1	Muraki et al. 2004
Chlorhexidine	CLK1, CLK3	Younis et al. 2010
Substituted 6-arylquinazolin-4-amines	CLK4, CLK1	Mott et al. 2009

Regulation of LAMMER Kinases

Aside from the alternative splicing of exon 4 in mammalian CLK1-4 kinases described above, relatively little is published concerning the regulation of the LAMMER protein kinases. In *tobacco*, the PK12 kinase is activated both transcriptionally and catalytically by the hormone ethylene (Table 5). In *Drosophila* two *Doa* isoforms are transcriptionally induced by the steroid hormone ecdysone during pupariation.

Phosphorylation and activation of CLK1 by AKT occurs in response to insulin signaling, consistent with CLK2 activation and stabilization described above in conjunction with its role as a suppressor of gluconeogenesis. One recent study demonstrated increased cell survival following phosphorylation of CLK2 by AKT in response to ionizing radiation.

LAMMER Kinases on Drugs

Several inhibitors of LAMMER/CLK kinases have been developed which have altering degrees of specificity for the individual family members (Table 6 and references therein). The first of these, TG003, is a benzothiazole that inhibits CLK1 kinase in tests of its ability to alter splicing in vitro and in mammalian cells, as well as in phosphorylation of ASF/SF2. TG003 also blocked CLK1-induced dissociation of nuclear speckles, as well as defects induced due to presumed excess CLK activity in *Xenopus* embryos that had been injected with mRNA for one of the *Xenopus CLK* kinases. No toxic effects on growth or cell-cycles of COS-7 cells were observed. TG003 has high specificity for both murine CLK1 (IC_{50}: 20 ηM) and CLK4 (IC_{50}: 15 ηM), whereas CLK2 and CLK3 were less affected (IC_{50} 200 ηM and >10 μM, respectively).

Chlorhexidine was identified as a second CLK inhibitor in a high-throughput screen for modulators of alternative splicing in mammalian cells using a luciferase splicing-reporter construct. Chlorhexidine inhibited splicing of the reporter and also a specific subset of a panel of alternatively spliced exons on DNA chips. CLK4 and CLK3 were most sensitive (IC_{50}: 10 and 50 μM, respectively), while CLK2 and CLK1 were less inhibited (IC_{50}: 25 and 50 μM). Inhibition of CLKs by chlorhexidine in mammalian cells resulted in specific reduction in phosphorylation of SRp75, SRp55, and SRp30, while the phosphorylation of SRp20, SF2/ASF, and SRm160 was not noticeably affected. Other SR protein kinases, such as SRPK, were not inhibited by chlorhexidine.

Finally, substituted 6-arylquinazolin-4-amines were identified as potent inhibitors of CLK4, with varying degrees of effects on the other family members, apparently via competitive inhibition of ATP binding. These compounds bind the kinases at the "hinge" region separating the two lobes of the catalytic domain. Interestingly, one of these compound specifically poisoned *Doa* mutant flies in comparison with wild type in preliminary tests, although no mutant phenotypes were observed (L. Rabinow and D. Auld unpublished).

Summary

Additional analysis of the mammalian LAMMER kinases, CLK1-4, may reveal multiple isoforms, as suggested by their occurrence in *Drosophila*. These alternative isoforms represent differentiable functions in flies, as evidenced by their genetic dissection. While it is clear that one of the major functions of the LAMMER protein kinases is the regulation of alternative splicing through the phosphorylation of SR and SR-like proteins, it also is equally apparent that additional classes of substrates exist, including other protein kinases and phosphatases, chromatin, and other proteins. Given the recently demonstrated involvement of the insulin pathway in the regulation of CLK1 and CLK2 activity, the LAMMER kinases would appear to lie at the intersection of one or more signaling cascades. Finally, despite their well-documented roles in the regulation of splicing, the ancestral function of the LAMMER kinases remains to be determined given the existence of a family member in the yeast *S. cerevisiae*.

Acknowledgments I apologize to the colleagues working on the LAMMER protein kinases whose excellent work could not be completely described or cited due to severe limitations on space and the number of references allowed. Wherever possible I have cited the most recent reference from each group to allow readers to identify prior citations of interest. Work in the laboratory of LR is supported by the University of Paris Sud and the CNRS.

References

Bard F, Casano L, Bard F, Mallabiabarrena A, Wallace E, Saito K, Kitayama H, Guizzunti G, Hu Y, Wendler F, Dasgupta R, Perrimon N, Malhotra V. Functional genomics reveals genes involved in protein secretion and Golgi organization. Nature 2006;439(7076):604–7.

Bender J, Fink GR. *AFC1*, a LAMMER kinase from *Arabidopsis thaliana*, activates STE12-dependent processes in yeast. Proc Natl Acad Sci. USA 1994;91:12105–9.

Bettencourt-Dias M, Giet R, Sinka R, Mazumdar A, Lock WG, Balloux F, Zafiropoulos PJ, Yamaguchi S, Winter S, Carthew RW, Cooper M, Jones D, Frenz L, Glover DM. Genome-wide survey of protein kinases required for cell cycle progression. Nature. 2004;432(7020):980–7.

Björklund M, Taipale M, Varjosalo M, Saharinen J, Lahdenperä J, Taipale J. Nature. 2006;439:1009.

Bullock AN, Das S, Debreczeni JE, Rellos P, Fedorov O, Niesen FH, et al. Kinase domain insertions define distinct roles of CLK kinases in SR protein phosphorylation. Structure. 2009;17(3):352–62.

Colwill K, Feng LL, Yeakley JM, Gish GD, Caceres JF, Pawson T, Fu XD. SRPK1 and Clk/STY protein kinases show distinct substrate specificities for Serine/Arginine-rich splicing factors. J Biol Chem. 1996a;271:24569–75.

Colwill K, Pawson T, Andrews B, Prasad J, Manley JL, Bell JC, Duncan PI. The Clk/STY protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. EMBO J. 1996b;15:265–75.

Du C, McGuffin ME, Dauwalder B, Rabinow L, Mattox W. Protein phosphorylation plays an essential role in the regulation of alternative splicing and sex determination in Drosophila. Mol Cell. 1998;2:741–50.

Edwards AC, Rollmann SM, Morgan TJ, Mackay TF. Quantitative genomics of aggressive behavior in *Drosophila melanogaster*. PLoS Genetics 2006;2:e154.

Fan Y, Schlierf M, Cuervo-Gaspar A, Dreux C, Kpebe A, Chaney L, Mathieu A, Hitte C, Gremy O, Sarot E, Horn M, Zhao Y, Kinzy TG, Rabinow L. Drosophila Translational Elongation Factor-1γ is Modified in Response to DOA Kinase Activity and is Essential For Cellular Viability. Genetics 2010;184:141–54.

Garcia-Sacristan A, Fernandez-Nestosa MJ, Hernandez P, Schvartzman JB, Krimer DB. Protein kinase clk/STY is differentially regulated during erythroleukemia cell differentiation: a bias toward the skipped splice variant characterizes postcommitment stages. Cell Res. 2005;15(7):495–503.

Giamas G, Hirner H, Shoshiashvili L, Grothey A, Gessert S, Kühl M, Henne-Bruns D, Vorgias CE, Knippschild U. Phosphorylation of CK1δ: identification of Ser370 as the major phosphorylation site targeted by PKA in vitro and in vivo. Biochem J. 2007;406(3):389–98.

Gorski SM, Chittaranjan S, Chittaranjan S, Pleasance ED, Freeman JD, Anderson CL, Varhol RJ, Coughlin SM, Zuyderduyn SD, Jones SJ, Marra MA. A SAGE Approach to Discovery of Genes Involved in Autophagic Cell Death. Curr Biol. 2003;13:358–63.

Hartman H, Federov A. The origin of the eukaryotic cell: a genomic investigation. Proc Natl Acad Sci USA. 2002;99:1420–5.

Hillman RT, Green RE, Brenner SE. An unappreciated role for RNA surveillance. Genome Biol. 2004;5(2):R8.

Jiang K, Patel NA, Watson JE, Apostolatos H, Kleiman E, Hanson O, Hagiwara M, Cooper DR. Akt2 regulation of Cdc2-like kinases (Clk/Sty), serine/arginine-rich (SR) protein phosphorylation, and insulin-induced alternative

splicing of PKCbetaII messenger ribonucleic acid. Endocrinology. 2009;150(5):2087–97.
Kang WH, Park YD, Hwang JS, Park HM. RNA-binding protein Csx1 is phosphorylated by LAMMER kinase, Lkh1, in reponse to oxidative stress in *Schizosaccharomyces pombe*. FEBS Letters 2007;581:3473.
Kang WH, Park YH, Park HM. The LAMMER kinase homolog, Lkh1, regulates Tup transcriptional repressors through phosphorylation in *Schizosaccharomyces pombe*. J Biol Chem. 2010;285(18):13797–806.
Katsu R, Onogi H, Wada K, Kawaguchi Y, Hagiwara M. Novel SR-rich-related Protein Clasp Specifically Interacts with Inactivated Clk4 and Induces the Exon EB Inclusion of Clk. J Biol Chem. 2002;277(46):44220–8.
Kojima T, Zama T, Wada K, Onogi H, Hagiwara M. Cloning of human PRP4 reveals interaction with Clk1. J Biol Chem. 2001;276(34):32247–56.
Kpebe A, Rabinow L. Dissection of DOA kinase isoform functions in Drosophila. Genetics. 2008;179:1973–87.
Lee K, Du C, Horn M, Rabinow L. Activity and autophosphorylation of LAMMER protein kinases. J Biol Chem. 1996;271:27299-303.
Lee CY, Clough EA, Yellon P, Teslovich TM, Stephan DA, Baehrecke EH. Curr Biol. 2003;13:350.
Menegay H, Moeslein F, Landreth G. The dual specificity protein kinase CLK3 is abundantly expressed in mature mouse spermatozoa. Exp Cell Res. 1999;253:463–73.
Menegay HJ, Myers MP, Moeslein FM, Landreth GE. Biochemical characterization and localization of the dual specificity kinase CLK1. J Cell Sci. 2000;113:3241–53.
Moeslein FM, Myers MP, Landreth GE. The CLK family kinases CLK1 and CLK2 phosphoryate and activate the tyrosine phosphatase PTP-1B. J Biol Chem. 1999;274: 26697–704.
Morris JZ, Navarro C, Lehmann R. Identification and analysis of mutations in bob, Doa and eight new genes required for oocyte specification and development in *Drosophila melanogaster*. Genetics. 2003;164(4):1435–46.
Mott BT, Tanega C, Shen M, Maloney DJ, Shinn P, Leister W, Marugan JJ, Inglese J, Austin CP, Misteli T, Auld DS, Thomas CJ. Evaluation of substituted 6-arylquinazolin-4-amines as potent and selective inhibitors of cdc2-like kinases (Clk). Bioorg Med Chem Lett. 2009;19(23):6700–5.
Muraki M, Ohkawara B, Hosoya T, Onogi H, Koizumi J, Koizumi T, Sumi K, Yomoda J, Murray MV, Kimura H, Furuichi K, Shibuya H, Krainer AR, Suzuki M, Hagiwara M. Manipulation of alternative splicing by a newly developed inhibitor of Clks. J Biol Chem. 2004; 279(23):24246–54.
Murata S, Yoshiara T, Lim CR, Sugino M, Kogure M, Ohnuki T, et al. Psychophysiological stress-regulated gene expression in mice. FEBS Lett. 2005;579(10):2137–42.
Nam SY, Seo HH, Park HS, An S, Kim JY, Yang KH, Kim CS, Jeong M, Jin YW. Phosphorylation of CLK2 at serine 34 and threonine 127 by AKT controls cell survival after ionizing radiation. J Biol Chem. 2010;285(41):31157–63.
Nayler O, Stamm S, Ullrich A. Characterization and comparison of four serine- and arginine-rich (SR) protein kinases. Biochem J. 1997;326:693–700.
Nayler O, Schnorrer F, Stamm S, Ullrich A. The cellular localization of the murine Serine/Arginine-rich protein kinase CLK2 is regulated by Serine 141 autophosphorylation. J Biol Chem. 1998;273:34341–8.
Nihira K, Taira N, Miki Y, Yoshida K. TTK/Mps1 controls nuclear targeting of c-Abl by 14–3–3-coupled phosphorylation in response to oxidative stress. Oncogene. 2008;27: 7285–95.
Nikolakaki E, Du C, Lai J, Cantley L, Giannakouros T, Rabinow L. Phosphorylation by LAMMER protein kinases: determination of a consensus site, identification of in vitro substrates and implications for substrate preferences. Biochemistry. 2002;41:2055–66.
Padmanabha R, Gehrung S, Snyder M. The *KNS1* gene of *Saccharomyces cerevisiae* encodes a nonessential protein kinase homologue that is distantly related to members of the *CDC28*/*cdc2* gene family. Mol Gen Genet. 1991; 229:1–9.
Park JW, Parisky K, Celotto AM, Reenan RA, Graveley BR. Identification of alternative splicing regulators by RNA interference in Drosophila. Proc Natl Acad Sci USA. 2004;101: 15974–9.
Prasad J, Manley JL. Regulation and substrate specificity of the SR protein kinase Clk/Sty. Mol Cell Biol. 2003; 23:4139–49.
Rabinow L, Birchler JA. A dosage-sensitive modifier of retrotransposon- induced alleles of the Drosophila white locus. EMBO J. 1989;8:879–89.
Rabinow L, Samson ML. The role of the Drosophila LAMMER protein kinase DOA in somatic sex determination. J Genet. 2010;89(3):271–7.
Rabinow L, Chiang SL, Birchler JA. Mutations at the *Darkener of apricot* locus modulate transcript levels of *copia* and *copia*-induced mutations in *Drosophila melanogaster*. Genetics. 1993;134:1175–85.
Rodgers JT, Haas W, Gygi SP, Puigserver P. Cdc2-like kinase 2 is an insulin-regulated suppressor of hepatic gluconeogenesis. Cell Metab. 2010;11(1):23–34.
Savaldi-Goldstein S, Aviv D, Davydov O, Fluhr R. Alternative splicing modulation by a LAMMER kinase impinges on developmental and transcriptome expression. Plant Cell. 2003;15(4):926–38.
Sessa G, Raz V, Savaldi S, Fluhr R. PK12, a plant dual-specificity protein kinase of the LAMMER family, is regulated by the hormone ethylene. Plant Cell. 1996;8:2223–34.
Stodjl DF, Bell JC. SR protein kinases: the splice of life. Biochem Cell Biol. 1999;77:293–8.
Velazquez-Dones A, Hagopian JC, Ma CT, Zhong XY, Zhou H, Ghosh G, Fu XD, Adams JA. Mass spectrometric and kinetic analysis of ASF/SF2 phosphorylation by SRPK1 and Clk/ Sty. J Biol Chem. 2005;280(50):41761–8.
Yamamoto A, Zwarts L, Callaerts P, Norga K, Mackay TF, Anholt RR. Neurogenetic networks for startle-induced locomotion in *Drosophila melanogaster*. Proc Natl Acad Sci USA. 2008;105(34):12393–8.
Younis I, Berg M, Kaida D, Dittmar K, Wang C, Dreyfuss G. Rapid-response splicing reporter screens identify differential regulators of constitutive and alternative splicing. Mol Cell Biol. 2010;30(7):1718–28.
Yun B, Farkas R, Lee K, Rabinow L. The *Doa* locus encodes a member of a new protein kinase family, and is essential for eye and embryonic development in *Drosophila melanogaster*. Genes Dev. 1994;8:1160–73.

CLK Kinases

▶ CLK

CMKBR1

▶ Chemokine Receptor CCR1

CMT2B

▶ Rab7a in Endocytosis and Signaling

c-Myb

Scott A. Ness
Department of Molecular Genetics & Microbiology, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

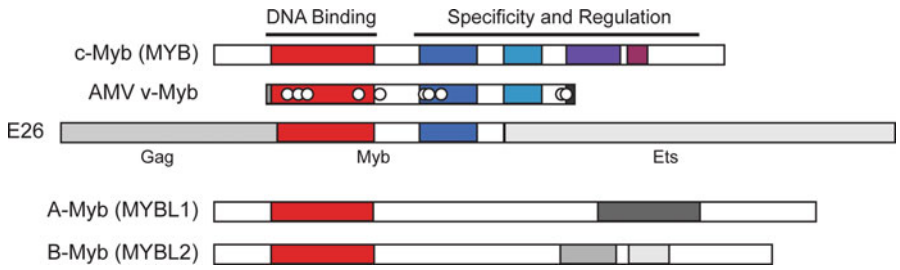
Synonyms

MYB; v-myb myeloblastosis viral oncogene homolog

Historical Background

The c-Myb protein is a DNA-binding transcription factor that regulates the expression of other genes, controls the proliferation and differentiation of hematopoietic, epithelial, and neural cells, and can be mutated into an oncogenic form that induces leukemias and other tumors in animals and humans (Zhou and Ness 2011) (Note: I use c-Myb and c-*myb* to distinguish between the protein and gene, respectively. Readers are encouraged to consult the cited review articles for links to the primary literature, which cannot be listed here due to limitations on space and the number of citations). The c-*myb* gene (MYB) is the normal counterpart to two v-*myb* oncogenes encoded by avian leukemia viruses that induce leukemias in animals and transform immature hematopoietic cells in tissue culture (Lipsick and Wang 1999; Ness 1996; Zhou and Ness 2011). Avian myeloblastosis virus (AMV) encodes a v-Myb protein that when compared to c-Myb (Fig. 1) is truncated at both the N-terminal and C-terminal ends and has several point mutations leading to changes in amino acid residues, which turn out to be critical for controlling the activity of the v-Myb protein and for altering the spectrum of target genes that it regulates (Lipsick and Wang 1999; Ness 2003; Zhou and Ness 2011). The E26 virus has a more complex structure, with an N-terminal domain comprised of 272 amino acids of the retroviral Gag protein fused to 285 amino acids of Myb, fused in turn to a C-terminal domain derived from 491 amino acids of another transcription factor, Ets-1 (Ness 1996; Zhou and Ness 2011). Both v-Myb proteins contain the DNA binding domain of c-Myb, which recognizes specific, but degenerate sites in DNA (Biedenkapp et al. 1988) and is required for the transforming and transcriptional activation functions of the Myb proteins (Lipsick and Wang 1999; Ness 2003; Zhou and Ness 2011).

The c-Myb DNA binding domain is highly conserved and defines a family of related transcription factors. In addition to c-Myb, vertebrates also express A-Myb (MYBL1) and B-Myb (MYBL2) proteins, which have highly related structures including nearly identical DNA binding domains near their N-termini and large, unique C-terminal domains (Fig. 1). The three Myb proteins are often co-expressed, can bind the same sets of DNA sequences and can activate the same reporter genes in transfection assays, but they activate different sets of target genes and have non-overlapping functions in differentiation and development (Gonda et al. 2008; Greig et al. 2008; Lipsick et al. 2001; Ness 2003; Ramsay 2005; Ramsay and Gonda 2008; Sala 2005; Zhou and Ness 2011), suggesting that the conserved DNA binding domains are not sufficient to determine the target gene specificities or activities of the Myb proteins (Ness 2003; Zhou and Ness 2011). All three Myb proteins are probably derived from a primordial precursor similar to the one D-Myb protein found in Drosophila, which appears to be most closely related in function to the vertebrate B-Myb protein (Lipsick 1996; Lipsick et al. 2001). Interestingly, plants have a much larger family of Myb-related transcription factors, involved in controlling the expression of differentiation-specific genes (Allan et al. 2008; Du et al. 2009).



c-Myb, Fig. 1 *Conserved domains in Myb proteins*. The structures of the c-Myb protein and the v-Myb proteins encoded by AMV and E26 viruses are diagrammed, with the most highly conserved domains shaded. The highly conserved DNA binding domain is shaded *red*, and the *dots* indicate the locations of point mutations in the AMV v-Myb protein. The structures of the A-Myb and B-Myb proteins, which have highly related DNA binding domains but different biological activities, are diagrammed below

The c-Myb DNA binding domain is composed of three "Myb Repeats," only two of which are retained in the v-Myb proteins, which have structures that resemble Homeo domains. The structures of the Myb repeats have been solved using both solution and crystallographic methods, providing important information about the locations of the v-Myb mutations that affect transcriptional activity (Ness 1996). One of the most interesting features of the Myb proteins is that the Myb DNA binding domain has been so highly conserved – even the surface-exposed residues that do not contact DNA are conserved. This conservation suggests that the surface-exposed residues of c-Myb are important for protein-protein interactions. Indeed, the DNA binding domain of c-Myb has been found to interact with a large number of protein partners (discussed below), suggesting that the so-called DNA binding domain is actually an important DNA- and protein-binding domain (Ness 1996, 1999; Zhou and Ness 2011). DNA-binding proteins with Myb repeats are found in microorganisms, insects, plants, and vertebrates (Lipsick 1996). Recently, some laboratories have begun referring to the Myb repeats as "SANT" domains, to reflect their relatedness to several other regulatory proteins that also contain complete or partial Myb domains (Boyer et al. 2002).

c-Myb in Stem Cells and Non-hematopoietic Tissues

The v-Myb oncoproteins transform hematopoietic cells, and the c-Myb protein has been linked to the regulation of hematopoietic cell differentiation and proliferation, both through anti-sense knockdown experiments demonstrating that c-Myb is required for hematopoietic progenitor cell differentiation in vitro and through mouse knockout experiments showing that c-Myb deficient mice die in utero due to a defect in definitive erythropoiesis. More recent experiments using conditional knockout strategies demonstrated that c-Myb function is required for the development of most, if not all myeloid and lymphoid cells and that c-Myb plays an important role in many other tissues, including breast and colon epithelia and some neural tissues (Ramsay 2005; Ramsay and Gonda 2008; Zhou and Ness 2011). Thus, although it is often thought to be a hematopoietic cell–specific transcription factor, c-Myb is actually expressed in a wide variety of tissues, especially in cells that are proliferating and/or differentiating, and likely plays an important regulatory role in many cell types.

With the understanding that c-Myb is expressed in and plays an important role in many other cell types has come the realization that mutated, rearranged, and/or overexpressed versions of c-*myb* play an important role in the development of many types of human tumors. Once thought of as an oncogene that was specific for leukemias and lymphomas, c-Myb is now recognized as an important oncogene in a variety of human malignancies, including colon and breast tumors and some neural and head and neck tumors (Gonda et al. 2008; Ramsay and Gonda 2008; Zhou and Ness 2011). Although the perceived importance of c-Myb in human tumorigenesis has grown, the role that c-Myb plays in cell transformation and tumor formation has yet to be delineated. For example, it is not known what genes c-Myb regulates in different types of tumors, or even if c-Myb regulates the same genes in hematopoietic, epithelial or other tumor types.

The activities of c-Myb appear to be complex and context-specific, and it is not yet clear how those activities play a role in human malignancies (Gonda et al. 2008; Ramsay and Gonda 2008; Zhou and Ness 2011).

Multiple Pathways Regulate c-Myb Expression

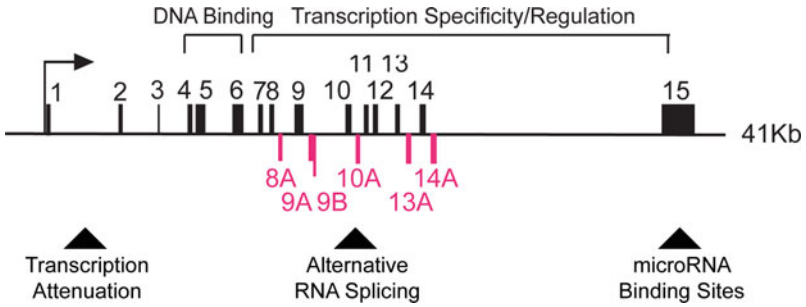
Expression of the c-*myb* gene is complex and controlled at the transcriptional and posttranscriptional levels. The promoter of the c-*myb* gene is a CpG island and resembles a "housekeeping gene" promoter, suggesting that it is relatively steadily expressed in cells that have the gene activated. However, a major regulator of c-*myb* transcription lies in the highly conserved first intron (Fig. 2), where multiple proteins bind and control the efficiency of RNA elongation. Several laboratories have studied c-*myb* transcriptional attenuation, which appears to be a major mechanism for controlling c-*myb* mRNA synthesis, and which is tightly regulated by a number of transcriptional regulators including estrogen receptors (Gonda et al. 2008; Ramsay et al. 2003).

The c-*myb* gene is also subject to dramatic and highly regulated alternative RNA splicing, which primarily involves the region of the gene (Fig. 2) that encodes the large C-terminal regulatory domain of c-Myb protein (Fig. 1). Recent studies have shown that the c-*myb* gene can generate as many as 60 distinct splice variants in some cell types. The variants are formed through the use of alternative exons (e.g., 8A, 9A, 9B), by skipping exons, by using alternative splice donor sites that effectively create "short" versions of some exons (e.g., 9 S), and through the combinatorial mixing of these mechanisms in single mRNA molecules. The levels of alternative splicing can be quite variable, but in some leukemia samples the alternatively spliced variants represent the majority of the c-*myb* transcripts. Interestingly, the alternative splicing does not affect the part of the c-*myb* gene that encodes the highly conserved DNA binding domain, but leads to changes in the large C-terminal domains of c-Myb protein that affect regulation and activity. Thus, the alternative splicing results in the synthesis of a family of c-Myb proteins with the same DNA binding domains, but different C-terminal domains and distinct activities. Alternative RNA splicing allows the c-*myb* gene to generate numerous, slightly different c-Myb transcription factors with altered specificities. Context-specific regulation of alternative splicing could lead to the production of specialized versions of c-Myb proteins capable of regulating different target genes, for example during T-cell or B-cell differentiation (Ness 2003; Zhou and Ness 2011).

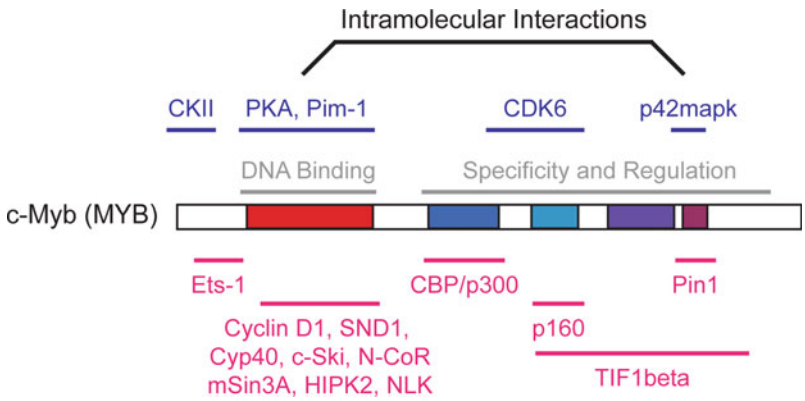
Mechanisms Controlling the Specificity and Activities of c-Myb

Much of the research into c-Myb has focused on the regulation of its activities and the differences that distinguish c-Myb from the oncogenic v-Myb. Early studies showed that c-Myb lacked the transforming or leukemia-inducing activities of the v-Myb proteins, and that truncations of the C-terminal domains were required to unleash the oncogenic potential (Lipsick and Wang 1999; Ness 1996). The C-terminus of c-Myb is the site of phosphorylation, ubiquitinylation, and sumoylation and is the location of peptide sequences that make the protein unstable and lead to its degradation. Thus, truncation of the C-terminus stabilizes the protein, which is one mechanism that leads to higher transforming activity by v-Myb. Other studies have shown that c-Myb undergoes intramolecular interactions that regulate its activity (Fig. 3). These interactions are likely regulated by one or more kinases, including ▶ Casein Kinase II (CKII), Protein Kinase A (PKA), and mitogen-activated protein kinases (▶ MAP Kinases) that phosphorylate c-Myb at several sites along the protein (Fig. 3). Phosphorylation at the N-terminus and in the DNA binding domain can affect DNA binding and some of the phosphorylation sites are altered by the truncations and mutations in AMV v-Myb, suggesting that phosphorylation, at least at some sites, could be involved in a negative regulatory loop that is disabled in the oncogenic variant (Ness 1999; Ness 2003; Zhou and Ness 2011).

The c-Myb protein has also been shown to interact with numerous proteins that are likely to modify or regulate its activity or help control which target genes are regulated and in which situations (Fig. 3). Some of these interacting proteins include ▶ Pim-1, an oncogenic protein kinase that binds and phosphorylates the c-Myb DNA binding domain and that is regulated in turn by upstream JAK-STAT signaling pathways, cyclin D1 and the cyclin-dependent kinases CDK4



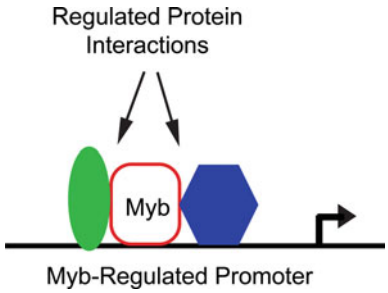
c-Myb, Fig. 2 *Regulation of c-myb gene expression*. The c-*myb* gene structure is summarized, with the start site of transcription shown at *left*, the normal exons shown above the line in *black*, and the alternative exons shown below the line in *pink*. Transcription attenuation occurs between exons 1 and 2, alternative RNA splicing occurs primarily between exons 7–14 and the large exon 15 contains numerous binding sites for regulatory microRNAs (miRs) and RNA binding proteins that affect mRNA stability and efficiency of translation



c-Myb, Fig. 3 *Multiple regulatory pathways affect c-Myb protein*. The diagram summarizes the structure of the c-Myb protein, with the most highly conserved domains shaded (see Fig. 1). The N- and C-terminal ends of c-Myb form intramolecular interactions, which is likely involved in the regulation of c-Myb DNA binding and/or transcriptional activation activity. Several protein kinases that have been reported to phosphorylate c-Myb are shown in *blue* and above the c-Myb diagram, and a subset of the proteins shown to interact with c-Myb are shown in *pink* and below the diagram, organized according to the domains of c-Myb that they interact with

and CDK6, which could regulate c-Myb during the cell cycle (Quintana et al. 2011), and the proline isomerases Cyp40 and ▶ Pin1, which could induce changes in conformation of the c-Myb protein (Ness 1999; Ness 2003). Several recent studies have identified regulatory complexes or mechanisms that involve Homeodomain-interacting protein kinase 2 (HIPK2) and simultaneous interactions with both the DNA binding and the C-terminal domains, suggesting that c-Myb is likely folded into one or more different conformational states that influence which partner proteins it is able to interact with and consequently which genes it can regulate (Zhou and Ness 2011).

Many of the best-characterized Myb-regulated promoters require cooperation or combinatorial interactions between c-Myb and other transcription factors, such as C/EBPbeta (Ness 1996, 1999; Ness 2003). The DNA sequences recognized by the Myb DNA binding domains are degenerate and are present at least three million times in the human genome, so combinatorial or cooperative interactions at promoters are likely required to increase the specificity of c-Myb for specific binding sites and specific target genes (Fig. 4). However, protein-protein interactions are subject to rapid and dramatic changes through regulatory mechanisms such as posttranslational modifications, which can be affected by upstream signaling pathways. This offers an attractive mechanism for how c-Myb target gene specificity can be regulated by upstream pathways. For example, activation of upstream protein



c-Myb, Fig. 4 *Combinatorial and cooperative binding at target promoters.* The c-Myb protein requires interactions with other proteins to determine its specificity, but protein-protein interactions are subject to regulation by posttranslational modifications and other mechanisms

kinases such as FMS-like tyrosine kinase receptor-3 (FLT3), c-KIT, AKT (also known as Protein Kinase B), or Epidermal Growth Factor (EGF) receptor could lead to the activation of downstream protein kinases that phosphorylate c-Myb or one or more of the interacting co-regulators, leading to changes in protein-protein interactions and a redirection of c-Myb and its co-regulators to different promoters. This mechanism also provides an explanation for how c-Myb could participate in oncogenesis, if upstream oncogenic kinases result in the retargeting of c-Myb to different promoters, such as genes that stimulate proliferation instead of differentiation. There is evidence that c-Myb moves to different sets of target genes during the cell cycle (Quintana et al. 2011), consistent with the proposal that c-Myb specificity is regulatable and changes as a result of upstream signals. Finding drugs or interventions that retarget c-Myb to differentiation-inducing genes in cancer cells would be a novel treatment strategy that could take advantage of the flexibility in c-Myb activity.

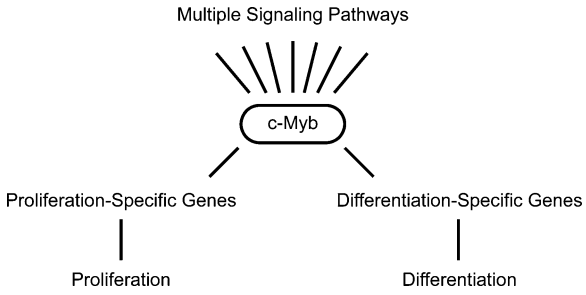
Myb Functions at the Decision Point Between Proliferation and Differentiation

A number of studies have linked the activity of c-Myb to the proliferation and differentiation of hematopoietic, epithelial, and neural stem cells (Ramsay 2005; Ramsay and Gonda 2008; Zhou and Ness 2011). The results suggest that c-Myb plays a critical role in the initial differentiation of stem cells to the most immature committed progenitor cells, where c-Myb probably plays an important role in regulating proliferation and the cell cycle. This highlights one of the most important and most difficult to understand properties of c-Myb: It is involved in the regulation of both proliferation and differentiation, two processes that are often thought of as opposite and contradictory. The oncogenic v-Myb proteins have been described as transforming cells by "blocking" differentiation. But experiments using temperature-sensitive variants of v-Myb showed that reactivating or reintroducing the v-*myb* oncogene into differentiated cells induced the cells to "de-differentiate," showing that the v-Myb proteins actually induced a transformed phenotype, probably by inducing the expression of target genes that are specific for immature, proliferating cells, which resulted in increased proliferation.

The oncogenic activity of c-Myb appears to be linked to the role it plays regulating the balance between differentiation and proliferation. Since c-Myb has the ability, in the right conditions, to induce either proliferation or differentiation, relatively modest changes in its activity can lead to dramatic changes in cell fate. For example, activating the appropriate upstream signaling pathway that leads indirectly to posttranslational modifications in c-Myb could push the cells out of proliferation and into terminal differentiation, or vice versa. The oncogenic v-Myb proteins appear to be variants of c-Myb that are locked, through mutations, into a proliferation-inducing activity, which is why they are able to transform cells and to induce leukemias. Judging from the large number of proteins and kinases that can interact with or phosphorylate c-Myb (Fig. 3), it seems likely that c-Myb lies downstream of multiple signaling pathways, perhaps acting as an integrator of upstream signals (Fig. 5), converting them into changes in gene expression based upon a complex protein-interaction code (Ness 2003) and resulting in cell fate decisions (e.g., proliferation vs. differentiation).

The Importance of c-Myb as a Human Oncogene

As an oncogene, c-*myb* was first identified in the context of avian leukemia virus-induced leukemias, but mapping the integration sites in retrovirus-induced leukemias in mice showed early on that the c-*myb* gene was a preferred target for activation and that it was an important mammalian oncogene



c-Myb, Fig. 5 *Myb as an integrator of upstream signals.* The c-Myb protein lies downstream of multiple signaling pathways, and likely acts as an integrator of signals, converting a protein-protein interaction code, which is affected by posttranslational modifications, into choices about which target genes get regulated and whether the cell proliferates or enters a differentiation pathway

(Ness 1996). Nevertheless, despite high levels of expression in most leukemias and lymphomas and many other tumors, convincing evidence that c-*myb* plays a causal role in human tumorigenesis was only discovered relatively recently. The c-*myb* gene was found to be involved in recurrent chromosomal translocations in some types of human T-cell acute leukemia (T-ALL) and in carcinomas of the breast and head and neck (Ramsay and Gonda 2008; Zhou and Ness 2011). These results showed conclusively that activation of the c-*myb* gene by amplification and/or rearrangement is associated with specific types of human cancers, demonstrating definitively that c-*myb* is an important oncogene in human hematopoietic and solid tumors.

Although there are no longer any arguments about whether c-*myb* is a human oncogene, there is still relatively little information about how c-*myb* transforms cells, either in human or animal systems. The transforming activities of v-Myb require both the DNA binding and transcriptional activation domains, suggesting that v-Myb transforms cells by activating specific target genes that lead to transformation and oncogenesis (Lipsick and Wang 1999; Ness 2003). However, the identities of those genes have not been elucidated. One of the most intriguing results came from the use of microarrays to compare the patterns of gene expression induced by the normal c-Myb and oncogenic v-Myb proteins. Surprisingly, expression of the c-Myb and v-Myb proteins in human cells led to the activation of totally different sets of target genes. Thus, v-Myb is not merely a more active or deregulated version of c-Myb, but instead activates an entirely different set of target genes (Ness 2003; Zhou and Ness 2011). Furthermore, the two proteins activated almost totally different sets of genes when expressed in different cell types, showing that their activities were extremely context-specific. Thus, it seems likely that mutated or alternatively spliced variants of c-Myb expressed in tumor cells probably activate totally different sets of target genes than the normal "wild-type" c-Myb protein. In addition, "normal" c-Myb expressed in cells that have constitutively activated upstream signaling pathways, as often happens in tumors, is likely to regulate different target genes than it would in normal cells.

Summary and Future Directions

The c-Myb protein is a transcription factor with many activities that change in response to numerous upstream signals. It is subject to complex regulation at the level of expression, alternative splicing, stability, and through posttranslational modifications. The oncogenic activity of c-Myb may be due to its role as an integrator of many different upstream signals. This flexibility, even "plasticity" also makes it vulnerable to activation through mutations or when the upstream signaling pathways that regulate it become corrupted. However, understanding how the activities of c-Myb change and respond to various signals may also offer a potential means of regulating it. Small molecules targeted to c-Myb might be able to shift its activity to induce tumor cells to stop proliferating and instead to differentiate. Clearly, the most important things to do are to identify and characterize the target genes that c-Myb regulates in different situations, in different cell types, in response to different upstream signals and in normal vs. transformed cells. This information will unleash new types of assays for determining how c-Myb is regulated and how its activity might be influenced as a novel therapeutic strategy.

References

Allan AC, Hellens RP, Laing WA. MYB transcription factors that colour our fruit. Trends Plant Sci. 2008;13(3):99–102.

Biedenkapp H, Borgmeyer U, Sippel AE, Klempnauer K-H. Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature. 1988;335:835–7.

Boyer LA, Langer MR, Crowley KA, Tan S, Denu JM, Peterson CL. Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. Mol Cell. 2002;10(4):935–42.
Du H, Zhang L, Liu L, Tang XF, Yang WJ, Wu YM, et al. Biochemical and molecular characterization of plant MYB transcription factor family. Biochemistry (Mosc). 2009;74(1):1–11.
Gonda TJ, Leo P, Ramsay RG. Estrogen and MYB in breast cancer: potential for new therapies. Expert Opin Biol Ther. 2008;8(6):713–7.
Greig KT, Carotta S, Nutt SL. Critical roles for c-Myb in hematopoietic progenitor cells. Semin Immunol. 2008;20(4):247–56.
Lipsick JS. One billion years of Myb. Oncogene. 1996;13:223–35 (review).
Lipsick JS, Wang DM. Transformation by v-Myb. Oncogene. 1999;18(19):3047–55 (review).
Lipsick JS, Manak J, Mitiku N, Chen CK, Fogarty P, Guthrie E. Functional evolution of the Myb oncogene family. Blood Cells Mol Dis. 2001;27(2):456–8 (review).
Ness SA. The myb oncoprotein: regulating a regulator. Biochim Biophys Acta. 1996;1288(3):F123–39 (review).
Ness SA. Myb Binding Proteins: regulators and Cohorts in Transformation. Oncogene. 1999;18:3039–46 (review).
Ness SA. Myb Protein Specificity: evidence of a Context-Specific Transcription Factor Code. Blood Cells Mol Dis. 2003;31(2):192–200 (review).
Quintana AM, Zhou YE, Pena JJ, O'Rourke J, Ness SA. Dramatic repositioning of c-Myb to different promoters during the cell cycle observed by combining cell sorting with chromatin immunoprecipitation. PLoS One. 2011;6(2):e17362. 2011 February 22.
Ramsay RG. c-Myb a stem-progenitor cell regulator in multiple tissue compartments. Growth Factors. 2005;23(4):253–61.
Ramsay RG, Gonda TJ. MYB function in normal and cancer cells. Nat Rev Cancer. 2008;8(7):523–34 (review).
Ramsay RG, Barton AL, Gonda TJ. Targeting c-Myb expression in human disease. Expert Opin Ther Targets. 2003;7(2):235–48.
Sala A. B-MYB, a transcription factor implicated in regulating cell cycle, apoptosis and cancer. Eur J Cancer. 2005;41(16):2479–84.
Zhou Y, Ness SA. Myb proteins: angels and demons in normal and transformed cells. Front Biosci. 2011;16:1109–31 (review).

c-MYC

► Myc

c-N-Ras

► RAS (H-, K-, N-RAS)

Collagen-Binding Factor Endo180

► MRC2

Concertina (*Drosophila melanogaster* Homolog)

► G Protein Alpha 12

Copine

Carl E. Creutz
Department of Pharmacology, University of Virginia, Virginia, VA, USA

Historical Background

The entry of the calcium ion into the cytoplasm of cells from the extracellular medium or from intracellular stores plays an important signaling role. Cytoplasmic calcium acts as a signal by binding to high-affinity calcium-binding proteins. These proteins in turn act as transducers of the signal by activating other proteins, or may be activated directly to carry out enzymatic or structural changes. In this way, many extracellular signals are converted into intracellular activities.

An important subset of these intracellular calcium-binding proteins are proteins that also interact with membranes in a calcium-regulated fashion. In the resting cell, many of these proteins are freely soluble in the cytoplasm (or nucleoplasm). However, when calcium enters the cell, these proteins move onto membrane surfaces. In this way, they make fundamental changes in the character of the membrane surface. Some, such as the annexins (Gerke et al. 2005), are so abundant that up to one half of the intracellular surface of all membrane systems may be covered by annexins in stimulated cells. This influences the organization of the lipids in the membrane, may promote membrane–membrane contacts, and may regulate ion channel activities or the properties of other peripheral or integral membrane proteins.

One of the most interesting members of this class of calcium-dependent, membrane-binding proteins was not characterized until the membrane-binding proteins of the important model secretory cell, *Paramecium tetraurelia* were studied (Creutz et al. 1998; Tomsig and Creutz 2002). In contrast to either mammals or green plants, in which the majority of membrane-binding proteins are annexins, Paramecium extracts contain only a single major membrane-binding protein. Sequencing of this protein revealed that it was not an annexin, but a protein that bound to membranes through a pair of C2 domains, homologous to the domain of protein kinase C that binds calcium and phospholipids. Further sequence analyses revealed that EST and genomic sequencing databases included many copies of homologous sequences from plants, animals, and slime molds, although the encoded proteins had not been previously studied or described, possibly because they are of much lower abundance than annexins in mammalian or plant cells. This new class of proteins was named "copines," derived from the French word *copine* (pronounced ko-peen'), which means a *friend*, because of the association (poetically, the friendship) of these proteins with membranes. Interestingly, not only are copines almost ubiquitously present in eukaryotes (although absent from yeast and *Drosophila*), they are generally present in a given organism as a family of multiple homologs – for example, humans have 9 copines, nematodes 6, *Dictyostelium* 6, and plants at least 3. This high degree of conservation and multiplicity of this protein family strongly suggests that the copines play a fundamentally important role in cell biology.

Nomenclature of Copine Family Members in Diverse Organisms

The broad distribution of copines in various organisms has led to the use of a variety of names for these proteins. Important examples are listed below.

In Humans: copine I, copine II, copine III, copine IV, copine V, copine VI, copine VII, copine VIII, copine IX

In Paramecium: *CPN1, CPN2*

In Arabidopsis: copine 1, copine 2, copine 3; bon1, bon2, bon3

In Dictyostelium: copine A, copine B, copine C, copine D, copine E, copine F; cpnA, cpnB, cpnC, cpnD, cpnE, cpnF

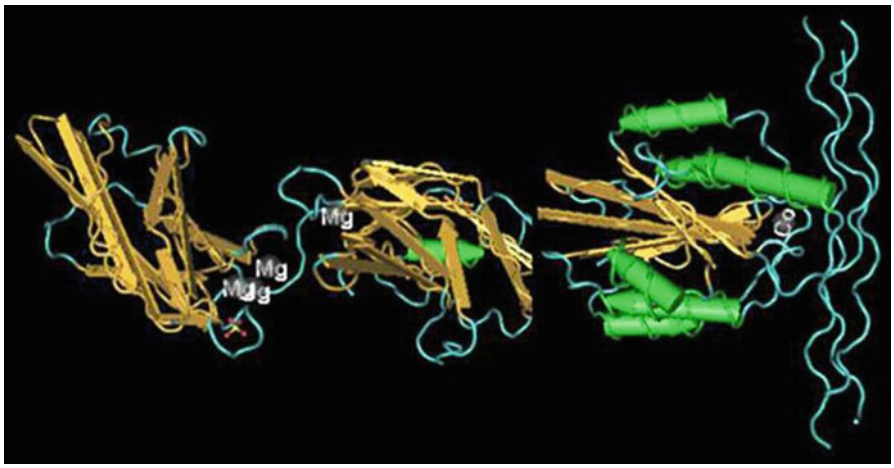
In Caenorhabditis: gem-4 copine, NRA-1 copine

Domain Structure of Copines

Analysis of the full sequences of the copines revealed an interesting domain structure (see Fig. 1). The N-terminal half of a copine protein contains the two C2 domains that are responsible for binding calcium and membranes. The C-terminal half shows a distant relationship to the "A domain" in the extracellular portion of integrins (or von Willebrand domain) (Lee et al. 1995). In the integrin protein family, this domain is responsible for the binding of extracellular matrix proteins. Although the sequence identity is only 23%, there is additional evidence that the copine domain is similar to the integrin domain. First of all, the integrin domain is known to bind magnesium and manganese, and the residues that coordinate magnesium are known from the X-ray structure of the integrin domain (Lee et al. 1995). These same residues are conserved in the copine domain (Creutz et al. 1998). In addition, it was demonstrated that copine binds magnesium or manganese (Tomsig and Creutz 2000). The secondary structure predictions for the copine A domain parallel closely the known secondary structure of the integrin domain – a pattern of alternating alpha helices and beta sheets (Creutz et al. 1998).

Functions of Copines

A key to understanding the biological functions of the copines may be to understand the role of their unique A domain. The similarity to the integrin A domain suggests two rather distinct possibilities. First, the integrin A domain has the classic form, called a Rossman fold, that is present in many nucleotide-binding proteins (Lee et al. 1995). This suggests the copines might require a nucleotide cofactor in order to function. There is one report in the literature that copine III has an associated protein kinase activity (Caudell et al. 2000), although the specific enzymatic activity was extraordinarily low and has not been reported for other members of the protein family.



Copine, Fig. 1 Schematic illustration of the domain structure of copine. The pair of N-terminal C2 domains are on the left. The A domain is on the right and is bound to a "target" protein (wavy vertical lines). This figure was assembled using the known structure of the C2 domains of synaptotagmin on the left, and the known structure of the integrin A domain bound to a triple helical collagen peptide on the right (Emsley et al. 2000). The actual structures of the copine domains, or their relative orientations in space, are not known

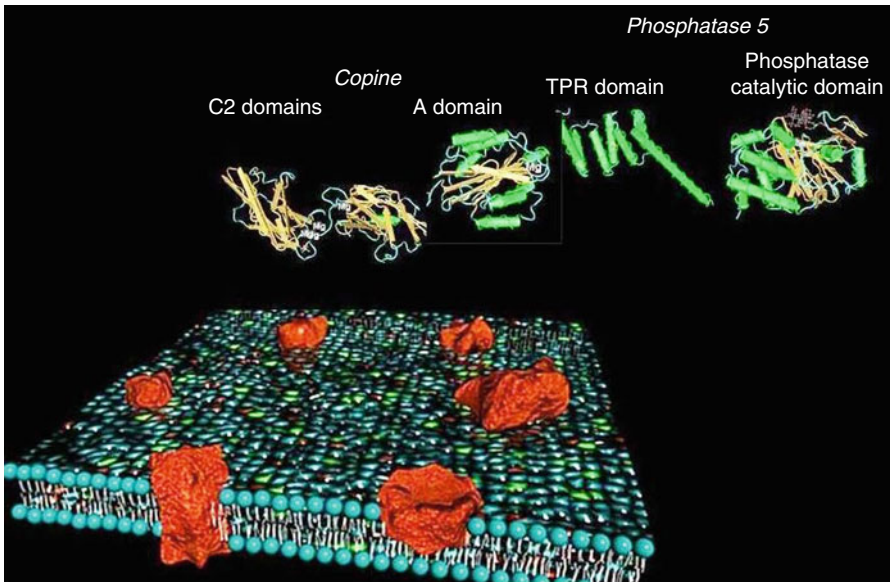
The other hypothesis suggested by the similarity of the copine A domain to the integrin A domain is that the copine A domain is a site for protein–protein interactions. This hypothesis was tested by screening for proteins that interact with the copine A domain. Using a yeast two-hybrid system with the A domains of three human copines as baits, a number of proteins were identified that interact with the copine A domain (Tomsig et al. 2003). The ability of these proteins to interact directly with copine was tested in in vitro assays using recombinant proteins in which the interactions were found to be of very high affinity, stable to extensive washing. Furthermore, the copines were found to be able to recruit the target proteins to phospholipid surfaces in vitro. In contrast to the integrin A domain, the proteins that were found to interact with the copine A domain are almost exclusively intracellular proteins (Tomsig et al. 2003).

Interaction of Copines with Signaling Proteins

The most interesting characteristic of the potential targets identified for copines is that the majority are proteins that are involved in intracellular signal transduction pathways. These targets could be grouped into several categories (Tomsig et al. 2003):

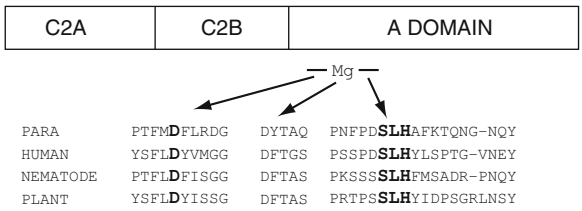
1. Regulators of phosphorylation: MEK1, protein phosphatase 5, CDC42 binding kinase (homolog of the myotonic dystrophy kinase), TAB2 (TAK1 kinase-binding protein).
2. Regulators of transcription: Generally these potential targets are not DNA-binding transcription factors but act as regulators of known transcription factors - Myc-binding protein (PAM), Sno proto-oncogene product, Wilm's tumor associated protein, BcoR (BCL-6 corepressor).
3. Calcium-binding proteins: Copine I (target of copine IV), ALG2.
4. Regulators of ubiquitination/NEDDylation: UBC12, E2-230.
5. Cytoskeletal regulation: Radixin, BICD2 (dynamitin-binding protein).

Although there is obviously considerable variety in the structures and functions of these targets, it was found that in the majority of cases the binding site for the copine could be mapped to a coiled-coil region of the target protein. Significant specificity for the particular coiled-coil domains in these targets was evident since the copines did not bind coiled-coils of abundant structural or fibrous proteins such as keratins or myosins. A consensus sequence for the coiled-coil copine target motif was determined and found to have predictive value for identifying new copine targets, in particular, the kinase MEK1 (Tomsig et al. 2003).



Copine, Fig. 2 Schematic illustration of the hypothetical role of copine in recruiting a signaling protein to a membrane surface. The A domain of copine binds the TPR domain of protein phosphatase 5. The C2 domains of copine migrate to the membrane surface in a calcium-dependent manner, therefore recruiting the phosphatase to the membrane

This array of potential targets suggests the copines may be involved generally in providing calcium regulation of intracellular signaling pathways. In this respect, the copines may be compared to the universal calcium transducer calmodulin. Calmodulin activates a number of enzymes, ion channels, or other "targets" when calcium activates calmodulin and causes it to bind other proteins. The "targets" for copine might be similarly regulated. Indeed, the binding of copine to the N-terminal domain of protein phosphatase 5 enhances the activity of the phosphatase (Tomsig et al. 2003). In addition, by virtue of binding to membranes as well as target proteins, the copines may be able to specifically localize signaling pathway components to certain membranes in the cell (see Fig. 2). This could enhance the assembly of signaling complexes involved in signaling across the plasma membrane. Alternatively, factors involved in regulating gene transcription could be sequestered and downregulated by binding to membranes through copine action.



Copine, Fig. 3 Novel, conserved sequence features of the copine A domain. The domain structure of copine is illustrated at the top. Underneath, the sequences near a magnesium-binding site of the A domains from Paramecium, human, nematode, and Arabidopsis copines are aligned. The SLH motif downstream from the magnesium-chelating residues is conserved in all species, as is a unique aspartate (D) upstream from the magnesium-chelating residues

A Dual-Function Hypothesis

It is possible that the copines may perform both of the hypothetical roles outlined above: They may bind certain target proteins as well as possess an enzymatic activity. This dual-function hypothesis is suggested by the presence of a very highly conserved sequence motif in the A domain whose presence is not explained either by the protein-binding activity or the calcium and phospholipid-binding activity of copine. Just C-terminal of certain critical residues involved in chelation of magnesium by the A domain is a sequence motif conserved from plants to humans (Creutz et al. 1998; see Fig. 3). Theoretically, this portion of the protein would be part of the A domain since it is flanked on either side by features conserved in integrin-like A domains in other proteins. However, this particular motif cannot be aligned with other A domain features and appears to represent an extended loop or fold of the protein between beta strands. Since this highly conserved motif contains an absolutely conserved serine and histidine, and there is

an absolutely conserved aspartate nearby, it is possible that these residues could be involved in forming a catalytic triad of serine, histidine, and aspartate. Such triads are frequently found in hydrolytic enzyme families of diverse substrate specificities and overall structures. If this speculation is correct and the copines possess enzymatic activity, it is possible that copines might regulate other signaling proteins by altering posttranslational modifications, either through the addition of a modifying group or the removal of such a group by hydrolysis.

Additional Diverse Studies of the Biology of Copines

Recent studies in animal and plant systems have implicated copines in a variety of functional contexts. Although the mechanistic roles of the copines in these systems are not well defined, they presumably rely on the ability of calcium to regulate the C2 domains and on the functions of target proteins interacting with the copines.

In the brain, copine VI (or N-copine for neural copine) is one of a number of proteins that is upregulated in the hippocampus after nerve stimulation with kainic acid (Nakayama et al. 1998). It has therefore been speculated that copine may be involved in synaptic potentiation. In addition, it was determined by two-hybrid analysis that the second C2 domain of copine VI interacts with OS-9, a protein of unknown function that is upregulated in certain cancers (Nakayama et al. 1999).

A copine has been identified in *C. elegans* that interacts genetically with a plasma membrane cation channel (Church and Lambie 2003). The copine may be involved in the trafficking or insertion of the ion channel into the cell membrane since mutation of the copine gene suppresses certain phenotypes of mutations in the ion channel gene.

A *C. elegans* copine is also associated in a multiprotein complex with the nicotinic acetylcholine receptor (Gottschalk et al. 2005). Furthermore, mutation of the copine, or downregulation of its expression by siRNA, reduces the number of receptors in the cell membrane therefore implicating the copine in the process of receptor trafficking or membrane insertion.

Genetic experiments in the green plant *Arabidopsis* have demonstrated that copine plays important roles in controlling cell growth and the hypersensitivity response. In a random screen for mutations causing dwarfism, it was found that plants with a copine mutation grow to a small size (hence the proposed name BON1 (Bonsai) for the copine gene) (Hua et al. 2001). In independent studies, a mutant *Arabidopsis* plant was isolated that was hypersensitive to low humidity and had enhanced apoptotic responses to stress (Jambunathan et al. 2001). The mutation was mapped to the same copine gene responsible for dwarfism. Green plants have multiple copine genes, similar to the situation in mammals. Therefore, the observation that modification of a single plant copine gene has significant phenotypic effects suggests that copines are not redundant in function.

Dictyostelium discoidem has six copine genes that are differentially regulated during development, indicating the corresponding gene products may each have unique functions (Damer et al. 2005). A knockout of the gene for copine A results in defects in cytokinesis and contractile vacuole function, suggesting roles in membrane biogenesis or function (Damer et al. 2007). In addition, the knockout model has a partial arrest in a late developmental stage.

Copine I appears to regulate signaling from the TNF-α receptor (Tomsig et al. 2004). ▶ TNF-α is a potent cytokine that elicits critical biological responses such as inflammation and apoptosis. One specific role of copine in this context is apparently to confer calcium sensitivity on the TNF-α//NF- κB signaling pathway. This leads to cross talk with signaling pathways that introduce calcium into the cytoplasm of cells such as activation of muscarinic receptors coupled to phospholipase C.

Copine III has recently been found in association with ErB2, the human epidermal growth factor receptor 2 in breast cancer cells, where it may play a role in regulation of cancer cell motility (Heinrich et al. 2010).

Summary

The copines are a family of calcium-dependent, membrane-binding proteins that consists of nine different gene products in humans. Homologous proteins are expressed in most plants, animals, and protists. The copines are soluble proteins that associate with

negatively charged phospholipids in a calcium-dependent fashion through the action of two C2 domains in the N-terminal half of the molecule. The C-terminal half of the copine molecule has an "A domain," or Von Willebrand domain, that may bind to and recruit target proteins to membrane surfaces. A hypothetical function for the copines is to provide a mechanism for the regulation of various signaling pathways by recruiting components of these pathways to membranes in a calcium-dependent fashion. Additional studies are needed to define the full set of copine-interacting proteins and to determine the effects on these proteins of relocalization or modification by copines.

References

Caudell EG, Caudell JJ, Tang CH, Yu TK, Frederick MJ, Grimm EA. Characterization of human copine III as a phosphoprotein with associated kinase activity. Biochemistry. 2000;39:13034–43.

Church DL, Lambie EJ. The promotion of gonadal dell divisions by the *Caenorhabditis elegans* TRPM cation channel GON-2 is antagonized by GEM-4 copine. Genetics. 2003;165:563–74.

Creutz CE, Tomsig JL, Snyder SL, Skouri F, Beisson J, Cohen J. The copines: a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from Paramecium to humans. J Biol Chem. 1998;273:1393–402.

Damer CK, Bayeva M, Hahn ES, Rivera J, Socec CI. Copine A, a calcium-dependent membrane-binding protein, transiently localizes to the plasma membrane and intracellular vacuoles in Dictyostelium. BMC Cell Biol. 2005;6:46.

Damer CK, Bayeva M, Kim PS, Ho LK, Eberhardt ES, Socec CI, Lee LS, Bruce EA, Goldman-Yassen AE, Naliboff LC. Copine A is required for cytokinesis, contractile vacuole function, and development in Dictyostelium. Eukaryot Cell. 2007;6:430–42.

Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin alpha2beta1. Cell. 2000;101:47–56.

Gerke V, Creutz CE, Moss SE. Annexins: linking Ca2+ signalling to membrane dynamics. Nat Rev Mol Cell Biol. 2005;6:449–61.

Gottschalk A, Almedom RB, Schedletzky T, Anderson SD, Yates 3rd JR, Schafer WR. Identification and characterization of novel nicotinic receptor-associated proteins in *Caenorhabditis elegans*. EMBO J. 2005;24:2566–78.

Heinrich C, Keller C, Boulay A, Vecchi M, Bianchi M, Sack R, Lienhard S, Duss S, Hofsteenge J, Hynes NE. Copine-III interacts with ErbB2 and promotes tumor cell migration. Oncogene. 2010;29:1598–610.

Hua J, Grisafi P, Cheng SH, Fink GR. Plant growth homeostasis is controlled by the Arabidopsis BON1 and BAP1 genes. Genes Dev. 2001;15:2263–72.

Jambunathan N, Siani JM, McNellis TW. A humidity-sensitive Arabidopsis copine mutant exhibits precocious cell death and increased disease resistance. Plant Cell. 2001;13:2225–40.

Lee JO, Rieu P, Arnaout MA, Liddington R. Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). Cell. 1995;80:631–8.

Nakayama T, Yaoi T, Yasui M, Kuwajima G. N-copine: a novel two C2-domain-containing protein with neuronal activity-regulated expression. FEBS Lett. 1998;428:80–4.

Nakayama T, Yaoi T, Kuwajima G, Yoshie O, Sakata T. Ca2(+)-dependent interaction of N-copine, a member of the two C2 domain protein family, with OS-9, the product of a gene frequently amplified in osteosarcoma. FEBS Lett. 1999;453:77–80.

Tomsig JL, Creutz CE. Biochemical characterization of copine: a ubiquitous calcium-dependent, phospholipid-binding protein. Biochemistry. 2000;39:16163–75.

Tomsig JL, Creutz CE. Copines: a ubiquitous family of Calcium-dependent, phospholipid-binding proteins. Cell Mol Life Sci. 2002;59:1467–77.

Tomsig JL, Snyder SL, Creutz CE. Identification of targets for calcium signalling through the copine family of proteins. Characterization of a coiled-coil copine-binding motif. J Biol Chem. 2003;278:10048–54.

Tomsig JL, Sohma H, Creutz CE. Calcium-dependent regulation of tumour necrosis factor-alpha receptor signalling by copine. Biochem J. 2004;378:1089–94.

COT

► TPL2

cPLA2

► Phospholipase A_2

c-Raf

► RAF-1 (C-RAF)

c-Raf-1

► RAF-1 (C-RAF)

CREB

Nan Yagishita-Kyo[1], Masatoshi Inoue[1], Mio Nonaka[1], Hiroyuki Okuno[1] and Haruhiko Bito[1,2]
[1]Department of Neurochemistry, The University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan
[2]CREST–Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

Synonyms

CREB1

Historical Background

Cyclic AMP-responsive element binding protein (CREB) is a transcription factor that was originally discovered and characterized from studies of hormone-induced cAMP-dependent regulation of cellular functions. In particular, CREB was found to be critical for stimulus-induced somatostatin upregulation downstream of glucagon and epinephrine. The mechanism of hormone-induced cAMP-dependent increase in somatostatin gene transcription was narrowed down to a 8-bp element in the somatostatin promoter region, which was named cAMP-responsive element (CRE). CREB was identified as a protein factor that bound to CRE sequence, and was purified to homogeneity from nuclear extracts using a CRE affinity chromatography. CREB binds to a specific palindromic CRE sequence, 5′-TGACGTCA-3′ as well as a half-site CRE motif (5′-TGACG-3′) (Montminy and Bilezikjian 1987; Mayr and Montminy 2001). Early experiments identified that CRE-bound CREB was in a dimerized state through binding of the C-terminal basic and leucine zipper domain (bZIP), and when the intracellular level of the second-messenger cAMP increased upon various hormonal stimuli, this in turn triggered cAMP-dependent gene transcription in the nucleus via cAMP/PKA-dependent phosphorylation of CREB (Gonzales and Montminy 1989; Mayr and Montminy 2001). Several other intracellular signaling pathways, such as CaMKs and several MAPK-dependent cascades, have also been shown to determine CREB phosphorylation status (Shaywitz and Greenberg 1999; Bito and Takemoto-Kimura 2003). Through regulation of CRE-dependent gene expression, CREB mediates cell growth, survival, death, proliferation, and differentiation, in response to a variety of stimuli in many different cell types. CREB function has especially been studied in the nervous system, among other organs. In the central nervous system, CREB is considered to have a major role in long-term memory formation.

More than 5% of mammalian genes appear to be potentially regulated by CREB-dependent transcription, as determined by genome-wide analyses of CREB-bound promoters (Impey et al. 2004; Zhang et al. 2005). The presence of CRE sequences have been reported within putative promoter elements that drives transcription of numerous functional proteins such as transcription factors (e.g., c-Fos, Egr-1, Per1, and CCAAT-enhancer-binding protein β), cellular metabolic enzymes (e.g., cytochrome c, phosphoenolpyruvate carboxykinase, cyclooxygenase-2, superoxide dismutase 2, and Bcl-2), growth factors and neuropeptides (e.g., somatostatin, enkephalin, brain-derived neurotrophic factor, insulin-like growth factor, fibroblast growth factor 6, and vasopressin), as well as many neuronal proteins (e.g., synapsin I, TrkB).

Gene and Protein Structure

Creb1 gene consists of at least 12 exons, several of which are alternatively spliced, resulting in a variety of isoforms. CREB expression is rather ubiquitous. The major isoforms of CREB are α- and Δ-CREB in most tissues. The β isoform is also expressed but at much lower levels, and can be upregulated when other isoforms are downregulated. α-CREB is longer than Δ-CREB, by the presence of 14-residue-long insert, termed α-peptide. Alternative splicing of several 5′-exons generates β-CREB, which lacks the first 40 amino acid residues of CREB protein. α-CREB, Δ-CREB, and β-CREB are all able of activating CRE-dependent transcription in response to cAMP increase, while several other splice variants, lacking the C-terminal bZIP domain, have been shown to generate inhibitory forms of CREB.

The characteristic functional domains of CREB are: an N-terminal glutamine-rich domain (Q1), a kinase-inducible domain (KID) with a phosphorylation site at Ser-133, another glutamine-rich domain (Q2), and a C-terminal bZIP domain (Mayr and Montminy 2001).

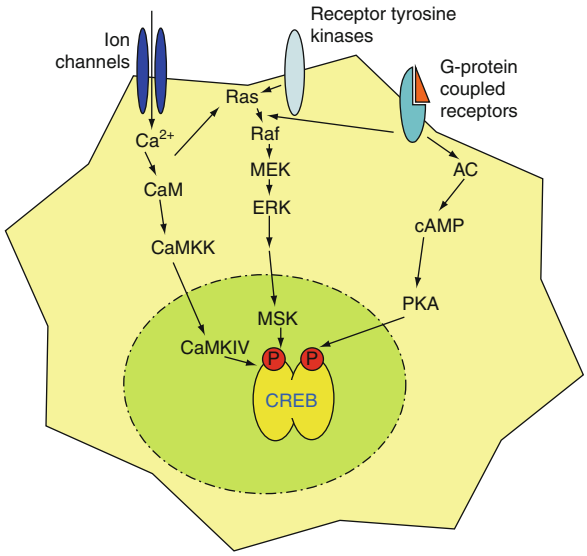
Activating transcription factor 1 (ATF1) and cAMP-responsive element modulator (CREM) share structural and functional similarities with CREB as regulators of CRE-dependent gene transcription, and form a CREB/ATF family.

CREM has an extensive sequence identity with CREB and has a notably interesting function. This gene generates both activator and repressor forms of CREM from the same gene by alternative splicing. The CREM isoforms α, β, and γ function as inhibitors of CREB- and cAMP-mediated transcription. However, another variant which is most similar to CREB with regards to its amino acid sequence, CREMτ, functions as an activator of CRE-mediated transcription.

Mechanistic Basis of CREB-Mediated Transcriptional Activation

CREB activity, as a transcription factor, is predominantly upregulated by phosphorylation of Ser-133 (amino acid residue number based on the full-length α-CREB) (Gonzalez and Montminy 1989). In the nucleus, a sizable amount of CREB is constitutively bound to CRE sites in the chromatin, regardless of cellular activity. The Ser-133 residue is located in the KID domain, and phosphorylation of this residue favors a structural conformation that allows high-affinity interaction with a KID-interacting (KIX) domain of the transcriptional coactivator CREB-binding protein (CBP), thus enabling an efficient docking of the transcriptional preinitiation complex (Chrivia et al. 1993). The signaling events that lead to phosphorylation of Ser-133 have been intensively investigated in many cell types and tissues which were exposed to many physiological and pathological stimuli (Mayr and Montminy 2001). The identity of a CREB kinase in a given context heavily depends on the stimulus conditions and cell types. Demonstrated functional CREB kinase pathways include: cAMP-dependent protein kinase (PKA), protein kinase C (PKC), RSK, MSK, MAP kinase-activated protein-kinases (MAPKAPK1/2), AKT, and CaMKI, CaMKII, and CaMKIV (Gonzales and Montminy 1989; Bito et al. 1996; Shaywitz and Greenberg 1999) (Fig. 1).

Compared with the variety of kinase-signaling pathways activating CREB, much less information is available about phosphatases that dephosphorylate pSer133. Either Ser/Thr protein phosphatase 1 (PP-1)

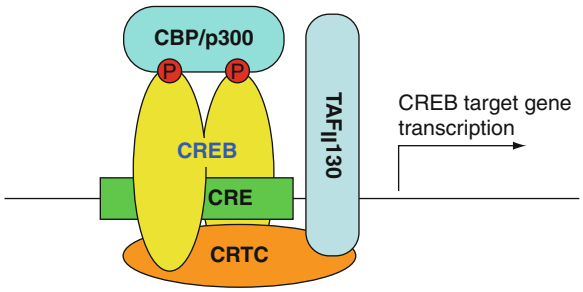


CREB, Fig. 1 *Signaling from the cell surface to nuclear CREB.* Cell growth, hormonal or neurotransmitter signals trigger CREB phosphorylation at residue Ser-133 through activation of either Ras-Raf-MEK-ERK-MSK, adenylate cyclase (AC)-cAMP-PKA or Ca^{2+}/CaM-CaMKK-CaMKIV cascades, respectively. This phosphorylation allows recruitment and stable anchoring of a CBP-containing transcription initiation complex near CRE sites

or PP-2A are shown to dephosphorylate CREB directly at Ser133 after pCREB dissociates from CBP, depending on the cell types. Several studies indicate that other phosphatases such as calcineurin (PP2B), PTEN, and protein tyrosine phosphatase 1B (PTP1B) may indirectly regulate dephosphorylation of phospho-Ser133, either by suppressing upstream CREB kinases or activating CREB phosphatases.

CREB Coactivators and CREB-Interacting Proteins

It has been shown that CREB constitutively binds functional CRE sites of many genes, even prior to cAMP elevation or Ser-133 phosphorylation events. Thus, the dominant effect of CREB phosphorylation at Ser-133 is to potentiate its transcriptional activity via enhanced interaction of CREB with CBP and its paralog p300. The binding of CBP/p300 to CREB leads to the recruitment of the RNA polymerase II complex to initiate transcription from transcriptional start sites that are adjacent to the CRE sites. Hence, the regulated CREB-CBP interaction via the KID and KIX domains plays a major



CREB, Fig. 2 *A CREB complex binds CRE to initiate transcription.* CREB homodimerizes and binds to a well-conserved DNA element named CRE. CREB Ser-133 is phosphorylated by several kinases in an activity-dependent manner, and this accelerates binding of CBP/p300 to CREB. The CREB coactivator CRTC binds to the bZIP domain of CREB. Recruitment of an RNA polymerase II complex may be facilitated by a higher affinity of CBP/p300 to phospho-CREB or by CRTC via $TAF_{II}130$

role in determining the level of CRE-dependent transcription activity. Additionally, the CREB Q2 domain recruits $TAF_{II}130$ present in the TFIID complex and may contribute to regulating CRE transcription in a rather stimulus-independent manner (Ferreri et al. 1994). An N-terminal glutamine-rich domain (Q1) may also contribute to transcription activity, but the extent of this has not been extensively demonstrated. While CREMτ, a transcription activator among the CREM isoforms, and which is highly similar to CREB protein, includes both Q1 and Q2 domains, CREM repressor isoforms possess no domains sharing sequence identities with either Q1 or Q2 regions. These contrasting facts also support the notion that both Q1 and Q2 may play additional roles in CREB transcription activation.

Both CREB homodimerization and CREB binding to the double-stranded DNA of CRE loci are mediated by the C-terminal bZIP domain. However, this bZIP domain also binds to CREB-regulated transcription coactivators (CRTCs, also known as transducers of regulated CREB activity, or TORCs) (Conkright et al. 2003; Iourgenko et al. 2003). Structurally, the Arg-314 residue in the bZIP domain of CREB is absolutely required to associate with the N-terminal coiled-coil region of CRTC, and this N-terminal end of CRTC may also be important for its tetramerization. CRTC also appears to recruit $TAF_{II}130$ and this may be another potential mechanism for enhancing CREB-regulated gene expression, though a detailed picture of how CRTC activates CREB is yet missing (Fig. 2).

The CREB coactivator role of CRTC is stimulated by CRTC dephosphorylation, which enables its nuclear entry. On the other hand, phosphorylated CRTC, which is excluded from the nucleus, localizes to the cytoplasm and remains in an inactive state. The nucleocytoplasmic shuttling of CRTC is strongly regulated by a balance between calcineurin-dependent dephosphorylation that occurs as a result of cellular calcium mobilization, and various regulated kinases (such as SIK and AMPK) that inhibit CRTC function (Screaton et al. 2004; Bittinger et al. 2004). Thus, CRTC appears to be a valuable sensor of intracellular milieu that can influence CREB function in a manner that is independent of phospho-CREB formation.

Physiological Functions of CREB

Physiological functions of CREB have been investigated in various organs using genetically modified model animals. In particular, in vivo CREB actions have been intensively studied in the developing and mature nervous and immune systems, as well as in the control of hepatic gluconeogenesis, insulin resistance in obesity, and oncogenesis (Silva et al. 1998; Kandel 2001; Bito and Takemoto-Kimura 2003; Conkright and Montminy 2005; Nonaka 2009).

A full CREB-null phenotype was obtained in knockout mice in which the bZIP domain of CREB gene was deleted. These CREB-null mutants were smaller than wild-type littermates and died immediately after birth due to respiratory distress. The commissural fibers in the brain were markedly reduced. There were also severe impairments in the development of T cells of the α/β lineage but not those of the γ/δ lineage (Lonze et al. 2002). A double mutant mouse line harboring mutations in both CREB and CREM genes showed a more severe neurodegeneration phenotype during brain development, suggesting that CREM gene products may compensate for the loss of CREB gene, especially in the developing central nervous system (Mantamadiotis et al. 2002). A dominant negative CREB inhibitory peptide (A-CREB) that interferes with the bZIP domain function of CREB (Ahn et al. 1998) has also been successfully used to probe CREB function in various organs such as liver, adipocyte, bone, skin, and brain, using tissue-specific promoters. In particular, liver-targeted A-CREB

expression has demonstrated the critical involvement of CREB in regulating hepatic gluconeogenesis (Dentin et al. 2007).

Interestingly, a CREB mutant mouse line, in which the exon 1 of CREB1 gene was deleted, showed a loss of both α- and Δ-isoforms of CREB, but a compensatory expression of both β-CREB (transcribed in this case from a cryptic alternate promoter) and CREMτ was found in a wide range of organs, except for the brain. These CREB mutant mice showed no obvious developmental abnormality in the body and also no obvious anatomical disorders in the brain. However, when hippocampus-dependent memory tasks were carried out, these adult mice exhibited a profound specific impairment in long-term memory while the short-term memory remained unaltered (Silva et al. 1998).

In order to investigate the roles of CREB activity in hippocampus, brain-targeted VP16-CREB transgenic mice have been generated (Barco et al. 2002). The hippocampal CA1 neurons of these transgenic mice exhibited a strong facilitation of a persistent late phase of long-term potentiation (L-LTP), which could be elicited even with weak stimuli that would otherwise only induce an early phase of LTP (E-LTP) in the wild-type mice. The results indicate that elevation of CRE-driven gene products in the brain might be sufficient to accelerate consolidation of LTP, and are consistent with a "synaptic tagging and capture" hypothesis.

Finally, from recent studies, it appears that aberrant CREB activity may be linked to oncogenesis and the etiology of certain leukemia (Conkright and Montminy 2005). Enhanced CREB expression or dominant active mutation in CRTC1 was found in primary human cancer or leukemic cells. Consistently, a transgenic mouse line, which overexpressed CREB specifically in the macrophage/monocyte lineage, exhibited increased proliferation of bone marrow cell in a growth factor–independent manner.

Summary

CREB is a well-studied transcription factor that is ubiquitously expressed, and is activated by a variety of cellular signaling pathways, downstream of cell growth, hormonal or neurotransmitter stimuli. Mobilization of various second-messenger pathways leads to CREB activation via phosphorylation of Ser-133, and this event is regulated by many kinases such as PKA, CaMKIV, or MSK, as a function of the activation context. CREB has many coactivators that potentiate its transcriptional activity. The phosphorylation of Ser-133 in the KID domain of CREB facilitates the binding of the KIX domain of CBP/p300 to KID, thereby stabilizing the recruitment of RNA polymerase II complex and allowing transcriptional initiation. CRTC is another important coactivator and interacting protein of CREB. CREB plays a key role in determining cellular proliferation in the developing nervous and immune systems, while also controlling protein-synthesis-dependent forms of long-term memory in the mature brain. Furthermore, CREB is essential in the control of hepatic gluconeogenesis, insulin resistance in obesity, and also in oncogenesis.

References

Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C. A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol. 1998;18:967–77.

Barco A, Alarcon JM, Kandel ER. Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell. 2002;108:689–703.

Bito H, Takemoto-Kimura S. Ca^{2+}/CREB/CBP-dependent gene regulation: a shared mechanism critical in long-term synaptic plasticity and neuronal survival. Cell Calcium. 2003; 34:425–30.

Bito H, Deisseroth K, Tsien RW. CREB phosphorylation and dephosphorylation: a Ca^{2+}- and stimulus duration-dependent switch for hippocampal gene expression. Cell. 1996;87:1203–14.

Bittinger MA, McWhinnie E, Meltzer J, Iourgenko V, Latario B, Liu X, Chen CH, Song C, Garza D, Labow M. Activation of cAMP response element-mediated gene expression by regulated nuclear transport of TORC proteins. Curr Biol. 2004;14:2156–61.

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature. 1993;365:855–9.

Conkright MD, Montminy M. CREB: the unindicted cancer co-conspirator. Trends Cell Biol. 2005;15:457–9.

Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M. TORCs: transducers of regulated CREB activity. Mol Cell. 2003;12:413–23.

Dentin R, Liu Y, Koo SH, Hedrick S, Vargas T, Heredia J, Yates 3rd J, Montminy M. Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. Nature. 2007;449:366–9.

Ferreri K, Gill G, Montminy M. The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. Proc Natl Acad Sci USA. 1994;91:1210–3.

Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell. 1989;59:675–80.

Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, McWeeney S, Dunn JJ, Mandel G, Goodman RH. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell. 2004; 119:1041–54.
Iourgenko V, Zhang W, Mickanin C, Daly I, Jiang C, Hexham JM, Orth AP, Miraglia L, Meltzer J, Garza D, Chirn GW, McWhinnie E, Cohen D, Skelton J, Terry R, Yu Y, Bodian D, Buxton FP, Zhu J, Song C, Labow MA. Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. Proc Natl Acad Sci USA. 2003; 100:12147–52.
Kandel ER. The molecular biology of memory storage: a dialogue between genes and synapses. Science. 2001;294:1030–8.
Lonze BE, Riccio A, Cohen S, Ginty DD. Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron. 2002;34:371–85.
Mantamadiotis T, Lemberger T, Bleckmann SC, Kern H, Kretz O, Martin Villalba A, Tronche F, Kellendonk C, Gau D, Kapfhammer J, Otto C, Schmid W, Schütz G. Disruption of CREB function in brain leads to neurodegeneration. Nat Genet. 2002;31:47–54.
Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol. 2001;2:599–609.
Montminy MR, Bilezikjian LM. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature. 1987;328:175–8.
Nonaka M. A Janus-like role of CREB protein: enhancement of synaptic property in mature neurons and suppression of synaptogenesis and reduced network synchrony in early development. J Neurosci. 2009;29:6389–91.
Screaton RA, Conkright MD, Katoh Y, Best JL, Canettieri G, Jeffries S, Guzman E, Niessen S, Yates 3rd JR, Takemori H, Okamoto M, Montminy M. The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. Cell. 2004;119:61–74.
Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem. 1999;68:821–61.
Silva AJ, Kogan JH, Frankland PW, Kida S. CREB and memory. Annu Rev Neurosci. 1998;21:127–48.
Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci USA. 2005;102:4459–64.

CREB (Cyclic AMP-Responsive Element-Binding Protein)

► p38 MAPK Family of Signal Transduction Proteins

CREB1

► CREB

crg-2

► CXCL10

CRG-5

► ATF3 Activating Transcription Factor 3

CRHSP-28 (Rat)

► TPD52 (Tumor Protein D52)

Crk-Associated Substrate (CAS)

► p130Cas

Csk

Masato Okada
Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

Synonyms

C-Src kinase; C-terminal Src kinase

Historical Background

The activity of the ► Src family tyrosine kinases (SFKs), known as representative proto-oncogene products, is negatively regulated by the phosphorylation at

their C-terminal regulatory tyrosine (Brown and Cooper 1996; Cooper et al. 1986). The protein tyrosine kinase Csk was identified as a specific kinase that directs the negative regulatory sites of SFKs (Nada et al. 1991; Okada and Nakagawa 1988). Analysis of Csk-deficient mice and cells provided evidence that Csk functions as an indispensable negative regulator of SFKs (Nada et al. 1991, 1993). The molecular basis of Csk-SFK interaction is recently verified by the crystal structure of Csk/c-Src complex (Levinson et al. 2008). Csk is expressed ubiquitously, but is highly concentrated in developing nervous system and the immune system (Okada et al. 1991). Csk is highly conserved in animal kingdom from the unicellular choanoflagellate to human in parallel with SFKs (Segawa et al. 2006). Csk is a cytosolic protein, consisting of the kinase domain and the conserved domains responsible for the protein-protein interaction (Ogawa et al. 2002) (Fig. 1). Several Csk-binding proteins, for example, Cbp/PAG1 and paxillin, were identified as specific scaffolds that recruit Csk to the sites where SFK is activated (Kawabuchi et al. 2000; Oneyama et al. 2008a; Sabe et al. 1994). The Csk-mediated inhibition of SFKs is crucial for suppressing oncogenic ability of SFKs (Oneyama et al. 2008b).

Structure of Csk

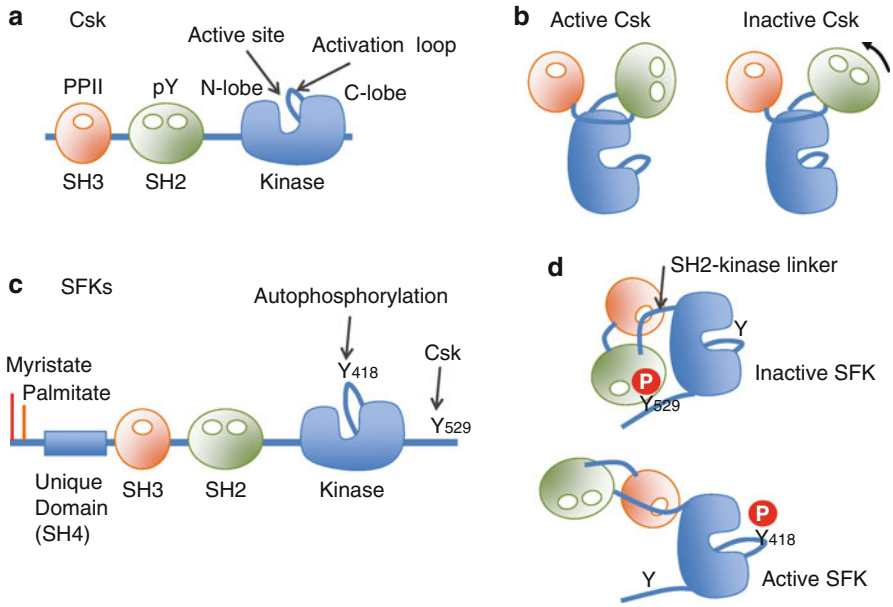
Csk is a non-receptor type of protein tyrosine kinase with a molecular mass of 50 kDa. It contains a Src homology 3 (SH3) and a ▶ Src homology 2 (SH2) domains in its N-terminal half and a kinase domain in its C-terminus. This primary structural arrangement of functional domains is similar to that of SFKs, but Csk lacks the N-terminal fatty acylation sites, the autophosphorylation site in the activation loop, and the C-terminal negative regulatory sites, all of which are crucial for regulating SFK activity (Fig. 1). The lack of autophosphorylation is a unique feature as a protein tyrosine kinase. The crystal structures of inactive and active forms of Src reveal the regulatory mechanism of SFKs (Cowan-Jacob et al. 2005; Xu et al. 1997). Upon phosphorylation at the C-terminal tyrosine, SFKs adopt the inactive conformation stabilized by two intramolecular inhibitory interactions: (1) binding of the C-terminal phosphotyrosine to the SH2 domain and (2) binding of the SH2-kinase linker to the SH3 domain. The dephosphorylation of the C-terminal tyrosine results in an open structure where the kinase domain adopts an active conformation (Fig. 1).

The crystal structure of Csk reveals significantly different dispositions of the functional domains from those of SFKs (Ogawa et al. 2002), indicating that Csk is regulated differently to SFKs (Fig. 1). The most intriguing difference in the domain organization between Csk and SFKs is that the binding pockets of SH3 and SH2 domains of Csk are oriented outward, enabling the intermolecular interactions. The crystal structure of Csk further predicts that Csk can adopt active and inactive conformations. It is suggested that the kinase domain of Csk is intrinsically inactive, but the direct interaction with the SH2 domain induces conformational change, resulting in an upregulation of the kinase activity (Wong et al. 2005).

Function of Csk

Accumulated evidence shows that SFKs are the major physiological substrates of Csk. Csk-deficient mice exhibit early embryonic lethality, accompanied by a constitutive activation of c-Src, Fyn, and Lyn (Nada et al. 1993). Conditional mutagenesis of the *csk gene* in specific tissues causes severe defects that are associated with constitutive activation of SFKs. Loss of Csk induces dysfunction in acute inflammatory responses (Thomas et al. 2004) and T-cell development (Schmedt et al. 1998), hyperplasia of the epidermis (Yagi et al. 2007), and defects in cell adhesion and migration (Nada et al. 1994). Even in invertebrates, such as *Drosophila* and *C. elegans*, the loss of Csk leads to constitutive activation of SFKs, causing hyperproliferation and defective cytoskeletal function, respectively (Read et al. 2004; Takata et al. 2009). These findings indicate that Csk is an indispensable regulator of SFKs.

As a protein tyrosine kinase, Csk has an exceptionally high specificity for the C-terminal regulatory tyrosine (Y527) of SFKs. The surrounding sequence of the regulatory site of SFKs (QYQ) is unique and well conserved among SFKs, but biochemical and structural studies reveal that a region (aa 504–525) located distantly from Y527 is rather crucial for specific recognition by Csk (Lee et al. 2003, 2006; Levinson et al. 2008). In addition to SFKs, several signaling proteins have been reported to serves as substrates of Csk.

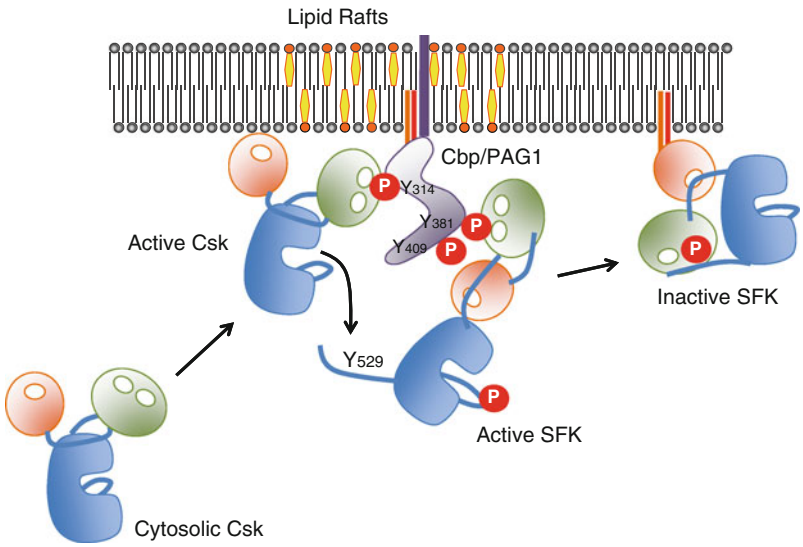


Csk, Fig. 1 Structural features of SFKs and Csk. (**a**) Domain organization of Csk. SH3; Src homology 3 domain, SH2; Src homology 2 domain. The ligands of SH3 and SH2 domains, PPII and pY, respectively are indicated. PPII; the polyproline type II helical structures, pY; the phosphotyrosine-containing protein ligands. The kinase domain consists of N-lobe and C-lobe. Active site and activation loop are indicated. (**b**) Schematic representation of the crystal structure of Csk. The binding pockets of SH3 and SH2 domains of Csk are oriented outward enabling the intermolecular interactions. Csk adopts active and inactive conformations. The 60° rotation of the SH2 domain, associated with the active-inactive form transition, is indicated by an *arrow*. (**c**) Domain organization of SFKs. SFKs has fatty acyl moieties (myristate and/or palmitate) and the non-conserved unique domains (SH4) in the N-terminal region. The autophosphorylation site (Y418) in the activation loop and the C-terminal negative regulatory site (Y529) are indicated. (**d**) Schematic representation of the inactive and active structures of SFKs based on the crystal structures of c-Src and Hck. The intramolecular interactions between pY529 and SH2 domain and between SH2-kinase linker and SH3 domain stabilize the inactive conformation. Dephosphorylation of pY529 unlocks the inactive conformation and the trans-autophosphorylation at Y418 makes the enzyme fully active

Those include paxillin (Sabe et al. 1994), $P2X_3$ receptor (D'Arco et al. 2009), c-Jun (Zhu et al. 2006), and Lats (Stewart et al. 2003). However, the physiological relevance of the phosphorylation of these proteins still remains unclear.

Regulation of Csk

Csk is predominantly present in cytosol due to the lack of fatty acyl modification, while its substrate SFKs are anchored to the membrane via the N-terminal myristate and palmitate moieties. Thus, the translocation of Csk to the membrane, where SFKs are activated, is one of the critical steps of Csk regulation (Howell and Cooper 1994). So far several scaffolding proteins have been shown to serve as anchors of Csk to the membrane. A well-characterized example is Cbp/PAG1 (Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched membrane) that is a transmembrane protein having both myristoyl and palmitoyl modifications like SFKs. Cbp/PAG1 is highly concentrated in the cholesterol-enriched membrane microdomain "lipid rafts" and serves an excellent substrate of SFKs. Upon phosphorylation of Y314 of Cbp/PAG1 by the activated SFKs, pY314 binds to the SH2 domain of Csk, and this in turn recruits Csk to the plasma membrane. Csk on the plasma membrane then efficiently inactivates SFKs that are also recruited to Cbp/PAG1. This negative feedback loop is crucial in preventing tumorigenesis and in controlling the cell signaling evoked by the activation of growth factor receptors (Fig. 2). Other Csk-binding proteins, such as paxillin (Sabe et al. 1994) and caveorin-1 (Cao et al. 2002), are also phosphorylated by SFKs and are involved in the negative regulation of SFKs by recruiting Csk to the sites where SFKs are activated in a manner similar to Cbp/PAG1.



Csk, Fig. 2 Negative regulatory loop of SFK via Cbp/PAG1 on lipid rafts. When SFKs are activated in response to cell stimulation, they phosphorylate Cbp/PAG1 residing in lipid rafts to create a binding site of Csk (pY314) and their own binding sites (pY381/409). Inactive Csk in cytosol is then recruited to the phosphorylated Cbp/PAG1, and the activated Csk efficiently phosphorylates Y529 of SFKs that are also recruited to Cbp/PAG1. The phosphorylated SFKs are released from Cbp/PAG1 by restoring the intramolecular interaction between SH2 domain and pY529

The binding of scaffolds to the SH2 domain of Csk can also activate the enzyme activity of Csk. The occupation of the SH2 domain of Csk by phosphorylated Cbp affects conformation of the catalytic domain, thereby increasing the activity toward SFKs (Takeuchi et al. 1993; Wong et al. 2004, 2005). Thus, it is likely that the scaffold proteins positively regulate Csk functions not only by recruiting Csk to the membrane but also by directly activating Csk.

It is also reported that the activity of Csk can be regulated by the oxidation state of the disulfide bond in the SH2 domain, suggesting the regulation mechanism by the redox state (Mills et al. 2007). Furthermore, there is a report indicating that Csk is phosphorylated by PKA at S364, resulting in an increase in kinase activity (Yaqub et al. 2003). However, their physiological relevance has not yet been addressed. Although the expression of Csk is substantially high in the developing nervous system and lymphoid cells, the mechanisms underlying the regulation of Csk at the expression levels are thoroughly unknown.

Csk in Diseases

Since Csk has a tumor-suppressive function by inhibiting oncogenic activity of SFKs, it is reasonable that Csk is involved in human cancer. Although there are some reports suggesting that Csk is downregulated in some cancers (Masaki et al. 1999), it seems that Csk downregulation is rather rare and it is more likely that Csk is expressed in various cancer cells at a comparable level as a housekeeping protein. In contrast, it is clear that the expression of Cbp/PAG1 is appreciably downregulated in a variety of cancer cells (Oneyama et al. 2008a), potentially via the epigenetic mechanism (Suzuki et al. 2011). The downregulation of Cbp/PAG1 may interfere with the translocation of Csk to the membrane, thereby upregulating SFK functions. This mechanism may account for the upregulation of SFKs in some cancer cells. Thus, the further analysis of Cbp-/PAG1-mediated regulatory system would provide new opportunities for therapeutic intervention in cancer.

Summary

The non-receptor tyrosine kinase Csk serves as an indispensable negative regulator of SFKs, by specifically phosphorylating the negative regulatory site of SFKs to suppress their oncogenic potential. Csk is mainly regulated through its SH2 domain, which is required for membrane translocation of Csk via

binding to scaffold proteins such as Cbp/PAG. The binding of scaffolds to the SH2 domain can also upregulate the kinase activity. These regulatory features are mostly clarified by the analysis of Csk structure at atomic levels. Although Csk itself is not directly relevant to human cancer, the perturbation of the regulation system of SFKs, which consists of Csk, Cbp/PAG1, or other scaffolds, and some tyrosine phosphatases, would be attributed to the upregulation of SFKs which is frequently observed in human cancers.

References

Brown MT, Cooper JA. Regulation, substrates and functions of src. Biochim Biophys Acta. 1996;1287:121–49.

Cao H, Courchesne WE, Mastick CC. A phosphotyrosine-dependent protein interaction screen reveals a role for phosphorylation of caveolin-1 on tyrosine 14: recruitment of C-terminal Src kinase. J Biol Chem. 2002;277:8771–4.

Cooper JA, Gould KL, Cartwright CA, Hunter T. Tyr527 is phosphorylated in pp 60c-src: implications for regulation. Science. 1986;231:1431–4.

Cowan-Jacob SW, Fendrich G, Manley PW, Jahnke W, Fabbro D, Liebetanz J, Meyer T. The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. Structure. 2005;13:861–71.

D'Arco M, Giniatullin R, Leone V, Carloni P, Birsa N, Nair A, Nistri A, Fabbretti E. The C-terminal Src inhibitory kinase (Csk)-mediated tyrosine phosphorylation is a novel molecular mechanism to limit P2X3 receptor function in mouse sensory neurons. J Biol Chem. 2009;284:21393–401.

Howell BW, Cooper JA. Csk suppression of Src involves movement of Csk to sites of Src activity. Mol Cell Biol. 1994;14:5402–11.

Kawabuchi M, Satomi Y, Takao T, Shimonishi Y, Nada S, Nagai K, Tarakhovsky A, Okada M. Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature. 2000;404:999–1003.

Lee S, Lin X, Nam NH, Parang K, Sun G. Determination of the substrate-docking site of protein tyrosine kinase C-terminal Src kinase. Proc Natl Acad Sci U S A. 2003;100:14707–12.

Lee S, Ayrapetov MK, Kemble DJ, Parang K, Sun G. Docking-based substrate recognition by the catalytic domain of a protein tyrosine kinase, C-terminal Src kinase (Csk). J Biol Chem. 2006;281:8183–9.

Levinson NM, Seeliger MA, Cole PA, Kuriyan J. Structural basis for the recognition of c-Src by its inactivator Csk. Cell. 2008;134:124–34.

Masaki T, Okada M, Tokuda M, Shiratori Y, Hatase O, Shirai M, Nishioka M, Omata M. Reduced C-terminal Src kinase (Csk) activities in hepatocellular carcinoma. Hepatology. 1999;29:379–84.

Mills JE, Whitford PC, Shaffer J, Onuchic JN, Adams JA, Jennings PA. A novel disulfide bond in the SH2 domain of the C-terminal Src kinase controls catalytic activity. J Mol Biol. 2007;365:1460–8.

Nada S, Okada M, MacAuley A, Cooper JA, Nakagawa H. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. Nature. 1991;351:69–72.

Nada S, Yagi T, Takeda H, Tokunaga T, Nakagawa H, Ikawa Y, Okada M, Aizawa S. Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell. 1993;73:1125–35.

Nada S, Okada M, Aizawa S, Nakagawa H. Identification of major tyrosine-phosphorylated proteins in Csk-deficient cells. Oncogene. 1994;9:3571–8.

Ogawa A, Takayama Y, Sakai H, Chong KT, Takeuchi S, Nakagawa A, Nada S, Okada M, Tsukihara T. Structure of the carboxyl-terminal Src kinase, Csk. J Biol Chem. 2002;277:14351–4.

Okada M, Nakagawa H. Identification of a novel protein tyrosine kinase that phosphorylates pp 60c-src and regulates its activity in neonatal rat brain. Biochem Biophys Res Commun. 1988;154:796–802.

Okada M, Nada S, Yamanashi Y, Yamamoto T, Nakagawa H. CSK: a protein-tyrosine kinase involved in regulation of src family kinases. J Biol Chem. 1991;266:24249–52.

Oneyama C, Hikita T, Enya K, Dobenecker MW, Saito K, Nada S, Tarakhovsky A, Okada M. The lipid raft-anchored adaptor protein Cbp controls the oncogenic potential of c-Src. Mol Cell. 2008a;30:426–36.

Oneyama C, Hikita T, Nada S, Okada M. Functional dissection of transformation by c-Src and v-Src. Genes Cells. 2008b;13:1–12.

Read RD, Bach EA, Cagan RL. Drosophila C-terminal Src kinase negatively regulates organ growth and cell proliferation through inhibition of the Src, Jun N-terminal kinase, and STAT pathways. Mol Cell Biol. 2004;24:6676–89.

Sabe H, Hata A, Okada M, Nakagawa H, Hanafusa H. Analysis of the binding of the Src homology 2 domain of Csk to tyrosine-phosphorylated proteins in the suppression and mitotic activation of c-Src. Proc Natl Acad Sci U S A. 1994;91:3984–8.

Schmedt C, Saijo K, Niidome T, Kuhn R, Aizawa S, Tarakhovsky A. Csk controls antigen receptor-mediated development and selection of T-lineage cells. Nature. 1998;394:901–4.

Segawa Y, Suga H, Iwabe N, Oneyama C, Akagi T, Miyata T, Okada M. Functional development of Src tyrosine kinases during evolution from a unicellular ancestor to multicellular animals. Proc Natl Acad Sci U S A. 2006;103:12021–6.

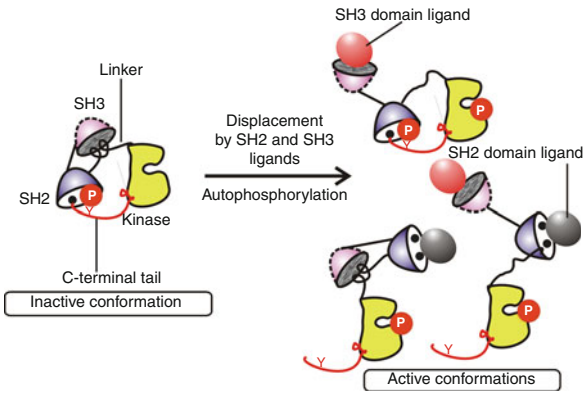
Stewart RA, Li DM, Huang H, Xu T. A genetic screen for modifiers of the lats tumor suppressor gene identifies C-terminal Src kinase as a regulator of cell proliferation in Drosophila. Oncogene. 2003;22:6436–44.

Suzuki K, Oneyama C, Kimura H, Tajima S, Okada M. Downregulation of the tumor suppressor Cbp/PAG1 is mediated by epigenetic histone modifications via the MAPK/PI3K pathway. J Biol Chem. 2011;286(18):15698–706.

Takata N, Itoh B, Misaki K, Hirose T, Yonemura S, Okada M. Non-receptor tyrosine kinase CSK-1 controls pharyngeal muscle organization in *Caenorhabditis elegans*. Genes Cells. 2009;14:381–93.

Takeuchi M, Kuramochi S, Fusaki N, Nada S, Kawamura-Tsuzuku J, Matsuda S, Semba K, Toyoshima K, Okada M, Yamamoto T. Functional and physical interaction of

protein-tyrosine kinases Fyn and Csk in the T-cell signaling system. J Biol Chem. 1993;268:27413–9.
Thomas RM, Schmedt C, Novelli M, Choi BK, Skok J, Tarakhovsky A, Roes J. C-terminal SRC kinase controls acute inflammation and granulocyte adhesion. Immunity. 2004;20:181–91.
Wong L, Lieser S, Chie-Leon B, Miyashita O, Aubol B, Shaffer J, Onuchic JN, Jennings PA, Woods Jr VL, Adams JA. Dynamic coupling between the SH2 domain and active site of the COOH terminal Src kinase, Csk. J Mol Biol. 2004;341:93–106.
Wong L, Lieser SA, Miyashita O, Miller M, Tasken K, Onuchic JN, Adams JA, Woods Jr VL, Jennings PA. Coupled motions in the SH2 and kinase domains of Csk control Src phosphorylation. J Mol Biol. 2005;351:131–43.
Xu W, Harrison SC, Eck MJ. Three-dimensional structure of the tyrosine kinase c-Src. Nature. 1997;385:595–602.
Yagi R, Waguri S, Sumikawa Y, Nada S, Oneyama C, Itami S, Schmedt C, Uchiyama Y, Okada M. C-terminal Src kinase controls development and maintenance of mouse squamous epithelia. EMBO J. 2007;26:1234–44.
Yaqub S, Abrahamsen H, Zimmerman B, Kholod N, Torgersen KM, Mustelin T, Herberg FW, Tasken K, Vang T. Activation of C-terminal Src kinase (Csk) by phosphorylation at serine-364 depends on the Csk-Src homology 3 domain. Biochem J. 2003;372:271–8.
Zhu F, Choi BY, Ma WY, Zhao Z, Zhang Y, Cho YY, Choi HS, Imamoto A, Bode AM, Dong Z. COOH-terminal Src kinase-mediated c-Jun phosphorylation promotes c-Jun degradation and inhibits cell transformation. Cancer Res. 2006;66:5729–36.



CSK-Homologous Kinase, Fig. 1 A model of activation of Src-family kinases (SFKs). Upon phosphorylation of the C-terminal regulatory tyrosine by CSK and CTK, the SFK molecule adopts the inactive conformation. In this conformation, the kinase domain is stabilized in the inactive configuration by two inhibitory intramolecular interactions: (**a**) binding of the SH2 domain to the phosphorylated C-terminal regulatory tyrosine and (**b**) binding of the SH2-kinase linker (linker) to the SH3 domain. Ligands of SH2 and SH3 domains disrupt these interactions and activate SFKs. The activated SFKs then undergo autophosphorylation which further stabilizes the kinase domain in the active configuration

CSK-Homologous Kinase

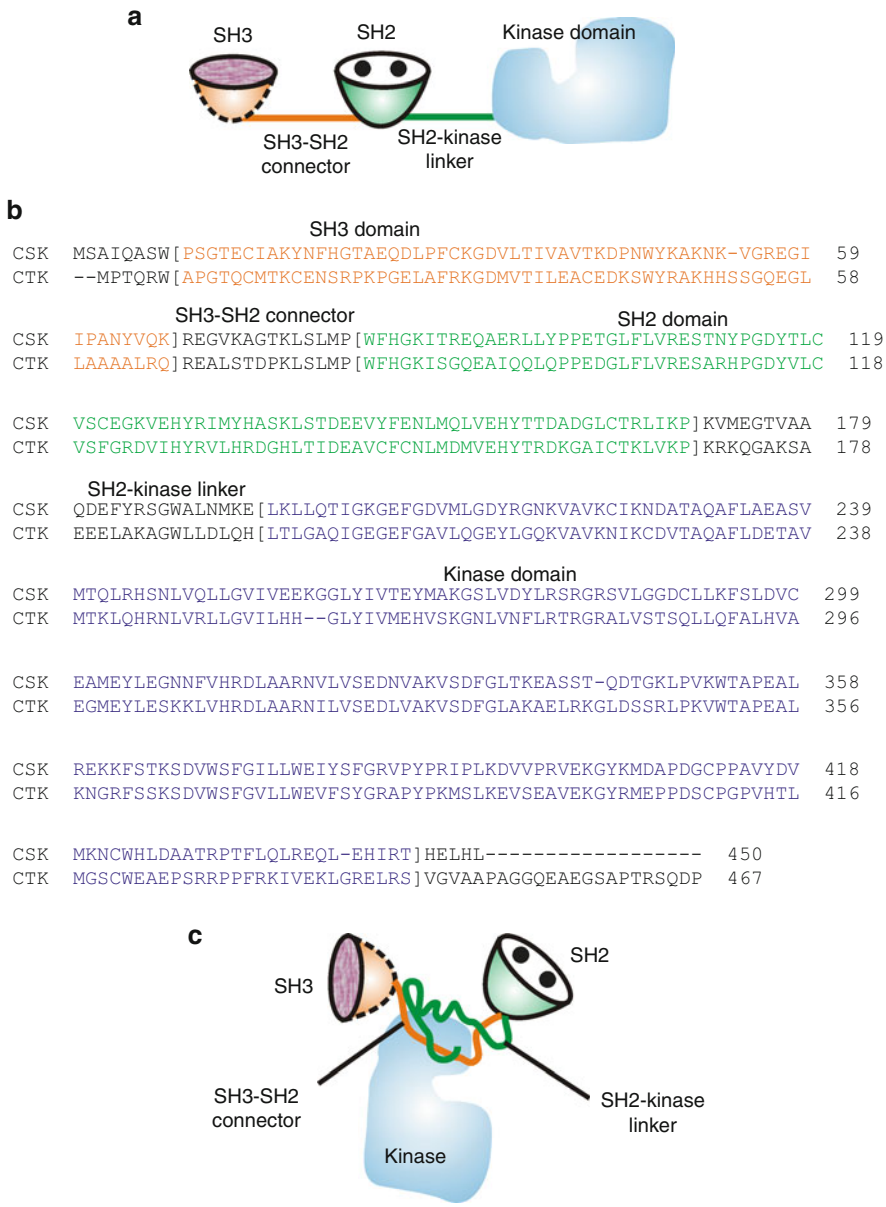
Heung-Chin Cheng[1], Mohammed Iqbal Hossain[1], Mohd Aizuddin Kamaruddin[1] and Yuh-Ping Chong[2]
[1]Department of Biochemistry and Molecular Biology, University of Melbourne Bio21 Molecular Science and Biotechnology Institute, Parkville, VIC, Australia
[2]Edinburgh Cancer Research Centre, Western General Hospital, Edinburgh, UK

Synonyms

BatK; CHK; CTK; Hyl; Lsk; Matk; Ntk

Historical Background

C-terminal Src kinase (► CSK) and CSK-homologous kinase (also referred to as CSK-type kinase, CTK) are the endogenous inhibitors of ► Src family of protein tyrosine kinases (SFKs) including ► c-Src, Fyn, Yes, Lyn, Hck, Blk, Fgr, and Lck. Both inhibitors inactivate SFKs by phosphorylating their consensus C-terminal inhibitory tyrosine (corresponds to Tyr-527 in c-Src) that stabilizes the inactive SFK conformation (Fig. 1). The discovery of CSK can be traced back to an important study performed two decades ago (Okada and Nakagawa 1989). The study showed that Tyr-527 of c-Src did not undergo autophosphorylation. Rather, it was phosphorylated by a putative upstream tyrosine kinase that was originally termed neonatal brain type of protein tyrosine kinase (N-PTK) isolated from the membrane fraction of neonatal rat brain. Okada and Nakagawa then conducted experiments to characterize the specificity of phosphorylation of c-Src by Ntk. First, c-Src phosphorylated by Ntk was subjected to sequential proteolytic digestion by trypsin and α-chymotrypsin. Phosphopeptide mapping revealed two major phosphopeptide fragments. Among them, one was recognized by an antibody raised against the C-terminal (residues 524–533) in c-Src. Further biochemical analysis of this fragment identified Tyr-527 as the phosphorylation site. Hence, Ntk was later referred to as the Carboxyl terminal Src Kinase (CSK).



CSK-Homologous Kinase, Fig. 2 Comparison of amino acid sequences of CSK and CTK. (**a**) Domain organization of CTK. From the N-terminal end, CTK contains a SH3 domain. It is linked to the SH2 domain by the SH3–SH2 connector. The SH2 domain is linked to the kinase domain by the SH2-kinase linker. (**b**) Alignment of sequences of CSK and CTK. (**c**) A model of the disposition of the functional domains of CSK. The three-dimensional structure of CSK shows that the SH2 and SH3 domains are located near the top of the N-lobe of the kinase domain. The SH3–SH2 connector and SH2-kinase linker interact with structures in the N-lobe of the kinase domain. Given the high degree of sequence homology of CSK and CTK, it is logical to predict that CTK also adopts a similar structure in solution

The cDNA sequence of *CSK* was later isolated (Nada et al. 1991). The sequence revealed that it encodes a protein with an SH3 domain, an SH2 domain and a kinase domain (Fig. 2). The homologue of CSK termed CSK-homologous kinase (also referred to as *C*SK-*T*ype *K*inase, CTK) was discovered a few years after identification of CSK. Depending on its structural features and the origins of the cDNA libraries, CTK was also named Hyl, BatK, Matk, CHK, and Ntk ((Cheng et al. 2006) and references quoted therein). CTK shares 54% identity with its closely related kinase CSK and retains similar structural organization and functional domains with CSK. Similar to CSK, CTK resides predominantly in cytosol. Unlike CSK, CTK expression is less ubiquitous. Its expression is mainly confined to neuronal and hemopoietic cells. In addition, Zhu et al. reported expression of CTK in normal colon epithelial cells (Zhu et al. 2008).

Alternative splicing of the *CTK* transcript gives rise to two CTK isoforms of 56 kDa and 52 kDa. The 56 kDa isoform contains an additional 41-residue segment at the N-terminus (reviewed in (Cheng et al. 2006) and references quoted therein). Northern blot analyses demonstrated that the 56 kDa CTK isoform is expressed in normal colon cells and hematopoietic cells of megakaryocyte lineage while the 52 kDa CTK isoform is expressed in brain cells ((Cheng et al. 2006; Zhu et al. 2008) and references quoted therein). Whether and how the two isoforms differ in their regulatory properties and functions remain unknown.

Structure and Domain Organization of CTK

CTK has similar domain organization like its CSK counterpart. Namely, it has a Src-homology 3 (SH3), an SH2 and a kinase domain (Okada and Nakagawa 1989). In addition, SH3 and SH2 domains are connected by the SH3–SH2 connector while SH2 and kinase domain through the SH2-kinase linker (Fig. 2). But unlike c-Src it lacks an N-terminal fatty acid acylation site as well as autophosphorylation site and C-terminal regulatory tyrosine phosphorylation site (reviewed in (Chong et al. 2005a)). Because of the absence of the N-terminal fatty acid acylation motif which targets cellular proteins to plasma membrane, CTK mostly localizes in the cytoplasm (reviewed in (Chong et al. 2005a)). It is well established that intramolecular interactions of the CSK kinase domain with the SH2 and SH3 domains play a significant role in regulating CSK kinase activity ((Lin et al. 2005) and references quoted therein). Given the similarity in the domain organizations of CSK and CTK, it is logical to predict that both the SH2 and SH3 domains are involved in regulating the kinase activity of CTK.

Comparison of the sequences of the SH2 and SH3 domains of CTK and CSK reveal significant differences in the residues dictating the binding of ligands. For example, Glu-127 which dictates the binding specificity of the CSK SH2 domain is substituted by Ile-126 at the homologous position in the CTK SH2 domain. These structural differences account for the striking difference in binding specificity of the CTK and CSK SH2 domains (Ayrapetov et al. 2005). In this entry, how these structural differences govern the differences in functions and regulation of CSK and CTK is discussed.

Much is known about the three-dimensional structure of CSK – the structures of CSK kinase domain, full-length CSK protein, and CSK kinase domain complexed with the c-Src kinase domains have been solved (Fig. 4) (reviewed in (Ia et al. 2010)). In contrast, only the structure of SH2 domain of CTK has been solved. Exactly how the various functional domains arrange in the three-dimensional structure of CTK remains unclear. Owing to the similarity of organizations of SH3, SH2, and kinase domains in the CSK and CTK sequence, it is logical to predict that their three-dimensional structures are very similar. Unlike SFKs, the SH2 and SH3 domains of CSK are located above the minor lobe of the kinase domain with both the SH3–SH2 connector (referred to as connector) and the SH2-kinase linker (linker) sitting atop the α-helix C (Fig. 1 and 2c). Such an arrangement allows both the SH3 and SH2 domains to control the conformation of kinase domain via the connector and linker which make intimate contacts with the kinase domain (Fig. 2c) (reviewed in (Ia et al. 2010; Chong et al. 2005b)). It is likely that a similar arrangement of the connector, linker, and the three functional domains can be found in the three-dimensional structure of CTK. With this arrangement, binding of the cognate ligands to SH2 and SH3 domains is expected to modulate kinase domain conformation and hence the kinase activity of CTK.

Phenotypes of *CTK*-Deficient Mice

Three groups of researchers reported the generation of *CTK* knockout mice (reviewed in (Cheng et al. 2006)). They all reported that the $CTK^{-/-}$ mice displayed no defect in hematopoiesis. However, upon stimulation with interleukin-7, bone marrow cells from $CTK^{-/-}$ mice display enhanced proliferation in culture, resulting in a higher number of pre-B cells. Lee *et al.* suggested that the phenotype is attributed to the ability of CTK to act as a negative regulator of pre-B cell proliferation (Lee et al. 2006). In addition, T-cells from $CTK^{-/-}$ mice exhibited impaired interferon γ production in culture. Taken together, the phenotypes revealed the unique role played by CTK in immune function. Furthermore, the milder-than-expected phenotypes of $CTK^{-/-}$ mice indicate significant functional overlap between CTK and CSK in cells expressing both enzymes.

SFK-Dependent and SFK-Independent Functions of CTK

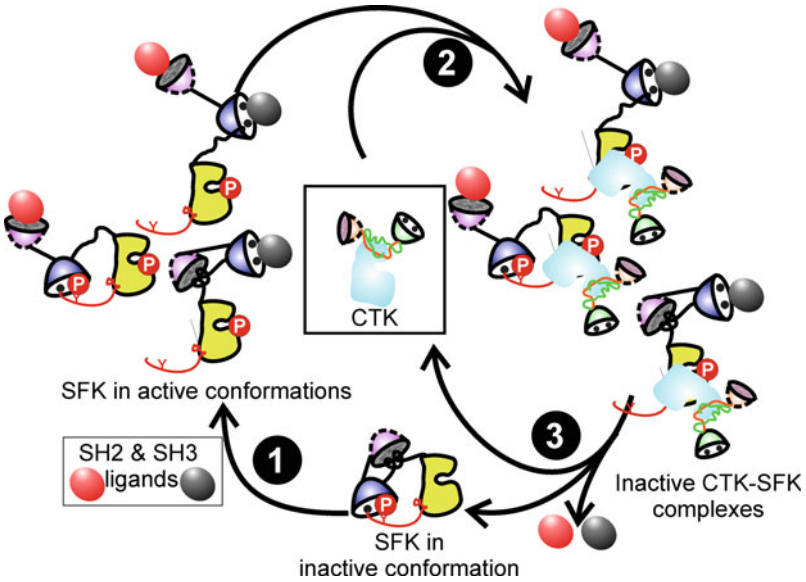
Since aberrant activation of SFKs contributes to formation of cancer ((Cheng et al. 2006; O'Hare et al. 2008) and reference therein), in normal cells, they are kept at the inactive conformation mainly by CSK and CTK. As shown in Fig. 1, SFKs in the inactive conformation are phosphorylated at the C-terminal tail tyrosine by CSK or CTK. Two intramolecular inhibitory interactions are required to keep the kinases in the inactive state: (1) binding of the phosphorylated C-terminal tail tyrosine to SH2 domain and (2) binding of the SH2-kinase linker to the SH3 domain. These interactions stabilize the kinase domain in the catalytically inactive state. Disruption of one or both inhibitory interactions activates SFKs. The active SFKs then undergo autophosphorylation at a conserved tyrosine (e.g., Tyr-416 of c-Src) in the kinase domain and/or dephosphorylation of the phosphorylated C-terminal tail tyrosine by a phosphatase. Upon autophosphorylation, SFKs prefer to adopt the active conformation (reviewed in (Chong et al. 2005b)).

Previous studies by Chong *et al*. demonstrated that CTK suppresses the activity of SFKs by two mechanisms (Fig. 3). The first mechanism, referred to as the catalytic mechanism, involves specific phosphorylation of the C-terminal regulatory tyrosine of SFKs (reviewed in (Cheng et al. 2006; Chong et al. 2005b, 2006) and references quoted therein). Upon phosphorylation, SFKs adopt the inactive conformation. The second mechanism, referred to the non-catalytic inhibitory mechanism, involves direct binding of CTK to the kinase domain of SFKs adopting different active conformations; and the binding alone is sufficient to suppress SFK activity (Chong et al. 2004; 2006). Furthermore, this unique non-catalytic inhibitory mechanism is reliant upon the ability of CTK to bind tightly to SFKs to form stable CTK/SFK complexes (Fig. 3) (Chong et al. 2006). In contrast, CSK fails to exhibit this non-catalytic inhibitory mechanism to suppress SFK activity.

How might CTK recognize SFKs as its physiological substrates? Although the three-dimensional structures of CTK/SFK complexes have not been solved, the structure of CSK/c-Src complex solved by Levinson *et al*. provides the conceptual framework that predicts how CTK selects SFKs as the substrates (Levinson et al. 2008) (Fig. 5a). In the CSK/c-Src structure, the two proteins are engaged in extensive contacts at an interface formed by the αD-helix and αF–αG loop of CSK (Fig. 4) and the segment consisting of residues 504–525 in the αI helix near the C-terminal regulatory motif of c-Src (Fig. 5b and c). Among the interactions between CSK and c-Src at the interface, the most notable are (1) the electrostatic interactions between the five basic residues (R279, R281, R283 of the αD helix and R384 and R389 of the αF–αG loop) of CSK and several acidic residues (E504, R510, E517, D518) of c-Src; and (2) the hydrophobic interaction between R279 of CSK with Y511 of c-Src (Fig. 5b). It is noteworthy that the sequences of the αD helices and αF–αG loops of CSK and CTK are highly conserved. Thus, it is logical to predict that CTK interaction with SFKs also involves the basic residues in the αD helices and αF–αG loops of CTK and acidic and hydrophobic residues near or in the αI helix of the SFK kinase domains. These predicted contacts may direct the active site of CTK to exclusively phosphorylate the C-terminal regulatory tyrosine of SFKs.

How might CTK bind SFKs and suppress their activity? Relevant to this question, CTK was found to bind active forms of SFKs while CSK exhibited binding with much lower affinity or no binding at all (Chong et al. 2006; Levinson et al. 2008). This suggests that in addition to the interactions of basic residues in the αD helices and αF–αG loops of CTK with acidic and hydrophobic residues in the αI helix of SFKs, additional motifs exist in both CTK and SFKs to mediate the tight binding to form the stable CTK/SFK complexes. Furthermore, it is logical to predict that the interactions of CTK with SFKs via these additional motifs underpin the ability of CTK to suppress SFK activity by the non-catalytic inhibitory mechanism. Elucidation of the structural basis of the CTK non-catalytic inhibitory mechanism awaits definition of these unknown additional interaction motifs in CTK and SFKs. The best approach to define these motifs is to determine the crystal structures of the CTK/SFK complexes.

Being a major endogenous inhibitor of SFKs, CTK is a potential tumor suppressor preventing aberrant activation of the oncogenic SFKs. Of relevance, CTK expression is suppressed in colon carcinoma cell lines and biopsies from patients suffering from colon cancer. Furthermore, expression of recombinant CTK in colon cancer cells suppresses SFK activity, anchorage-independent growth, and cell invasion (Zhu et al.



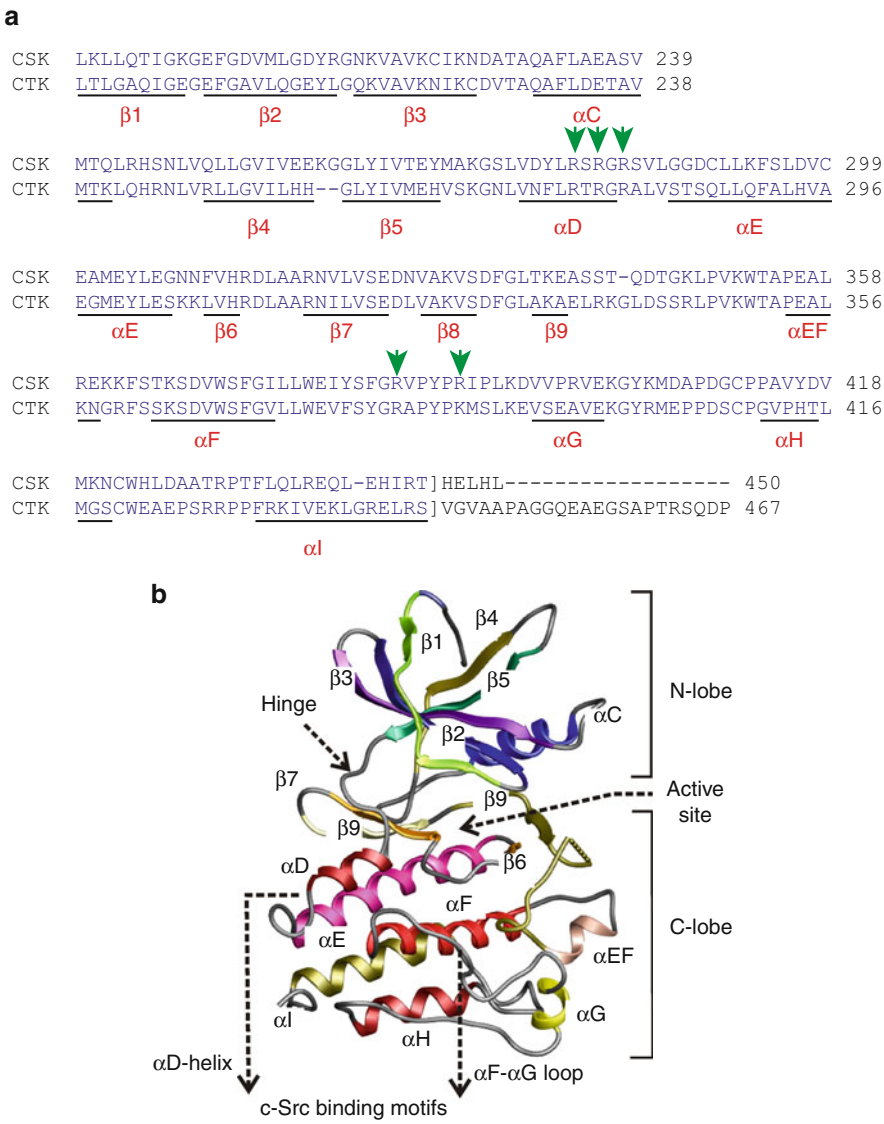
CSK-Homologous Kinase, Fig. 3 A model of inhibition of SFKs by CTK. Step 1: Activation of the inactive SFK by disruption of the two inhibitory intramolecular interactions by SH2 and SH3 domain ligands. Depending on binding of ligands to the SH2 and/or SH3 domains, SFK adopts different active conformations. The active SFK then undergoes autophosphorylation at the conserved tyrosine in the activation loop of the kinase domain. Step 2: CTK employs the non-catalytic inhibitory mechanism to suppress the activity of SFKs. This mechanism involves binding of CTK to the active forms of SFKs to form stable CTK/SFK complexes. Step 3: CTK phosphorylates the C-terminal regulatory tyrosine. The autophosphorylation site of SFKs is dephosphorylated by phosphatases. Upon dissociation of CTK, the SFK molecules with the phosphorylated C-terminal tyrosine adopt the inactive conformation

2008). In addition, overexpression of CTK was found to suppress growth and proliferation of human breast carcinoma MCF-7 cells (reviewed in (Cheng et al. 2006; Chong et al. 2005a)).

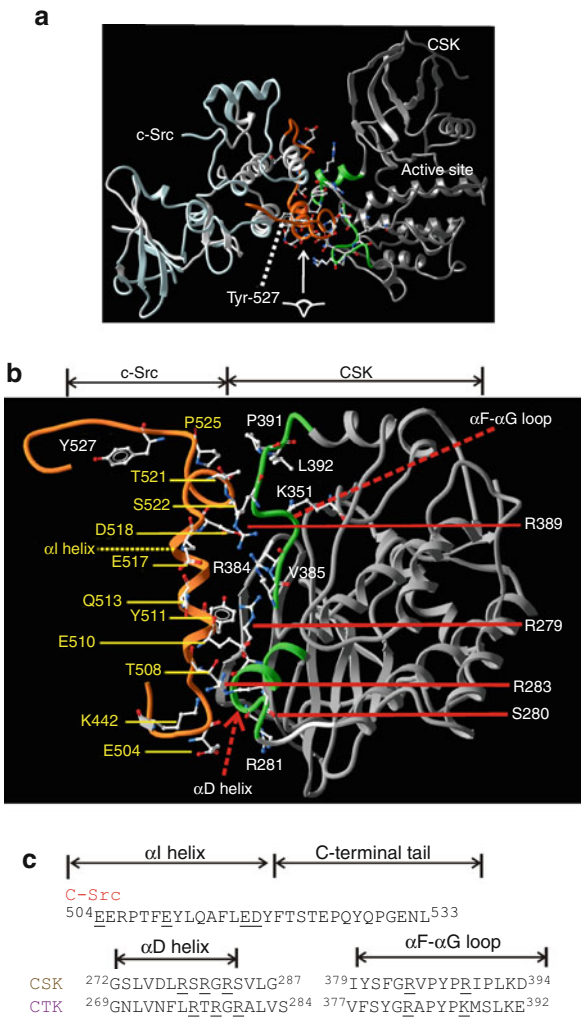
SFKs are not the only physiological substrates of CTK. There is a growing body of evidence suggesting that CTK is capable of phosphorylating other cellular proteins to perform SFK-independent functions. For example, CTK expression causes activation of MAP kinase signaling pathway in $SFY^{-/-}$ cells which lack SFKs (Zagozdzon et al. 2006), suggesting that the activation was governed by an SFK-independent mechanism. Relevant to these findings, CTK overexpression could induce phosphorylation of an immunoglobulin superfamily protein called the tyrosine-protein phosphatase non-receptor substrate 1 (SHPS-1) in PC12 cells (reviewed in (Ia et al. 2010)). The authors provided data suggesting that CTK binds to SHPS-1 and directly phosphorylate it at Tyr-428, Tyr-452, Tyr-469, and Tyr-495. In addition to SFKs and SHPS1, CTK may also phosphorylate other cellular proteins. The KESTREL method (Cohen and Knebel 2006), which has been used for searching and identifying potential physiological substrates of a number of protein kinases, will be useful for identifying potential physiological substrates of CTK.

The Roles of SH3 and SH2 Domains in Regulation of Kinase Activity and Subcellular Localization of CTK

Unlike SFKs, CTK lacks the N-terminal fatty acid acylation domain. For this reason, CTK resides predominantly in the cytosol in a number of cell types. Apart from directly binding to SFKs to form stable protein complexes (Chong et al. 2006), CTK interacts with three transmembrane receptor tyrosine kinases including ErbB2, c-kit, and TrkA, the focal adhesion kinase-related kinase Pyk2/RAFTK and the scaffolding protein paxillin (reviewed in Chong et al. 2005a, b). Since SFKs are localized to plasma membrane, endosomes, and perinuclear regions (Seong et al. 2009), CTK needs to be recruited from cytosol to these organelles to inhibit SFKs. Both the SH2 and



CSK-Homologous Kinase, Fig. 4 Structure and functional motifs of the CSK kinase domain. (**a**) Amino acid sequence of the CSK kinase domain (residues 192–445) and CTK kinase domain (residues 191–467). The secondary structures of the kinase domain are indicated below the corresponding segments. The structural elements critical for catalysis, regulation, and binding of substrates include: (1) the phosphate-binding loop (P-loop) of the consensus motif (GxGxΦG, where x is any amino acid residue and Φ represents a hydrophobic residue) in the β1–β 2 loop responsible for interacting with the β-phosphate of ATP; (2) the β3 strand Lys (Lys-222 of CSK and Lys-221 of CTK) which, upon formation of an ion pair with (3) the αC-helix glutamate (Glu-236 of CSK and Glu-235 of CTK), interacts with the α- and β-phosphates of ATP; (4) the "hinge" motif that links the N-terminal lobe (residues 192–267 of CSK) to the C-terminal lobe (274–445 of CSK) of the kinase domain; (5) the catalytic loop (HRLAARN) containing the conserved aspartate that functions as the general base in catalysis; (6) the activation segment beginning with the DFG motif and ending with the APE motif; and (7) the arginine residues (Arg-279, Arg-281, Arg-283, Arg-384, Arg-389 of CSK and Arg-276, Arg-278, Arg-280, Arg-382, and Lys-387 of CSK) in the αD-helix and αF–αG loop critical for binding to c-Src (indicated by green arrow heads). (**b**) Structure of CSK kinase domain in the active configuration (PDB entry:3D7T). All β-strands and α-helices are labeled. The αD-helix and αF–αG loop forming the c-Src-binding motifs are shown. The crevice between the N-lobe and C-lobe of the kinase domain forms the active site

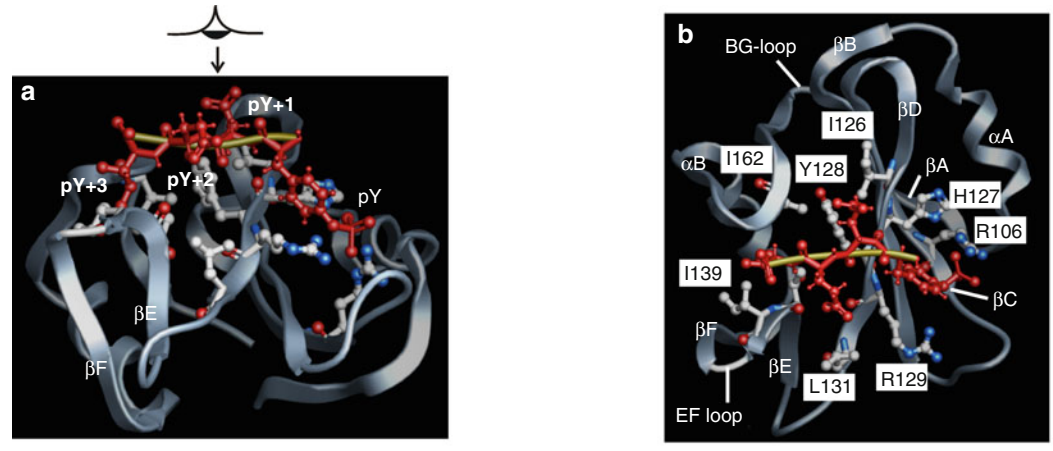


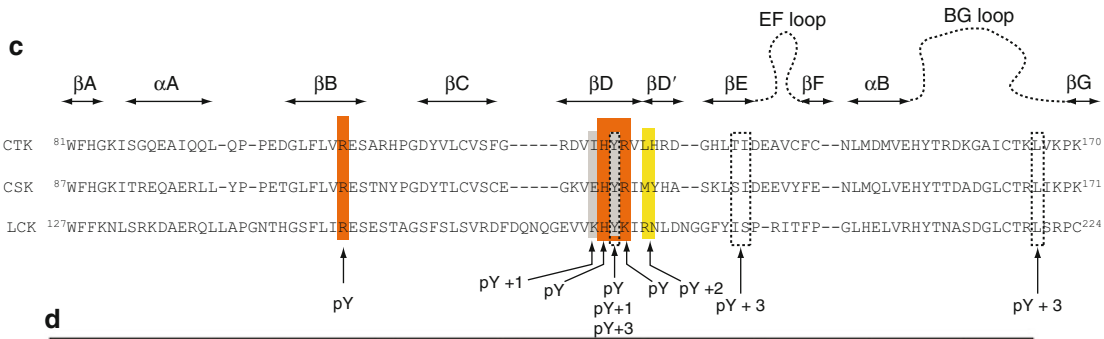
CSK-Homologous Kinase, Fig. 5 Structure of the CSK/c-Src complex. (**a**) The structure of the CSK/c-Src complex (3D7U). (**b**) The close-up view from the bottom of panel A. The conserved residues (D504, E510, Y511, E517, and D518) in the αI helix near the C-terminus of c-Src interacting with the basic residues (R279, R281, R283, R384, and R389) residing in or near the αD-helix and αF–αG loop in CSK kinase domain are shown. (**c**) The Src-binding motifs of CSK and the homologous regions in CTK. R279, R281, and R283 of αD-helix and R384 and R389 of αF–αG loop of CSK and their homologues in CTK are underlined

SH3 domains are involved in targeting CTK to specific subcellular compartments to perform its physiological functions.

CTK SH2 domain crystal structure has been solved by Murthy and Webster in 2001 (PDB: 1JWO; deposited in 2001, modified in 2009). This structure was superimposed to the structure of Lck SH2 domain with the bound pYEEI peptide ligand to generate a hypothetical structure consisting of the CTK SH2 domain with the pYEEI peptide docked to its ligand-binding sites (Fig. 6a and b). The hypothetical structure reveals the amino acid residues of CHK SH2 domain essential for interacting with the phosphotyrosine (pY) and residues at the first, second, and third positions (pY + 1, pY + 2, and pY + 3 positions) from the C-terminal end of pY in the peptide ligand. As illustrated in Fig. 6c, the main structural features on SH2 domain is the presence of two groups of hydrophobic residues in EF loop and BG loop (thr-138 and Ile-139 of the EF loop and Ile-162 of the BG loop). Together with Tyr-128 of the βD strand, these two groups of EF loop and BG loop hydrophobic residues form the binding pocket for the pY + 3 residue in peptide ligand. Indeed, Huang et al. found that CTK SH2 domain prefers phosphopeptide ligands with hydrophobic residues at the pY + 3 position (Huang et al. 2008). For binding to pY in the peptide ligand, the interacting residues include the basic residues Arg-106, His-127, and Arg-129, as well as Tyr-128. Screening with oriented peptide library (OPL) revealed that the CHK SH2 domain preferred a hydrophobic pY + 3 residue in the ligand (Fig. 6d) (Huang et al. 2008). For the pY + 1, Y + 2, and Y + 4 positions, the preferred residues in the peptide ligand are also hydrophobic residues (Fig. 6b, c). This finding is in agreement with the previous prediction by Ayrapetov et al. that Ile-126 in the βD strand governs the preference of the CHK SH2 domain for hydrophobic pY + 1 residue in the peptide ligand (Ayrapetov et al. 2005). Using the SMALI algorithm developed by Huang et al. (2008), a number of tyrosine-phosphorylated cellular proteins were predicted to be recognized by CTK SH2 domain. Among them are the growth factor receptor kinases ErbB2 and c-Kit, which were previously identified to be ligands of the CTK SH2 domain (reviewed in (Chong et al. 2005a)).

Although the ligands of CTK SH3 domain have yet to be identified, evidence provided by Hirao et al. suggests that the CTK SH3 domain can interact with an unknown adaptor protein located on the plasma membrane in megakaryocytic Dami cells. The binding recruits CTK to the plasma membrane. The recruitment is associated with inhibition of the SFK member Lyn and may contribute to suppression of fibronectin-stimulated cell spreading (reviewed in (Chong et al. 2005a)). Little is known about CTK SH3 domain binding specificity. Similar to CSK SH3 domain, binding of





d

Position in the SH2 domain binding motif	−2	−1	pY	+1	+2	+3	+4
Interacting residues in SH2 domain of CTK			R106 (βD) H127 (βD) Y128 (βD) R129 (βD)	I126 (βD) Y128 (βD)	L131 (βD′)	Y128 (βD) T138 (βD′) I140 (βD′) I162 (BG-loop)	
Preferred residues by OPL screen	I/V/M/L	M/E	pY	Y/A/E/T	M	A/V/MI	M
pY1248 of ErbB2	P	P	pY	L	G	L	D
pY794 of TrkA	P	S	pY	L	D	V	L
pY568 of c-Kit	N	N	pY	V	Y	I	D
pY794 of TrkA	Y	V	pY	I	D	P	T

CSK-Homologous Kinase, Fig. 6 Structure of the SH2 domain of CTK. (**a**) Three-dimensional structure of CTK SH2 domain (1JWO) with the pYEEI peptide ligand (1LKL) docked to the ligand-binding site. Residues located at the N-terminal side of the phosphotyrosine are referred to as pY-1, pY-2, pY-3, etc. Those located at the C-terminal side of the phosphotyrosine are referred to as pY + 1, pY + 2, pY + 3, etc. (**b**) View of the structure of CTK SH2 domain complexed with the pYEEI from the top of the structure shown in panel A. (**c**) Alignment of the sequences of the SH2 domains of CTK, CSK, and Lck. Orange: residues interacting with the phosphotyrosine. Gray: residues intercating with the pY + 1 residue of the ligand. Yellow: residues interacting with the pY + 2 residue of the ligand. Dotted square: residues interacting with the pY + 3 residue of the ligand. (**d**) Residues in the CTK SH2 domain predicted to interact with the phosphotyrosine, pY + 1, pY + 2, and pY + 3 residues in the ligand. The CTK SH2 domain-preferred residues located at the pY-2 to pY-4 positions of the peptide ligand revealed by the oriented peptide library (OPL) screen (second row) (Huang et al. 2008). Sequences of the CTK-binding motifs of phosphorylated ErbB2, Trak A, and c-Kit

CTK SH3 domain to its ligand is expected to govern the subcellular localization of CTK. Identification of the ligands targeting the CTK SH3 domain will provide further insight into regulation of subcellular localization of CTK. Most SH3 domains bind peptide ligands with the PxxP motif, the two prolines in the motif interact with four conserved hydrophobic aromatic residues in the SH3 domain. In CSK, they are Tyr-18, Phe-20, Trp-47, and Tyr-64 (Fig. 2b). Intriguingly, three out of four of these residues are substituted by the less hydrophobic residues (Cys-17, Asn-19, and Ala-63) in CTK (Fig. 2b). It is unclear whether the lack of hydrophobic aromatic residues at the PxxP motif-binding pocket affects the specificity of CTK SH3 domain binding to cellular protein ligands. In addition to binding ligand, the SH3 domain was demonstrated to mediate dimerization of CSK (Levinson et al. 2009). In contrast to CSK, CTK exists as a monomer in solution (Chan et al. 2010), indicating that the CTK SH3 domain does not govern dimerization of the enzyme. How might the SH3 and SH2 domains of CTK govern its kinase activity? Chan et al. (2010) compared the specific enzymatic activities of recombinant CTK and CSK and found that they exhibit similar efficiency in phosphorylating the SFK member Lyn, indicating that the basal activity of both kinases are similar. Other than this, little is known about the regulation of CTK activity. In contrast, it is well documented that the SH2 and SH3 domains play significant roles in governing the kinase activity of CSK (reviewed in (Ia et al. 2010)). Owing to the high degree of sequence homology between CTK and CSK (Fig. 2 and 4), structural comparison of both kinases allows one to make predictions of CTK regulation. The presence of SH2 and SH3 domains suggests that CTK kinase activity is likely to be regulated by protein ligands that bind to these two domains (Fig. 3). The activity of many protein kinases is governed by the orientation of a conserved structural motif in the kinase domain called the α-helix C (Fig. 3) (see (Ia et al. 2010) for review). Results by biochemical analysis by Mikkola and Bergman (reviewed in (Chong et al. 2005b)) suggest that the SH2-kinase linker interacts with the α-helix C and controls CTK kinase activity. Their results imply that protein ligands that specifically bind to the SH2 domain of CTK can regulate CTK activity by modulating this interaction. Of relevance, the SH2 domain of CTK was reported to bind to a motif containing the phosphorylated Tyr-1248 of ErbB2 (Fig. 6). It will be worthwhile to examine if and how binding of CTK to the phosphorylated ErbB2 affects CTK kinase activity and its efficiency in inhibiting SFKs. Unlike many other protein kinases, CTK lacks the conserved autophosphorylation tyrosine in the activation loop of the kinase domain, suggesting that it is not regulated by tyrosine phosphorylation of the activation loop.

Summary

Studies of the phenotypes of $CTK^{-/-}$ mice and biochemical studies of CTK functions in cancer cell lines demonstrated that CTK plays unique roles in modulating immune cell signaling and functions as a tumor suppressor. Presumably, these functions of CHK are attributed to its ability to suppress the activity of SFKs. In addition to hematopoietic cells, neurons also express high level of CTK. Little is known about the function of CTK in neurons. Future investigation should focus on elucidating the mechanism of regulation and functions of CTK in neurons. Even though CTK is a major endogenous inhibitor of SFKs, little is known about the structure, regulation, and the structural basis of its inhibition of SFKs. There are two outstanding questions concerning the structure and function of CTK: What is the structural basis of the non-catalytic inhibitory mechanism employed by CTK to inhibit SFK activity? What are the other physiological substrates of CTK?

Deciphering the structural basis of CTK inhibition of SFKs by the non-catalytic inhibitory mechanism requires mapping of the SFK-binding determinants in CTK and the CTK-binding determinants in SFK. A previous study demonstrated that these determinants reside in the kinase domain of both CTK and SFKs (Chong et al. 2006). As shown in Fig. 5, similar to CSK, the conserved basic residues in the αD-helix and αF–αG loop of CTK are likely SFK-binding determinants. However, the binding of CTK to SFKs is much tighter than that of CSK to SFKs (Chong et al. 2006). It is logical to predict that in addition to these determinants in the αD-helix and αF–αG loop, determinants residing in other regions of CTK kinase domain are required for its tight binding to SFKs. Further, biochemical analyses are needed to map these determinants. Finally, determination of the three-dimensional structures of CTK and CTK/c-Src

complex will provide valuable insights into the structural basis of this non-catalytic inhibitory mechanism of CTK.

There is no doubt that small-molecule inhibitors specifically inhibiting SFKs are needed for development as therapeutics for cancer treatment. A number of inhibitors targeting the ATP-binding pocket of the active SFKs have been developed for clinical use or for preclinical studies. Among them, Dasatinib, AZD0530, and SKI-606 are currently in clinical trials for the treatment of different forms of cancer. The use of Dasatinib for the treatment of drug-resistant chronic myelogenous leukemia has been approved. Owing to the ability of these small molecule compounds to inhibit other protein kinases and possibly some non-protein kinase enzymes, use of these compounds for the treatment of cancer may cause significant side effects in patients receiving the treatment. Thus, the second-generation chemical inhibitors which target SFKs with exquisite selectivity are urgently needed. Since CTK can employ the non-catalytic inhibitory mechanism to specifically suppress the activity of SFKs adopting the active conformations. Future investigation to define the structural basis of the non-catalytic inhibitory mechanism of CTK will benefit the development of this new generation of inhibitors.

References

Ayrapetov MK, Nam NH, Ye G, Kumar A, Parang K, Sun G. Functional diversity of Csk, Chk, and Src SH2 domains due to a single residue variation. J Biol Chem. 2005;280:25780–7.

Chan KC, Lio DS, Dobson RCJ, Jarasrassamee B, Hossain MI, Roslee AK, Ia KK, Perugini MA, Cheng H-C. Development of the procedures for high yield expression and rapid purification of active recombinant Csk-Homologous Kinase (CHK) – Comparison of the catalytic activities of CHK and CSK. Protein Expr Purif. 2010. doi:10.1016/j.pep.2010.07.007.

Cheng H-C, Chong YP, Ia KK, Tan OA, Mulhern TD. Csk homologous kinase. UCSD-Nature Molecule Pages. 2006. doi:doi:10.1038/mp.a000705.01.

Chong YP, Mulhern TD, Zhu HJ, Fujita DJ, Bjorge JD, Tantiongco JP, Sotirellis N, Lio DS, Scholz G, Cheng HC. A novel non-catalytic mechanism employed by the C-terminal Src-homologous kinase to inhibit Src-family kinase activity. J Biol Chem. 2004;279:20752–66.

Chong YP, Ia KK, Mulhern TD, Cheng HC. Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases. Biochim Biophys Acta. 2005a;1754:210–20.

Chong YP, Mulhern TD, Cheng HC. C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)–endogenous negative regulators of Src-family protein kinases. Growth Factors. 2005b;23:233–44.

Chong YP, Chan AS, Chan KC, Williamson NA, Lerner EC, Smithgall TE, Bjorge JD, Fujita DJ, Purcell AW, Scholz G, Mulhern TD, Cheng HC. C-terminal Src kinase-homologous kinase (CHK), a unique inhibitor inactivating multiple active conformations of Src family tyrosine kinases. J Biol Chem. 2006;281:32988–99.

Cohen P, Knebel A. KESTREL: a powerful method for identifying the physiological substrates of protein kinases. Biochem J. 2006;393:1–6.

Huang H, Li L, Wu C, Schibli D, Colwill K, Ma S, Li C, Roy P, Ho K, Songyang Z, Pawson T, Gao Y, Li SS. Defining the specificity space of the human SRC homology 2 domain. Mol Cell Proteomics. 2008;7:768–84.

Ia KK, Mills RD, Hossain MI, Chan K-C, Jarasrassamee B, Jorissen RN, Cheng H-C. Structural elements and allosteric mechanisms governing regulation and catalysis of CSK-family kinases and their inhibition of Src-family kinases. Growth Factors. 2010;28(5):329–50.

Lee BC, Avraham S, Imamoto A, Avraham HK. Identification of the nonreceptor tyrosine kinase MATK/CHK as an essential regulator of immune cells using Matk/CHK-deficient mice. Blood. 2006;108:904–7.

Levinson NM, Seeliger MA, Cole PA, Kuriyan J. Structural basis for the recognition of c-Src by its inactivator CSK. Cell. 2008;134:124–34.

Levinson NM, Visperas PR, Kuriyan J. The tyrosine kinase CSK dimerizes through Its SH3 domain. PLoS ONE. 2009;4:e7683.

Lin X, Ayrapetov MK, Lee S, Parang K, Sun G. Probing the communication between the regulatory and catalytic domains of a protein tyrosine kinase, CSK. Biochemistry. 2005;44:1561–7.

Nada S, Okada M, MacAuley A, Cooper JA, Nakagawa H. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. Nature. 1991;351:69–72.

O'Hare T, Eide CA, Deininger MW. Persistent LYN signaling in imatinib-resistant, BCR-ABL-independent chronic myelogenous leukemia. J Natl Cancer Inst. 2008;100:908–9.

Okada M, Nakagawa H. A protein tyrosine kinase involved in regulation of pp 60c-src function. J Biol Chem. 1989;264:20886–93.

Seong J, Lu S, Ouyang M, Huang H, Zhang J, Frame MC, Wang Y. Visualization of Src activity at different compartments of the plasma membrane by FRET imaging. Chem Biol. 2009;16:48–57.

Zagozdzon R, Kaminski R, Fu Y, Fu W, Bougeret C, Avraham HK. Csk homologous kinase (CHK), unlike CSK, enhances MAPK activation via Ras-mediated signaling in a Src-independent manner. Cell Signal. 2006;18:871–81.

Zhu S, Bjorge JD, Cheng HC, Fujita DJ. Decreased CHK protein levels are associated with Src activation in colon cancer cells. Oncogene. 2008;27:2027–34.

Csnk2

► Casein Kinase II

CSPP28 (Rabbit)

▶ TPD52 (Tumor Protein D52)

c-SRC

▶ c-Src Family of Tyrosine Kinases>

c-Src Family of Tyrosine Kinases

Banibrata Sen[1] and Faye M. Johnson[1,2]
[1]Department of Thoracic/Head and Neck Medical Oncology, Unit 432, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
[2]The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX, USA

Synonyms

ASV; c-SRC; p60-Src; SRC; SRC1

Historical Background

In 1911, pathologist Francis Peyton Rous isolated a virus from a Plymouth Rock chicken that has continued to bear his name, the Rous sarcoma virus (RSV) (Rous 1911). Rous sarcoma virus is the archetypal retrovirus, capable of causing tumors in chickens and rapidly transforming cells in culture with high efficiency through production of the protein viral sarcoma (v-Src), the first identified transforming protein. In 1976, Bishop and Varmus demonstrated that the *v-Src* gene has a normal cellular homolog gene (protooncogene), *c-Src*, and that the *v-Src* gene product, pp60v-Src or v-Src, is a phosphoprotein with an apparent molecular mass of 60 kDa with intrinsic protein kinase activity (Stehelin et al. 1976). Sequencing of the chicken *c-Src* gene and the RSV *v-Src* gene demonstrated that the two genes are closely related except at the *C*-terminal end, and it is this structural difference that leads to constitutive activation of *v-Src*, which underlies its transforming capacity (Takeya and Hanafusa 1983).

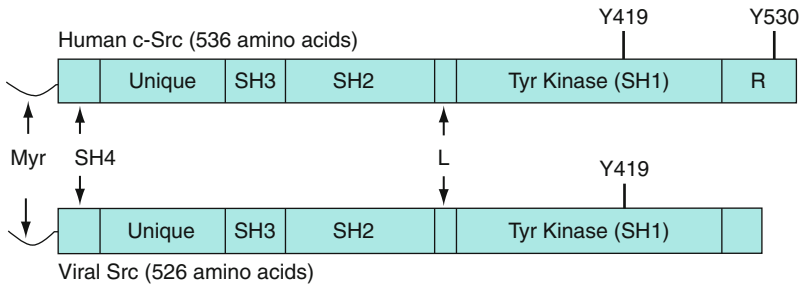
c-Src is the prototypic member of a family of non-receptor, membrane-associated tyrosine kinases comprising 11 c-Src family kinases (SFKs); the other ten are Fyn, Yes, Blk, Yrk, Frk (also known as Rak), Fgr, Hck, Lck, Srm, and Lyn (Sen and Johnson 2011). The human genome contains a Yes pseudogene known as *YESps*. c-Src, Yes, and Fyn are ubiquitously expressed in a variety of tissues. Srm is found in keratinocytes, whereas Blk, Fgr, Hck, Lck, and Lyn are found primarily in hematopoietic cells. Frk occurs chiefly in bladder, breast, brain, colon, and lymphoid cells. In fact, Frk has been shown to be a nuclear protein with growth-inhibitory effects when ectopically expressed in breast cancer cells. Blk occurs chiefly in colon, prostate, and small intestine cells, but it was initially isolated from a breast cancer cell line.

SFKs are activated by cytokine receptors, receptor protein tyrosine kinase, G-protein coupled receptors, and integrins. They promote cancer cell proliferation, survival, motility, and invasiveness (Summy and Gallick 2003).

Structure

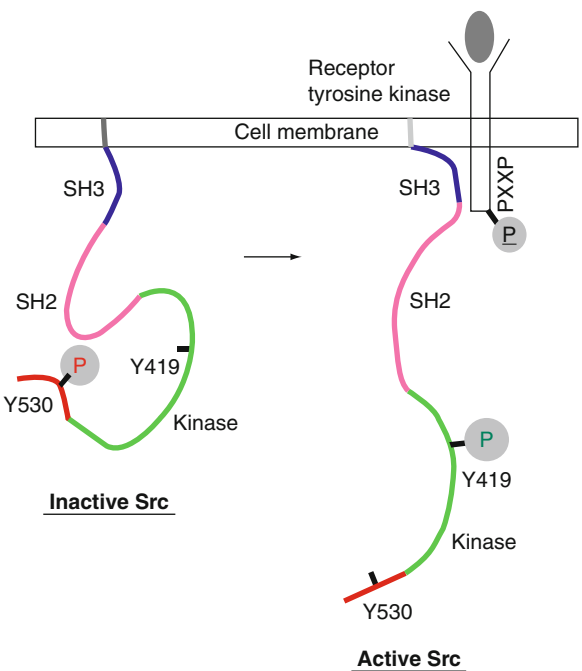
SFKs are proteins of approximately 60 kDa composed of several functional domains: (a) a myristic acid moiety at the *N*-terminal region responsible for localization at the inner surface of the cell membrane; (b) a unique domain that provides unique functions and specificity to each member of the SFK family; (c) an SH3 domain that is able to bind proline-rich sequences to mediate both intracellular and intercellular interactions; (d) an SH2 domain that binds phosphorylated tyrosine residues on the SFK molecule itself or its substrates; (e) a linker domain that binds to the SH3 domain; and (f) a catalytic domain that is composed of two lobes separated by a catalytic cleft in which the ATP and substrate-binding sites reside and phosphate transfer occurs. The cleft forms an activation loop that contains tyrosine (Y419, c-Src, human) which is a positive regulatory site responsible for maximum kinase activity. The SFKs also include (g) the *C*-terminal tail, which contains a negative regulatory tyrosine (Y530, c-Src, human) residue (Fig. 1 and reviewed in Johnson and Gallick 2010).

Upon phosphorylation of Y530, the SFK attains a "closed" or inactive conformation by binding its



c-Src Family of Tyrosine Kinases, Fig. 1 Cartoon representation of the structural domain of human-Src and viral-Src. Src is composed of an N′-terminal myristic acid chain attached to SH4 domain, a unique region followed by SH3 and SH2 domain. A short linker domain (L) followed by tyrosine kinase domain (SH1) harboring Tyr 419 and a C′-terminal regulatory domain (R) harboring Tyr 530. Viral Src differ from human Src in a number of ways with one major difference being the lack of a regulatory domain (R) at its C′-terminal end

C-terminal region to its SH2 domain. In this closed conformation, the activation loop adopts a compact structure, which fills the catalytic cleft and precludes access of ATP and substrate. The closed conformation masks the Y419 residue, furthermore, preventing activation by autophosphorylation (Fig. 2). Although c-Src and v-Src exhibit several single amino acid differences, the most striking difference is the substitution of the most *C*-terminal 19 amino acids of chicken c-Src (533 amino acids) by 12 completely different amino acids in RSV v-Src (526 amino acids) (Takeya and Hanafusa 1983). Following loss of the *C*-terminal negative regulatory Tyr residue, v-Src and v-Yes are no longer regulated by intramolecular interaction and become constitutively active.



c-Src Family of Tyrosine Kinases, Fig. 2 Schematic representation of Src kinase regulation by differential phosphorylation at kinase domain (Tyr 419) and C′-terminal regulatory domain (Tyr 530)

Regulation of c-Src Kinase Activity

v-Src is a constitutively activated form of c-Src capable of eliciting many dramatic biological responses, including adhesion, migration, invasion, proliferation, differentiation, and survival. Because of the extensive involvement of c-Src in multiple cellular processes (described below), investigators have searched for the mechanisms behind c-Src activation and discovered that many different processes can alter c-Src kinase activity.

Structure of the C-terminal Regulatory Domain

Phosphorylation of the *C*-terminal regulatory residue (Y530, c-Src) may be mediated by any of several kinases and phosphatases. As already described, phosphorylation of Y530, deletion or mutation of the *C*-terminal regulatory region, and displacement of the SH3 or SH2 domain mediated by intramolecular interactions regulate c-Src activity in cells. Because of the loss of the *C*-terminal negative regulatory Tyr residue, v-Src and v-Yes are no longer regulated by intermolecular interactions. Rare activating mutations in c-Src have been reported in some cases of advanced colon and endometrial cancers

(Irby et al. 1999). These mutations result in a stop codon at 531, one residue beyond the Y530, resulting in a truncated c-Src that is unable to form the closed conformation.

Cytoplasmic Kinases

Two important protein tyrosine kinases in the phosphorylation of SFKs are c-Src kinase (▶ CSK) and its homolog CSK-homologous kinase (▶ CHK), which are both able to phosphorylate Y530. Substantial evidence suggests that CSK and CHK are negative regulators of SFKs that play distinct roles during development of the nervous system. Their distinct biological effects may be due to distinct signaling effects. Specifically, CHK was shown to enhance MAPK signaling, while the role of CSK was mediated predominantly by c-Src regulation. In cancer cells, CHK was able to downregulate ErbB-2/neu-activated Src kinases. CSK is structurally similar to c-Src, comprising an SH2 domain, an SH3 domain, and a tyrosine kinase domain, but it lacks the regulatory tyrosine residue at its *C*-terminal end. A number of proteins that specifically bind CSK and regulate its activity toward c-Src have been identified, including tyrosine-protein phosphatase non-receptor type 12 with a *C*-terminal PEST motif (PTP-PEST), which could potentially counteract the activity of CSK by dephosphorylating c-Src at Y530. Another mechanism of CSK regulation is through the transmembrane adapter protein Cbp (CSK-binding protein OR protein associated with glycosphingolipid-enriched microdomains (PAG)), which is a c-Src substrate. Following phosphorylation by c-Src, Cbp can bind to the SH2 domain of CSK, thus allowing its recruitment to the plasma membrane where active c-Src resides, creating a negative regulatory loop (Sen and Johnson 2011).

Protein Tyrosine Phosphatases

Several protein tyrosine phosphatases (PTPs) have been implicated in regulation of c-Src kinase activity, including PTP-alpha, PTP-gamma, SHP-1, SHP-2, and PTP-1B. PTP-alpha is ubiquitously expressed and enriched in brain tissue and is able to dephosphorylate both Y419 and Y530 in vitro in cancer cells (Zheng et al. 1992). However, it is unclear whether PTP-alpha acts as an activator or repressor molecule. Both c-Yes and c-Fyn are dephosphorylated and activated by PTP-alpha. In contrast, PTP-gamma is capable of dephosphorylating Y530 and is responsible for elevated c-Src kinase activity (Bjorge et al. 2000).

SHP-1 (also known as PTP-1 C) and SHP-2 are cytosolic, SH2 domain-containing PTPs expressed in epithelial and hematopoetic cells. They can dephosphorylate Y530 and subsequently increase c-Src kinase activity. The SH2 domain of SHP-2 binds to the SH3 domain of c-Src, which results in allosteric regulation of c-Src.

PTP-1B (also known as PTP-N1) was purified from breast cancer cells as a phosphatase that can dephosphorylate a Y530-containing peptide (Bjorge et al. 2000). Biochemical analysis showed that cancer cells have elevated levels of PTP activity, which correlates with reduced phosphorylation of the *C*-terminal residue of c-Src and may have an important role in controlling c-Src kinase activity.

Membrane-Associated Receptors and Cytoplasmic Kinases

c-Src can act as an upstream or downstream modulator of receptor molecules, including receptor tyrosine kinases (RTKs), steroid hormone receptors, and G-protein coupled receptors (GPCR). c-Src can also be activated by non-receptor tyrosine kinases.

SFKs physically interact with activated RTKs, creating a positive regulatory loop that contributes to robustness of RTK signaling. Upon stimulation by its ligands, RTKs cause receptor dimerization and autophosphorylation of tyrosine residues of the cytoplasmic domain. The resulting phosphorylation acts as a docking site to recruit and activate c-Src, which in turn phosphorylates other RTKs and creates other SH2 binding recruitment sites, allowing binding of the Grb2-SOS complex leading to activation of downstream signaling of the Ras-MAPK and PI3K signaling pathways. c-Src can associate with overexpressed epidermal growth factor receptor (EGFR) to cause synergistic mitogenicity. c-Src activity can be stimulated by EGFR autophosphorylation of Tyr 845. EGF has been shown to stimulate invasion and metastasis of carcinoma cells through Src-mediated activation of ▶ p^{130}Cas. RTKs and integrins also act synergistically in promoting cell survival, proliferation, cytoskeletal reorganization, and invasion through signaling mediated by c-Src.

The binding of SFKs to various proteins plays important roles in SFK regulation. Several structural features of SFKs facilitate their interactions with

proteins that regulate them. X-ray crystal structure analysis revealed that the inactive Src conformation can be achieved by interaction between the SH2 domain and the *C*-terminal Tyr residue or between the SH3 domain and the SH2 kinase linker. A variety of Src-binding proteins can compete for binding in this cleft, disrupting the intramolecular interaction and activating c-Src. Platelet-derived growth factor receptor (PDGFR) and focal adhesion kinase (FAK) can bind to the c-Src SH2 domain and activate c-Src and Hck (another member of SFK). p^{130}Cas can bind to the c-Src SH2 and SH3 domains, activating c-Src.

Increased Expression and Altered Protein Stability

Recent evidence suggests that c-Src is subject to ubiquitin-dependent degradation. Specifically, activated forms of c-Src are turned over more rapidly than inactive c-Src. One possible explanation is that c-Src and Fyn bind to and phosphorylate c-Cbl. Upon binding with c-Src, c-Cbl acts as an E3-ubiquitin ligase, leading to c-Src degradation (Frame 2002).

SFK Regulation of Cancer Cell Function

SFKs are important in various aspects of tumor development, including proliferation, migration, invasiveness, apoptosis, and angiogenesis. It is well documented that c-Src is responsible for dynamic regulation of the actin cytoskeleton, resulting in cell motility, cell membrane adhesion, and cell-cell adhesion. c-Src acts as one of the central components of the focal adhesion complex. The c-Src SH3 domain associates with actin filaments upon activation, which then drive translocation of c-Src to cell-cell and cell-matrix adhesion sites where c-Src can interact with plasma membrane-bound molecular partners to take part in two major transduction events: signaling from RTKs, which mainly affects cell growth, proliferation, and migration; and signaling from adhesion receptors such as integrins and E-cadherin, which mainly regulates cytoskeletal functions (Fig. 3 and reviewed in (Johnson and Gallick 2010)).

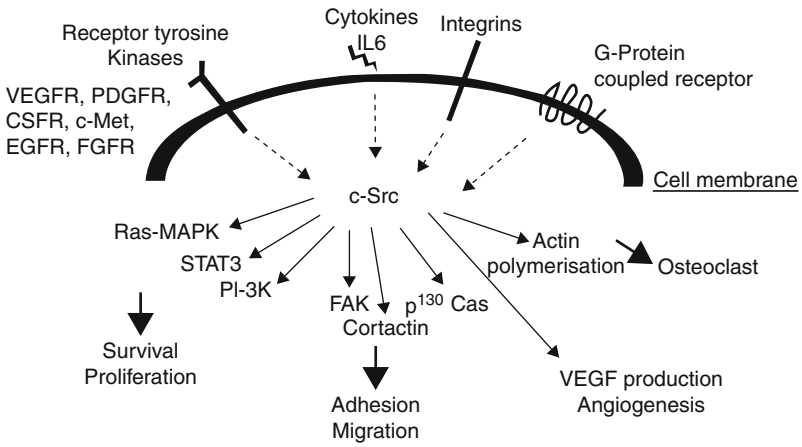
SFKs are frequently overexpressed and/or aberrantly activated in a variety of cancers. The contributions of c-Src to cell regulation and cancer development were discussed by Summy and Gallick (2003). Activation is very common in colorectal and breast cancers and less frequent in melanomas and ovarian, gastric, head and neck, pancreatic, lung, brain, and hematologic cancers. The extent of increased SFK activity often correlates with malignant features and patient survival.

Colon Cancer

Involvement of c-Src in cancer development and progression has been studied more extensively in colon cancer than in any other human cancer. c-Src kinase activity is elevated in premalignant ulcerative colitis lesions, and lesions with the highest extent of dysplasia have the greatest potential for progression to carcinoma. Surprisingly, adenomas displayed stronger immunohistochemical staining for active c-Src than advanced adenocarcinomas (Summy and Gallick 2003).

c-Src-specific kinase activity via phosphorylation is elevated in colon cancer cell lines. Although c-Src is frequently activated in human colon cancers, it is not the only SFK whose activation corresponds with malignant progression. c-Yes is also frequently activated in colon cancers. c-Yes kinase activity was elevated in three of five colon cancer cell lines and 10 of 21 primary colon carcinomas relative to normal colonic mucosa (Summy and Gallick 2003). Because of the high homology between c-Src and c-Yes, it has been assumed that both kinases perform similar functions in cancer development, but c-Yes is evidently unable to compensate fully for the lack of c-Src in these processes because of its substrate specificity among different SFKs. Regulation of actin cytoskeleton dynamics is crucial to the motility, invasiveness, and metastatic spread of tumor cells, and thus the contribution of c-Yes to tumor development may differ from that of c-Src. Interestingly, Lck is also expressed in colon cancer as a result of abnormal activation of the *Lck* promoter due to loss of its transcriptional repressor.

Treatment of a human colon tumor cell line with tyrosine kinase inhibitor herbimycin-A caused a reduction in c-Src kinase activity followed by subsequent reduction in colon cancer cell growth. c-Src kinase activity as measured by phosphorylated Y419 is important for proliferation and growth of primary tumors derived from human colon cancer cells. SFKs may contribute to the invasiveness of colon carcinoma cells through dynamic regulation of the actin cytoskeleton and activation of matrix proteases. c-Src expression also regulates the cadherin-catenin association, thereby regulating cell-cell contact. Another report suggested that the level of vascular endothelial



c-Src Family of Tyrosine Kinases, Fig. 3 Cartoon representation of Src signaling in tumor development and progression

growth factor (VEGF) varied directly with c-Src level in a colon cancer cell line (Johnson and Gallick 2010; Summy and Gallick 2003).

Breast Carcinoma

Considerable data support a role for SFKs in the progression of breast cancer. Several independent reports have demonstrated elevated levels of c-Src kinase activity in breast carcinoma tissue in comparison to normal breast epithelium. Lehrer et al. showed that tumors expressing the progesterone receptor generally displayed higher kinase activity than those that did not express progesterone receptor and that 70% of tyrosine kinase activity in breast cancer could be attributed to c-Src (Lehrer et al. 1989). Female mice with forced expression of active c-Src frequently developed epithelial hyperplasia, which occasionally progressed to full neoplasia. As with colon cancer, c-Src is important in the induction of VEGF transcription in breast cancer, suggesting the importance of c-Src in cancer angiogenesis.

Zhang et al. provided both clinical and experimental evidence that c-Src plays a critical role in establishment of latent bone metastasis in breast cancer. Using a bioinformatic approach, they identified a c-Src activity gene expression signature that was highly associated with the late onset of bone metastasis in breast cancer (Zhang et al. 2009).

Melanoma

Human melanoma is one of the few cancers in which c-Yes plays a more crucial role in the cancer development than c-Src. c-Yes kinase activity is increased in melanoma cells compared to normal melanocytes, but c-Src kinase activity is not upregulated. Likewise, c-Yes is activated in the presence of nerve growth factor, whereas c-Src is not (Johnson and Gallick 2007).

Head and Neck Cancer

c-Src is expressed in Barrett's esophagus and esophageal adenocarcinoma as well as other head and neck cancers. c-Src is overexpressed in hyperproliferating regions of head and neck squamous cell carcinoma (HNSCC), dysplastic epithelium, papillomas, and inflamed normal tissues (Summy and Gallick 2003). Inhibition of c-Src leads to a universal decrease in HNSCC cell invasion, with more modest and variable effects on cell cycle arrest and apoptosis (Johnson et al. 2005). c-Src inhibition in HNSCC leads to reactivation of pSTAT3 expression, which is considered a mechanism for cellular resistance toward c-Src inhibitors (Sen et al. 2009). c-Src activity regulates invadopodia formation in HNSCC cell lines, thereby increasing invasiveness (Kelley et al. 2010). It has been shown that c-Src along with c-Met plays an important role in survival of head and neck cancer cells and that c-Met acts as direct c-Src substrate in HNSCC, which suggest that Src-dependent cell survival is also regulated by c-Met receptor activation (Sen et al. 2011).

Pancreatic Cancer

c-Src overexpression and increased c-Src kinase activity were observed in pancreatic cancer cell lines but not in normal pancreatic cells. Activated c-Src expression in pancreatic carcinoma cells results in elevated expression of the insulin-like growth factor-1 (IGF-1) receptor, which leads to increased IGF-1-dependent cell proliferation. c-Src-mediated reciprocal regulation

of E-cadherin expression also correlated with growth and progression of human pancreatic cancers (Summy and Gallick 2003).

Ovarian Cancer

c-Src may play very specific roles in the progression of ovarian cancer. c-Src expression is required for anchorage-independent growth and angiogenesis in ovarian cancer. c-Src activity appears to be important for SHC phosphorylation and Erk1/2 phosphorylation downstream of CXCR1/2 receptor stimulation in ovarian cancer (Summy and Gallick 2003). It has also been reported that c-Src inhibition enhanced paclitaxel cytotoxicity in ovarian cancer cells by caspase 9-independent activation of caspase 3. Recent data show the importance of c-Src and protein kinase G-alpha interaction in promoting DNA synthesis and cell proliferation in human ovarian cancer cells (Leung et al. 2010).

Bladder Cancer

c-Src kinase activity is upregulated in human bladder carcinoma cells. c-Src is involved in the epithelial-to-mesenchymal transition of bladder cancer cells in a rat bladder carcinoma model. Increased caveoline-1 expression and decreased c-Src expression and kinase activity correlated with bladder tumor aggressiveness (Thomas et al. 2011).

Gastric Cancer

c-Src kinase activity is greater in gastric carcinoma tissues than in normal mucosal samples. In a subset of gastric cancer cell lines, c-Src inhibition led to increased cell cycle arrest and apoptosis. The resistant gastric carcinoma cell lines had *c-Met* amplification, suggesting that this pathway is a possible mechanism of resistance (Okamoto et al. 2010).

Lung Cancer

Increased expression of c-Src has been reported in 60–80% of adenocarcinomas and bronchioloalveolar cancers and in 50% of squamous cell carcinomas isolated from patients with non-small cell lung cancer (NSCLC). High levels of c-Src kinase activity in NSCLC correlate with tumor size. The mitogenic effects of both nicotine and asbestos are mediated through c-Src. c-Src-mediated constitutive ▶ STAT3 activity has been found in multiple NSCLC cell lines. c-Src inhibition leads to STAT3 activation in multiple NSCLC cell lines, which is believed to be the alternative resistance mechanism for NSCLC cell survival upon c-Src inhibition (Byers et al. 2009). Studies have shown that activation of STAT3 and FAK by c-Src is required for anchorage-dependent and -independent growth in a range of human tumors, including NSCLC. Furthermore, stimulation of STAT3 by EGF, IL6, and hepatocyte growth factor in NSCLC all required c-Src activity. c-Src also activates the VEGF pathway via STAT3 (Johnson and Gallick 2007).

In human NSCLC, c-Src activity is associated with inhibition of anoikis, a form of cell death induced by detachment of adherent cells from the substratum. Following detachment from the primary tumor, c-Src activity is increased, which is able to compensate for the loss of survival signals from cell matrix. Under hypoxic conditions, SFK-dependent transcriptional upregulation of the endothelial PAS-domain protein-1 was observed. These data indicate that SFKs may be involved in regulation of signaling pathways that govern multiple aspects of lung cancer progression (Johnson and Gallick 2007; Summy and Gallick 2003).

Leukemia and Lymphoma

The SFK Lyn is expressed in lymphocytes and monocytes. Lyn was specifically activated in myeloid leukemia cell lines in response to IL3. Lyn is also responsible for phosphorylation of B-cell receptor (BCR) and its coreceptors Ig-alpha and Ig-beta. Lyn is also involved in IL6-mediated cell proliferation. Lck is expressed in T lymphocytes and plays an important role in T-cell hematopoiesis, proliferation, and receptor signaling (Johnson and Gallick 2007).

Clinical Trials with c-Src Inhibitors

Because of the extensive literature supporting the importance of c-Src in tumor progression, angiogenesis, and metastasis, as well as positive correlations between c-Src expression and cancer progression, c-Src is emerging as a promising target for anticancer therapy. Several small-molecule inhibitors of c-Src kinases are undergoing clinical trials after promising preclinical studies, such as the ATP-binding competitive inhibitors dasatinib (BMS-354825, Sprycel), bosutinib (SKI-606), ponatinib (AP24534), and bafetinib (INNO-406), and the substrate binding-site inhibitor Kxo-I (KX2-391) (Sen and Johnson 2011).

Dasatinib (BMS-354825; Bristol-Myers Squibb, New York, NY), an oral inhibitor of Abl, cKit, SFKs, PDGFR, Btk, Ephrin receptor A2 (EphA2), and other kinases, is currently used in the treatment of Bcr-Abl-positive leukemia and gastrointestinal stromal tumor. It is well tolerated by humans. It suppresses the invasion of HNSCC, NSCLC, and other epithelial cancers in vitro (Johnson and Gallick 2010). It is also able to greatly inhibit development of metastasis in an orthotopic mouse model of pancreatic cancer. The triple-negative subtype and EGFR-overexpressing breast cancer cell lines were particularly sensitive to dasatinib (Rothschild et al. 2010; Sen and Johnson 2011). On the basis of promising results of a phase I/II trial of the combination of dasatinib and docetaxel in patients with castration-resistant prostate cancer, this combination is now being studied in a phase III clinical trial (Rothschild et al. 2010). A phase I study of dasatinib in combination with capecitabine or paclitaxel has shown promising results in breast cancer patients. In a phase II study with dasatinib as a single agent in NSCLC had modest clinical activity that was lower than that generally observed in patients who receive standard chemotherapy. Marked activity in one patient and prolonged stable disease in four others suggested a potential subpopulation of patients with dasatinib-sensitive NSCLC (Johnson et al. 2010). Partial responses have been observed in a phase I/II study of dasatinib in combination with erlotinib in advanced NSCLC (Haura et al. 2010). Single-agent dasatinib failed to demonstrate significant activity in patients with advanced HNSCC, despite durable c-Src inhibition (Brooks et al. 2011).

Bosutinib (SKI 606; Wyeth Pharmaceuticals Inc., Madison, NJ) is an oral inhibitor of SFKs and Abl, with a lower affinity for cKit and PDGFR than dasatinib. It showed promising results in a colon cancer murine model and was well tolerated. In preclinical studies, bosutinib resulted in a dose-dependent reduction in proliferation, invasion, and migration of breast cancer cells. In a breast cancer mouse model, bosutinib significantly reduced metastasis to liver, spleen, and lung (Rothschild et al. 2010). In phase II trials, bosutinib either as single agent or in combination with exemestane, letrozole/capecitabine, or zolendronic acid has been well tolerated in patients with advanced breast cancer and has shown promising results (Sen and Johnson 2011).

Saracatinib (AZD 0530; AstraZeneca Pharmaceuticals, London, UK) is an oral inhibitor of SFKs and Abl. Despite promising preclinical studies, AZD0530 was withdrawn from further clinical development, likely because of its lack of clinical efficacy in multiple studies.

Other ATP-competitive tyrosine kinase inhibitors aimed at multiple targets, including SFKs, are being evaluated. XL999 is an oral inhibitor of SFKs, VEGFR, PDGFR, fibroblast growth factor receptor (FGFR), and fms-related tyrosine kinase 3 (FLT3) and has shown activity against solid tumors in phase I and phase II trials. XL228 targets the IGF receptor 1, aurora kinase, FGFR, and Abl. M475271 is another oral kinase inhibitor of c-Src and VEGFR that showed preclinical activity in lung adenocarcinoma cell lines. KX2-391 is an SFK inhibitor that targets the peptide substrate-binding site rather than the ATP-binding site of c-Src. It is not a tyrosine kinase inhibitor. It appears to have a wide spectrum of antitumor activity that is distinct from that of other tyrosine kinase inhibitors. In a phase I trial in patients with advanced solid tumors, KX2-391 was well tolerated (Rothschild et al. 2010).

Summary

A wealth of data indicates the importance of SFKs in the growth and development of various types of human cancers. The structure of c-Src and the mode of its regulation have been studied extensively since its discovery. To clarify and fully elucidate the normal physiological functions of c-Src and other SFKs, their interactions with specific substrates and binding partners in different subcellular environments should be characterized in detail. Phase I and phase II studies of c-Src inhibitors as single agents or in combination regimens in lung cancer, breast cancer, and prostate cancer are promising and warrant further investigation. A detailed comprehensive understanding is needed for the available inhibitors to improve our current approach to cancer therapy.

References

Bjorge JD, Pang A, Fujita DJ. Identification of protein-tyrosine phosphatase 1B as the major tyrosine phosphatase activity capable of dephosphorylating and activating c-Src in several human breast cancer cell lines. J Biol Chem. 2000;275(52):41439–46.

Brooks HD, Glisson BS, Bekele BN, Johnson FM, Ginsberg LE, El-Naggar A, Culotta KS, Takebe N, Wright J, Tran HT, Papadimitrakopoulou VA. Phase 2 study of dasatinib in the treatment of head and neck squamous cell carcinoma. Cancer. 2011;117(10):2112–9.

Byers LA, Sen B, Saigal B, Diao L, Wang J, Nanjundan M, Cascone T, Mills GB, Heymach JV, Johnson FM. Reciprocal regulation of c-Src and STAT3 in non-small cell lung cancer. Clin Cancer Res. 2009;15(22):6852–61.

Frame MC. Src in cancer: deregulation and consequences for cell behaviour. Biochim Biophys Acta. 2002;1602(2):114–30.

Haura EB, Tanvetyanon T, Chiappori A, Williams C, Simon G, Antonia S, Gray J, Litschauer S, Tetteh L, Neuger A, Song L, Rawal B, Schell MJ, Bepler G. Phase I/II study of the Src inhibitor dasatinib in combination with erlotinib in advanced non-small-cell lung cancer. J Clin Oncol. 2010;28(8):1387–94.

Irby RB, Mao W, Coppola D, Kang J, Loubeau JM, Trudeau W, Karl R, Fujita DJ, Jove R, Yeatman TJ. Activating SRC mutation in a subset of advanced human colon cancers. Nat Genet. 1999;21(2):187–90.

Johnson FM, Gallick GE. SRC family nonreceptor tyrosine kinases as molecular targets for cancer therapy. Anticancer Agents Med Chem. 2007;7(6):651–9.

Johnson FM, Gallick GE. Src family kinase inhibitors in cancer therapy. In: Georgiev B, Markovski S, editors. Serpins and protein kinase inhibitors: novel functions, structural features and molecular mechanisms, vol. 6. New York: Nova Science; 2010. p. 125–53.

Johnson FM, Saigal B, Talpaz M, Donato NJ. Dasatinib (BMS-354825) tyrosine kinase inhibitor suppresses invasion and induces cell cycle arrest and apoptosis of head and neck squamous cell carcinoma and non-small cell lung cancer cells. Clin Cancer Res. 2005;11(19 Pt 1):6924–32.

Johnson FM, Bekele BN, Feng L, Wistuba I, Tang XM, Tran HT, Erasmus JJ, Hwang LL, Takebe N, Blumenschein GR, Lippman SM, Stewart DJ. Phase II study of dasatinib in patients with advanced non-small-cell lung cancer. J Clin Oncol. 2010;28(30):4609–15.

Kelley LC, Ammer AG, Hayes KE, Martin KH, Machida K, Jia L, Mayer BJ, Weed SA. Oncogenic Src requires a wild-type counterpart to regulate invadopodia maturation. J Cell Sci. 2010;123(Pt 22):3923–32.

Lehrer S, O'Shaughnessy J, Song HK, Levine E, Savoretti P, Dalton J, Lipsztein R, Kalnicki S, Bloomer WD. Activity of pp 60c-src protein kinase in human breast cancer. Mt Sinai J Med. 1989;56(2):83–5.

Leung EL, Wong JC, Johlfs MG, Tsang BK, Fiscus RR. Protein kinase G type Ialpha activity in human ovarian cancer cells significantly contributes to enhanced Src activation and DNA synthesis/cell proliferation. Mol Cancer Res. 2010;8(4):578–91.

Okamoto W, Okamoto I, Yoshida T, Okamoto K, Takezawa K, Hatashita E, Yamada Y, Kuwata K, Arao T, Yanagihara K, Fukuoka M, Nishio K, Nakagawa K. Identification of c-Src as a potential therapeutic target for gastric cancer and of MET activation as a cause of resistance to c-Src inhibition. Mol Cancer Ther. 2010;9(5):1188–97.

Rothschild SI, Gautschi O, Haura EB, Johnson FM. Src inhibitors in lung cancer: current status and future directions. Clin Lung Cancer. 2010;11(4):238–42.

Rous P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J Exp Med. 1911;13(4):397–411.

Sen B, Johnson FM. Regulation of Src family kinases in human cancers. J Signal Transduct. 2011; 1–14.

Sen B, Saigal B, Parikh N, Gallick G, Johnson FM. Sustained Src inhibition results in signal transducer and activator of transcription 3 (STAT3) activation and cancer cell survival via altered Janus-activated kinase-STAT3 binding. Cancer Res. 2009;69(5):1958–65.

Sen B, Peng S, Saigal B, Williams MD, Johnson FM. Distinct interactions between c-Src and c-Met in mediating resistance to c-Src inhibition in head and neck cancer. Clin Cancer Res. 2011;17(3):514–24.

Stehelin D, Varmus HE, Bishop JM, Vogt PK. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature. 1976;260(5547):170–3.

Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev. 2003;22(4):337–58.

Takeya T, Hanafusa H. Structure and sequence of the cellular gene homologous to the RSV src gene and the mechanism for generating the transforming virus. Cell. 1983;32(3):881–90.

Thomas S, Overdevest JB, Nitz MD, Williams PD, Owens CR, Sanchez-Carbayo M, Frierson HF, Schwartz MA, Theodorescu D. Src and caveolin-1 reciprocally regulate metastasis via a common downstream signaling pathway in bladder cancer. Cancer Res. 2011;71(3):832–41.

Zhang XH, Wang Q, Gerald W, Hudis CA, Norton L, Smid M, Foekens JA, Massague J. Latent bone metastasis in breast cancer tied to Src-dependent survival signals. Cancer Cell. 2009;16(1):67–78.

Zheng XM, Wang Y, Pallen CJ. Cell transformation and activation of pp 60c-Src by overexpression of a protein tyrosine phosphatase. Nature. 1992;359(6393):336–9.

C-Src Kinase

► Csk

C-Terminal Src Kinase

► Csk

CTK

► CSK-Homologous Kinase

Ctnnb

► Beta-Catenin

CTNNB1

▶ Beta-Catenin

C-type (Calcium-Dependent, Carbohydrate Recognition Domain) Lectin Superfamily Member 9

▶ CLEC4E

C-type (Calcium-Dependent, Carbohydrate-Recognition Domain) lectin, Superfamily Member 5

▶ CLEC5A

C-type Lectin Domain Family 4 Member E

▶ CLEC4E

C-type Lectin-Domain Family 1 Member A

▶ CLEC-1

C-type Lectin-Domain Family 1, Member a

▶ CLEC-1

C-type Lectin-Domain Family 1, Member b

▶ CLEC-2

C-type Lectin-Domain Family 5, Member A

▶ CLEC5A

C-type Lectin-Like Receptor 1

▶ CLEC-1

C-type Lectin-Like Receptor 2

▶ CLEC-2

C-Type Mannose Receptor 2

▶ MRC2

Cupidin

▶ Homer

CX3CL1

Paula A. Pino and Astrid E. Cardona
Department of Biology and South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, TX, USA

Synonyms

Fractalkine; Neurotactin

Historical Background

Human CX3CL1 was first cloned in 1997 and the mouse homolog in 1998. CX3CL1 is a relatively

large protein consisting of an amino-terminal domain, a mucin-like stalk attached to a transmembrane region that connects the molecule to the plasma membrane, followed by the intracellular domain. CX3CL1 is biologically active either as a membrane-bound protein or as a soluble protein upon proteolytic cleavage from cell membranes. CX3CL1 actions are mediated through interaction with its unique G-protein coupled receptor CX3CR1 (previously called chemokine receptor CKRBRL1, RBS11, or V28). Both CX3CL1 and CX3CR1 are highly abundant in central nervous system (CNS) tissues; CX3CL1 is produced by neurons and CX3CR1 is present on microglial cells. In the periphery, CX3CL1 is produced mostly by endothelial cells and CX3CR1 is expressed on peripheral leukocytes. CX3CL1 plays a role in chemotaxis, cell adhesion, and cellular activation. Notably, CX3CL1 and CX3CR1 have been implicated in modulation of microglial activation and are involved in a wide spectrum of biological functions in various tissues and pathologies.

CX3CL1 Expression Patterns

CX3CL1 and its unique receptor CX3CR1 are abundant in CNS tissues. Unlike most chemokines, CX3CL1 is expressed as a transmembrane protein present predominantly in neurons and on the surface of endothelial cells of selected tissues including heart, lung, kidney, and pancreas. Both human coronary artery and umbilical vein endothelial cells (ECs) express CX3CL1 mRNA and protein; however, CX3CL1 mRNA has not been detected in brain vasculature in healthy mice or upon autoimmune inflammation (Sunnemark et al. 2005). Treatment of human umbilical vein endothelial cell cultures with recombinant CX3CL1 caused proliferation, migration and tube formation suggesting an effect on angiogenesis. In HUVEC cells CX3CL1 induced phosphorylation of ERK, Akt, and eNOS as well as an increase of nitric oxide (NO) production (Lee et al. 2006), indicative of the activating effects upon CX3CR1 engagement.

Proteolytic cleavage is essential for regulation of the available pools of membrane-bound versus soluble CX3CL1, and in turn for defining its adhesive versus chemotactic properties. In the healthy CNS, low levels of CX3CL1 are detected in a constitutive fashion. Upon inflammation, both CX3CL1 and its receptor are upregulated in the inflamed CNS tissues during EAE (Huang et al. 2006) as well as in several other pathologies (Cardona et al. 2006; Savarin-Vuaillat et al. 2007). More specifically, TNF significantly increased CX3CL1 mRNA (>100-fold) and protein expression, which was associated with increased shedding of CX3CL1 from the cell membranes (Hurst et al. 2009). CCL2 also appeared to regulate CX3CL1 functions by regulating receptor expression. More specifically, monocytes stimulation with CCL2 increased their adhesion to immobilized CX3CL1, in a p38 MAPK dependent pathway (Green et al. 2006). Although in vitro studies suggest that lipopolysaccharide (LPS) can regulate expression of both CX3CR1 and CX3CL1 (Mizuno et al. 2003), the exact signaling pathways and players involved in the transcriptional and posttranslational regulation of CX3CL1 by cytokines in vivo remain to be clarified.

Release of CX3CL1 from cellular membranes is mediated by the alpha-secretase activity of two disintegrins and metalloproteases (MMPs), ADAM10 (Gough et al. 2004) and ADAM17 (also known as tumor necrosis factor converting enzyme, TACE) (Garton et al. 2001). ADAM10 mediates constitutive and ionomycin-induced cleavage of CX3CL1, whereas ADAM17 catalyzes the inducible release of the soluble domain of CX3CL1 upon phorbol myristate acetate (PMA) stimulation. Most recently it was demonstrated that cathepsins S and MMP2 are also involved in CX3CL1 shedding. Of interest is the report of CX3CL1 release by apoptotic lymphocytes, providing the first demonstration of chemokine/chemokine-receptor activity in the mobilization of macrophages toward apoptotic cells, suggesting a mechanism by which macrophages infiltrate tissues containing apoptotic lymphocytes (Truman et al. 2008).

Dendritic cells (DCs) also express CX3CL1, which functions to control NK cell activation (Pallandre et al. 2008). Recently, a new role of CX3CL1 receptor in DCs differentiation was reported (Lyszkiewicz et al. 2011). Using competitive adoptive transfer experiments it was demonstrated that CX3CR1 expression on hematopoietic progenitors promotes development of DCs and myeloid cells. Interestingly, this model was performed transferring precursor cells into non-irradiated and irradiated recipients to delineate the real role of CX3CR1 under steady-state conditions, compared to inflammatory conditions induced by irradiation (Chen et al. 2007; Xun et al. 1994). It is

proposed that irradiation might have a negative effect on the contribution of CX3CR1 signaling to the generation of DCs and monocytes/macrophages (Auffray et al. 2009; Lyszkiewicz et al. 2011). Based on the above, the role of CX3CR1 is mainly restricted to the steady-state generation of myeloid cells. Additionally, competitive adoptive transfer experiments were developed transferring CX3CR1-deficient precursors directly into the lymphoid target organs such as spleen and thymus, demonstrating similar competitive disadvantage when compared to CX3CR1-sufficient precursor cells. These data implicate CX3CR1 in developmental processes rather than homing of developing cells to lymphoid organs.

In mice the gene encoding CX3CL1 is located in chromosome 8. In humans, CX3CL1 is clustered on chromosome 16q13 together with monocyte derived chemokine (MDC, CCL22) and TARC (CCL17) (Hiroyama et al. 2001). The three human genes are separated by 6.3- and 28.5-kb intervening sequences, respectively, and reside in a head-to-tail orientation with respect to each other (GenBank acc. no. AC004382).

The CX3CL1 receptor (CX3CR1) is a membrane-bound protein and member of the G-protein coupled receptor family mainly produced by microglia. Outside the CNS, CX3CR1 resides mainly on specific leukocyte populations (Jung et al. 2000) including monocytes, NK cells, dendritic cells, and subpopulations of T cells.

CX3CL1/CX3CR1 in Microglial Function

Microglia are the resident macrophage of the CNS, which under resting conditions, constantly survey their microenvironment supporting their critical role in CNS homeostasis (Nimmerjahn et al. 2005). Under inflammatory conditions, microglial cells become activated and upregulate CD45, MHC and co-stimulatory molecule expression, phagocytic activity and are able to stimulate the proliferation of Th1 (▶ IFN-gamma-producing) and Th2 (Interleukin-4-producing) $CD4^+$ T cell lines (Carson et al. 1998). Activated microglia are responsible for the removal of cellular debris and pathogens during CNS injury, inflammation, and infections. In vitro studies have suggested that phagocytosis of myelin by microglia and macrophages could enhance neuroinflammation based on the release of pro-inflammatory cytokines and nitric oxide by these cells (Williams et al. 1994). Additionally, it has been suggested a dual role for microglial phagocytosis based on the need of myelin debris removal for remyelination (Neumann et al. 2009, Chastain et al. 2011).

Meucci et al. in 2000 showed for the first time the neuroprotective functions of CX3CL1 using hippocampal neurons. CX3CL1 conferred neuronal protection from HIV-1 gp120 induced neurotoxicity. Subsequently it was demonstrated that CX3CL1 inhibited release of pro-inflammatory mediators by microglia both in vitro and in vivo. Two knockout mouse models for CX3CL1 and four for CX3CR1 have been reported. These mice develop normally, and under healthy conditions, microglia of CX3CR1-deficient mice did not differ phenotypically from wild type microglia. However upon inflammation or neurodegeneration a distinct phenotype linked to defective CX3CR1 signaling was revealed. In response to systemic lipopolysaccharide injections *Cx3cr1*$^{-/-}$ microglia became highly activated and released pro-inflammatory mediators such as IL-1 that correlated with neuronal damage. Detrimental effects of CX3CR1-deficiency were also observed in models of Parkinson's disease, amyotrophic lateral sclerosis (Cardona et al. 2006), and most recently the absence of CX3CR1 was found to modulate beta-amyloid deposition in Alzheimer's disease models (Lee et al. 2010). Therefore, CX3CR1 appeared to be a key molecule involved in modulation of microglial activation. Interestingly, in a model of transient focal ischemia, Cx3cl1$^{-/-}$ mice showed a 28% reduction in infaction size and lower mortality rate when compared to wild type mice.

Recently, it has been found that soluble CX3CL1 (sCX3CL1), released from mouse cortical neurons damaged by glutamate excitotoxicity, enhances phagocytic uptake of neuronal debris by microglia (Noda et al. 2011). In this study, phagocytosis-related factors expressed by microglia in response to sCX3CL1 such as the PS receptor MFG-E8, was identified as the opsonin involved in clearance of apoptotic cells (Fuller et al. 2008). It was found that the enhanced phagocytosis and the clearance of damaged neurons by CX3CL1-treated microglia in turn promoted neuronal survival. Another finding was the activation of intracellular signaling pathways by sCX3CL1, such as ERK and JNK MAPK, being the last one an important signaling to drive the expression of the antioxidant

enzyme heme oxygenase-1 (HO-1) by microglia via Nrf2 nuclear translocation factor (Noda et al. 2011). Therefore, CX3CL1 plays an important neuroprotective role signaling through CX3CR1 on microglial cells.

CX3CL1/CX3CR1 in Peripheral Leukocytes

In peripheral blood CX3CR1 helps to distinguish "resident" monocytes (identified as LFA-1^{+}, L-Sel^{-}, Ly6C^{-}, *CCR2*$^{-}$, *CX3CR1*$^{+}$), whereas CCR2 marks the "inflammatory" monocyte subset (LFA-1^{-}, L-Sel^{+}, Ly6C^{+}, *CCR2*$^{+}$, *CX3CR1*$^{-}$) (Geissmann et al. 2008). During EAE, monocyte populations mirror this peripheral pattern (Saederup et al. 2010). It was demonstrated that CCR2 expression is controlled at a posttranscriptional stage in some Ly6C^{lo} monocytes and NK and T cells. Furthermore, Ly6C^{hi} monocytes fail to enter the CNS of CCR2-deficient mice during EAE and are mainly replaced by granulocytic cells, not Ly6C^{lo} monocytes. These findings suggest that CCR2+ Ly6C^{hi} monocytes initiate and maintain neuroinflammatory responses, while tissue remodeling is mediated by CX3CR1+ microglia. These studies provide further evidence of differential expression of CX3CR1 and CCR2 to distinguish monocyte/macrophage subsets and discriminate between resident microglia and infiltrating macrophages and DCs in the brain (Saederup et al. 2010, Mizutani et al. 2011).

Notably, multiple sclerosis (MS) patients show lower expression of CX3CR1 in peripheral NK cells when compared to healthy controls (Infante-Duarte et al. 2005) and a deficiency of CX3CR1^{+} PBMC correlated with disease activity. It was also shown the existence of distinct NK cell phenotypes depending on the magnitude of CX3CR1 expression (Hamann et al. 2011). The NK cell phenotypes defined by CX3CR1 expression differ in their cytolytic activity, cytokine profile, proliferative response, and their impact on monocyte functionality. Moreover, three different stages of NK cell maturation were identified based on the expression of CX3CR1 and CD56. While the CX3CR1^{neg} CD56^{bright} phenotype is exclusively characteristic for immature NK cells, the magnitude of CX3CR1 expression on CD56^{dim} NK cells discriminates between intermediary CX3CR1^{neg} CD56^{dim} and fully mature CX3CR1^{high} CD56^{dim} NK cells. Hence, these studies demonstrates that CX3CR1 can be used in conjunction with CD56 and with other novel maturation markers to delineate NK cell phenotypes characteristic for the sequential stages of human NK cell maturation.

Human CX3CR1 in Chronic Inflammation

The studies using CX3CR1-deficient mice become of particular relevance for humans as two single nucleotide polymorphisms in the human CX3CR1 loci produce four allelic receptor variants. Most individuals carry CX3CR1$^{V249/T280}$, and CX3CR1$^{I249/M280}$ is present in >20% of the population. These changes decrease CX3CL1 affinity and correlate with protection from atherosclerosis (Nassar et al. 2008; McDermott et al. 2001; Moatti et al. 2001; McDermott et al. 2003) making CX3CR1 a potential attractive target for therapeutic intervention in cardiac disease. Contrasting this protective effect in atherosclerosis, variant M280 receptor was reported to correlate with enhanced susceptibility to age-related macular degeneration (Chan et al. 2005). Interestingly, CX3CR1 clears its ligand from circulation and tissues acting as a scavenger receptor. Therefore, CX3CL1 elevation could possibly function as a biomarker to assess CX3CR1 dysfunction. CX3CL1/CX3CR1 have also been implicated in the pathology of autoimmune diseases such as systemic lupus erythematosus and patients with neuropsychiatric involvement showed higher serum levels of CX3CL1 (Yajima et al. 2005). Similar findings were reported in rheumatoid vasculitis and osteoarthritis patients (Klosowska et al. 2009).

CX3CL1/CX3CR1 in Cancer

Binding of CX3CL1 to CX3CR1 present on prostate cancer cells led to activation of anti-apoptotic signaling pathways, thereby enhancing survival and persistence of malignant cells. Therefore, CX3CR1 might support tumorigenic responses which translate into worse prognosis. In addition CX3CR1 signaling on tumor cells mediated cytotoxicity of malignant cells against endothelial cells expressing membrane-bound CX3CL1 and therefore enhancing pathology. CX3CR1 on tumor cells from patients with pancreatic ductal adenocarcinoma (PDAC) mediated chemotactic migration of PDAC cells toward CX3CL1 in vitro, as

well as adhesion to neural cells expressing the ligand CX3CL1. High CX3CR1 expression was associated with perineural invasion and with earlier local tumor recurrence in PDAC patients correlating with worse prognosis (Marchesi et al. 2010). In a separate context, combination of CX3CL1 and IL2 gene transfer into tumor cells was shown to reduce tumor size and liver metastasis in an animal model of neuroblastoma. Similarly, expression of CX3CL1 on the surface of tumor cells, via a chimeric immunoglobulin-chemokine construct, reduced incidence and size of lymphoma (Lavergne et al. 2003). These results suggest that the oncogenic effects of CX3CL1 toward tumor cells vary depending on the predominant expression of CX3CL1 or the receptor by the malignant cell type.

Summary

CX3CL1 is a unique chemokine that plays important roles in myeloid cells and in CNS microglia. CX3CL1 functions go beyond the original proposed role of chemokines in cellular recruitment. CX3CL1/CX3CR1 are key modulators of microglial function, play important roles in the development of myeloid cells and expression levels appeared altered in inflammatory conditions including autoimmune inflammation, atherosclerosis, and cancer. CX3CL1/CX3CR1 provides an example of neuronal/microglial communication. Due to its unique peripheral pattern of expression, this chemokine/receptor pair might confer a CNS/peripheral communication system whose tight functions are now recognized as critical for myeloid cells and its effect in other cell types are yet to be defined.

References

Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol. 2009;27:669–92.

Cardona A, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, et al. Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci. 2006;9(7):917–24.

Carson MJ, Reilly CR, Sutcliffe JG, Lo D. Mature microglia resemble immature antigen-presenting cells. Glia. 1998; 22(1):72–85.

Chan CC, Tuo J, Bojanowski CM, Csaky KG, Green WR. Detection of CX3CR1 single nucleotide polymorphism and expression on archived eyes with age-related macular degeneration. Histol Histopathol. 2005;20(3):857–63.

Chastain EM, Duncan DS, Rodgers JM, Miller SD. The role of antigen presenting cells in multiple sclerosis. Biochim Biophys Acta. 2011;1812(2):265–74.

Chen CJ, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat Med. 2007;13(7):851–6.

Fuller AD and Van Eldik LJ. MFG-E8 regulates microglial phagocytosis of apoptotic neurons. J Neuroimmune Pharmacol. 2008;3(4):246–56.

Garton KJ, Gough PJ, Blobel CP, Murphy G, Greaves DR, Dempsey PJ, et al. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). J Biol Chem. 2001;276(41): 37993–8001.

Geissmann F, Auffray C, Palframan R, Wirrig C, Ciocca A, Campisi L, et al. Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunol Cell Biol. 2008;86(5):398–408.

Gough PJ, Garton KJ, Wille PT, Rychlewski M, Dempsey PJ, Raines EW. A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. J Immunol. 2004;172(6):3678–85.

Green SR, Han KH, Chen Y, Almazan F, Charo IF, Miller YI, et al. The CC chemokine MCP-1 stimulates surface expression of CX3CR1 and enhances the adhesion of monocytes to fractalkine/CX3CL1 via p38 MAPK. J Immunol. 2006;176(12):7412–20.

Hamann I, Unterwalder N, Cardona AE, Meisel C, Zipp F, Ransohoff RM, et al. Analyses of phenotypic and functional characteristics of CX3CR1-expressing natural killer cells. Immunology. 2011;133(1):62–73.

Hiroyama T, Iwama A, Nakamura Y, Nakauchi H. Fractalkine shares signal sequence with TARC: gene structures and expression profiles of two chemokine genes. Genomics. 2001;75(1–3):3–5.

Huang D, Shi FD, Jung S, Pien GC, Wang J, Salazar-Mather TP, et al. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. FASEB J. 2006;20(7):896–905.

Hurst LA, Bunning RA, Couraud PO, Romero IA, Weksler BB, Sharrack B, et al. Expression of ADAM-17, TIMP-3 and fractalkine in the human adult brain endothelial cell line, hCMEC/D3, following pro-inflammatory cytokine treatment. J Neuroimmunol. 2009;210(1–2):108–12.

Infante-Duarte C, Weber A, Kratzschmar J, Prozorovski T, Pikol S, Hamann I, et al. Frequency of blood CX3CR1-positive natural killer cells correlates with disease activity in multiple sclerosis patients. FASEB J. 2005;19(13):1902–4.

Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol Cell Biol. 2000;20(11):4106–14.

Klosowska K, Volin MV, Huynh N, Chong KK, Halloran MM, Woods JM. Fractalkine functions as a chemoattractant for osteoarthritis synovial fibroblasts and stimulates phosphorylation of mitogen-activated protein kinases and Akt. Clin Exp Immunol. 2009;156(2):312–9.

Lavergne E, Combadiere B, Bonduelle O, Iga M, Gao JL, Maho M, et al. Fractalkine mediates natural killer-dependent antitumor responses in vivo. Cancer Res. 2003;63(21): 7468–74.
Lee SJ, Namkoong S, Kim YM, Kim CK, Lee H, Ha KS, et al. Fractalkine stimulates angiogenesis by activating the Raf-1/MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways. Am J Physiol Heart Circ Physiol. 2006;291(6):H2836–46.
Lee S, Varvel N, Konerth M, Xu G, Cardona AE, Ransohoff RM, et al. CX3CR1 deficiency alters microglial activation and reduces beta-amyloid deposition in two Alzheimer's disease models. Am J Pathol. 2010;177:2549–62.
Lyszkiewicz M, Witzlau K, Pommerencke J, Krueger A. Chemokine receptor CX3CR1 promotes dendritic cell development under steady-state conditions. Eur J Immunol. 2011;41(4):1256–65.
Marchesi F, Locatelli M, Solinas G, Erreni M, Allavena P, Mantovani A. Role of CX3CR1/CX3CL1 axis in primary and secondary involvement of the nervous system by cancer. J Neuroimmunol. 2010;224(1–2):39–44.
McDermott DH, Halcox JP, Schenke WH, Waclawiw MA, Merrell MN, Epstein N, et al. Association between polymorphism in the chemokine receptor CX3CR1 and coronary vascular endothelial dysfunction and atherosclerosis. Circ Res. 2001;89(5):401–7.
McDermott DH, Fong AM, Yang Q, Sechler JM, Cupples LA, Merrell MN, et al. Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. J Clin Invest. 2003;111(8):1241–50.
Mizuno T, Kawanokuchi J, Numata K, Suzumura A. Production and neuroprotective functions of fractalkine in the central nervous system. Brain Res. 2003;979(1–2):65–70.
Mizutani M, Pino A, Saederup N, Charo I, Ransohoff RM and Cardona AE. The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. J Immunol. 2011 (In press).
Moatti D, Faure S, Fumeron F, Amara M, Seknadji P, McDermott DH, et al. Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. Blood. 2001;97(7):1925–8.
Nassar BA, Nanji AA, Ransom TP, Rockwood K, Kirkland SA, Macpherson K, et al. Role of the fractalkine receptor CX3CR1 polymorphisms V249I and T280M as risk factors for early-onset coronary artery disease in patients with no classic risk factors. Scand J Clin Lab Invest. 2008;68(4):286–91.
Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science. 2005;308(5726):1314–8.
Noda M, Doi Y, Liang J, Kawanokuchi J, Sonobe Y, Takeuchi H, et al. Fractalkine attenuates excito-neurotoxicity via microglial clearance of damaged neurons and antioxidant enzyme heme oxygenase-1 expression. J Biol Chem. 2011;286(3):2308–19.
Neumann H, Kotter, MR and Franklin JM. Debris clearance by microglia: an essential link between degeneration and regeneration. Brain. 2009;132(Pt2):288-95.
Pallandre JR, Krzewski K, Bedel R, Ryffel B, Caignard A, Rohrlich PS, et al. Dendritic cell and natural killer cell cross-talk: a pivotal role of CX3CL1 in NK cytoskeleton organization and activation. Blood. 2008;112(12):4420–4.
Saederup N, Cardona AE, Croft K, Mizutani M, Cotleur AC, Tsou CL, et al. Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. PLoS One. 2010;5(10):e13693.
Savarin-Vuaillat C, Ransohoff RM. Chemokines and chemokine receptors in neurological disease: raise, retain, or reduce? Neurotherapeutics. 2007;4(4):590–601.
Sunnemark D, Eltayeb S, Nilsson M, Wallstrom E, Lassmann H, Olsson T, et al. CX3CL1 (fractalkine) and CX3CR1 expression in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis: kinetics and cellular origin. J Neuroinflammation. 2005;2:17.
Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood. 2008;112(13):5026–36.
Williams K, Ulvestad E, Waage A, Antel JP and McLaurin J. Activation of adult human derived microglia by myelin phagocytosis in vitro. J Neurosci Res. 1994;38(4):433–43.
Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. Blood. 1994;83(8):2360–7.
Yajima N, Kasama T, Isozaki T, Odai T, Matsunawa M, Negishi M, et al. Elevated levels of soluble fractalkine in active systemic lupus erythematosus: potential involvement in neuropsychiatric manifestations. Arthritis Rheum. 2005;52(6):1670–5.

CXCL10

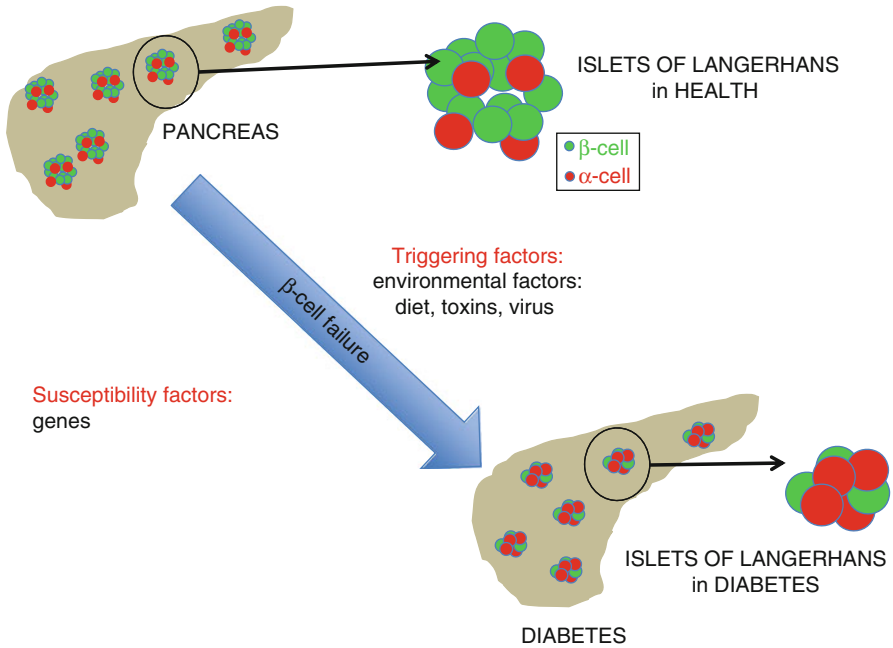
Federico Paroni and Kathrin Maedler
Center for Biomolecular Interactions,
University of Bremen, Bremen, Germany

Synonyms

C7; Chemokine (C-X-C motif) ligand 10; crg-2; gIP-10; IFI10; INP10; IP-10; mob-1; SCYB10

Historical Background

Chemokines/chemoattractant cytokines are small proteins with a mass of 8–10 kDa. Cytokines and chemokines are effector molecules that play a pivotal role in orchestrating both the innate and acquired immune responses. Additionally, they are involved in cell differentiation, division, and repair. The chemokine's nomenclature has been established



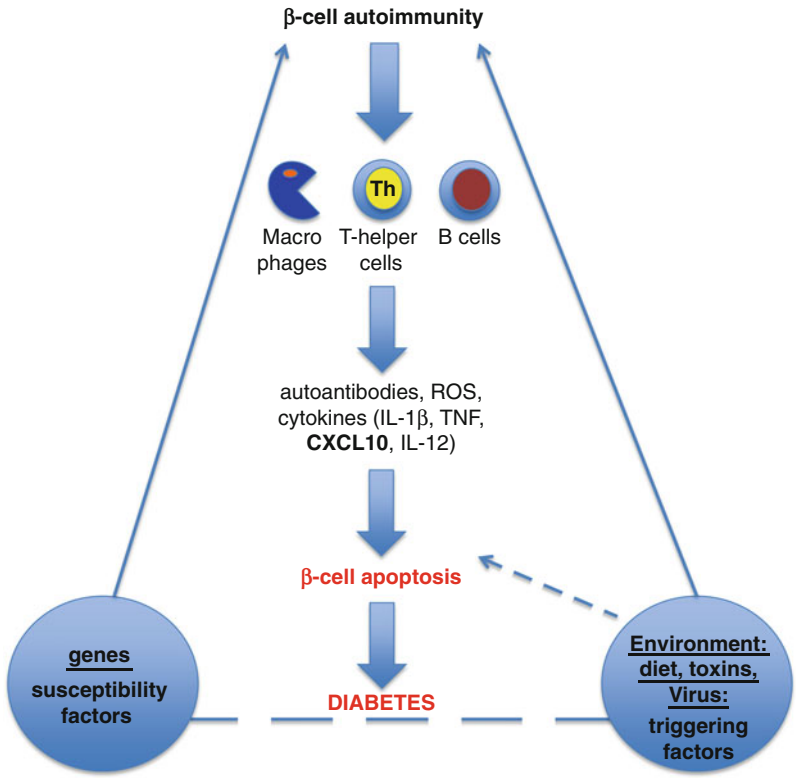
CXCL10, Fig. 1 Diabetes is caused by β-cell destruction triggered by environmental factors in genetically predisposed individuals

in the early 1990s at the International Symposium on Chemotactic Cytokines in Baden (Lindley et al. 1993) on the base of the N-term conserved cysteine motif. Chemokines are classified into four families: C, CC, CXC, CX3C, where X represent any amino acid residue. CXCL10, also called interferon-γ-inducible protein 10 (IP-10), has been initially identified as a chemokine induced by interferon-γ and secreted by a variety of tissues, for example, endothelial cells, monocytes, fibroblasts, and keratinocytes (Luster and Ravetch 1987). CXCL10 has been classified as inflammatory chemokine due to its ability to strongly attract lymphocytes, lack of the ELR motif (glutamic acid-leucine-arginine) which abolishes neovascularization, and function as angiostatic chemokine (Strieter et al. 1995). CXCL10 as well as CXCL9 (MIG) and CXCL11 (ITAC) exerts its action of immune response activator through its natural receptor CXCR3, but CXCL10 also binds to a member of the Toll-like receptor family, TLR4, shown in pancreatic β-cells (Schulthess, Paroni et al. 2009). Over the past few years, both serum levels and tissue expression of CXCL10 were monitored and correlated with various autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, autoimmune thyroid disease, and diabetes mellitus (Hanaoka et al. 2003; Rotondi et al. 2007). Diabetes is a metabolic disease in which the body is unable to produce sufficient amounts of insulin to maintain normoglycemia. Diabetes was reported by Greek physicians already in 250 B.C. and is the Greek word for "syphon," referring to the severe condition of polyuria, the production of large amounts of urine. The complete term "diabetes mellitus" was established later in the seventeenth century. Mellitus is Latin for honey, which is how the physician Thomas Willis described the taste of urine in patients.

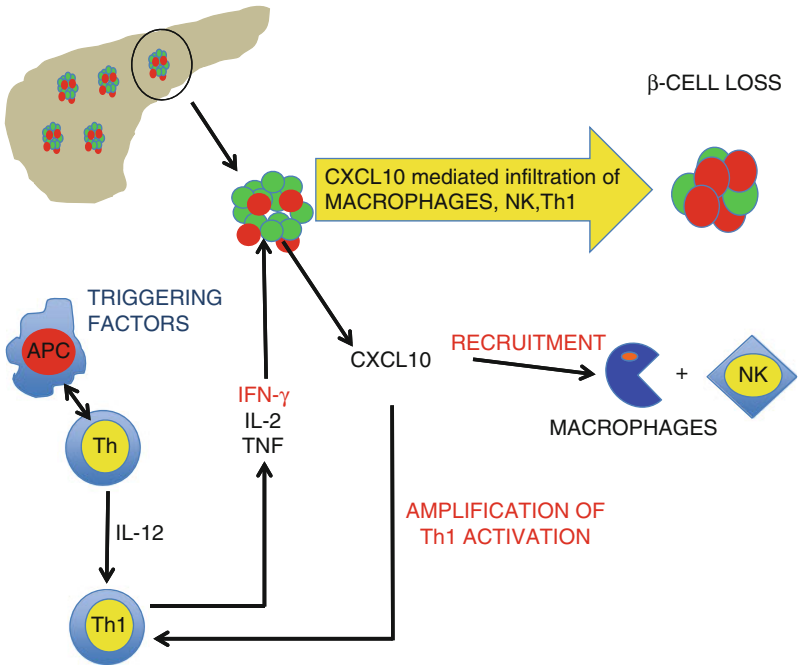
Blood glucose levels are controlled by pancreatic hormones produced by different cell types within the organized structures of the islets of Langerhans that form the endocrine portion of the pancreas. In particular, the hormone insulin, produced by the β-cells, is responsible for decreasing blood glucose by inducing its uptake into target tissues after meals. Diabetes manifests when β-cells fail to produce sufficient amounts of insulin, due to a loss of function and the loss of β-cells themselves. A number of studies over the years, either performed on mouse models or by investigating autopsy material from human pancreata, show that a hallmark of diabetes in both autoimmune type 1 diabetes (T1DM) as well as obesity-related type 2 diabetes (T2DM) is the loss of insulin-producing β-cells by apoptosis (Donath and Halban 2004) (Fig. 1).

CXCL10 in T1DM

In T1DM, β-cell apoptosis is the result of an autoimmune attack. Interaction between antigen-presenting cells and T-cells leads to a prolonged presence of intra-islet inflammatory mediators (cytokines, chemokines, reactive oxygen species (ROS)), finally resulting in β-cell destruction (Paroni et al. 2009). T-helper 1 (Th1) cells are suggested to be crucial in triggering and amplifying such immune attack (Delovitch and Singh 1997) (Fig. 2). The production of IL-12 promotes the development of Th1 cells that produce IFN-γ, IL-2, and TNF-β (Frigerio et al. 2002). IFN-γ activates the transcription, production, and secretion of CXCL10 on target cells that in turn enhances the activation of the immune system (Th1 cells) in a paracrine way (Christen and Von Herrath 2004). $CXCR3^+$ T-cells are recruited to β-cells within the islets. Increased CXCL10 serum levels in patients with T1DM as well as in at-risk individuals were shown in several studies (Shimada et al. 2001; Nicoletti et al. 2002). In contrast, one study reported no statistically significant differences in CXCL10 serum levels in a similar patient collective (Rotondi et al. 2003a, 2003b). In pancreatic tissue from a patient with recent onset of T1DM, CXCL10 expression was measured within islets and lymphocytes (Roep et al. 2010). A positive correlation has been shown between CXCL10 and IFN-γ serum levels and GAD-reactive-IFN-γ producing $CD4^+$ cells (Shimada et al. 2001). In contrast, disease duration and CXCL10 serum levels are negatively correlated (Shimada et al. 2001), similarly to most of the immune markers, which cannot or can rarely be detected in long-standing T1DM. Measurements of inflammatory markers at different time points after diagnosis of the disease may be one reason for the data variation in different studies (Fig. 3). In the non-obese diabetic (NOD) mouse model for T1DM, CXCL10 is produced in pancreatic islets even before detectable insulitis (Cardozo et al. 2003; Li et al. 2005). Inhibition of CXCL10 delayed immune-mediated diabetes in the



CXCL10, Fig. 2 β-cell destruction in T1DM is triggered by inflammatory mediators. Activation of the immune system leads to increased levels of reactive oxygen species, autoantibodies and cytokines. Secreted cytokines act on the β-cell and/or via potentiation of the immune system activation



CXCL10, Fig. 3 Potentiation of the immune system activation by CXCL10. Triggering factors (virus, toxins) lead to T-cell differentiation and maturation. Cytokines produced by Th1 activated cells (IFN-γ, TNFα and IL-12) target β-cells leading to CXCL10 secretion. CXCL10 production and secretion is then boosted through a positive feedback leading to a further recruitment of macrophages and NK cells within the islets. Enhanced activation of the immune system leads to the immuno-mediated β-cell loss

NOD mouse (Morimoto et al. 2004) and transgenic mice expressing CXCL10 in β-cells show spontaneous infiltration of lymphocytes as well as impairment of β-cell function (Rhode et al. 2005).

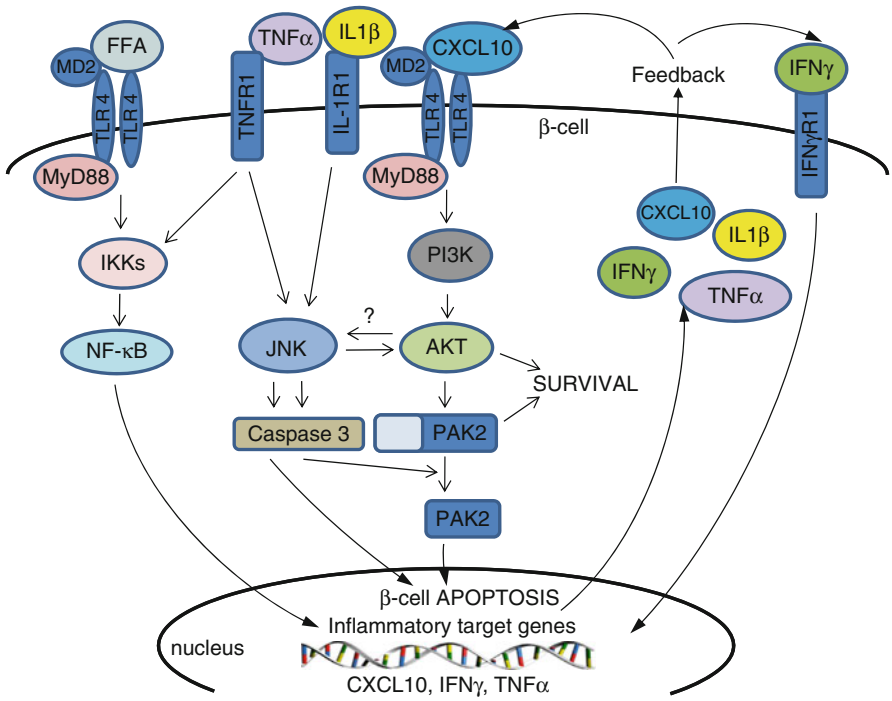
CXCL10 in Virus-Mediated Diabetes

Basic and still unresolved questions regarding the onset of diabetes are how endogenous β-cell antigens become immunogenic and whether environmental factors such as viruses play a causative role in this process. Infection with viruses with a tropism for pancreatic islets highly increases CXCL10 levels both in vivo and in vitro. Mouse studies suggest a pivotal role of CXCL10 during virus-induced diabetes (Christen et al. 2003; Morimoto et al. 2004). Abolishing CXCL10 signaling using specific antibodies leads to a reduction of diabetes incidence (Morimoto et al. 2004). The reduction of diabetes incidence in mice is rather chemokine-specific than a redundant immune system effect. The protective effect is due to less lymphocyte infiltration combined with a reduction of CD8+ lymphocyte activation. In contrast, CXCL10 expression in mice is insufficient to trigger significant autoimmune damage of β-cells. CXCL10 was able to abrogate autoimmunity when it was expressed outside the pancreas (Christen and Von Herrath 2004).

CXCL10 in T2DM

Recently, the presence of immune cell infiltration within islets was also observed in T2DM (Ehses et al. 2007); elevated levels of cytokines in the serum and within islets and apoptotic β-cells characterize the disease. High serum levels of the chemokine CXCL10 have been found in patients with manifest T2DM and with a high risk to develop the disease (Nicoletti et al. 2002; Xu et al. 2005; Herder et al. 2006). CXCL10 does not only act as chemoattractant for the immune system but also triggers β-cell apoptosis in isolated human islets (Schulthess et al. 2009). CXCL10 expression was observed in islets in diabetes as well as in islets from obese patients (Schulthess et al. 2009) and in high-fat diet-fed mice (unpublished observation). Also in adipose tissue from obese individuals (Herder et al. 2007a) and obese mice, CXCL10 is increased.

Obesity is characterized by high levels of circulating free fatty acids (FFA) that stimulate the production of cytokines including CXCL10 that can be



CXCL10, Fig. 4 Hypothetical model of CXCL10 mediated β-cell apoptosis. CXCL10, as well as IFN-γ, IL-1β and TNF-α potentiate their effect via a self activation loop that can be further enhanced by the free fatty acids (FFA). CXCL10 mediated activation of TLR4 leads to activation of the PI3K/Akt pathway followed by JNK and PAK2 cleavage, which reverses the signaling from survival to apoptosis (Adapted from Paroni et al. (2009)

further amplified by IFN-γ which is also elevated in obesity (Shimabukuro et al. 1998; Herder et al. 2007b). In β-cells CXCL10 acts as pro-apoptotic effector through the alternative receptor TLR4 (Schulthess, Paroni et al. 2009). The canonical signaling pathway PI3K/Akt is essential for β-cell survival. Upon CXCL10/TLR4 interaction, Akt is at first activated and promotes β-cell survival together with transcription and secretion of cytokines like TNF-α, IFN-γ, IL-1β, and CXCL10, which then initiate a cascade and potentiate the apoptotic effect through a paracrine/autocrine effect. The CXCL10-TLR4 cascade activates JNK, induces caspase 3 cleavage, which in turn cleaves activated protein kinase 2 (PAK-2), which is downstream of Akt and reverses Akt signaling from proliferation/survival to apoptosis (Fig. 4). These data implicate an important role of CXCL10 in the disease progression of diabetes and suggest blocking CXCL10 signaling as a new therapeutic strategy for diabetes.

Summary

There is strong evidence that CXCL10 plays a causative role for the onset and development of diabetes. However, serum levels of CXCL10 during the development of the disease are discordant among studies. Such discrepancy can be produced by limitation of the analysis as well as the comparison groups (sex, age and especially duration of the autoimmune disease). Serum levels of CXCL10 may not reflect its expression within the islet of Langerhans, observed in obesity, T1DM and T2DM. Different tissue vascularization and time and duration of CXCL10 expression during the disease are important considerable variables. The dual effect of initiation/maintenance or abolition of the immune response and its relation to time and location of CXCL10 expression was already described in mice during virus-induced diabetes (Christen and Von Herrath 2004). Such a complex scenario is strengthening the role of CXCL10 during the onset of both T1DM and T2DM. Due to its potential role as diagnostic marker as well as its intriguing apoptotic activity, further studies are required in order to: (1) establish a consistent analysis method to bring uniformity in the results among different studies, (2) further elucidate the activation pathways that lead β-cells to undergo apoptosis upon CXCL10 signaling activation, and (3) clarify the extent of the contribution of CXCL10 in vivo during the onset of autoimmune disease.

This work was supported by the European Research Council (ERC), the German Research Foundation (DFG), and the Juvenile Diabetes Research Foundation (JDRF).

References

Cardozo AK, Proost P, et al. IL-1beta and IFN-gamma induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice. Diabetologia. 2003;46(2):255–66.

Christen U, Von Herrath MG. IP-10 and type 1 diabetes: a question of time and location. Autoimmunity. 2004;37(5):273–82.

Christen U, McGavern DB, et al. Among CXCR3 chemokines, IFN-gamma-inducible protein of 10 kDa (CXC chemokine ligand (CXCL) 10) but not monokine induced by IFN-gamma (CXCL9) imprints a pattern for the subsequent development of autoimmune disease. J Immunol. 2003;171(12):6838–45.

Delovitch TL, Singh B. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. Immunity. 1997;7(6):727–38.

Donath MY, Halban PA. Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. Diabetologia. 2004;47:581–9.

Ehses JA, Perren A, et al. Increased number of islet-associated macrophages in type 2 diabetes. Diabetes. 2007;56(9):2356–70.

Frigerio S, Junt T, et al. Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulitis. Nat Med. 2002;8(12):1414–20.

Hanaoka R, Kasama T, et al. A novel mechanism for the regulation of IFN-gamma inducible protein-10 expression in rheumatoid arthritis. Arthritis Res Ther. 2003;5(2):R74–81.

Herder C, Baumert J, et al. Chemokines as risk factors for type 2 diabetes: results from the MONICA/KORA Augsburg study, 1984–2002. Diabetologia. 2006;49(5):921–9.

Herder C, Hauner H, et al. Constitutive and regulated expression and secretion of interferon-gamma-inducible protein 10 (IP-10/CXCL10) in human adipocytes. Int J Obes (Lond). 2007a;31(3):403–10.

Herder C, Schneitler S, et al. Low-grade inflammation, obesity, and insulin resistance in adolescents. J Clin Endocrinol Metab. 2007b;92(12):4569–74.

Li D, Zhu SW, et al. Expression of interferon inducible protein-10 in pancreas of mice. World J Gastroenterol. 2005;11(30):4750–2.

Lindley IJD, Westwick J, Kunkel SL. Nomenclature announcement: the chemokines. Immunol Today. 1993;14:24.

Luster AD, Ravetch JV. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). J Exp Med. 1987;166(4):1084–97.

Morimoto J, Yoneyama H, et al. CXC chemokine ligand 10 neutralization suppresses the occurrence of diabetes in nonobese diabetic mice through enhanced beta cell proliferation without affecting insulitis. J Immunol. 2004;173(11):7017–24.

Nicoletti F, Conget I, et al. Serum concentrations of the interferon-gamma-inducible chemokine IP-10/CXCL10 are augmented in both newly diagnosed type I diabetes mellitus patients and subjects at risk of developing the disease. Diabetologia. 2002;45(8):1107–10.

Paroni F, Domsgen E, et al. CXCL10- a path to beta-cell death. Islets. 2009;1(3):256–9.

Rhode A, Pauza ME, et al. Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. J Immunol. 2005;175(6):3516–24.

Roep BO, Kleijwegt FS, et al. Islet inflammation and CXCL10 in recent-onset type 1 diabetes. Clin Exp Immunol. 2010;159(3):338–43.

Rotondi M, Lazzeri E, et al. Role for interferon-gamma inducible chemokines in endocrine autoimmunity: an expanding field. J Endocrinol Invest. 2003a;26(2):177–80.

Rotondi M, Romagnani P, et al. to: Nicoletti F, Conget L, Di Mauro M et al. (2002) Serum concentrations of the interferon-alpha-inducible chemokine IP-10/CXCL10 are augmented in both newly-diagnosed Type I diabetes mellitus patients and subjects at risk of developing the disease. Diabetologia 45:1107–1110. Diabetologia. 2003b;46(7):1020–1.

Rotondi M, Chiovato L, et al. Role of chemokines in endocrine autoimmune diseases. Endocr Rev. 2007;28(5):492–520.

Schulthess FT, Paroni F, et al. CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. Cell Metab. 2009;9(2):125–39.

Shimabukuro M, Zhou YT, et al. Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. Proc Natl Acad Sci U S A. 1998;95(5):2498–502.

Shimada A, Morimoto J, et al. Elevated serum IP-10 levels observed in type 1 diabetes. Diabetes Care. 2001;24(3):510–5.

Strieter RM, Polverini PJ, et al. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J Biol Chem. 1995;270(45):27348–57.

Xu H, Nakayama K, et al. Elevated plasma concentration of IP-10 in patients with type 2 diabetes mellitus. Nihon Jinzo Gakkai Shi. 2005;47(5):524–30.

Cyclic ADP-Ribose Hydrolase

► CD38

Cyclin A

Randy Y. C. Poon
Division of Life Science, Hong Kong University of Science and Technology, Kowloon, Hong Kong

Synonyms

CCNA1; CCNA2

Historical Background

Cyclins are first discovered as proteins that varied in abundance during the cell cycle (Hunt, 2004). Cyclin A is one of the first members of the cyclin family to be cloned. Classical cyclins are activating subunits for cyclin-dependent kinases (CDKs) and are essential components of the cell cycle engine (Morgan 2007). Some members of the cyclin family are also known to perform functions unrelated to cell cycle control.

Two cyclin A are present in vertebrates. Cyclin A1 is the "embryonic" form, expressing mainly in early zygotes and testis. Cyclin A2 is the "somatic" form and is widely expressed in most growing cells. In the literature, "cyclin A" generally refers to cyclin A2. Notable exceptions are papers on *Xenopus* embryonic cells, in which "cyclin A" generally refers to cyclin A1.

Cyclin A is expressed periodically during the cell cycle, accumulating from early S phase and disappearing during mitosis. It is generally accepted that cyclin A functions in both S phase and mitosis. Two CDKs, CDK1 (also called CDC2) and CDK2, are catalytically activated by cyclin A. Cyclin A is also believed to play important roles in targeting CDKs to their specific substrates.

Cyclin A1

The only physiological function of cyclin A1 seems to be for spermatogenesis (Fung and Poon 2006). Disruption of cyclin A1 in mice leads to a block of the first meiotic division in male mice. These mice have significantly smaller testes than the wild type littermates and are sterile because of an arrest of spermatogenesis at the latter stages of meiotic prophase. Spermatocytes also undergo apoptosis in the absence of cyclin A1, in part through ▶ p53-dependent mechanisms.

Cyclin A1 is implicated in the tumorigenesis of several cancers, including myeloid leukemia, testicular cancer, breast cancer, and prostate cancer (Yam et al. 2002). As the expression of cyclin A1 is normally suppressed in most somatic cells, reactivation of cyclin A1 is believed to deregulate cell proliferation. Ectopic expression of cyclin A1 has also been reported to induce apoptosis.

Cyclin A2

Cyclin A2 is ubiquitously expressed in proliferating somatic cells and plays essential functions during the cell cycle. Disruption of cyclin A2 in mice causes early embryonic lethality. Cyclin A2-null embryos develop normally until about day 5.5, possibly because of the persistence of a maternal pool of cyclin A2 or compensation by cyclin A1 during early embryo development. Experiments with conditional ablation of cyclin A2 indicated that while cyclin A2 is redundant in fibroblasts, it is essential for cell cycle progression in hematopoietic and embryonic stem cells (Kalaszczynska et al. 2009).

Deregulated expression of cyclin A2 is believed to contribute to tumorigenesis. Although an increased expression of cyclin A2 has been detected in many types of cancer, it is uncertain if this merely reflects the highly proliferative nature of cancers (Yam et al. 2002).

Functions of Cyclin A

Unique among the vertebrate cyclins, cyclin A is both an S-phase cyclin and an M-phase cyclin, functioning in both S phase and mitosis, respectively. Furthermore, both CDK1 and CDK2 can be activated by binding to cyclin A.

Activation of CDKs

CDK1 and CDK2 are expressed at a constant level during the cell cycle. Their activity is determined predominantly by the fluctuation of the cyclin subunit. Activation of cyclin A-CDK complexes also requires the phosphorylation of a residue on the T-loop of the kinase subunit (Thr161 in CDK1; Thr160 in CDK2). Nevertheless, the activity of the enzyme responsible for this phosphorylation (CDK-activating kinase, CAK) does not appear to be regulated during the cell cycle (Morgan 2007).

Under several checkpoint conditions, such as after DNA damage and replication block, the activity of cyclin A-CDK is suppressed (Wohlbold and Fisher 2009). This is carried out by the phosphorylation of Thr14 and Tyr15 of the CDK subunit by WEE1 and MYT1. While WEE1 specifically phosphorylates Tyr15, MYT1 displays a stronger preference for Thr14. During unperturbed cell cycle, cyclin

A2-CDK1, but not cyclin A2-CDK2, is regulated by Thr14/Tyr15 phosphorylation. After checkpoint activation, however, both CDK1 and CDK2 complexes are inactivated by Thr14/Tyr15 phosphorylation (Chow et al. 2003). Inhibition of cyclin A-CDK2 complexes is also carried out by binding to members of the $p21^{CIP1/WAF1}$ family of CDK inhibitors ($p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$). Members of this family of CDK inhibitors share a highly conserved N-terminal region, which interacts with both cyclin A and CDK2 at the same time.

DNA Replication

Cyclin A is predominantly localized to the nucleus. A proportion of cyclin A is more specifically detected at sites of DNA replication. In fact, cyclin A shuttles between the nucleus and the cytoplasm, but nuclear export is slower than the import, resulting in the nuclear localization of the protein.

DNA replication in eukaryotes involves first the formation of pre-replicative complexes (components include ORC proteins, CDC6, CDT1, and MCM proteins) on the origins of replication. Components of the pre-replicative complexes are then phosphorylated by protein kinase complexes including cyclin A-CDK2 and ▶ DBF4-CDC7, allowing the loading of CDC45 onto the origins. The pre-replicative complexes are disassembled after the firing of the origins, thereby preventing the re-firing of the same origin during the same cell cycle.

Cyclin A is implicated in two steps during DNA replication (Woo and Poon 2003). The first step involves the initiation of DNA replication, presumably for the loading of CDC45 onto origins of replication. The second step involves the inhibition of assembly of the pre-replicative complex to ensure that origins are fired only once per cell cycle. Several components of the pre-replicative complex including CDC6, ORC1, MCM4, and CDT1 are phosphorylated by cyclin A-CDK2, thereby preventing them from forming the pre-replicative complex. The underlying mechanisms include both proteolysis and nuclear export. For instance, phosphorylation of CDC6 promotes its nuclear export by a CRM1-dependent mechanism. On the other hand, phosphorylation of ORC1 and CDT1 promotes their degradation by SCF^{SKP2} complexes. Phosphorylation of MCM4 also directly inhibits the helicase activity of the MCM complexes.

Mitosis

Although cyclin A is clearly required for mitosis, its precise role is not fully understood. One hypothesis is that cyclin A itself is a component of M-phase-promoting factor (MPF). The alternative hypothesis is that cyclin A is part of the machinery that triggers the activation of MPF (Lindqvist et al. 2009). Once a portion of MPF is activated, it is essentially an auto-amplifying system.

Other Functions

Other functions attributed to cyclin A include the regulation of several transcription factors, DNA double-strand break repair, the p53-response pathway, and centrosome duplication (Poon and Fung 2007).

Regulation of Cyclin A During the Cell Cycle

Expression of cyclin A is highly regulated during the cell cycle. Cyclin A protein starts to accumulate at early S phase, continues through S phase and G_2 phase, and disappears during mitosis. In relation to other cyclins during the cell cycle, cyclin A is synthesized and destroyed after cyclin E, but is slightly earlier than cyclin B. The periodic expression of cyclin A is regulated at the levels of transcription and proteolysis.

Transcription

Cyclin A mRNA is regulated during development as well as during the cell cycle. The cyclin A promoter is repressed during G_1 phase and highly active during S phase and G_2/M (Fung and Poon 2005).

Cyclin A1 promoter contains GC boxes, which bind to members of the SP1 family. Transactivation of the cyclin A1 promoter by B-MYB also depends on the SP1 binding sites. B-MYB can be phosphorylated and activated by cyclin A1–CDK2, suggesting the possibility of a positive-feedback regulation. The cyclin A1 promoter can also be transactivated by ▶ C-MYB, which interacts with the MYB-binding sites in the promoter.

One of the mechanisms that controls developmental-specific expression of cyclin A1 may involve methylation. The CpG islands of cyclin A1 promoter are highly methylated in certain tissues and somatic cell lines. However, although methylation of cyclin A1 promoter is correlated with gene silencing in somatic

cell lines, it has little correlation with the tissue-specific repression of the promoter. In early embryos, cyclin A1 expression is also regulated at the level of mRNA stability. The maternal cyclin A1 mRNA is destabilized once transcription is initiated in the zygote. The destabilization of cyclin A1 mRNA is due to deadenylation and relies on the 3′-untranslated region of the maternal transcript. In addition, deadenylation also causes direct translational repression of the cyclin A1 mRNA.

Several regulatory elements in cyclin A2 promoter (CCAAT boxes, NF-Y and B-MYB binding, and CHR) are also found in promoters of other genes involved in G_2/M control, including cyclin B, CDK1, and CDC25C. The promoter of cyclin A2 contains CCAAT boxes, which bind the transcriptional activator NF-Y. Expression of cyclin A2 is also stimulated by B-MYB, a transcription factor controlled by E2F during G_1/S transition. Expression of B-MYB is further enhanced during S phase through phosphorylation by cyclin A2–CDK2.

During G_1 phase, cyclin A2 promoter is repressed by the occupation of a repressor element containing an E2F-binding site. E2F associates with pRb and two closely related proteins p107 and p130 during G_1. These pRb-related proteins repress E2F-responsive promoters by recruiting the histone deacetylase HDAC and the chromatin remodeling complex SWI/SNF to the promoter. Interestingly, HDAC activity is dispensable but ▶ SWI/SNF is required for pRb to repress the cyclin A2 promoter. The activation of cyclin D/E-CDK complexes during G_1 leads to the hyperphosphorylation of pRb, releasing E2F and allowing the activation of the cyclin A2 promoter.

Transcription factors other than E2F are also known to regulate cyclin A2 transcription. In addition to the E2F-binding repressor element, another element termed the cell cycle genes homology region (CHR) is also important for the repression of cyclin A2 transcription in G_1 (Muller and Engeland 2010). Only putative CHR-binding activities, which are unrelated to E2F, have been identified so far. TAFII250, a subunit of TFIID, can stimulate cyclin A2 transcription through an enhancer element. Transcription of cyclin A2 can be negatively regulated by p53, but probably not through the direct interaction of p53 with its cognate consensus sequence. Finally, cyclin A2 transcription can be stimulated by cAMP or repressed by transforming growth factor-β through an activating transcription factor/cAMP-response-element-binding protein (ATF/▶ CREB) site in the promoter.

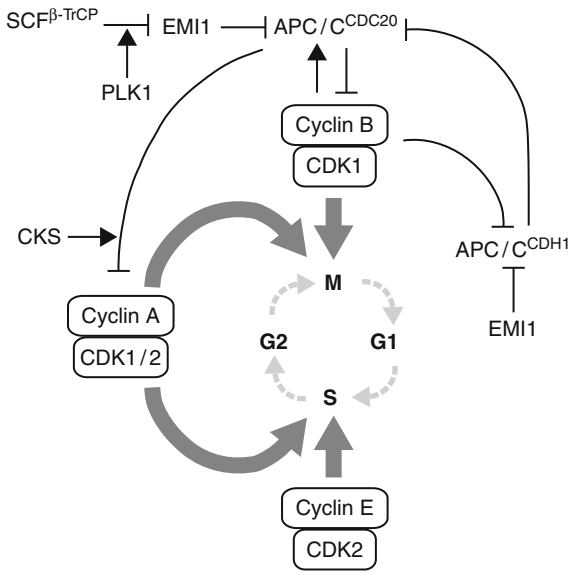
In addition to transcriptional control, the expression of cyclin A2 is also regulated at the level of mRNA stability. For example, the Wilms tumor 1-associating protein (WTAP) interacts with the 3′-untranslated region of cyclin A2 mRNA and stabilizes the mRNA.

Degradation

Degradation of cyclin A2 starts during prometaphase and is complete at metaphase. This differs from that of cyclin B, which is degraded only before the onset of anaphase. The timely destruction of cyclin A2 is in part conferred by a short sequence at the N-terminal region known as the destruction box (D-box). D-box-containing proteins are targeted for ubiquitination by a ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C) (Skaar and Pagano 2009). Degradation of cyclin A2 during mitosis involves the APC/C targeting subunit CDC20 as well as the CDK-interacting protein CKS.

In contrast to the well-established mechanism for cyclin B degradation, precisely how the degradation of cyclin A2 by APC/C is regulated is less well understood. In fact, there are important differences between the degradation processes of the two mitotic cyclins, including the extent of the sequences required for D-box function. During unperturbed mitosis, cyclin A2 is destroyed slightly earlier than cyclin B. Another hallmark of cyclin A2 is that its destruction can occur even when the APC/C is inhibited by the spindle-assembly checkpoint (Di Fiore and Pines 2010).

After the cell exits mitosis, APC/C becomes associated with another targeting subunit called CDH1. Unlike APC/C^{CDC20}, APC/C^{CDH1} is activated only after mitosis because its formation is suppressed by CDK-dependent phosphorylation during mitosis. The activated APC/C^{CDH1} then degrades CDC20 and is important for curbing the unscheduled accumulation of cyclins during G_1 phase. During late G_1 to early S phase, the re-accumulation of cyclin A2-CDK complexes causes the phosphorylation of CDH1 and prevents its association with the APC/C core. The inactivation of APC/C^{CDH1} during S phase then allows the re-accumulation of regulators of the subsequent mitosis, including proteins such as cyclin B and PLK1. How cyclin A2 can accumulate in the presence of APC/C^{CDH1} is an important issue. One mechanism



Cyclin A, Fig. 1 Cyclin A and the cell cycle. Together with cyclin E-CDK2 and other kinases, cyclin A-CDK1/2 are important for the initiation of DNA replication and prevention of re-replication. Cyclin A-CDK1/2 are also essential for mitosis, probably as part of the mechanism for kick-starting the main mitotic engine (cyclin B-CDK1). During mitosis, APC/C^{CDC20} is activated by cyclin B-CDK1 and is responsible for degrading cyclin A, a process also involves CKS. Cyclin B itself is degraded later once the constraint of the APC/C^{CDC20} from the spindle-assembly checkpoint is lifted, thereby driving mitotic exit. At the same time, the inhibition of APC/C^{CDH1} by cyclin B-CDK1 is removed. The high activity of APC/C^{CDH1} during G_1 phase keeps the expression of cyclin A and cyclin B low. This continues until late G_1, when APC/C^{CDH1} is turned off by EMI1. EMI1 is eventually degraded by a PLK1- and SCF-dependent mechanism, allowing the mitotic cyclins to accumulate for the next mitosis

may involve the self-destruction of the APC/C E2 ligase UbcH10. Another mechanism is through the inhibition of APC/C^{CDH1} by a protein called EMI1, which begins to accumulate at the G_1/S transition. During mitosis, EMI1 is targeted to ubiquitin-mediated degradation by SCF$^{\beta\text{-TrCP}}$ and PLK1-dependent phosphorylation, thereby resetting the APC/C system for the next cell cycle (Lindqvist et al. 2009). Figure 1 summarizes the relationship between cyclin A and the pathways for its degradation.

Summary

Cyclin A is an essential regulator of the cell cycle. Cyclin A binds and activates the cyclin-dependent kinases CDK1 and CDK2. The complexes are important for the regulation of DNA replication as well as mitosis. During S phase, cyclin A-CDK complexes are critical for the loading of replication factors for the initiation of replication. Cyclin A-CDK complexes have a further role in S phase in preventing the re-firing of the same origins of replication. The functions of cyclin A in mitosis is less understood, but it may be involved in kick-starting the main mitotic engine. Cyclin A starts to accumulate at early S phase and disappears during mitosis. The periodic regulation of cyclin A during the cell cycle is achieved through transcriptional control and proteolysis. Transcriptional control of cyclin A promoter involves multiple transcriptional factors that display cell cycle-dependent activities. Destruction of cyclin A during mitosis is carried out by APC/C.

More works are required to provide a comprehensive picture of the functions of cyclin A. In particular, the precise role of cyclin A during mitosis should be deciphered. In this connection, many substrates of cyclin A-CDK remain to be discovered. Understanding a more complete repertoire of cyclin A-CDK's targets will be the key to unravel the mysteries surrounding cyclin A. Also very little is known about the epigenetic control of the promoters of cyclin A1 and cyclin A2. As both cyclin A1 and cyclin A2 are frequently deregulated in cancers, it is vital to understand how they are controlled during tumorigenesis. Cyclin A is likely to be useful as a target for cancer diagnostic and treatment.

References

Chow JP, Siu WY, Ho HT, Ma KH, Ho CC, Poon RYC. Differential contribution of inhibitory phosphorylation of CDC2 and CDK2 for unperturbed cell cycle control and DNA integrity checkpoints. J Biol Chem. 2003;278:40815–28.

Di Fiore B, Pines J. How cyclin A destruction escapes the spindle assembly checkpoint. J Cell Biol. 2010;190:501–9.

Fung TK, Poon RYC. A roller coaster ride with the mitotic cyclins. Semin Cell Dev Biol. 2005;16:335–42.

Fung TK, Poon RYC. Cyclin A1. UCSD-nature molecular pages. 2006. doi:10.1038/mp.a000716.01.

Hunt T. The discovery of cyclin (I). Cell. 2004;116:S63–4. 1 p following S65.

Kalaszczynska I, Geng Y, Iino T, et al. Cyclin A is redundant in fibroblasts but essential in hematopoietic and embryonic stem cells. Cell. 2009;138:352–65.

Lindqvist A, Rodriguez-Bravo V, Medema RH. The decision to enter mitosis: feedback and redundancy in the mitotic entry network. J Cell Biol. 2009;185:193–202.

Morgan DO. The cell cycle: principles of control. Oxford: Oxford University Press; 2007.
Muller GA, Engeland K. The central role of CDE/CHR promoter elements in the regulation of cell cycle-dependent gene transcription. FEBS J. 2010;277:877–93.
Poon RYC, Fung TK. Cyclin A2. UCSD-nature molecular pages. 2007. doi:10.1038/mp.a000717.01.
Skaar JR, Pagano M. Control of cell growth by the SCF and APC/C ubiquitin ligases. Curr Opin Cell Biol. 2009;21:816–24.
Wohlbold L, Fisher RP. Behind the wheel and under the hood: functions of cyclin-dependent kinases in response to DNA damage. DNA Repair Amst. 2009;8:1018–24.
Woo RA, Poon RYC. Cyclin-dependent kinases and S phase control in mammalian cells. Cell Cycle. 2003;2:316–24.
Yam CH, Fung TK, Poon RYC. Cyclin A in call cycle control and cancer. Cell Mol Life Sci. 2002;59:1317–26.

Cysteine Sulfinic Acid Reductase

▶ Sulfiredoxin

cyt-PTPe

▶ PTPe (RPTPe and Cyt-PTPe)

D

D2

▶ NCAM1

Dachsous

▶ Cadherins

DANCE

▶ Fibulins

DAP Kinase

Thomas J. Lukas
Deptartment of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, IL, USA

Synonyms

DAPK; Death-associated protein kinase

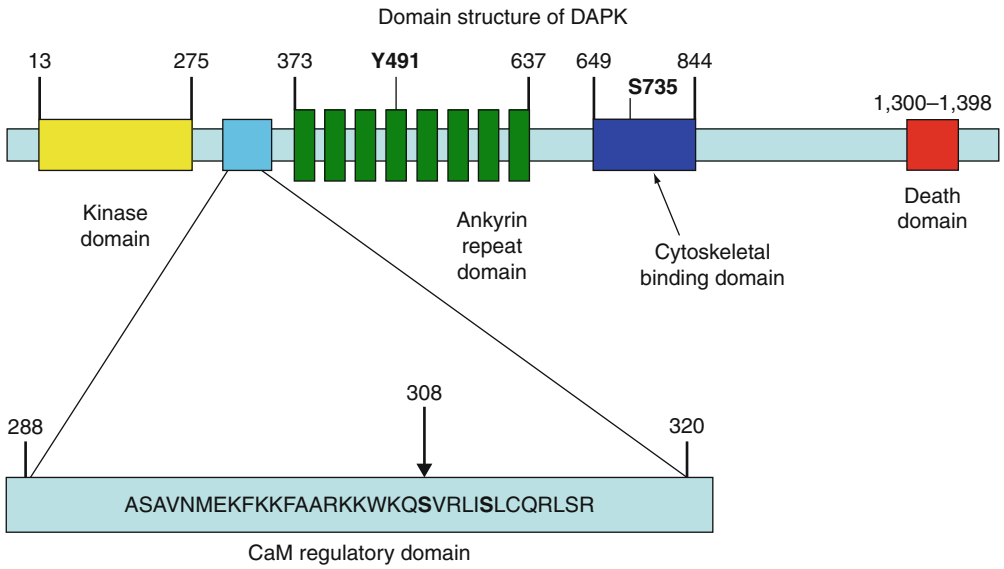
Historical Background

Death-associated protein kinase (DAPK) is the inaugural member of a class of Ser/Thr protein kinases whose members exhibit homologous catalytic domains as well as share cell death-associated functions (Bialik and Kimchi 2006). DAPK was discovered by Kimchi and co-workers as a tumor suppressor gene whose expression is lost in multiple tumor types (Cohen and Kimchi 2001). This attracted the interest of several investigators resulting in an impressive body of literature concerning its function, regulation, and involvement in various diseases and conditions. For example, DAPK participates in apoptotic pathways initiated by interferon-γ, TNFα, activated Fas, and loss of attachment to the extracellular matrix (Bialik and Kimchi 2006). By activating p53 in a p19ARF-dependent fashion, DAPK is an intrinsic tumor suppressor that opposes early stage oncogenic transformation (Raveh et al. 2001). However, hypermethylation of the DAPK promoter inactivates this pathway in tumorigenesis such as found in multiple myeloma (Chim et al. 2007) and other cancers (Michie et al. 2010).

Domain Structure of DAPK and Regulation of Enzymatic Activity

DAPK has several functional domains (Fig. 1). These include a series of ankyrin-repeat domains as well as a "death" domain and catalytic and calmodulin-regulatory domains that are similar to other calmodulin-dependent protein kinases (Lukas et al. 1998). DAPK activation requires calmodulin (CaM), but it is further modulated by phosphorylation at sites within and outside of the CaM regulatory domain (Bialik and Kimchi 2006). Phosphorylation at two sites decrease DAPK activity, Ser-308 (CaM regulatory domain) and Tyr-491/492 in one of the ankyrin repeat

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

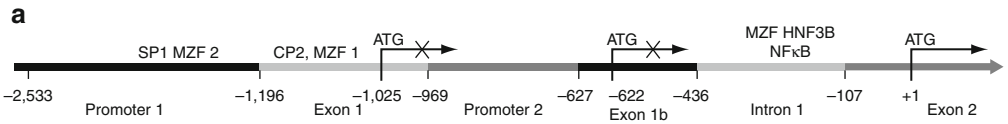


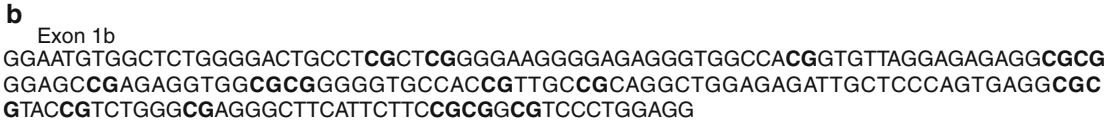
DAP Kinase, Fig. 1 Domain structure of DAPK. Shown are the locations of the kinase catalytic (*yellow*), calmodulin regulatory (*light blue*), ankyrin repeats (*green*), cytoskeletal binding (*blue*), and death domain (*red*). Phosphorylation sites at residues 491 and 735 are shown in *bold*. The calmodulin regulatory domain is expanded to show the amino acid sequence and the location of the Ser-308 phosphosphorylation site

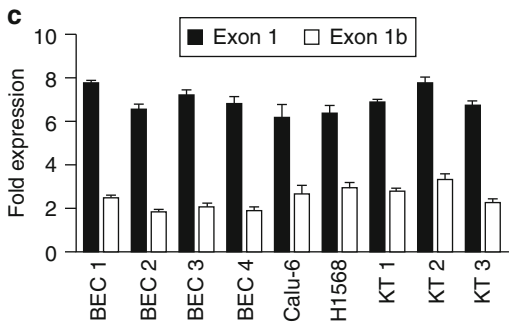
domains (Fig. 1). As with smooth muscle/nonmuscle myosin light chain kinase (MLCK), phosphorylation within the CaM regulatory domain (Ser-308) of DAPK desensitizes CaM activation. Ser-308 is dephosphorylated by the protein phosphatase PP2a (Gozuacik et al. 2008), allowing CaM activation. Phosphorylation of Ser-735 by activation of ▶ MAP kinase (ERK1 & 2) results in an increase in DAPK activity (Michie et al. 2010). It is thought that this tight control over DAPK activation is one of the checkpoints for committing a cell to an apoptotic/necrotic death.

Regulation of DAPK Expression

The upstream genetic sequences that contain the promoter(s) for the human DAPK gene are diagrammed in Fig. 2a. There are two transcriptional start sites that are controlled by independent promoters. Two alternative translational start sites at -1,025 and -622 are followed by termination codons within exon 1 and 1b, respectively, rendering them nonfunctional. These sites precede the active start site in exon 2. There is no TATA box in either promoter, but there are several of transcription factor binding sites including AP2, E-box, CAAT box, AP1, and nuclear factor kappa B (NF-kB) (Pulling et al. 2009). A CpG island of 590 bp containing 46 CpG dinucleotides is directly upstream of the translational start site. An additional 100 CpGs are located 1,000 bp upstream of this region. Exon 1b contains 17 CpG dinucleotides that may also be methylated in cancer cells (Fig. 2b). Both promoters are active in multiple cell types. Promoter 1 (Exon 1) activity was 40–50% higher than promoter 2- Exon 1b-intron promoter using reporter constructs (Fig. 2c) (Pulling et al. 2009). Methylation analysis in 5 of 15 tumor cell lines revealed that 51–91% of the CpGs in the promoter 1-exon 1 region were methylated and associated with a complete loss of transcription from exon 1. Similarly, there was a good correlation with methylation of promoter 2-exon 1b-intron constructs and loss of expression in 9 of 15 tumor cell lines. The methylation status and location within the DAPK promoters varies greatly in cultured cells but methylation at multiple sites correlates with a decrease or loss of expression. Because of the dual promoters, DAPK expression in one cell type may be more susceptible to methylation than another cell type where the alternative promoter is used. This perhaps explains variability in DAPK promoter hypermethylation in tissue biopsy samples where a mixture of cell types (metastatic vs nonmetastatic) is analyzed. However, the association







MZF1, MZF2 - myeloid zinc finger transcription factor SP1 - transcription factor SP1
CP2 - alpha globin transcriptional element, Gene = TFCP2 NFkB - nuclear factor kappa beta
HNF3B - hepatocyte nuclear factor 3 beta, Gene = FOXA2

DAP Kinase, Fig. 2 DAPK genomic structure, exon 1b sequence, and expression of exon 1 and 1b transcripts. (**a**) Schematic diagram depicting the location of promoter 1, exon 1 and 1b, promoter 2, and intron 1 in relation to the translational start site within exon 2 is shown. *Shaded* areas and numbers depict the boundaries and location of each region. The location of the ATGs in exon 1 and 1b that are not translational start sites due to stop codons in these exons are shown, as are the location of transcription factor-binding sites that affect reporter activity. (**b**) Sequence of the 186 bp region designated exon 1b with the 17 CpG dinucleotides (*bolded)* is shown. (**c**) Quantitative expression of DAPK exon 1 and 1b transcripts in H1568, Calu-6, BEC, and keratinocyte (KT) cell lines. Fold expression between exon 1 and 1b transcripts is compared. Reproduced from Pulling et al. (2009) with permission. Transcription factor descriptions were added

of DAPK methylation with tumor aggressiveness and disease progression (poor prognosis) is significant (Pulling et al. 2009; Katzenellenbogen et al. 1999; Chim et al. 2007).

Signal Transduction Pathways Involving DAPK and Its Protein Substrates

Because of its multiple protein interacting domains, DAPK is involved in several cellular processes. Two primary areas of investigative focus are apoptosis and autophagy (Bialik and Kimchi 2006). A summary of the known DAPK substrates is given in Table 1. DAPK phosphorylates regulatory myosin light chains and is involved in membrane blebbing that occurs during programmed cell death (apoptosis) (Bialik and Kimchi 2006). Calmodulin-regulated kinase kinase (CaMKK) is a neuronal protein substrate of DAPK. Its phosphorylation site (Ser-511) is near the CaM recognition domain and results in an attenuation of CaM stimulated activity (Schumacher et al. 2004). Another neuronal substrate is Syntaxin 1A where phosphorylation of Ser-188 is proposed to decrease binding of syntaxin-1A to Munc18-1, a syntaxin binding protein that regulates a complex (known as the SNARE complex) that is necessary for synaptic vesicle docking and secretion (Tian et al. 2003). Thus, if DAPK is activated in presynaptic neurons, two processes may be affected that relate to cellular homeostasis. DAPK phosphorylation of CaMKK is proapoptotic because this kinase is responsible for activating survival pathways through phosphorylation of the kinase known as AMPK (Fogarty et al. 2010), while phosphorylation of syntaxin-1A can lead to inhibition of neurotransmitter secretion (Bialik and Kimchi 2006). DAPK

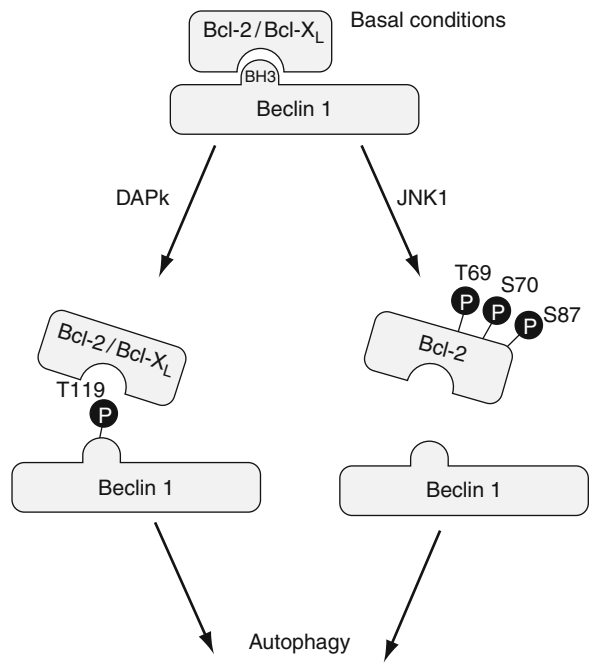
DAP Kinase, Table 1 Functional substrates of DAPK

Substrate	Sequence	In vivo?	Function	Reference
DAPK	RKKWKQ-**S**-VRL	Yes	Inhibits activation	(Shohat et al. 2001)
MLC	RPQRAT-**S**-NVF or RPQRA-**T**-SNVF	Yes	Activates myosin/membrane blebbing	(Bialik and Kimchi 2006)
Zip Kinase	RRRLK-**T**-RL YTIK-**S**-H-**S**-**S**-L PNN-**S**-YADFERF-**S**-K	Yes	Localization and Dimerization, proapopototic	(Shani et al. 2004)
Syntaxin 1A	GHMDSSI-**S**-KQA	Yes	Synaptic vesicle membrane fusion	(Bialik and Kimchi 2006)
CaMKK	RREERSL-**S**-APG	Yes	Inhibits activation	(Schumacher et al. 2004)
Beclin 1A	SRRLKV-**T**-GDLF	Yes	Dissociates Beclin from Bcl-xL proteins	(Zalckvar et al. 2009)
MCM3	TIERRY-**S**-DLT	?	Unknown	(Bialik et al. 2008)

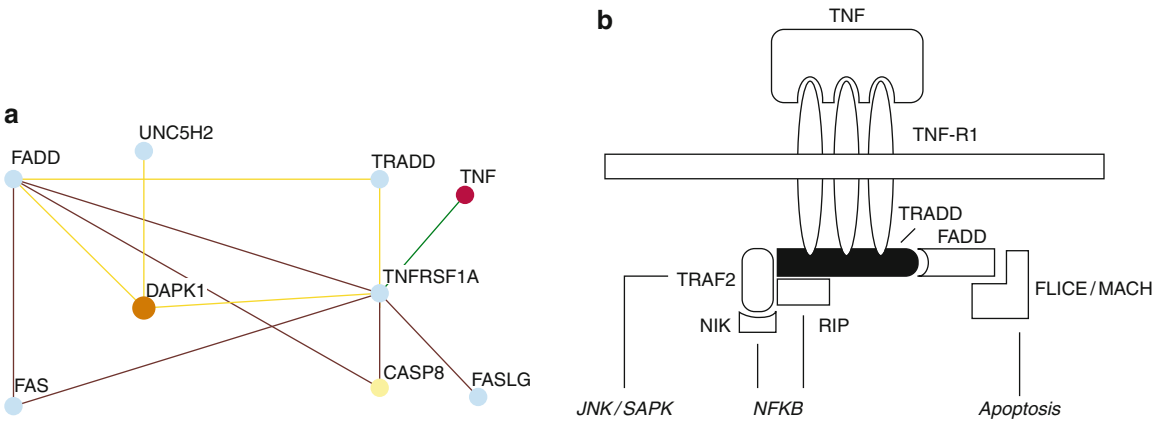
phosphorylation of Zip kinase (DAPK3) activates this kinase leading to changes in cell morphology characteristic of apoptotic cells (Shani et al. 2004). Finally, in a screening assay, DAPK was found to phosphorylate MCM3, a protein that may be involved in the regulation of DNA replication (Bialik et al. 2008).

DAPK, Autophagy and Apoptosis

Figure 3 summarizes one phase of autophagy and the role that DAPK and its substrate Beclin 1 play in this process. Autophagy is a highly conserved process that is characterized by the formation of membrane enclosed "autophagosomes" that function to engulf intracellular organelles and other constituents and deliver them to the lysosomes for degradation. Thus, autophagy is the cell's intrinsic "recycling" machinery that serves to provide material for cell metabolism during periods when extracellular sources of nutrients are low. Beclin 1 is an essential autophagic protein that binds ▶ BcL-2 family proteins through its BH3 domain. The phosphorylation site for DAPK (Thr-119) is located within the BH3 domain, and phosphorylation promotes dissociation of Beclin 1 from proteins Bcl-XL and BcL-2. Dissociated BcL-XL promotes activation of autophagic machinery by interacting with a complex centered upon phosphatidylinositol-3-kinase. This multiprotein complex participates in autophagosome nucleation. Phosphorylation of Bcl-2 by JNK1 kinase also leads to autophagy by a similar mechanism. Beclin 1, itself, has been proposed to interact with several autophagy activators in the complex including AMBRA1, UVRAG, and Bif-1 (Zalckvar et al. 2009). Chronic activation of autophagy can lead to cell death through nutrient depletion (Zalckvar et al. 2009).



DAP Kinase, Fig. 3 A model showing two phosphorylation events which regulate the interaction between Beclin 1 and its inhibitors. DAPK-mediated phosphorylation on Beclin 1's T119 residue, and JNK1-mediated phosphorylation on residues T69, S70, and S87 of Bcl-2, each reduces Beclin 1's interaction with its inhibitors leading to autophagy. Reproduced from Zalckvar et al. (2009) by permission from the Authors



DAP Kinase, Fig. 4 DAPK and apoptosis. (**a**) DAPK engages several proteins in a complex that receives inputs from potential death signals (FADD, TNF receptor 1) as well as survival signals (UNC5H2 –Netrin receptor). (**b**) Schematic representation of TNF-R1 (TNF receptor 1) and its signal transducers. Upon ligand-induced trimerization, the adaptor TRADD and the downstream signal transducers FADD, TRAF2, and RIP are rapidly recruited to TNF-R1. The JNK/SAPK pathway is associated with cellular stress response, NFκβ with cytoprotection, and the FLICE/MACH1 complex directly activates Caspase 8, an apoptosis initiating protease. Used with permission from Natoli et al. (1998)

DAPK is linked through direct binding to a number of proteins that participate in apoptosis by one or more pathways (Cohen et al. 1999) outlined in Fig. 4a. Not all of these pathways are functional in a given cell type. The first pathway is through the binding of Fas to the death-initiator, FADD. DAPK interacts with FADD by way of its death domain (Fig. 1). This interaction results in the downstream activation of caspases that leads to cell death (Fig. 4a). The involvement of DAPK was established in multiple ways. (1) Expression of a fragment of DAPK containing the death domain protects cells from Fas-mediated cell death; (2) Expression of a DAPK mutant lacking the death domain does not promote cell death; (3) DAPK mutants that lack CaM regulation (deletion of CaM segment) resulted in massive apoptosis (Shohat et al. 2001). Thus, both the death domain and DAPK catalytic activity are necessary for Fas-mediated cell death. Apoptosis mediated by DAPK is also initiated from other signals such as UNC5H2. UNC52B (netrin 1 receptor) when bound to ligand blocks DAPK activation (Llambi et al. 2005). However, when netrin is absent, UNC5H2 reduces the phosphorylation of DAPK at Ser-308 which induces its activation by Ca^{2+} CaM (Shohat et al. 2001). Thus, netrin1/UNC5H2 functions as a DAPK switch. (Llambi et al. 2005). In the second pathway, apoptosis is mediated by the ▶ TNFα pathway which has multiple branches (Fig. 4b). DAPK activity is not strictly required in this pathway (Bialik and Kimchi 2006), but, DAPK is part of a large complex that includes the TNFα receptor and several accessory proteins including TRADD, FADD, and FLICE2/MACH (Natoli et al. 1998). Note that none of these proteins are known substrates of DAPK (Table 1). Thus, the function of DAPK in this pathway may be related to its ability to serve as a scaffold for the assembly of proteins in the TNFα receptor complex and facilitate the induction of apoptosis. In some cell types, however, depletion of DAPK by mRNA interference can promote apoptosis (Jin and Gallagher 2003). This may be mediated by a DAPK isoform that has a truncation near the C-terminus of the protein that inhibits the activity of the death domain (Bialik and Kimchi 2006). It is not clear what mediates the expression of this isoform called DAPKβ, but additional work in this area is needed to reveal the complexity of DAPK expression and activity.

Summary

DAPK is a fascinating kinase with respect to its involvement in multiple cellular processes that have not been fully studied. For example, although some substrates have been identified, the timing and duration of DAPK activation may be critically important with respect to cellular commitment to apoptosis.

References

Bialik S, Kimchi A. The death-associated protein kinases: structure, function, and beyond. Annu Rev Biochem. 2006;75:189–210.

Bialik S, Berissi H, Kimchi A. A high throughput proteomics screen identifies novel substrates of death-associated protein kinase. Mol Cell Proteomics. 2008;7(6):1089–98.

Chim CS, Liang R, Fung TK, Choi CL, Kwong YL. Epigenetic dysregulation of the death-associated protein kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway in multiple myeloma. J Clin Pathol. 2007;60(6):664–9.

Cohen O, Kimchi A. DAP-kinase: from functional gene cloning to establishment of its role in apoptosis and cancer. Cell Death Differ. 2001;8(1):6–15.

Cohen O, Inbal B, Kissil JL, Raveh T, Berissi H, Spivak-Kroizaman T, et al. DAP-kinase participates in TNF-alpha- and Fas-induced apoptosis and its function requires the death domain. J Cell Biol. 1999;146(1):141–8.

Fogarty S, Hawley SA, Green KA, Saner N, Mustard KJ, Hardie DG. Calmodulin-dependent protein kinase kinase-beta activates AMPK without forming a stable complex: synergistic effects of Ca2+ and AMP. Biochem J. 2010;426(1):109–18.

Gozuacik D, Bialik S, Raveh T, Mitou G, Shohat G, Sabanay H, et al. DAP-kinase is a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death. Cell Death Differ. 2008;15(12):1875–86.

Jin Y, Gallagher PJ. Antisense depletion of death-associated protein kinase promotes apoptosis. J Biol Chem. 2003;278(51):51587–93.

Katzenellenbogen RA, Baylin SB, Herman JG. Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. Blood. 1999;93(12):4347–53.

Llambi F, Lourenco FC, Gozuacik D, Guix C, Pays L, Del Rio G, et al. The dependence receptor UNC5H2 mediates apoptosis through DAP-kinase. EMBO J. 2005;24(6):1192–201.

Lukas TJ, Mirzoeva S, Watterson DM. Calmodulin-regulated protein kinases. In: Van Eldik LJ, Watterson DM, editors. Calmodulin and signal transduction. New York: Academic; 1998. p. 66–168.

Michie AM, McCaig AM, Nakagawa R, Vukovic M. Death-associated protein kinase (DAPK) and signal transduction: regulation in cancer. FEBS J. 2010;277(1):74–80.

Natoli G, Costanzo A, Guido F, Moretti F, Levrero M. Apoptotic, non-apoptotic, and anti-apoptotic pathways of tumor necrosis factor signalling. Biochem Pharmacol. 1998;56(8):915–20.

Pulling LC, Grimes MJ, Damiani LA, Juri DE, Do K, Tellez CS, et al. Dual promoter regulation of death-associated protein kinase gene leads to differentially silenced transcripts by methylation in cancer. Carcinogenesis. 2009;30(12):2023–30.

Raveh T, Droguett G, Horwitz MS, DePinho RA, Kimchi A. DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. Nat Cell Biol. 2001;3(1):1–7.

Schumacher AM, Schavocky JP, Velentza AV, Mirzoeva S, Watterson DM. A calmodulin-regulated protein kinase linked to neuron survival is a substrate for the calmodulin-regulated death-associated protein kinase. Biochemistry. 2004;43(25):8116–24.

Shani G, Marash L, Gozuacik D, Bialik S, Teitelbaum L, Shohat G, et al. Death-associated protein kinase phosphorylates ZIP kinase, forming a unique kinase hierarchy to activate its cell death functions. Mol Cell Biol. 2004;24(19):8611–26.

Shohat G, Spivak-Kroizman T, Cohen O, Bialik S, Shani G, Berrisi H, et al. The pro-apoptotic function of death-associated protein kinase is controlled by a unique inhibitory autophosphorylation-based mechanism. J Biol Chem. 2001;276(50):47460–7.

Tian JH, Das S, Sheng ZH. Ca2+-dependent phosphorylation of syntaxin-1A by the death-associated protein (DAP) kinase regulates its interaction with Munc18. J Biol Chem. 2003;278(28):26265–74.

Zalckvar E, Berissi H, Mizrachy L, Idelchuk Y, Koren I, Eisenstein M, et al. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep. 2009;10(3):285–92.

DAP Kinase-Related Apoptotic Kinase 2

► DRAK2

DAPK

► DAP Kinase

DARPP

Daniela V. Rosa[1], Luiz Alexandre V. Magno[1], Bruno R. Souza[2] and Marco A. Romano-Silva[3]
[1]INCT de Medicina Molecular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[2]Department of Cell & Systems Biology, Centre for the Analysis of Genome Evolution and Function, University of Toronto, Toronto, ON, Canada
[3]INCT de Medicina Molecular; Departamento de Saúde Mental, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Synonyms

Darpp32; DARPP-32; Dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein, 32 kDa; Dopamine and cAMP-regulated

phosphoprotein; Dopamine- and cAMP-regulated phosphoprotein, Mr32 kDa; Ppp1r1b; Protein phosphatase 1 DARPP-32 inhibitor protein; Protein phosphatase 1, regulatory (inhibitor) subunit 1B

Historical Background

Recent studies using behavioral analysis of animal models suggest that alterations in critical intracellular signaling pathways have an important role in the pathophysiology and treatment of complex neuropsychiatric disorders. The hypothesis is that, if the vast majority of psychiatric medications exert their primary therapeutic actions in the first week of treatment, the therapeutic effects involve transcriptional changes initiated and maintained by critical intracellular signaling pathways.

The dopaminergic neurotransmission system has been the focus of much research throughout the last few decades, including psychiatric and neurological disorders, and drug action mechanisms. Dopamine is associated with motor behavior, pleasure and reward, cognition, among other functions. It is well known that dopamine plays an important role in the coordination and regulation of the two output pathways by acting in a bidirectional manner. Five different dopamine receptors were cloned in humans, and classified in two subgroups: D_1 and D_2 receptors. Many electrophysiological and gene transcriptional data, obtained in vitro and in vivo, suggest that dopamine exerts stimulatory effects via D_1 receptors and inhibitory effects via D_2 receptors (West and Grace 2002). All of dopaminergic receptors are metabotropic and alter cAMP signaling: D_1 receptor subtypes (D_1, D_5) stimulate adenylyl cyclase, whereas D_2 receptor subtypes (D_2S, D_2L, D_3, D_4) inhibit adenylyl cyclase (Svenningsson et al. 2004).

Initially discovered as the major target for dopamine-activated adenylyl cyclase and PKA in striatum, DARPP-32 is now well known for playing a central role in the biology of dopaminoceptive neurons in the central and peripheral nervous system (reviewed by Reis et al. 2007). The phosphorylation by PKA at Thr34 converts DARPP-32 into a potent high-affinity inhibitor of the multifunctional serine/threonine protein phosphatase, PP-1 (Svenningsson et al. 2004). DARPP-32 is also phosphorylated at Thr75 by Cdk5 converting DARPP-32 into an inhibitor of PKA. Furthermore, the state of phosphorylation of DARPP-32 at Thr34 is fine-tuned by the phosphorylation state of two serine residues, Ser102 and Ser137, which are phosphorylated by CK2 and CK1, respectively. Thus, by the virtue of its unique ability to modulate the activity of both PP-1 and PKA, DARPP-32 is critically involved in regulating electrophysiological, transcriptional, and behavioral responses to physiological and pharmacological stimuli, including antidepressants, neuroleptics, and drugs of abuse (Svenningsson et al. 2004).

The serotonergic neurotransmitter system, together with the dopaminergic system, regulates emotion, mood, reward, and cognition. Detailed studies in striatal slices and whole animals have shown that serotonin causes increase in phosphorylation of DARPP-32 at Thr34 and Ser137, and decreased phosphorylation at Thr75. The actions of serotonin in regulating phosphorylation of DARPP-32 at Thr34 and Thr75 were mediated primarily via activation of 5-HT4 and 5-HT6 receptors, whereas the regulation of phosphorylaion at Ser137 was mediated primarily via 5-HT2 receptors. DARPP-32 phosphorylation is also modulated by glutamate, GABA, neuromodulators, and neuropeptides (Svenningsson et al. 2004).

DARPP-32 Knockout and Mutant Mice

The generation of DARPP-32 knockout (KO) mice (Fienberg et al. 1998) and mutant mice with point mutations in phosphorylation sites of DARPP-32 (Svenningsson et al. 2003) has provided a powerful tool for the search of the DARPP-32 roles in both behavioral and neurobiological basis of several diseases. Overall, studies have shown that DARPP-32 is required for the physiological actions of dopamine. A well-accepted molecular explanation for this function is the reduced induction of gene expression after treatment with D_1 receptor agonist in mice lacking DARPP-32 (Svenningsson et al. 2000).

In this context, studies using DARPP-32 KO mice have indicated that DARPP-32-dependent pathways are involved in the modulation of the short, and perhaps long, term actions of drugs of abuse (Svenningsson et al. 2005). In fact, addictive properties of most drugs of abuse are mediated via dopaminergic pathways, particularly through post-synaptic neurons within the striatum, which contains high levels

of DARPP-32. For example, cocaine preference (Zachariou et al. 2002), ethanol reward (Risinger et al. 2001), and stimulatory effects of caffeine on motor activity (Lindskog et al. 2002) are reduced in mice lacking DARPP-32. In addition, the exaggerated locomotor response to d-methamphetamine in mice lacking PDE1B was blocked in PDE1B x DARPP-32 double-KO mice (Ehrman et al. 2006). Similar results were reported showing distinct roles of phosphorylation sites of DARPP-32 in mediating behavioral and biochemical actions of cocaine in the Thr34 and Thr75 mutant mice (Zachariou et al. 2002).

Other lines of evidence have shown a critical role for DARPP-32 in the drug therapeutic actions. Beneficial responsiveness to fluoxetine, which increases phosphorylation of DARPP-32 at Thr34, was strongly reduced in DARPP-32 knockout mice (Svenningsson et al. 2002). In addition, there is evidence for a functional role of DARPP-32 in learning and memory (Heyser et al. 2000). For more detailed information, see Fienberg and Greengard (2000).

DARPP-32 and Human Genetics

Since the function of DARPP-32 is endogenously regulated by the action of neurotransmitters and neuromodulators, and exogenously by cocaine and therapeutic drugs, it has been long viewed as potential candidate gene for the most of psychiatric disorders. However, few studies have examined variations in the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), demonstrating a weak association between *PPP1R1B* genetic polymorphisms and the diseases studied. The initial evidence that a chromosomal region within 17q, which includes the *PPP1R1B* gene, increases risk for schizophrenia (Cardno et al. 2001) and bipolar disorder (Dick et al. 2003) has offered a useful starting point for genetic studies.

Several studies have reported abnormalities in DARPP-32 expression in the brain of psychiatric disorder patients (Reis et al. 2007). Because of this evidence, genetic association studies were performed with schizophrenia, bipolar disorder, nicotine dependency, cognitive ability, attention-deficit hyperactivity disorder (ADHD), and breast cancer. Among them, positive associations were found only with nicotine dependence (Beuten et al. 2007), reinforcement learning (Frank et al. 2007), and in one out of five schizophrenia studies (Meyer-Lindenberg et al. 2007). In these studies, weak-to-moderate associations were reported. However, the inconsistencies in the *PPP1R1B* genetic findings can be partially due to the different samples, particularly the ethnicity effects, and not assessing the same genetic alterations.

In a sample of 2,037 subjects, Beuten et al. (2007) found that a *PPP1R1B* haplotype formed by rs2271309-rs907094-rs3764352-rs3817160 was significantly associated with smoking quantity in European-Americans, but not in African-Americans (Beuten et al. 2007). This data not only suggest that nicotine addiction risk is predisposed by *PPP1R1B* genetic variations, but also that it may be influenced by ethnic diversity. Using a translational genetics approach, Meyer-Lindenberg et al. (2007) found that the same schizophrenia-related *PPP1R1B* variants that impact cognitive functions, also predict the expression of DARPP-32 mRNA. Interestingly, in an independent sample of healthy Caucasian subjects, these same polymorphisms were associated with different patterns of neostriatal morphology and function (Meyer-Lindenberg et al. 2007). Corroborating with this hypothesis, another finding provided evidence that rs907094, which was shown to be associated with striatal dopamine function (Meyer-Lindenberg et al. 2007), may predict mechanisms in human reinforcement learning as well (Frank et al. 2007). Thus, the ability of how genetic variations in the *PPP1R1B* affects learning and decision-making processes, as well as addiction and striatal function, may have substantial implications on several disorders such as ADHD and schizophrenia.

Psychiatric Disorders and Neurological Diseases

Abnormalities in the neurotransmitter systems are hallmark physiological features in the brain of patients with psychiatric disorders. However, the complexity of neuronal signaling is based not only in the regulation of receptors activity at the synaptic membrane, but also in the integration of many intracellular signaling cascades. Because of this, DARPP-32 emerged as strong candidate involved in the manifestation of psychiatric disorders. In the last decade, several studies reported abnormalities in the DARPP-32 expression in the brain of patients with schizophrenia, bipolar disorder, depression, ADHD, and Parkinson disease.

DARPP, Table 1 DARPP-32 and psychiatric and neurological disorders – summary of DARPP-32 expression patterns in samples from psychiatric and neurologic disorder patients

Disorder	Sample	DARPP-32 alteration
Schizophrenia	Dorsolateral prefrontal cortex	Decrease: protein (Albert et al. 2002; Ishikawa et al. 2007); mRNA in suicidal patients (Feldcamp et al. 2008) No alteration: mRNA (Baracskay et al. 2006)
	Anterior cingulate cortex	No alteration: mRNA (Baracskay et al. 2006)
	Thalamus	No alteration: mRNA (Clinton et al. 2005)
	Lymphocytes	Decrease: protein (Torres et al. 2009)
Bipolar disorder	Dorsolateral prefrontal cortex	Decrease: protein (Ishikawa et al. 2007)
	Lymphocytes	Decrease: protein (Torres et al. 2009)
Parkinson disease	Putamen	Decrease: protein (Cash et al. 1987); No alteration: protein (Girault et al. 1989)
	Substantia nigra pars reticulate	Decrease: protein (Cash et al. 1987)
	Substantia nigra pars compacta	Decrease: protein (Cash et al. 1987)
	Caudate nucleus	No alteration: protein (Girault et al. 1989)

Schizophrenia: Abnormalities in the dopamine and glutamate neurotransmitter systems are involved with schizophrenia. The DARPP-32 signaling pathway is regulated by both neurotransmitters' signaling. Several studies, using postmortem brain from drug treated schizophrenic patients, have reported alterations in the DARPP-32 levels (See Table 1). The studies using postmortem brain of patients demonstrated a decrease in the protein levels, but not mRNA, in the dorsolateral prefrontal cortex of patients, a region suggested to be associated with negative symptoms and cognitive impairments of schizophrenia. Also, no alterations were demonstrated in the mRNA levels in the anterior cingulated cortex and thalamus, regions respectively involved in the decision-making and in the integration of different neural circuit activities, of schizophrenic patients.

Several studies showed that blocking D_2 receptors, the main target of antipsychotics, can increase the phosphorylation of DARPP-32(Thr34), affecting motor behavior. This modulation of motor behavior by antipsychotics is impaired in DARPP-32 KO mice (reviewed by Reis et al. 2007). However, both in vitro and in vivo experiments demonstrated that antipsychotics do not regulate the expression of DARPP-32, suggesting that the decrease of DARRP-32 in the prefrontal cortex of schizophrenic patients is related to the disease, and not to the pharmacological treatment (Souza et al. 2008, 2010).

Furthermore, recently, levels of DARPP-32 were shown to be decreased in lymphocytes of drug treated schizophrenic patients, suggesting DARPP-32 as a potential biomarker for this illness (see Table 1).

Bipolar Disorder: Recently, two studies reported alterations of DARPP-32 levels in bipolar disorder patients. The levels of DARPP-32 are decreased in the dorsolateral prefrontal cortex of bipolar disorder patients, indicating that DARPP-32 might be involved in the neurotransmission imbalance in the patients' brain. Furthermore, DARPP-32 levels are decreased in the lymphocytes of patients, pointing to DARPP-32 as a putative biomarker for bipolar disorder (See Table 1).

Depression: Much evidence supports the involvement of dopamine and serotonin in depression, for example, pharmacological treatment and dopamine metabolite levels. Acute treatment with fluoxetine increases the phosphorylation of DARPP-32 in many regions of the mouse brain. Chronic treatment with lithium and antidepressants increases DARPP-32 levels in the prefrontal cortex of rats (reviewed by Reis et al. 2007). A recent study reported that chronic electroconvulsive stimulation, which is very effective for depression, increases the levels of DARPP-32 in rats' hippocampus and striatum (Rosa et al. 2007).

Attention Deficit and Hyperactivity Disorder (ADHD): Dopamine signaling is the main target of pharmacological treatment of ADHD. It was recently

reported that methylphenidate treatment regulates the expression of DARPP-32 in rats' brain. Interestingly, this regulation is dependent on drug posology and the age of the rats, and it is region specific as well (Souza et al. 2009). This regulation of DARPP-32 expression by methylphenidate might be important in the improvement of ADHD symptoms.

Parkinson Disease: It is well known that dopamine signaling abnormalities are involved in the Parkinson Disease. Two studies reported different results regarding the levels of DARPP-32 in the brain of Parkinson Disease patients. One reported decreased levels of DARPP-32 in the putamen and substantia nigra of patients. However, no alterations in DARPP-32 levels were found in the putamen and caudate nucleus of a different group of Parkinson Disease patients (reviewed by Reis et al. 2007) (see Table 1).

DARPP-32 and Drugs of Abuse

It is well established that the dopaminergic system plays an important role in reward-related behaviors, and drugs with reinforcing properties share the ability to increase dopaminergic transmission (Svenningsson et al. 2005).

Cannabis: The major psychoactive components of marihuana and hashish are cannabinoids. Fernández-Ruiz et al. (2010) showed that the effects of cannabinoids on dopamine transmission and dopamine-related behaviors are generally indirect and affected by the modulation of GABA and glutamate inputs received by dopaminergic neurons. Recent evidence suggests, however, that certain eicosanoid-derived cannabinoids may directly activate TRPV1 receptors. These receptors have been found in some dopaminergic pathways, what allows a direct regulation of DA function by cannabinoid signaling. In the brain, cannabinoids interact with neuronal CB1 receptors, thereby producing a marked reduction of motor activity. These receptors are coupled to Gs protein, that enhances cAMP levels and, consequently, leads to the phosphorylation of DARPP-32 at Thr34. Point mutation of Thr75 does not affect the behavioral response to CP55940, a selective CB1 receptor agonist. On the other hand, catalepsy induced by CP55940 is reduced in both DARPP-32 knockout mice and in Thr34-Ala DARPP-32 mutant mice. Activation of CB1 receptors, either by an agonist or by inhibition of reuptake of endogenous cannabinoids, stimulates phosphorylation at Thr34 (reviewed by Reis et al. 2007; Andersson et al. 2005).

Cocaine: Acute treatment with cocaine increases the phosphorylation of DARPP-32(Thr34) and decrease the phosphorylation ofDARPP-32(Thr75). On the other hand, chronic treatment with cocaine increases phosphorylation of DARPP-32(Thr75) and decreases phosphorylation of DARPP-32(Thr34) (Svenningsson et al. 2004).

Chen et al. (2009) demonstrated that acute stimulation with cocaine activates the dopamine D_1 receptors, consequently leading to DARPP-32(Thr34) phosphorylation in the striatum. Several studies have shown that DARPP-32 participates in the progressive development of behavioral sensitization to cocaine. Knockout of DARPP-32 or DARPP-32 mutation (threonine 34 replaced by alanine) in mice attenuated the hyperlocomotor activity induced by acute cocaine treatment. Moreover, chronic treatment with cocaine decreased phosphorylation at Thr34 but increased at Thr75. This latter effect was due to enhanced Cdk5.

Opiates: Opiates act on the dopaminergic system in the brain via the μ-receptor and modulates the expression of DARPP-32, which represents an exciting nexus for drug-induced changes in neural long-term synaptic plasticity. Mahajan et al. (2009) showed that heroin significantly increased both D_1 and DARPP-32 gene expression. Also, it was shown that gene silencing DARPP-32 by siRNA in cultured normal human astrocyte cells modulated the activity of downstream effector molecules, such as PP-1. Opiates, such as morphine, bind to opioid receptor subtypes (mu, delta, and kappa) that are abundant in striatum. Mu- and delta-receptors are coupled to Gi protein, which decreases phosphorylation of DARPP-32 at Thr34 and modulates both dopamine and adenosine receptor activation (reviewed by Reis et al. 2007).

Nicotine: Nicotine is the critical component in tobacco smoke that is involved in addiction. It has been shown that nicotine modulates dopaminergic neurotransmission mainly by enhancing dopamine release in nigrostriatal and mesolimbic dopaminergic systems. Abdolahi et al. (2010) demonstrated incubation of drug seeking following extended access to nicotine self-administration and suggested that enhanced PKA signaling in the insular cortex via phosphorylation of DARPP-32 at Thr34 is associated with this effect. At low concentrations, nicotine decreases

phosphorylation of DARPP-32 at Thr34 in mouse neostriatal slices. In the other hand, high concentrations of nicotine increased the phosphorylation of DARPP-32 at Thr34. Therefore, different concentrations of nicotine lead to different amounts of dopamine release and, consequently, distinct activation patterns of dopamine receptors (reviewed by Reis et al. 2007).

Ethanol: Ethanol does not have a clearly defined site of action. It can act directly as agonist of $GABA_A$ and antagonist of NMDA receptors, and indirectly as agonist of dopamine D_1 receptor. Studies with DARPP-32 knockout mice showed DARPP-32 involvement in ethanol reward induced behavior in both place preference and self-administration tests (Risinger et al. 2001). It has been demonstrated that moderate levels of ethanol increase phosphorylation of DARPP-32 at Thr34 in striatal slices. Ethanol administration was found to increase phosphorylation of DARPP-32(Thr34) in the nucleus accumbens (NAc) and amygdala (but not in the striatum) of wild-type and transgenic mice, with a greater effect in the amygdala of transgenic mice. It was found also to increase DARPP-32(Thr75) in the amygdala of the wild-type mice only, and in the NAc and striatum of both the transgenic and wild-type mice. The authors concluded that the effect of ethanol on the balance of DARPP-32 phosphorylation, especially in the amygdala, may contribute to differential motivational effects of ethanol (Goodman 2008).

Summary

The importance of DARPP-32 arises from its relationship with several different signaling systems/cascades involved in important intracellular functions such as gene expression, cell differentiation, metabolism, and neuronal plasticity. The protein is an integrator of cellular function, and as such a putative target to fine-tune those functions. In addition, research should be conducted to gather information from animal models such as the *Caenorhabditis elegans, Drosophyla melanogaster, Aplysia* sp. and *Zebrafish*. An example, in *Caenorhabditis elegans* "Area-Restricted Search" behavior is controlled by a dopaminergic response to food deprivation that modulated glutamatergic signaling. Dopaminergic pathway compounds are not entirely described, and, like humans, this behavior time course is on the order of minutes, so a similar process might explain, and a DARPP-32 like protein could be part of it.

Future research will certainly shed more light on the roles of DARPP-32 in different biological processes, as well as potential new functions. Nanotechnology is having an increasing impact in the healthcare industry. The combination of diagnostic (imaging) and therapeutic capability enables the "real-time" monitoring of therapeutic progression, thus bringing "personalized medicine" closer to clinical reality. Bonoiu et al. (2009) introduced a nanotechnology approach that utilizes gold nanorod-DARPP-32 siRNA complexes that target to dopaminergic signaling pathway in the brain. Gene silencing of the nanoplexes in dopaminergic neuronal cells was evidenced by the reduction in the expression of key proteins, as DARPP-32, belonging to this pathway, with no observed cytotoxicity. Since these nanoplexes were shown to transmigrate across an in vitro model of the blood–brain barrier, it appears to be suited for brain-specific delivery of appropriate siRNA for therapy of drug addiction and other brain diseases. Nevertheless, the available collection of evidence suggests that DARPP-32 lies at the nexus of multiple signaling pathways that modulate important signaling states of a given cell type, thus, assuring that it will continue to be an important molecule in quest to find new pharmacological targets.

References

Abdolahi A, Acosta G, Breslin FJ, Hemby SE, Lynch WJ. Incubation of nicotine seeking is associated with enhanced protein kinase A-regulated signaling of dopamine- and cAMP-regulated phosphoprotein of 32 kDa in the insular cortex. Eur J Neurosci. 2010;31(4):733–41.

Albert KA, Hemmings HC, Jr, Adamo AI, et al. Evidence for decreased DARPP-32 in the prefrontal cortex of patients with schizophrenia. Arch Gen Psychiat. 2002;59(8):705–12.

Andersson M, Usiello A, Borgkvist A, et al. Cannabinoid action depends on phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa at the protein kinase A site in striatal projection neurons. J Neurosci. 2005;25(37):8432–8.

Baracskay KL, Haroutunian V, Meador-Woodruff JH. Dopamine receptor signaling molecules are altered in elderly schizophrenic cortex. Synapse. 2006;60(4):271–9.

Beuten J, Ma JZ, Lou XY, Payne TJ, Li MD. Association analysis of the protein phosphatase 1 regulatory subunit 1B (PPP1R1B) gene with nicotine dependence in European- and African-American smokers. Am J Med Genet B Neuropsychiatr Genet. 2007;144B(3):285–90.

Bonoiu AC, Mahajan SD, Ding H, et al. Nanotechnology approach for drug addiction therapy: gene silencing using delivery of gold nanorod-siRNA nanoplex in dopaminergic neurons. Proc Natl Acad Sci USA. 2009;106(14): 5546–50.

Cardno AG, Holmans PA, Rees MI, et al. A genomewide linkage study of age at onset in schizophrenia. Am J Med Genet. 2001;105(5):439–45.

Cash R, Raisman R, Ploska A, Agid Y. Dopamine D-1 receptor and cyclic AMP-dependent phosphorylation in Parkinson's disease. J Neurochem. 1987;49(4):1075–83.

Chen JC, Chen PC, Chiang YC. Molecular mechanisms of psychostimulant addiction. Chang Gung Med J. 2009;32(2): 148–54.

Clinton SM, Ibrahim HM, Frey KA, et al. Dopaminergic abnormalities in select thalamic nuclei in schizophrenia: involvement of the intracellular signal integrating proteins calcyon and spinophilin. Am J Psychiat. 2005;162(10): 1859–71.

Dick DM, Foroud T, Flury L, et al. Genomewide linkage analyses of bipolar disorder: a new sample of 250 pedigrees from the National Institute of Mental Health Genetics Initiative. Am J Hum Genet. 2003;73(1):107–14.

Ehrman LA, Williams MT, Schaefer TL, et al. Phosphodiesterase 1B differentially modulates the effects of methamphetamine on locomotor activity and spatial learning through DARPP32-dependent pathways: evidence from PDE1B-DARPP32 double-knockout mice. Genes Brain Behav. 2006;5(7):540–51.

Feldcamp LA, Souza RP, Romano-Silva M, et al. Reduced prefrontal cortex DARPP-32 mRNA in completed suicide victims with schizophrenia. Schizophr Res. 2008;103(1–3): 192–200.

Fernandez-Ruiz J, Hernandez M, Ramos JA. Cannabinoid-dopamine interaction in the pathophysiology and treatment of CNS disorders. CNS Neurosci Ther. 2010;16(3):e72–91.

Fienberg AA, Greengard P. The DARPP-32 knockout mouse. Brain Res Brain Res Rev. 2000;31(2–3):313–9.

Fienberg AA, Hiroi N, Mermelstein PG, et al. DARPP-32: regulator of the efficacy of dopaminergic neurotransmission. Science. 1998;281(5378):838–42.

Frank MJ, Moustafa AA, Haughey HM, Curran T, Hutchison KE. Genetic triple dissociation reveals multiple roles for dopamine in reinforcement learning. Proc Natl Acad Sci USA. 2007; 104(41):16311–6.

Goodman A. Neurobiology of addiction. An integrative review. Biochem Pharmacol. 2008;75(1):266–322.

Girault JA, Hemmings HC, et al. Phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, by casein kinase II. J Biol Chem. 1989;264(36):21748–59.

Heyser CJ, Fienberg AA, Greengard P, Gold LH. DARPP-32 knockout mice exhibit impaired reversal learning in a discriminated operant task. Brain Res. 2000;867(1–2): 122–30.

Ishikawa M, Mizukami K, Iwakiri M, Asada T. Immunohistochemical and immunoblot analysis of Dopamine and cyclic AMP-regulated phosphoprotein, relative molecular mass 32,000 (DARPP-32) in the prefrontal cortex of subjects with schizophrenia and bipolar disorder. Prog Neuropsychopharmacol Biol Psychiat. 2007;31(6):1177–81.

Lindskog M, Svenningsson P, Pozzi L, et al. Involvement of DARPP-32 phosphorylation in the stimulant action of caffeine. Nature. 2002;418(6899):774–8.

Mahajan SD, Aalinkeel R, Reynolds JL, et al. Therapeutic targeting of "DARPP-32": a key signaling molecule in the dopiminergic pathway for the treatment of opiate addiction. Int Rev Neurobiol. 2009;88:199–222.

Meyer-Lindenberg A, Straub RE, Lipska BK, et al. Genetic evidence implicating DARPP-32 in human frontostriatal structure, function, and cognition. J Clin Invest. 2007; 117(3):672–82.

Reis HJ, Rosa DV, Guimaraes MM, et al. Is DARPP-32 a potential therapeutic target? Expert Opin Ther Targets. 2007;11(12):1649–61.

Risinger FO, Freeman PA, Greengard P, Fienberg AA. Motivational effects of ethanol in DARPP-32 knock-out mice. J Neurosci. 2001;21(1):340–8.

Rosa DV, Souza RP, Souza BR, et al. DARPP-32 expression in rat brain after electroconvulsive stimulation. Brain Res. 2007;1179:35–41.

Souza BR, Motta BS, Rosa DV, et al. DARPP-32 and NCS-1 expression is not altered in brains of rats treated with typical or atypical antipsychotics. Neurochem Res. 2008; 33(3):533–8.

Souza RP, Soares EC, Rosa DV, et al. Cerebral DARPP-32 expression after methylphenidate administration in young and adult rats. Int J Dev Neurosci. 2009;27(1):1–7.

Souza BR, Torres KC, Miranda DM, et al. Lack of effects of typical and atypical antipsychotics in DARPP-32 and NCS-1 levels in PC12 cells overexpressing NCS-1. J Negat Results Biomed. 2010;9:4.

Svenningsson P, Fienberg AA, Allen PB, et al. Dopamine D(1) receptor-induced gene transcription is modulated by DARPP-32. J Neurochem. 2000;75(1):248–57.

Svenningsson P, Nairn AC, Greengard P. DARPP-32 mediates the actions of multiple drugs of abuse. AAPS J. 2005;7(2): E353–60.

Svenningsson P, Nishi A, Fisone G, et al. DARPP-32: an integrator of neurotransmission. Annu Rev Pharmacol Toxicol. 2004;44:269–96.

Svenningsson P, Tzavara ET, Carruthers R, et al. Diverse psychotomimetics act through a common signaling pathway. Science. 2003;302(5649):1412–5.

Svenningsson P, Tzavara ET, Witkin JM, et al. Involvement of striatal and extrastriatal DARPP-32 in biochemical and behavioral effects of fluoxetine (Prozac). Proc Natl Acad Sci USA. 2002;99(5):3182–7.

Torres KC, Souza BR, Miranda DM, et al. The leukocytes expressing DARPP-32 are reduced in patients with schizophrenia and bipolar disorder. Prog Neuropsychopharmacol Biol Psychiat. 2009;33(2):214–9.

West AR, Grace AA. Opposite influences of endogenous dopamine D1 and D2 receptor activation on activity states and electrophysiological properties of striatal neurons: studies combining in vivo intracellular recordings and reverse microdialysis. J Neurosci. 2002;22(1):294–304.

Zachariou V, OitMarand M, Allen PB, et al. Reduction of cocaine place preference in mice lacking the protein phosphatase 1 inhibitors DARPP 32 or Inhibitor 1. Biol Psychiatry. 2002;51(8):612–20.

Darpp32

▶ DARPP

DARPP-32

▶ DARPP

Dbf4

Hisao Masai
Genome Dynamics Project, Department of Genome Medicine, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Synonyms

ASK (activator of S phase kinase)/huDbf4 (human); Chiffon (*Drosophila*); Dbf4 (dumbbell former 4)/DNA52 (*Saccharomyces cerevisiae*); Dbf4l1; Dfp1/Him1/Rad35 (*Schizosaccharomyces pombe*), Spo6 (a second Dbf4 homologue in *S. pombe*); Drf1/ASKL1 (a second activator of Cdc7 in human and *Xenopus*); nimO (*Aspergillus*)

Historical Background

dbf4 (dumbbell former 4) mutation was originally identified in the screening for budding yeast temperature-sensitive mutants which arrest with dumbbell-shaped phenotype at the nonpermissive temperature (Johnston and Thomas 1982). The terminal phenotypes of *dbf4*(ts) strain at a nonpermissive temperature were very similar to those of ▶ *cdc7*(ts), encoding a serine-threonine kinase known to be essential for initiation of DNA replication. Later, *dbf4* was rediscovered as a multi-copy suppressor of ▶ *cdc7*(ts), suggesting physical and functional interactions between ▶ Cdc7 and Dbf4 (Kitada et al. 1992). Following this finding, Dbf4 was shown to bind to ▶ Cdc7 and stimulate its kinase activity, establishing that Dbf4 is the activation subunit for ▶ Cdc7 kinase (Jackson et al. 1993). The presence of ▶ Cdc7 homologues in species other than budding yeast was first reported in fission yeast (*hsk1*; Masai et al. 1995). Following this discovery, an ortholog of Dbf4 was reported in fission yeast (Brown and Kelly 1998, Dfp1/Him1). The orthologs of Dbf4 from vertebrates were also identified and found to form complexes with cognate ▶ Cdc7 (Kumagai et al. 1999).

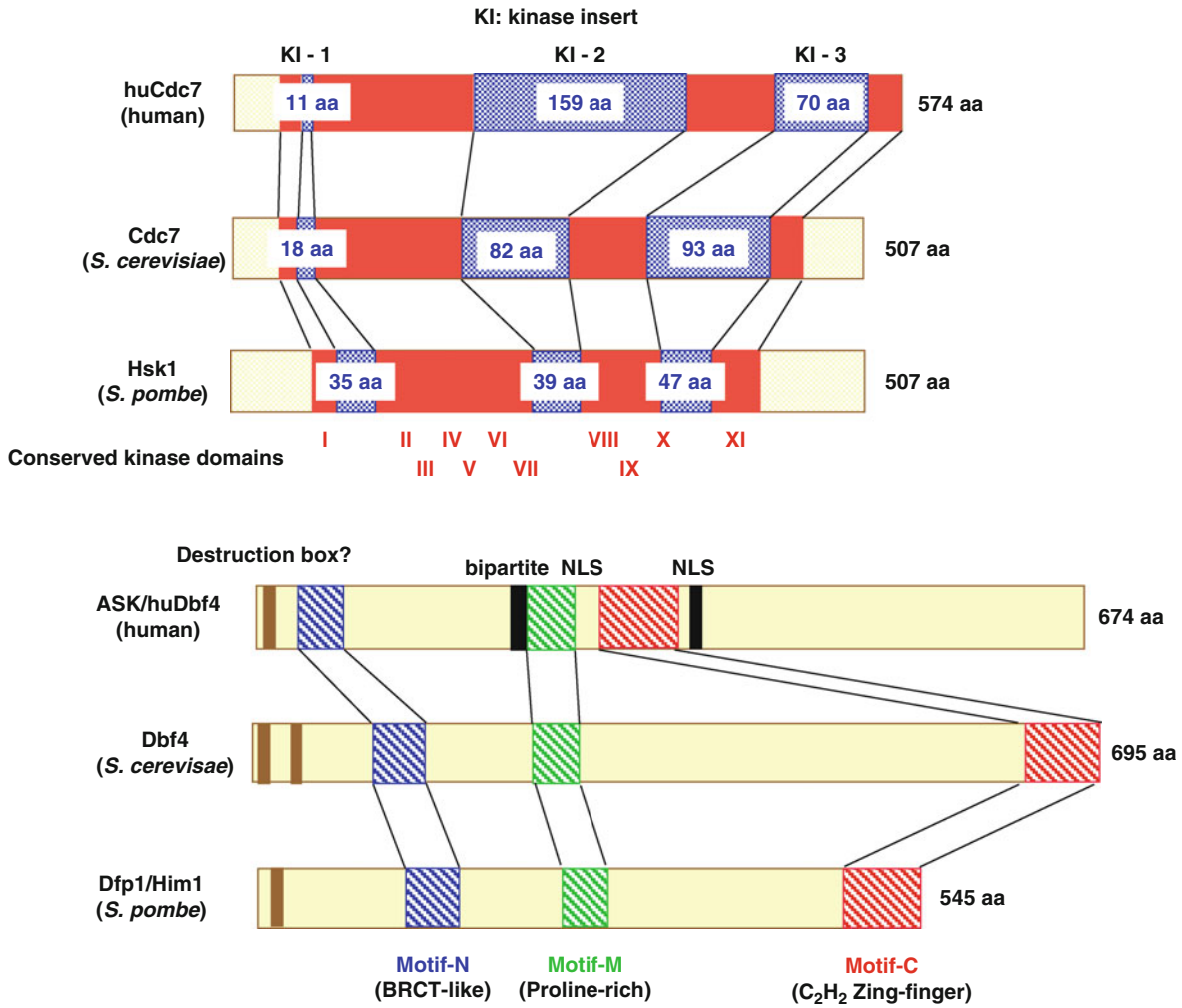
Identification of Orthologs of Dbf4 in Other Species

Purification of Hsk1 kinase from fission yeast led to identification of Dfp1, the fission yeast ortholog of Dbf4 (Brown and Kelly 1998). Two-hybrid screening with Hsk1 as a bait also led to the identification of Him1, identical to Dfp1. Interestingly, *dfp1/him1* was found to be allelic to *rad35*, a radiation-sensitive mutant, implicating Dbf4 in DNA damage response pathway (Takeda et al. 1999). In *Aspergillus*, a mutant called *nimO* was isolated and was shown to encode a Dbf4 homologue (James et al. 1999). Human homologue of Dbf4, ASK (activator of S phase kinase), was isolated by two-hybrid screening using huCdc7 as a bait (Kumagai et al. 1999).

A second Dbf4 subunit in human was identified and named Drf1 or ASKL1 (Montagnoli et al. 2002; Yoshizawa-Sugata et al. 2005). Drf1/ASKL1 can bind and activate huCdc7, but its role during cell cycle may be secondary. Drf1/ASKL1 is present also in *Xenopus*. In *Xenopus* egg extracts or during early development of *Xenopus*, XDrf1/ASKL1 rather than XDbf4 plays a major role as an activator of XeCdc7, whereas XDbf4 replaces the XDrf1/ASKL1 in somatic cells (Takahashi and Walter 2005). In fission yeast, a second set of ▶ Cdc7-Dbf4, Spo4-Spo6, is present which functions specifically during the sporulation/second meiotic division stage of meiosis (Nakamura et al. 2000). No functional homologues of Spo4-Spo6 have been identified in other species.

Activation of ▶ Cdc7 Kinase by Dbf4 Protein

Analysis of sequences of Dbf4 and its orthologs revealed the presence of three conserved motifs, motif-N, -M, and -C (Masai and Arai 2000; Fig. 1). Further analyses indicated that motif-M and -C can bind to ▶ Cdc7 independently. Binding of both motifs to ▶ Cdc7 is required for full activation of



Dbf4, Fig. 1 *Comparison of the primary structures of Cdc7 and Dbf4/ASK proteins from human, budding yeast, and fission yeast*. The sequences of human, *S. cerevisiae*, or *S. pombe* Cdc7 or Dbf4/ASK were aligned and the conserved segments are indicated. *Upper*: *Red* regions are kinase conserved domains which are similar between the three species. Blue regions are kinase insert sequences and are not conserved. *Lower*: Motif-N, -M and -C, shown in *blue*, *green*, and *red*, respectively, are conserved across the species

► Cdc7 kinase. The sequences and length connecting motif-M and motif-C can be varied without affecting the kinase activity, suggesting that they serve as a linker sequence (Ogino et al. 2001). Motif-M is a proline-rich motif with no apparent similarity to known motifs. Motif-C is a C_2H_2-type zinc finger-like structure. Motif-N is composed of a single copy of a BRCT-like sequence, which could be uniquely replaced by the BRCT motif from Rev1 (Harkins et al. 2009). Motif-M alone can sustain mitotic viability (Fung et al. 2002), but both motif-M and -C are required for viability through meiosis in fission yeast (Ogino et al. 2001). On the other hand, motif-N is not essential for viability, although it is involved in conferring resistance to genotoxic agents. In mammalian cells, motif-M and motif-C are sufficient for maximum kinase activity, but motif-N is also required for viability (Yamashita et al. 2005).

Functions of Dbf4/ASK During Cell Growth

A temperature-sensitive mutant of *dbf4* in budding yeast ceases growth at the onset of S phase, an identical position with that of ► *cdc7*(ts) cells. The arrested cells can reversibly enter the S phase upon return to

a permissive temperature. Analyses of a temperature-sensitive mutant of *nimO*, the *Aspergillus* homologue of Dbf4, showed that NimO is required for initiation of DNA synthesis and for efficient progression through S phase as well as for DNA replication checkpoint coupling S and M phases (James et al. 1999). *Drosophila* homologue of Dbf4, Chiffon, is required for chorion gene amplification. Hypomorphic mutant alleles of the chiffon gene cause thin, fragile chorions and female sterility. Null alleles of chiffon had the additional phenotypes of rough eyes and thin thoracic bristles, phenotypes often associated with disruption of normal cell cycle (Landis and Tower 1999).

Dbf4 and Dfp1/Him1 are hyperphosphorylated in response to replication stress (such as treatment with hydroxyurea, which inhibits cellular nucleotide reductase and depletes cellular nucleotide pool). This phosphorylation of Dbf4 or Dfp1/Him1 depends on both ▶ Cdc7/Hsk1 and checkpoint kinase Rad53 or Cds1, respectively (Takeda et al. 2001; Brown and Kelly 1999). It has been proposed that this phosphorylation somehow inhibits the function of Dbf4, which contributes to suppression of firing of late origins. However, the nature of this inhibition is still unknown. Extensive mutagenesis of potential phosphorylation sites on Dbf4 rendered the mutant Dbf4 refractory to the checkpoint inhibition and combination of the Dbf4 mutant with a similar phosphorylation site mutant of Sld3 abrogated the checkpoint inhibition of late origin firing, showing that Dbf4 is a critical target of DNA replication checkpoint (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). Human Dbf4/ASK may also be hyperphosphorylated by replication stress (Heffernan et al. 2007).

In mouse ES cells, conditional knockout of Dbf4/ASK is lethal and cells undergo cell death, as was observed in ES cells in which the ▶ *Cdc7* gene was conditionally knocked down.

Functions During Meiosis

Initial characterization of budding yeast ▶ *cdc7*(ts) indicated the essential role of ▶ Cdc7 for meiotic recombination, but not for premeiotic DNA replication. In fission yeast as well, cells are arrested with one nucleus in a *hsk1*(ts) cells. In both fission yeast and budding yeast, initiation of meiotic recombination, i.e., induction of DSB (double-stranded DNA breaks) does not occur in the absence of Hsk1/▶ Cdc7 (Ogino et al. 2006). Furthermore, Mer2, a factor essential for loading of DSB endonuclease Spo11, was identified to be a critical target of ▶ Cdc7 (Sasanuma et al. 2008; Wan et al. 2008). Premeiotic DNA replication can proceed albeit at slightly slower rate in *hsk1*(ts) mutant or *cdc7* as in which ▶ Cdc7 can be chemically inactivated.

On the other hand, repression of dbf4$^+$ expression regulated by the tet promoter suppressed premeiotic DNA replication in budding yeast when it was suppressed before meiosis was induced, but DNA replication was observed if dbf4$^+$ expression was suppressed later, presumably due to inadequate suppression of dbf4$^+$ expression. This result suggests that Dbf4 function is required for premeiotic DNA replication. In the latter cells, premeiotic S phase completed but the meiosis was still arrested before anaphase I. This arrest was relieved by *rec8* deletion, suggesting a crucial role of ▶ Cdc7 during meiotic chromosome segregation (Valentin et al. 2006). Indeed, Rec8 cleavage by separase is regulated by phosphorylation mediated by ▶ Cdc7 or casein kinase 1 (Katis et al. 2010). ▶ Cdc7 also regulates monopolar attachment of sister kinetochores by regulating the recruitment of the monopolin complex to kinetochores through phosphorylation of monopolin subunit Lrs4 (Matos et al. 2008). Thus, Dbf4, in a complex with ▶ Cdc7, may regulate multiple steps of meiotic cell cycle.

Other Dbf4-Related Molecules

Another ▶ Cdc7-Dbf4-related complex, the Spo4 (Cdc7-like)-Spo6(Dbf4-like) complex, is expressed in fission yeast during late meiosis and is specifically required for the sporulation stage (Nakamura et al. 2000). Kinase complexes related to Spo4-Spo6 have not been found in other species.

A second Dbf4/ASK-like molecule, Drf1/ASKL1 was identified in silico on the human genome and was shown to function as an activation subunit for human ▶ Cdc7 kinase (Montagnoli et al. 2002; Yoshizawa-Sugata et al. 2005). The expression level of Drf1/ASKL1 increases during late S/G2 and inhibition of its expression resulted in accumulation of late S-G2/M populations. In contrast to Dbf4/ASK, Drf1/ASKL1 is present mostly in the nuclear soluble fractions, not in chromatin-bound fractions. The homologue of Drf1/ASKL1 was indentified in *Xenopus* and was shown to be predominantly expressed in *Xenopus* egg extracts, while XDbf4 is the major form of the ▶ Cdc7

activation subunit in somatic cells (Takahashi and Walter 2005). Drf1/ASKL1 has been identified only in human and Xenopus.

Association of Dbf4 with Origin Sequences in Budding Yeast

One-hybrid assays showed that Dbf4 interacts with the replication origin sequences in budding yeast (Dowell et al. 1994). Mapping of the interacting segment on Dbf4 indicated motif-N as the origin-interacting domain. Thus, Dbf4 targets ▶ Cdc7 kinase at the origin of DNA replication. At the origin, ▶ Cdc7 targets pre-replicative complex (pre-RC) which is generated on chromatin during early G1. Among the components of pre-RC, MCM is the critical and conserved substrate of ▶ Cdc7 (Lei et al. 1997; Sato et al. 1997). See the section of "▶ Cdc7" for details on how ▶ Cdc7-mediated phosphorylation may activate initiation of DNA replication.

Interaction of Dbf4 with Other Replication Factors

Yeast two-hybrid analyses indicated the interaction of mouse Dbf4/ASK with Orc1, 2, 5, and 6 as well as with MCM2, 3, 4, and 7, consistent with the interaction of Dbf4 with pre-RC assembled at origins in budding yeast (Kneissl et al. 2003). In the same report, mouse ▶ Cdc7 was reported to interact with Orc1 and 6 and with MCM2, 4, 5 and 7 in two-hybrid assays.

Regulation of Expression of Dbf4 During the Cell Cycle

Expression of Dbf4 is cell cycle regulated. Regulation is generally on both transcription and protein levels. In budding yeast, transcription of Dbf4 is regulated during cell cycle, peaking during G1 (Chapman and Johnston 1989). The promoter contains a MluI cell cycle box (or MCB) and may be regulated by the MCB-binding factor (MBF). Furthermore, the budding yeast Dbf4 protein is degraded by APC during G1 phase. The protein is present during S through G2 phase, coinciding with the active ▶ Cdc7-Dbf4 kinase activity during this period. The potential degradation signal was identified in the N-terminal segment of Dbf4. Similar regulation is likely to operate for the fission yeast Dfp1/Him1 gene, which is expressed specifically during S through G2/M phase (Brown and Kelly 1998; Takeda et al. 1999).

Expression of mammalian Dbf4/ASK is also cell cycle regulated. The transcript is repressed in the quiescent cells and induced after growth stimulation. A 63-base pair ASK promoter segment was identified, which is sufficient for mediating growth stimulation (Yamada et al. 2002). This minimal promoter segment contains an Sp1 site but no canonical E2F site, but can be activated by ectopic E2F expression. Within the 63-base pair region, the Sp1 site as well as other elements are essential for stimulation by growth signals and by E2F, whereas an AT-rich sequence proximal to the coding region may serve as an element required for suppression in quiescence. Another report proposed the presence of MCB in the core promoter region of human Dbf4/ASK (Wu and Lee 2002). The Dbf4/ASK protein levels decrease during G1 phase, and a part of this may be attributed to cell cycle–dependent protein degradation.

Developmental Role of Dbf4

A role of Dbf4 in heart/eye development was suggested in *Xenopus* (Brott and Sokol 2005). Dbf4/ASK inhibits the canonical Wnt signaling pathway, possibly through interacting with Frodo. This role of Dbf4 does not involve its ability to activate Cdc7 kinase, since the Dbf4-motif-M, which is known to be essential for Cdc7 kinase activation, is not required for its role in heart development. Expression of Drf1/ASKL1 and Dbf4/ASK molecules is developmentally regulated in *Xenopus*. Drf1/ASKL1 is predominantly expressed in early development, but is later replaced by Dbf4/ASK in somatic cells (Takahashi and Walter 2005).

Those who are interested in learning more about eukaryotic DNA replication are recommended to read the reference (Masai et al. 2010).

Summary

Dbf4 is the activation subunit for Cdc7, a conserved kinase essential for initiation of DNA replication. Dbf4 is evolutionally conserved and carries three conserved motifs, motif-N, -M and, -C. Motif-M or -C interacts with Cdc7 on its own, but motif-M alone can support mitotic growth in yeast. Motif-C, the most conserved segment, is required together with motif-M for full kinase activation as well as for meiotic function of Cdc7-Dbf4. Motif-N of Dbf4 is involved in interaction with chromatin of the Cdc7-Dbf4 complex. Dbf4 may induce conformational change of Cdc7 and facilitate

its binding to ATP as well as its association with the critical substrate of the Cdc7-Dbf4 kinase complex (Kitamura et al. 2011). Second Dbf4, Drf1/ASKL1, has been discovered in human and *Xenopus*. Abundance of Dbf4 is cell cycle regulated and contributes to cell cycle oscillation of Cdc7 kinase activity.

References

Brott BK, Sokol SY. A vertebrate homolog of the cell cycle regulator Dbf4 is an inhibitor of Wnt signaling required for heart development. Dev Cell. 2005;8(5):703–15.

Brown GW, Kelly TJ. Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. J Biol Chem. 1998;273(34):22083–90.

Brown GW, Kelly TJ. Cell cycle regulation of Dfp1, an activator of the Hsk1 protein kinase. Proc Natl Acad Sci USA. 1999;96(15):8443–8.

Chapman JW, Johnston LH. The yeast gene, DBF4, essential for entry into S phase is cell cycle regulated. Exp Cell Res. 1989;180(2):419–28.

Dowell SJ, Romanowski P, Diffley JF. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science. 1994;265(5176):1243–6.

Fung AD, Ou J, Bueler S, Brown GW. A conserved domain of *Schizosaccharomyces pombe* dfp1(+) is uniquely required for chromosome stability following alkylation damage during S phase. Mol Cell Biol. 2002;22(13):4477–90.

Harkins V, Gabrielse C, Haste L, Weinreich M. Budding yeast Dbf4 sequences required for Cdc7 kinase activation and identification of a functional relationship between the Dbf4 and Rev1 BRCT domains. Genetics. 2009;183(4):1269–82.

Heffernan TP, Unsal-Kaçmaz K, Heinloth AN, Simpson DA, Paules RS, Sancar A, Cordeiro-Stone M, Kaufmann WK. Cdc7-Dbf4 and the human S checkpoint response to UVC. J Biol Chem. 2007;282(13):9458–68.

Jackson AL, Pahl PM, Harrison K, Rosamond J, Sclafani RA. Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol Cell Biol. 1993;13(5):2899–908.

James SW, Bullock KA, Gygax SE, Kraynack BA, Matura RA, MacLeod JA, McNeal KK, Prasauckas KA, Scacheri PC, Shenefiel HL, Tobin HM, Wade SD. nimO, an *Aspergillus* gene related to budding yeast Dbf4, is required for DNA synthesis and mitotic checkpoint control. J Cell Sci. 1999;112(Pt 9):1313–24.

Johnston LH, Thomas AP. A further two mutants defective in initiation of the S phase in the yeast *Saccharomyces cerevisiae*. Mol Gen Genet. 1982;186(3):445–8.

Katis VL, Lipp JJ, Imre R, Bogdanova A, Okaz E, Habermann B, Mechtler K, Nasmyth K, Zachariae W. Rec8 phosphorylation by casein kinase 1 and Cdc7-Dbf4 kinase regulates cohesin cleavage by separase during meiosis. Dev Cell. 2010;18(3):397–409.

Kitada K, Johnston LH, Sugino T, Sugino A. Temperature-sensitive cdc7 mutations of *Saccharomyces cerevisiae* are suppressed by the *DBF4* gene, which is required for the G1/S cell cycle transition. Genetics. 1992;131(1):21–9.

Kitamura R, Fukatsu R, Kakusho N, Cho Y-S, Taniyama C, Yamazaki S, Toh G-T, Yanagi K, Arai N, Chang, H-J, Masai H. Molecular mechanism of activation of human Cdc7 kinase: Bipartite interaction with Dbf4/ASK stimulates ATP binding and substrate recognition. J Biol Chem. 2011;286:23031–43.

Kneissl M, Pütter V, Szalay AA, Grummt F. Interaction and assembly of murine pre-replicative complex proteins in yeast and mouse cells. J Mol Biol. 2003;327(1):111–28.

Kumagai H, Sato N, Yamada M, Mahony D, Seghezzi W, Lees E, Arai K, Masai H. A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G1/S transition in mammalian cells. Mol Cell Biol. 1999;19(7):5083–95.

Landis G, Tower J. The *Drosophila chiffon* gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. Development. 1999;126(19):4281–93.

Lei M, Kawasaki Y, Young MR, Kihara M, Sugino A, Tye BK. Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev. 1997;11(24):3365–74.

Lopez-Mosqueda J, Maas NL, Jonsson ZO, Defazio-Eli LG, Wohlschlegel J, Toczyski DP. Damage-induced phosphorylation of Sld3 is important to block late origin firing. Nature. 2010;467(7314):479–83.

Masai H, Arai K. Dbf4 motifs: conserved motifs in activation subunits for Cdc7 kinases essential for S-phase. Biochem Biophys Res Commun. 2000;275(1):228–32.

Masai H, Miyake T, Arai K. hsk1+, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae* CDC7, is required for chromosomal replication. EMBO J. 1995;14(13):3094–104.

Masai H, Matsumoto S, You Z, Yoshizawa-Sugata N, Oda M. Eukaryotic chromosome DNA replication: where, when, and how? Annu Rev Biochem. 2010;79:89–130.

Matos J, Lipp JJ, Bogdanova A, Guillot S, Okaz E, Junqueira M, Shevchenko A, Zachariae W. Dbf4-dependent CDC7 kinase links DNA replication to the segregation of homologous chromosomes in meiosis I. Cell. 2008;135(4):662–78.

Montagnoli A, Bosotti R, Villa F, Rialland M, Brotherton D, Mercurio C, Berthelsen J, Santocanale C. Drf1, a novel regulatory subunit for human Cdc7 kinase. EMBO J. 2002;21(12):3171–81.

Nakamura T, Kishida M, Shimoda C. The *Schizosaccharomyces pombe spo6+* gene encoding a nuclear protein with sequence similarity to budding yeast Dbf4 is required for meiotic second division and sporulation. Genes Cells. 2000;5(6):463–79.

Ogino K, Takeda T, Matsui E, Iiyama H, Taniyama C, Arai K, Masai H. Bipartite binding of a kinase activator activates Cdc7-related kinase essential for S phase. J Biol Chem. 2001;276(33):31376–87.

Ogino K, Hirota K, Matsumoto S, Takeda T, Ohta K, Arai K, Masai H. Hsk1 kinase is required for induction of meiotic dsDNA breaks without involving checkpoint kinases in fission yeast. Proc Natl Acad Sci USA. 2006;103(21):8131–6.

Sasanuma H, Hirota K, Fukuda T, Kakusho N, Kugou K, Kawasaki Y, Shibata T, Masai H, Ohta K. Cdc7-dependent phosphorylation of Mer2 facilitates initiation of yeast meiotic recombination. Genes Dev. 2008;22(3):398–410.

Sato N, Arai K, Masai H. Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. EMBO J. 1997;16(14):4340–51.

Takahashi TS, Walter JC. Cdc7-Drf1 is a developmentally regulated protein kinase required for the initiation of vertebrate DNA replication. Genes Dev. 2005;19(19):2295–300.

Takeda T, Ogino K, Matsui E, Cho MK, Kumagai H, Miyake T, Arai K, Masai H. A fission yeast gene, him1(+)/dfp1(+), encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. Mol Cell Biol. 1999;19(8):5535–47.

Takeda T, Ogino K, Tatebayashi K, Ikeda H, Ki A, Masai H. Regulation of initiation of S phase, replication checkpoint signaling, and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. Mol Biol Cell. 2001;12(5):1257–74.

Valentin G, Schwob E, Della SF. Dual role of the Cdc7-regulatory protein Dbf4 during yeast meiosis. J Biol Chem. 2006;281(5):2828–34.

Wan L, Niu H, Futcher B, Zhang C, Shokat KM, Boulton SJ, Hollingsworth NM. Cdc28-Clb5 (CDK-S) and Cdc7-Dbf4 (DDK) collaborate to initiate meiotic recombination in yeast. Genes Dev. 2008;22(3):386–97.

Wu X, Lee H. Human Dbf4/ASK promoter is activated through the Sp1 and MluI cell-cycle box (MCB) transcription elements. Oncogene. 2002;21(51):7786–96.

Yamada M, Sato N, Taniyama C, Ohtani K, Arai K, Masai H. A 63-base pair DNA segment containing an Sp1 site but not a canonical E2F site can confer growth-dependent and E2F-mediated transcriptional stimulation of the human ASK gene encoding the regulatory subunit for human Cdc7-related kinase. J Biol Chem. 2002;277(31):27668–81.

Yamashita N, Kim JM, Koiwai O, Arai K, Masai H. Functional analyses of mouse ASK, an activation subunit for Cdc7 kinase, using conditional ASK knockout ES cells. Genes Cells. 2005;10(6):551–63.

Yoshizawa-Sugata N, Ishii A, Taniyama C, Matsui E, Arai K, Masai H. A second human Dbf4/ASK-related protein, Drf1/ASKL1, is required for efficient progression of S and M phases. J Biol Chem. 2005;280(13):13062–70.

Zegerman P, Diffley JF. Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. Nature. 2010;467(7314):474–8.

Dbf4 (Dumbbell Former 4)/DNA52 (*Saccharomyces cerevisiae*)

► Dbf4

Dbf4l1

► Dbf4

Death-Associated Protein Kinase

► DAP Kinase

Dectin-1

► CLEC7A

Delta Glutamate Receptor (*GluD1*, *GluD2*)

Kazuhisa Kohda, Wataru Kakegawa and Michisuke Yuzaki
Department of Neurophysiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan

Synonyms

GluRδ1; GluRδ2; GluD1; GluD2

Historical Background

The δ1 glutamate receptor (GluRδ1; GluD1) and the δ2 glutamate receptor (GluRδ2; GluD2) were cloned by homology screening in 1993 at the end of the "gold rush" for cloning of ionotropic glutamate receptor (iGluR) cDNA. They were regarded as orphan receptors for a long time since their endogenous ligands were unknown. GluD1 is highly expressed in hair cells of the auditory and vestibular systems in adult mice. Indeed, deletion of a gene encoding GluD1 (*grid1*) in mice leads to deficit in high-frequency hearing. In contrast, GluD2 is highly expressed in cerebellar Purkinje cells and deletion of a gene encoding GluD2 (*grid2*) results in cerebellar ataxia and characteristic phenotypes at parallel fiber (PF)–Purkinje cell

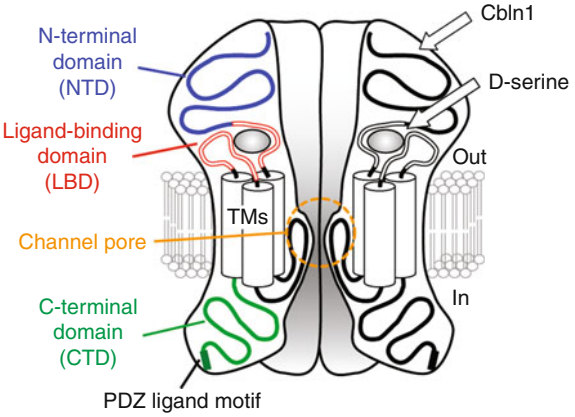
synapses. Functionally, long-term depression (LTD) of synaptic transmission, which is thought to underlie motor coordination and motor learning, is completely blunted. Morphologically, approximately 40% of dendritic spines of *grid2*-null Purkinje cells remain uninnervated by PF terminals. Although these characteristic phenotypes of *grid1*-null and *grid2*-null mice point to essential roles played by GluD1 and GluD2, mechanisms by which GluD1/2 mediate these functions have remained mostly unclear. For some original references, which have been omitted in this entry for the sake of space, please refer to reviews elsewhere (Yuzaki 2008).

Genes and Expression

The size of *grid1* and *grid2* genes is much larger (~760 kb for *grid1* and ~1.4 Mb for *grid2*) than sizes of genes encoding other iGluRs (~200 kb). This large size and a high percentage of purine nucleotides within the locus are thought to render *Grid2* susceptible to frequent spontaneous mutations in mice, called *hotfoot* (Yuzaki 2008). No spontaneous mutant mouse linked to the *grid1* locus is known so far. However, recent genome-wide association studies indicated linkage of the *GluD1* gene with bipolar disorder, major depressive disorder, and schizophrenia (Fallin et al. 2005; Guo et al. 2007; Treutlein et al. 2009). Furthermore, genome-wide copy number variation studies have implicated that *GRID1* may be associated with autism spectrum disorder (Glessner et al. 2009; Smith et al. 2009). No human diseases have been associated with the *GRID2* locus.

In addition to its high expression in hair cells, GluD1 mRNA is present at a low level in pyramidal and dentate granule cell layers of the hippocampus in adult mice. The mRNA level of GluD1 is much higher especially in caudate-putamen and anteroventral thalamic nucleus in younger mice, suggesting its role in neuronal development. Since GluD1-specific antibody is not available, there has been no immunohistochemical study describing GluD1 expression in the brain.

GluD2 expression is also not confined to cerebellar Purkinje cells. Immunohistochemical analyses of wild-type and *lurcher* mice indicate that GluD2 is expressed in several other brain regions, such as the midbrain-spinal cord (Yuzaki 2008). Quite recently, using Purkinje cell–specific *Grid2*-null mice, Yamasaki et al. reported that interneurons in the cerebellum expressed GluD2 (Yamasaki et al. 2011).



Delta Glutamate Receptor (*GluD1*, *GluD2*), Fig. 1 Presumed membrane topology of GluD2. The ligand-binding domain (LBD) is separated by transmembrane (TM) domains 1 through 3. The ligand-binding pocket, to which D-serine binds, is indicated by an *arrow*. Cbln1 binds to the most N-terminal domain (NTD) outside of the LBD (indicated by an *arrow*). The channel pore is indicated by a *dotted circle*. The most C-terminal domain (CTD) of GluD2 constitutes a PDZ ligand motif, to which PDZ proteins such as PSD-93, PTPMEG, S-SCAM, n-PIST, and delphilin bind

Structure and Function

On the basis of sequence homology with other iGluRs, the topology of GluD1/2 in the cell membrane is predicted to be similar to that of other iGluRs, which are composed of an N-terminal domain (NTD) and a bipartite ligand-binding domain (LBD) on the extracellular side of the plasma membrane, three transmembrane (TM) domains (TM1, TM3, and TM4), an ion channel-forming re-entrant loop segment (TM2 or P-loop), and a cytoplasmic C-terminal domain (CTD) (Fig. 1). GluD2 and GluD1 likely form a homomeric complex in vivo, although it is possible that a small proportion of GluD1 or GluD2 exists as a heteromer with other iGluRs, as observed in vitro (Yuzaki 2008).

Extracellular N-Terminal Domain (NTD)

The NTD of iGluRs contributes to receptor assembly and efficient surface transport of the receptor. Similarly, various small in-frame deletions in the NTD of GluD2 found in many *hotfoot* mutant mice impair the homomeric oligomerization of GluD2 and its

Delta Glutamate Receptor (*GluD1*, *GluD2*), Table 1 Summary of the phenotypes of *grid2*-null mice expressing various GluD2 transgenes

grid2 locus	Transgene	Disrupted function/deleted domain	Age	Method	Ataxia	Motor learning[a]	Synapse formation	LTD
WT	None		Young/ adult		√	$\surd^{e,r}$	√	√
grid2-null	None		Young/ adult		↓	$\downarrow^{e,r}$	↓	↓
	+GluD2^{WT}		Young/ adult	Virus vector/TG mouse	√	$\surd^{e,r}$	√	√
	+GluD2$^{\Delta NT}$	N-terminal domain (NTD)	Adult	Virus vector	√*	n.e.	↓	√
	+GluD2$^{R/K}$	Ligand-binding ability (LBD)	Adult	Virus vector/TG mouse	√	$\surd^{r}$	√	√
			Young	Virus vector/TG mouse	√	$\downarrow^{r}$	√	↓
	+GluD2$^{Q/R}$	Ca^{2+} permeability	Adult	TG mouse	√	n.e.	√*	√
	+GluD2$^{V/R}$	Channel function	Adult	Virus vector/	√	n.e.	√	√
	+GluD2$^{\Delta CT7}$	PDZ ligand (CTD)	Adult	Virus vector/TG mouse	√	$\downarrow^{e,r}$	√	↓

√ – rescued, √* – partially rescued, ↓ – not rescued, *n.e.* – not examined
[a]Motor learning was examined by the delayed eyeblink conditioning test (e) or the rotor-rod test (r)

subsequent exit from the endoplasmic reticulum to reach the cell surface (Yuzaki 2008). These findings suggest that, like other iGluRs, the NTD of GluD2 is essential for receptor assembly, and that unstable oligomers may be retained in the endoplasmic reticulum by the quality control mechanism.

Virally mediated expression of wild-type GluD2 or chimeric glutamate receptor 6 (GluK2) that had the NTD of GluD2 completely rescues impaired PF synaptogenesis in *grid2*-null mice, while expression of GluD2 lacking the NTD or the chimeric GluD2 with the NTD of GluK2 does not (Table 1). Thus, the NTD of GluD2 is necessary and sufficient for synapse formation between PFs and Purkinje cells (Kakegawa et al. 2009). Recently, the NTD of GluD2 was shown to bind to ▶ Cbln1, which is secreted from cerebellar granule cells (Matsuda et al. 2010). ▶ Cbln1 also binds to neurexin on the presynaptic terminals (Matsuda and Yuzaki 2011). These findings indicate that the NTD of GluD2 plays a crucial role in formation and maintenance of PF synapses by forming a tripartite complex consisting of GluD2, ▶ Cbln1, and neurexin at PF-Purkinje cell synapses. Since ▶ Cbln1 also bind to the NTD of GluD1 to promote synaptogenesis in cultured hippocampal neurons (Matsuda et al. 2010; Matsuda and Yuzaki 2011), the NTD of GluD1 may also play a similar role in neurons expressing GluD1.

Extracellular Ligand-Binding Domain (LBD)

An arginine (R) residue highly conserved in the LBD of iGluRs is essential for binding to amino acid ligands, including glutamate, aspartate, glutamine, glycine, lysine, serine, arginine, ornithine, and histidine. Surprisingly, the mutant GluD2 transgene (GluD2$^{R/K}$), in which the conserved arginine is replaced by lysine (K) to lose the ability of ligand binding, still rescues all the abnormal phenotypes of adult *grid2*-null mice (Table 1) (Hirai et al. 2005). Therefore, no L-glutamate analog binding is likely required for GluD2 to achieve its functions at least in adult mice.

The LBD of GluD2 was crystallized and shown to bind to D-serine (Fig. 1) in a manner dependent on the arginine residue (Naur et al. 2007). Thus, D-serine fails to bind to GluD2$^{R/K}$. Interestingly, *grid2*-null mice expressing GluD2$^{R/K}$ exhibited impaired LTD and motor dyscoordination during development (Kakegawa et al. 2011). Indeed, D-serine is released from Bergmann glia after the burst stimulation of PFs in immature, but not mature, cerebellum because of developmental upregulation of D-amino acid oxidase (DAO), a D-serine-degrading enzyme. These findings indicate that D-serine serves as an endogenous ligand for GluD2 in immature cerebellum. Although D-serine also binds to GluD1 (Yadav et al. 2011), its physiological significance remains to be determined.

Channel Pore-Forming Domain

A point mutation in the TM3 of GluD2 ($GluD2^{Lc}$) makes GluD2 channel constitutively open, causing Purkinje cell death in *lurcher* mice (Zuo et al. 1997). The Ca^{2+} permeability of $GluD2^{Lc}$ is abolished by replacing glutamine (Q) with arginine (R) at the Q/R site ($GluD2^{Q/R}$) (Kohda et al. 2000; Kakegawa et al. 2007a). However, when a mutant $GluD2^{Q/R}$ transgene is introduced into *grid2*-null Purkinje cells, LTD and other major abnormalities of *grid2*-null mice are rescued (Table 1). These finding indicate that although cerebellar LTD depends on Ca^{2+} influx, GluD2 unlikely serves as a Ca^{2+}-permeable channel (Kakegawa et al. 2007a).

The channel activity of $GluD2^{Lc}$ is abolished by replacing valine (V) with arginine (R) at one position upstream of the Q/R site ($GluD2^{V/R}$) (Kakegawa et al. 2007b). Surprisingly, expression of $GluD2^{V/R}$ in *grid2*-null Purkinje cells by Sindbis virus completely restored LTD and motor coordination (Table 1), indicating that channel activity of GluD2 is not required for inducing LTD. Together, although there is still a possibility that GluD2 might function as an ion channel under some circumstances, channel activities are not required to achieve major functions of GluD2 at PF-Purkinje cell synapses.

Cytoplasmic C-Terminal Domain (CTD)

The cytoplasmic CTD of GluD2 binds to many intracellular proteins such as Shank, PICK1, and the adaptor protein complex ▶ AP-4. In addition, the most CTD of GluD2 constitutes a PDZ ligand motif, to which PDZ proteins, such as PSD-93, PTPMEG, S-SCAM, n-PIST, and delphilin, bind (Yuzaki 2008). When the mutant GluD2 lacking the C-terminal seven amino acids ($GluD2^{\Delta CT7}$) is expressed in *grid2*-null PCs, it fails to rescue abrogated LTD and impaired delayed eyeblink conditioning, a cerebellum-dependent motor learning, in *grid2*-null mice (Table 1) (Kohda et al. 2007; Kakegawa et al. 2008). Furthermore, D-serine binding to GluD2 failed to induce LTD when the CTD is deleted (Kakegawa et al. 2011). In contrast, the mutant transgene $GluD2^{\Delta CT7}$ completely restores abnormal PF synapse formation (Kakegawa et al. 2008). These findings indicate that signaling via the CTD of GluD2 is not necessary for morphological integrity at PF synapses, but absolutely required for the induction of LTD and motor learning.

Summary

Although GluD2 was referred to an orphan receptor for a long time, it now has two unusual endogenous ligands – ▶ Cbln1 and D-serine. The extracellular NTD of GluD2 plays a crucial role in the formation and maintenance of PF synapses by forming a tripartite complex with ▶ Cbln1 and its presynaptic receptor neurexin in the cerebellum. On the other hand, the LBD of GluD2 binds to D-serine, which is released from cerebellar Bergman glia during early developmental period, to regulate LTD and motor learning. Although signaling via the CTD of GluD2 is required for the induction of LTD and motor learning, how this is achieved by intracellular proteins that bind to the CTD remains unclear. In addition, whether ▶ Cbln1 binding to the NTD and D-serine binding to the LBD interact with each other and induce conformational changes to the whole GluD2 complex remains to be determined. Finally, although ▶ Cbln1 (and Cbln2) and D-serine bind to GluD1, it is currently unclear whether GluD1 regulates synaptic functions similar to those exerted by GluD2 in various brain regions in vivo.

References

Fallin MD, Lasseter VK, Avramopoulos D, Nicodemus KK, Wolyniec PS, McGrath JA, et al. Bipolar I disorder and schizophrenia: a 440-single-nucleotide polymorphism screen of 64 candidate genes among Ashkenazi Jewish case-parent trios. Am J Hum Genet. 2005;77:918–36.

Glessner JT, Wang K, Cai G, Korvatska O, Kim CE, Wood S, et al. Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. Nature. 2009;459:569–73.

Guo SZ, Huang K, Shi YY, Tang W, Zhou J, Feng GY, et al. A case-control association study between the GRID1 gene and schizophrenia in the Chinese Northern Han population. Schizophr Res. 2007;93:385–90.

Hirai H, Miyazaki T, Kakegawa W, Matsuda S, Mishina M, Watanabe M, et al. Rescue of abnormal phenotypes of the delta2 glutamate receptor-null mice by mutant delta2 transgenes. EMBO Rep. 2005;6:90–5.

Kakegawa W, Miyazaki T, Hirai H, Motohashi J, Mishina M, Watanabe M, et al. Ca2+ permeability of the channel pore is not essential for the delta2 glutamate receptor to regulate synaptic plasticity and motor coordination. J Physiol. 2007a;579:729–35.

Kakegawa W, Kohda K, Yuzaki M. The delta2 'ionotropic' glutamate receptor functions as a non-ionotropic receptor to control cerebellar synaptic plasticity. J Physiol. 2007b; 584:89–96.

Kakegawa W, Miyazaki T, Emi K, Matsuda K, Kohda K, Motohashi J, et al. Differential regulation of synaptic plasticity and cerebellar motor learning by the C-terminal PDZ-binding motif of GluRdelta2. J Neurosci. 2008;28:1460–8.
Kakegawa W, Miyazaki T, Kohda K, Matsuda K, Emi K, Motohashi J, et al. The N-terminal domain of GluD2 (GluRdelta2) recruits presynaptic terminals and regulates synaptogenesis in the cerebellum in vivo. J Neurosci. 2009;29:5738–48.
Kakegawa W, Miyoshi Y, Hamase K, Matsuda S, Matsuda K, Kohda K, et al. D-serine regulates cerebellar LTD and motor coordination through the delta2 glutamate receptor. Nat Neurosci. 2011;14:603–11.
Kohda K, Wang Y, Yuzaki M. Mutation of a glutamate receptor motif reveals its role in gating and delta2 receptor channel properties. Nat Neurosci. 2000;3:315–22.
Kohda K, Kakegawa W, Matsuda S, Nakagami R, Kakiya N, Yuzaki M. The extreme C-terminus of GluRdelta2 is essential for induction of long-term depression in cerebellar slices. Eur J Neurosci. 2007;25:1357–62.
Matsuda K, Yuzaki M. Cbln family proteins promote synapse formation by regulating distinct neurexin signaling pathways in various brain regions. Eur J Neurosci. 2011;33:1447–61.
Matsuda K, Miura E, Miyazaki T, Kakegawa W, Emi K, Narumi S, et al. Cbln1 is a ligand for an orphan glutamate receptor delta2, a bidirectional synapse organizer. Science. 2010;328:363–8.
Naur P, Hansen KB, Kristensen AS, Dravid SM, Pickering DS, Olsen L, et al. Ionotropic glutamate-like receptor delta2 binds D-serine and glycine. Proc Natl Acad Sci USA. 2007;104:14116–21.
Smith M, Spence MA, Flodman P. Nuclear and mitochondrial genome defects in autisms. Ann NY Acad Sci. 2009;1151:102–32.
Treutlein J, Muhleisen TW, Frank J, Mattheisen M, Herms S, Ludwig KU, et al. Dissection of phenotype reveals possible association between schizophrenia and Glutamate Receptor Delta 1 (GRID1) gene promoter. Schizophr Res. 2009;111:123–30.
Yadav R, Rimerman R, Scofield MA, Dravid SM. Mutations in the transmembrane domain M3 generate spontaneously open orphan glutamate delta 1 receptor. Brain Res. 2011;1382:1–8.
Yamasaki M, Miyazaki T, Azechi H, Abe M, Natsume R, Hagiwara T, et al. Glutamate receptor delta2 is essential for input pathway-dependent regulation of synaptic AMPAR contents in cerebellar Purkinje cells. J Neurosci. 2011;31:3362–74.
Yuzaki M. Delta receptors. In: Gereau RW, Swanson GT, editors. The glutamate receptors. Totowa: Humana; 2008.
Zuo J, De Jager PL, Takahashi KA, Jiang W, Linden DJ, Heintz N. Neurodegeneration in Lurcher mice caused by mutation in delta2 glutamate receptor gene. Nature. 1997;388:769–73.

DEP Domain Containing 2

▶ P-Rex

DEP.2

▶ P-Rex

Depdc2

▶ P-Rex

DERK (*Drosophila melanogaster*)

▶ ERK1/ERK2

D-*erythro*-Sphingosine-1-phosphate

▶ Sphingosine-1-Phosphate

Destruction-Box, D-Box

▶ Monopolar Spindle 1 (Mps1)

Dfp1/Him1/Rad35 (*Schizosaccharomyces pombe*), Spo6 (a Second Dbf4 Homologue in *S. pombe*)

▶ Dbf4

Dfz1

▶ FZD (Frizzled)

Dfz2

▶ FZD (Frizzled)

DHHC Proteins

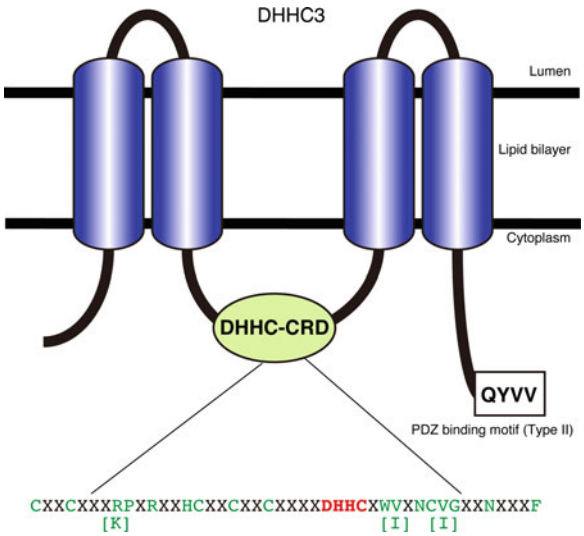
Shinichiro Oku, Yuko Fukata and
Masaki Fukata
Division of Membrane Physiology,
Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, Aichi, Japan
Department of Physiological Sciences,
School of Life Science, The Graduate University for Advanced Studies (SOKENDAI), Aichi, Japan

Synonyms

Palmitoyl acyl transferase (PAT); Palmitoylating enzyme; zDHHC proteins

Historical Background

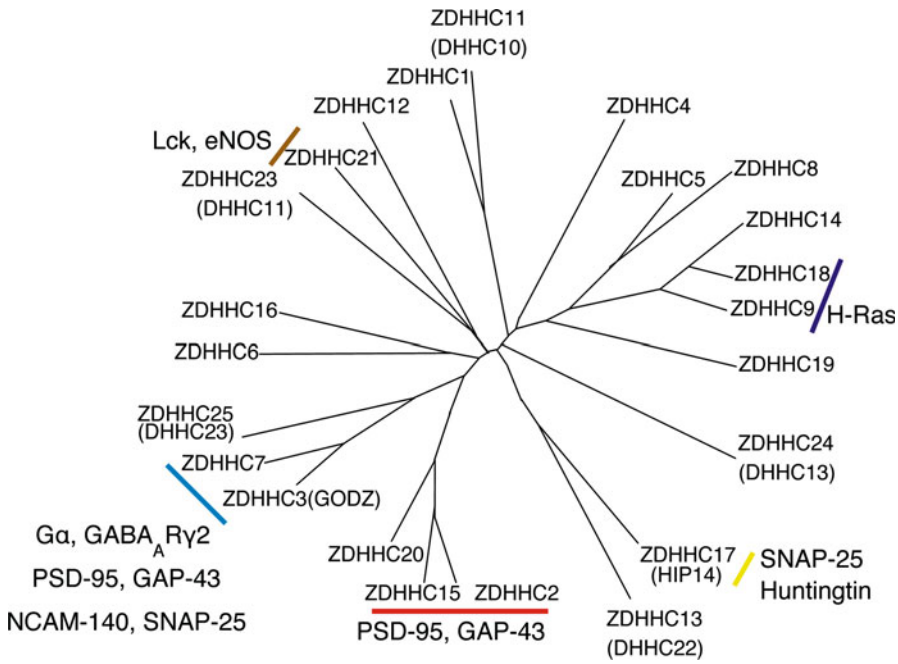
Protein palmitoylation is the first discovered and the most common lipid modification. This posttranslational change involves addition of the saturated 16-carbon palmitate to specific cysteine residues by a labile thioester linkage (Linder and Deschenes 2007). Although reversible palmitoylation was discovered over 30 years ago, the enzymes that add palmitate to proteins (palmitoyl acyl transferases, PATs) and those that cleave the thioester bond (palmitoyl protein thioesterases, PPTs) had been elusive. In 2002, genetic screening in yeast identified proteins that mediate PAT activity. Erf2/4 (Lobo et al. 2002) and Akr1 (Roth et al. 2002) were identified as PATs for yeast Ras2 and Yck2, respectively. Erf2 and Akr1 share a conserved DHHC (Asp-His-His-Cys) cysteine-rich domain (CRD) (Fig. 1) and have four or six transmembrane domains. The DHHC sequence and its surrounding CRD sequence are essential for their enzymatic activity. In 2004, 23 kinds of mammalian DHHC proteins were systematically isolated and some of them were characterized as PATs (Fukata et al. 2004). DHHC proteins have now emerged as evolutionally conserved PATs from plants, yeast (7 genes), *Caenorhabditis elegans* (15 genes), *Drosophila melanogaster* (22 genes) to mammals (24 genes).



DHHC Proteins, Fig. 1 The domain structure of DHHC3, a prototype of mammalian DHHC proteins. DHHC proteins contain four or six transmembrane domains and a conserved DHHC (Asp-His-His-Cys) motif in the cytoplasmic cysteine-rich domain (CRD). Some members of DHHC proteins have unique motif/domain, such as PDZ-binding motif (in DHHC3). The consensus sequence of DHHC-CRD is indicated (*green* and *red*). X, a variety of amino acids

Subfamily Classification of Mammalian DHHC Proteins

The large family of DHHC proteins can be classified into several subfamilies based on the homology of the DHHC-CRD core catalytic domains. DHHC2 and DHHC15 belong to one subfamily, while DHHC3 and DHHC7 form another subfamily. Importantly, DHHC proteins in the same subfamily often share their substrates (Fig. 2) (Fukata and Fukata 2010; Greaves and Chamberlain 2011). DHHC3/7 subfamily palmitoylates most of palmitoyl-proteins, such as PSD-95, GAP43, Gα, SNAP-25, NCAM-140, and $GABA_A$ receptor γ subunit. In contrast, DHHC2/15 subfamily more specifically palmitoylates PSD-95 and GAP43 (Fukata et al. 2004), but not Gα, NCAM-140 nor $GABA_A$ receptor γ subunit (Fig. 2). H/N-Ras is the only well-established substrate for DHHC9/18 subfamily. Because the number of identified substrate-enzyme pairs is limited, enzymatic activities of some DHHC proteins, such as DHHC1/10 subfamily, still remain undocumented.



DHHC Proteins, Fig. 2 Phylogenetic tree of mammalian DHHC protein family members. 23 DHHC proteins can be categorized into several subfamilies based on the homology of the catalytic DHHC domains. For example, DHHC2 and DHHC15 belong to one subfamily (in *red*), while DHHC3 and DHHC7 form another subfamily (in *blue*). The discovery of this mammalian DHHC protein family and the establishment of simple screening system using DHHC protein library have facilitated identification of palmitoyl substrate-enzyme pairs. Importantly, the same subfamily of DHHC proteins often shares substrates, for example, DHHC2/15 subfamily specifically palmitoylates PSD-95 and GAP-43. DHHC3/7 subfamily palmitoylates most of palmitoyl-proteins, such as PSD-95, GAP-43, Gα, GABA$_A$Rγ2, and SNAP-25. DHHC9/18 subfamily (in *purple*) palmitoylates H-Ras, DHHC21 (in *brown*) palmitoylates Lck and eNOS, and DHHC17 (in *yellow*) palmitoylates SNAP-25 and huntingtin. Note that some DHHC clone numbers initially collected by Fukata et al. (2004) are different from a current standard nomenclature (ZDHHC clone numbers) (Fukata and Fukata 2010). At present, 24 zDHHC genes have been annotated in the mouse genome (ZDHHC22 is recently annotated)

Enzymatic and Regulatory Mechanisms for DHHC Proteins

DHHC-CRD region is the catalytic core site as mutation of the cysteine in the DHHC motif makes DHHC proteins catalytically inactive. Cysteines in this DHHC-CRD region are often autopalmitoylated (Roth et al. 2002; Fukata et al. 2004). Two mechanisms for palmitate transfer to a substrate have been proposed: (1) DHHC protein directly transfers palmitate from palmitoyl-CoA to the substrate and (2) DHHC protein first forms a thioester intermediate with palmitate (i.e., autopalmitoylation of DHHC proteins), followed by the transfer of the palmitate to the substrate. Recent mutational analysis in yeast revealed that palmitoylation reaction by DHHC proteins, at least yeast Erf2, is mediated by the latter two-step reaction (Mitchell et al. 2010). In addition to the catalytically critical DHHC-CRD domain, some DHHC proteins have regulatory regions such as SH3 domain and ankyrin repeats at the C-terminal or N-terminal cytoplasmic regions. These domains may recruit specific substrate proteins to the DHHC enzyme. In fact, ankyrin repeats of DHHC17/HIP14 contribute to determining the substrate specificity (Huang et al. 2009).

DHHC3/7, 17, and 2/15 subfamily members do not require additional proteins (cofactors) for their PAT activity. In contrast, yeast Erf2 and its mammalian ortholog DHHC9 are inactive PATs in the absence of accessory proteins, Erf4 (for Erf2) or GCP16 (for DHHC9; Golgi complex protein of 16 kDa) (Swarthout et al. 2005). So far, the regulatory mechanism that directly activates or inactivates DHHC PAT activity is not known.

Akin to protein phosphorylation, protein palmitoylation is a reversible reaction that can be

regulated by specific extracellular signals, for example, β-adrenergic receptor activation accelerates depalmitoylation and palmitoylation levels of $G\alpha_s$ (Degtyarev et al. 1993; Wedegaertner and Bourne 1994). Palmitoylation of ▶ LAT, linker for activation of T-cells, is reduced by T-cell receptor activation by ionomycin (Hundt et al. 2006). Also, glutamate-induced synaptic activity induces depalmitoylation of PSD-95 scaffolding protein (El-Husseini et al. 2002) and Cdc42 small GTPase (Kang et al. 2008). It is conceivable that unidentified PPTs, rather than DHHC-PATs, might play dominant roles in these processes. In contrast, blockade of neuronal activity rapidly increases PSD-95 palmitoylation. This dynamic palmitoylation is mediated by synaptic translocation of a PSD-95 PAT, DHHC2. This contrasts with the constitutive PSD-95 palmitoylation mediated by Golgi-resident DHHC3. Thus, the large family of DHHC palmitoylating enzymes are differentially localized and regulated in polarized cells (Noritake et al. 2009).

Inhibitors of DHHC Proteins

Several lipid-based compounds including 2-bromopalmitate (2BP), tunicamycin, and cerulenin have been used to inhibit protein palmitoylation (Ducker et al. 2006). Among them, the palmitate analog 2BP has been the most widely used. 2BP irreversibly inhibits DHHC-mediated palmitoylation directly acting on DHHC proteins in vitro (Fukata et al. 2004; Jennings et al. 2009), although 2BP also inhibits fatty acyl-CoA ligase and other enzymes involved in lipid metabolism in cells. In addition, 2-(2-hydroxy-5-nitrobenzylidene)-benzo[b]thiophen-3-one (Compound V) reversibly inhibits DHHC-mediated palmitoylation (Jennings et al. 2009). Because these inhibitors are not specific for individual DHHC-PATs, development of DHHC member-specific inhibitors is awaited.

Pathophysiological Significance of DHHC Proteins

DHHC protein members are linked to several human diseases (Table 1). DHHC8 is one of susceptibility genes for schizophrenia. Originally, microdeletions of human chromosome 22q11.2 locus, containing the DHHC8 gene, were reported to cause cognitive deficits and be associated with a high risk of developing schizophrenia. DHHC8 knockout mice showed behavioral phenotypes, a deficiency in prepulse inhibition and decreased exploratory activity in a new environment, and abnormalities in dendritic spines of neurons (Mukai et al. 2004; Mukai et al. 2008). DHHC17/HIP14 is an associated protein with huntingtin protein, which is a causal gene for Huntington's disease. DHHC17 palmitoylates huntingtin and regulates its trafficking and function (Yanai et al. 2006). Polyglutamine expansion of huntingtin gene reduces interaction of huntingtin protein with DHHC17, resulting in decreased huntingtin palmitoylation. Knockout mice of DHHC17/HIP14 display behavioral, biochemical, and neuropathological defects that are reminiscent of Huntington's disease (Singaraja et al. 2011). Finally, mutations of DHHC9 and DHHC15 have been reported to be associated with X-linked mental retardation.

DHHC Proteins, Table 1 Human disorders associated with DHHC proteins

DHHC protein	Disorders associated with DHHC-PATs
DHHC2	Colorectal cancer
DHHC8	Schizophrenia
DHHC9	X-linked mental retardation
	Colorectal cancer
DHHC11	Bladder cancer
DHHC15	X-linked mental retardation
DHHC17/HIP14	Huntington's disease

Summary

DHHC proteins are evolutionally conserved palmitoylating enzymes and add palmitates to proteins at specific cysteine residues by a reversible thioester linkage. DHHC proteins are integral membrane proteins with DHHC-CRD domain as a catalytic domain. Enzymatic activity and subcellular distribution of DHHC proteins can be dynamically regulated by extracellular stimulations. However, signal transduction pathways from receptors on the plasma membrane to DHHC proteins are poorly understood. Because mutations of DHHC proteins are closely associated with human diseases like schizophrenia and cancers, it would be beneficial to develop drugs acting on a specific DHHC protein for human health.

References

Degtyarev MY, Spiegela M, Jones TL. Increased palmitoylation of the Gs protein alpha subunit after activation by the beta-adrenergic receptor or cholera toxin. J Biol Chem. 1993;268:23769–72.

Ducker CE, Griffel LK, Smith RA, Keller SN, Zhuang Y, Xia Z, Diller JD, Smith CD. Discovery and characterization of inhibitors of human palmitoyl acyltransferases. Mol Cancer Ther. 2006;5:1647–59.

El-Husseini Ael-D, Schnell E, Dakoji S, Sweeney N, Zhou Q, Prange O, Gauthier-Campbell C, Aguilera-Moreno A, Nicoll RA, Bredt DS. Synaptic strength regulated by palmitate cycling on PSD-95. Cell. 2002;108:849–63.

Fukata Y, Fukata M. Protein palmitoylation in neuronal development and synaptic plasticity. Nat Rev Neurosci. 2010;11:161–75.

Fukata M, Fukata Y, Adesnik H, Nicoll RA, Bredt DS. Identification of PSD-95 palmitoylating enzymes. Neuron. 2004;44:987–96.

Greaves J, Chamberlain LH. DHHC palmitoyl transferases: substrate interactions and (patho)physiology. Trends Biochem Sci. 2011;36:245–53.

Huang K, Sanders S, Singaraja R, Orban P, Cijsouw T, Arstikaitis P, Yanai A, Hayden MR, El-Husseini A. Neuronal palmitoyl acyl transferases exhibit distinct substrate specificity. FASEB J. 2009;23:2605–15.

Hundt M, Tabata H, Jeon M-S, Hayashi K, Tanaka Y, Krishna R, De Giorgio L, Liu Y-C, Fukata M, Altman A. Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. Immunity. 2006;24:513–22.

Jennings BC, Nadolski MJ, Ling Y, Baker MB, Harrison ML, Deschenes RJ, Linder ME. 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. J Lipid Res. 2009;50:233–42.

Kang R, Wan J, Arstikaitis P, Takahashi H, Huang K, Bailey AO, Thompson JX, Roth AF, Drisdel RC, Mastro R, Green WN, Yates JR, Davis NG, El-Husseini A. Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. Nature. 2008;456:904–9.

Linder ME, Deschenes RJ. Palmitoylation: policing protein stability and traffic. Nat Rev Mol Cell Biol. 2007;8:74–84.

Lobo S, Greentree WK, Linder ME, Deschenes RJ. Identification of a Ras palmitoyltransferase in Saccharomyces cerevisiae. J Biol Chem. 2002;277(43):41268–73.

Mitchell DA, Mitchell G, Ling Y, Budde C, Deschenes RJ. Mutational analysis of Saccharomyces cerevisiae Erf2 reveals a two-step reaction mechanism for protein palmitoylation by DHHC enzymes. J Biol Chem. 2010;285:38104–14.

Mukai J, Liu H, Burt RA, Swor DE, Lai W-S, Karayiorgou M, Gogos JA. Evidence that the gene encoding ZDHHC8 contributes to the risk of schizophrenia. Nature Genet. 2004;36:725–31.

Mukai J, Dhilla A, Drew LJ, Stark KL, Cao L, MacDermott AB, Karayiorgou M, Gogos JA. Palmitoylation-dependent neurodevelopmental deficits in a mouse model of 22q11 microdeletion. Nat Neurosci. 2008;11:1302–10.

Noritake J, Fukata Y, Iwanaga T, Hosomi N, Tsutsumi R, Matsuda N, Tani H, Iwanari H, Mochizuki Y, Kodama T, Matsuura Y, Bredt DS, Hamakubo T, Fukata M. Mobile DHHC palmitoylating enzyme mediates activity-sensitive synaptic targeting of PSD-95. J Cell Biol. 2009;186:147–60.

Roth AF, Feng Y, Chen L, Davis NG. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. J Cell Biol. 2002;159:23–8.

Singaraja RR, Huang K, Sanders SS, Milnerwood AJ, Hines R, Lerch JP, Franciosi S, Drisdel RC, Vaid K, Young FB, Doty C, Wan J, Bissada N, Henkelman RM, Green WN, Davis NG, Raymond LA, Hayden MR. Altered palmitoylation and neuropathological deficits in mice lacking HIP14. Hum Mol Genet. 2011;20(20):3899–909.

Swarthout JT, Lobo S, Farh L, Croke MR, Greentree WK, Deschenes RJ, Linder ME. DHHC9 and GCP16 constitute a human protein fatty acyltransferase with specificity for H- and N-Ras. J Biol Chem. 2005;280:31141–8.

Wedegaertner PB, Bourne HR. Activation and depalmitoylation of Gs alpha. Cell. 1994;77:1063–70.

Yanai A, Huang K, Kang R, Singaraja RR, Arstikaitis P, Gan L, Orban PC, Mullard A, Cowan CM, Raymond LA, Drisdel RC, Green WN, Ravikumar B, Rubinsztein DC, El-Husseini A, Hayden MR. Palmitoylation of huntingtin by HIP14 is essential for its trafficking and function. Nat Neurosci. 2006;9:824–31.

Diphtheria Toxin Receptor

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

DLK

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

DLK (Dual Leucine Zipper-Bearing Kinase)

Jean-Philippe Couture and Richard Blouin
Département de Biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada

Synonyms

DLK; Dual leucine zipper bearing kinase; Dual leucine zipper-bearing kinase; MAP kinase upstream kinase;

Map3k12; Mitogen-activated protein kinase kinase kinase 12; MUK; Zipper protein kinase; ZPK

Historical Background

DLK is a serine/threonine kinase that belongs to the mixed-lineage kinase (MLK) family of mitogen-activated protein kinase kinase kinases (MAPKKKs) (Gallo and Johnson 2002). It was discovered in 1994 as a protein differentially expressed during the retinoic-acid-induced neuronal differentiation of human NT2 teratocarcinoma cells and originally denoted zipper protein kinase (ZPK, Reddy and Pleasure 1994). Parallel and subsequent studies led to the identification and cloning of the mouse and rat homologs of ZPK, respectively termed DLK (Holzman et al. 1994) and MAP kinase upstream kinase (MUK, Hirai et al. 1996).

Structure, Expression, and Subcellular Localization

DLK is a 120 kDa protein that shares with other MLKs structural characteristics unique among the protein kinase family, namely a catalytic domain hybrid between those found in serine/threonine and tyrosine kinases, and two leucine zipper motifs involved in protein dimerization and activation (Gallo and Johnson 2002). DLK also possesses glycine-, serine-, and proline-rich sequences located upstream and downstream of the catalytic domain that are presumably important for mediating protein interactions and/or for controlling subcellular localization (Fig. 1).

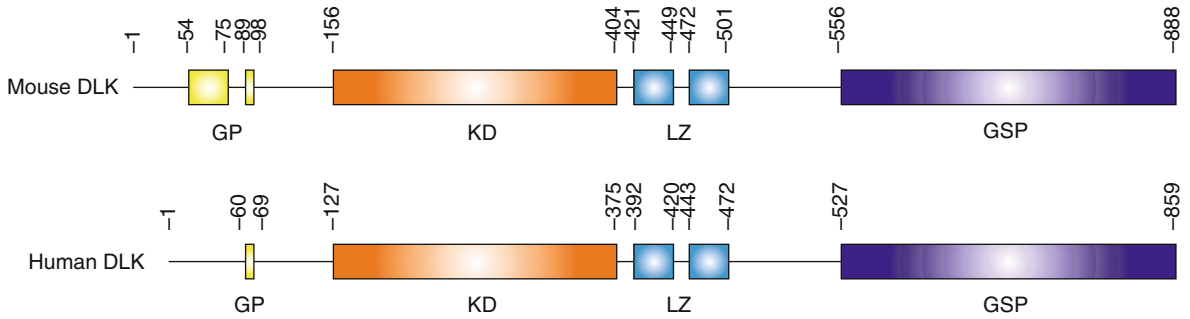
Based on Northern blot analysis of human and mouse tissues, the highest levels of DLK mRNA were observed in brain and kidney (Reddy and Pleasure 1994; Holzman et al. 1994). DLK mRNA was also detected by in situ hybridization in mouse skin, stomach, small intestine, liver, pancreas, and testis (Nadeau et al. 1997). In all these tissues, the expression of DLK mRNA increases with development and correlates with areas occupied by differentiated rather than proliferating cells (Nadeau et al. 1997). For example, in developing mouse skin, DLK mRNA expression was detected in the suprabasal cell layers of the epidermis but not in the innermost basal layer, which contains mitotically active cells. Consistently, the DLK protein is specifically expressed in the differentiated granular layer of human epidermis (Robitaille et al. 2005).

During mouse brain development, the DLK protein is most abundant in neurons of the subventricular zone and intermediate zone, and found to be preferentially localized in axons, where it is tightly associated with the microtubules (Hirai et al. 2002). DLK also localizes on Golgi apparatus in cultured embryonic cortical cells and NIH 3T3 fibroblasts (Hirai et al. 2002).

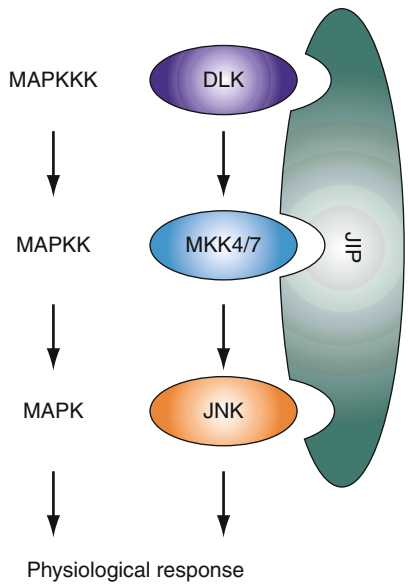
Signaling Properties and Regulation

DLK is a MAPKKK that serves as a pivotal component of the mitogen-activated protein kinase (MAPK) pathways, of which the best characterized in mammals are: extracellular signal-regulated kinases (ERKs), p38 kinases, and c-Jun N-terminal kinases (JNKs) (Gallo and Johnson 2002). The MAPKs are essential for transducing extracellular signals that regulate different cellular responses such as growth, differentiation, migration, survival, death, and metabolism (Gallo and Johnson 2002). Similar to other MLKs, DLK preferentially activates JNK, potentially by phosphorylating the JNK direct upstream activators MAPK kinase (MKK) 4 and/or 7 (Fig. 2) (Gallo and Johnson 2002). DLK also causes weak activation of p38 MAPK when overexpressed in COS and NIH 3T3 cells (Gallo and Johnson 2002). More recently, DLK was found to be required for platelet-derived growth factor (PDGF)-stimulated ERK activation in NIH 3T3 cells (Daviau et al. 2009).

To date, very little is known about the molecular mechanisms regulating DLK activation and signal suppression. Current evidence suggests that dimerization or oligomerization of DLK mediated by the leucine zipper motifs is a prerequisite for autophosphorylation, activation, and stimulation of the JNK pathway (Gallo and Johnson 2002). Another important mechanism by which DLK is proposed to be regulated is through changes in its phosphorylation status. This idea is supported by the observation that a fraction of cellular DLK is phosphorylated under normal conditions in vivo, and that phosphorylation increases substantially in response to okadaic acid, an inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A) (Gallo and Johnson 2002). Recent work also indicates that vanadate, a protein tyrosine phosphatase inhibitor, and



DLK (Dual Leucine Zipper-Bearing Kinase), Fig. 1 Primary structures of mouse and human DLK. Both proteins share 99% identity within their catalytic domains and 95% identity throughout their overall primary structure. The most significant difference between mouse and human DLK resides in the amino (N)-terminal extracatalytic region, where an additional stretch of 33 amino acids is found in the murine sequence. Numbers indicate positions relative to the first amino acid. *GP* Gly, Pro-rich domain; *KD* Kinase domain; *LZ* Leucine zipper motif; *GSP* Gly, Ser, Pro-rich domain



DLK (Dual Leucine Zipper-Bearing Kinase), Fig. 2 Schematic representation of the DLK-JNK signaling pathway. DLK mediates signals to JNK through phosphorylation and activation of MKK4 or MKK7. The scaffold protein JIP-1 negatively regulates DLK by preventing its oligomerization and activation

PDGF induce ▶ Src-dependent tyrosine phosphorylation and activation of endogenous DLK in mouse NIH 3T3 cells (Daviau et al. 2009).

Work from a number of laboratories has also established that the regulation of DLK is achieved by heterologous interactions with various cellular proteins. The binding of DLK to these proteins, in particular the scaffold JNK-interacting protein (JIP)-1 and MUK-binding inhibitory protein (MBIP), plays an important role in DLK regulation by preventing its dimerization and activation (Gallo and Johnson 2002; Fukuyama et al. 2000). Furthermore, other studies carried out in mouse fibroblasts have revealed that the active form of DLK interacts with C terminus of Hsc70-interacting protein (CHIP), a U-box-dependent E3 ubiquitin ligase that promotes its ubiquitination and subsequent degradation (Daviau et al. 2006). Ubiquitin-mediated degradation constitutes a significant mechanism for regulation of DLK levels, as its fly and worm orthologues are down-regulated by an E3 ubiquitin ligase, termed Highwire/RPM-1, which is required for synaptic growth (Nakata et al. 2005; Collins et al. 2006). Other mechanisms besides degradation may also play a role in controlling the steady-state level of DLK. For example, it was shown in cell lines from different origins that apoptotic stimuli promote the stabilization of JNK pathway components like ▶ MLK3, DLK, and JIP (Xu et al. 2005). Interestingly, inhibition of JNK activity or expression blocked this effect, suggesting that JNK contributes to MLK stabilization through a positive feedback loop mechanism. Taken together, these results suggest that the decision of whether DLK undergoes degradation or stabilization probably depends on the nature and duration of the stimulus, the cell type, as well as the identity of its signaling partners.

Biological Functions

In vitro studies with different types of cells, including neurons, fibroblasts, epithelial cells, and preadipocytes,

identified a role for DLK in regulation of apoptosis, differentiation, and axonal growth. For example, in rat pheochromocytoma PC12 cells and sympathetic neurons, ectopic expression of DLK induces apoptosis, whereas kinase-deficient DLK severely inhibits death caused by nerve growth factor deprivation (Xu et al. 2001). Additionally, down-regulation of DLK by RNA interference in mouse NIH 3T3 fibroblasts and human MDA-MB-231 breast cancer epithelial cells blocks the apoptotic response induced by calphostin C (Robitaille et al. 2008). As stated above, DLK exhibits a differentiation-associated localization in skin epidermis. Consistently, DLK overexpression in poorly differentiated normal keratinocytes is sufficient to induce phenotypic changes associated with keratinocyte terminal differentiation, including up-regulation of filaggrin expression, DNA fragmentation, activation of transglutaminases, and formation of corneocytes (Robitaille et al. 2005). Other evidence suggests that DLK may also play a role in adipocyte cell differentiation. In 3T3-L1 cells induced to undergo adipocyte differentiation, DLK expression is up-regulated, and its loss by RNA interference completely blocks the accumulation of lipid droplets as well as the expression of the adipogenic markers adiponectin and fatty acid synthase. In agreement with this, cells lacking DLK show significantly less expression of the master regulators of adipogenesis, peroxisome proliferator-activated receptor (PPAR)-γ2 and the CCAAT enhancer-binding protein (C/EBP)α (Couture et al. 2009). Finally, using knockdown approaches, it was recently observed that DLK is required for neurite extension in both PC12 cells and mouse primary cortical neurons (Eto et al. 2010). Taken together, these data indicate that DLK may fulfill different functions, depending on the cell type and/or the stimuli.

Considerable progress has also been made within the last few years in understanding the *in vivo* biological functions of DLK. In mice, the knockout of the *DLK* gene results in a lethal phenotype around birth (Hirai et al. 2006), suggesting that it might have a crucial role during embryogenesis and organogenesis. Embryos lacking DLK show abnormal development of the brain characterized by defects in anterior commissure formation and axon growth of neocortical pyramidal neurons (Hirai et al. 2006). In addition, these mice display reduced JNK activity and reduced phosphorylation of the microtubule-associated protein doublecortin. In parallel to the knockout studies, Miller et al. (2009) generated mice with a gene-trap mutation of *DLK* and showed that axons of this mouse were protected from degeneration after transection, demonstrating an absolute requirement for this enzyme in the neuronal response to injury. Interestingly, this function seems to be conserved throughout evolution, since a dramatic defect in axon degeneration or regeneration after injury has been noticed in *Drosophila* and *C. elegans* mutants defective in *Wallenda* and *DLK-1*, the fly and worm orthologues of DLK respectively (Miller et al. 2009; Hammarlund et al. 2009). Thus, DLK appears to be a pivotal signaling component for regulation of axon growth and stress response in neuronal cells.

Summary

DLK is a serine/threonine kinase that functions as an upstream activator of the MAPK pathways. It is expressed in a tissue-specific manner and regulated by mechanisms that involve phosphorylation, interactions with different protein partners, and ubiquitin-mediated degradation. The functions of DLK are diverse and include regulation of development, cell differentiation, and apoptosis. Recent findings from analyses of mouse, fly, and worm mutants defective in *DLK* or its invertebrate orthologues show interestingly that this enzyme may also be required for the neuronal response to injury. Thus, DLK seems to be a key regulator for various fundamental biological processes, although the precise molecular mechanisms by which it mediates such effects are still elusive.

Acknowledgments We thank the Natural Sciences and Engineering Research Council of Canada for its financial support. We also apologize to our colleagues whose work could not be cited due to space limitations.

References

Collins CA, Wairkar YP, Johnson SL, DiAntonio A. Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron. 2006;51:57–69.

Couture JP, Daviau A, Fradette J, Blouin R. The mixed-lineage kinase DLK is a key regulator of 3T3-L1 adipocyte differentiation. PLoS ONE. 2009;4:e4743.

Daviau A, Proulx R, Robitaille K, Di Fruscio M, Tanguay RM, Landry J, et al. Down-regulation of the mixed-lineage dual leucine zipper-bearing kinase by heat shock protein 70 and its co-chaperone CHIP. J Biol Chem. 2006;281:31467–77.

Daviau A, Di Fruscio M, Blouin R. The mixed-lineage kinase DLK undergoes Src-dependent tyrosine phosphorylation and activation in cells exposed to vanadate or platelet-derived growth factor (PDGF). Cell Signal. 2009;21:577–87.
Eto K, Kawauchi T, Osawa M, Tabata H, Nakajima K. Role of dual leucine zipper-bearing kinase (DLK/MUK/ZPK) in axonal growth. Neurosci Res. 2010;66:37–45.
Fukuyama K, Yoshida M, Yamashita A, Deyama T, Baba M, Suzuki A, et al. MAPK upstream kinase (MUK)-binding inhibitory protein, a negative regulator of MUK/dual leucine zipper-bearing kinase/leucine zipper protein kinase. J Biol Chem. 2000;275:21247–54.
Gallo KA, Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol. 2002;3:663–72.
Hammarlund M, Nix P, Hauth L, Jorgensen EM, Bastiani M. Axon regeneration requires a conserved MAP kinase pathway. Science. 2009;323:802–6.
Hirai S, Izawa M, Osada S, Spyrou G, Ohno S. Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. Oncogene. 1996;12:641–50.
Hirai S, Kawaguchi A, Hirasawa R, Baba M, Ohnishi T, Ohno S. MAPK-upstream protein kinase (MUK) regulates the radial migration of immature neurons in telencephalon of mouse embryo. Development. 2002;129:4483–95.
Hirai S, de Cui F, Miyata T, Ogawa M, Kiyonari H, Suda Y, et al. The c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. J Neurosci. 2006;26:11992–2002.
Holzman LB, Merritt SE, Fan G. Identification, molecular cloning, and characterization of dual leucine zipper bearing kinase. A novel serine/threonine protein kinase that defines a second subfamily of mixed lineage kinases. J Biol Chem. 1994;269:30808–17.
Miller BR, Press C, Daniels RW, Sasaki Y, Milbrandt J, DiAntonio A. A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci. 2009;12:387–9.
Nadeau A, Grondin G, Blouin R. In situ hybridization analysis of ZPK gene expression during murine embryogenesis. J Histochem Cytochem. 1997;45:107–18.
Nakata K, Abrams B, Grill B, Goncharov A, Huang X, Chisholm AD, et al. Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell. 2005;120:407–20.
Reddy UR, Pleasure D. Cloning of a novel putative protein kinase having a leucine zipper domain from human brain. Biochem Biophys Res Commun. 1994;202:613–20.
Robitaille H, Proulx R, Robitaille K, Blouin R, Germain L. The mitogen-activated protein kinase kinase kinase dual leucine zipper-bearing kinase (DLK) acts as a key regulator of keratinocyte terminal differentiation. J Biol Chem. 2005;280:12732–41.
Robitaille K, Daviau A, Lachance G, Couture JP, Blouin R. Calphostin C-induced apoptosis is mediated by a tissue transglutaminase-dependent mechanism involving the DLK/JNK signaling pathway. Cell Death Differ. 2008;15:1522–31.
Xu Z, Maroney AC, Dobrzanski P, Kukekov NV, Greene LA. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. Mol Cell Biol. 2001;21:4713–24.
Xu Z, Kukekov NV, Greene LA. Regulation of apoptotic c-Jun N-terminal kinase signaling by a stabilization-based feed-forward loop. Mol Cell Biol. 2005;25:9949–59.

Dmel

► Rab8

DOCK2; Dedicator of Cytokinesis 2

Hiroshi Nishihara
Graduate School of Medicine, Laboratory of Translational Pathology, Hokkaido University, Sapporo, Japan

Synonyms

FLJ46592; KIAA0209

Historical Background

DOCK2 was initially designated by Nishihara et al. in 1999 (Nishihara et al. 1999) as a hematopoietic cell-specific homologue of the CDM (*c*ed-5 of *Caenorhabditis elegans*, *D*OCK180 of humans, and *m*yoblast city of *Drosophila melanogaster*) family proteins. The name "DOCK" was originally designated as "*D*ownstream *o*f *CRK*" for DOCK180, an archetype of CDM family proteins in 1996 (Hasegawa et al. 1996).

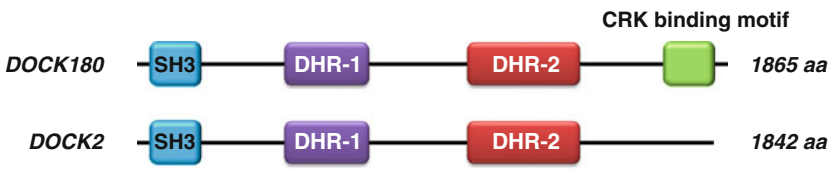
Molecular Mechanism of DOCK2 as a Rac-Specific GEF (Guanine Nucleotide Exchange Factor)

In mammals, 11 DOCK180-related proteins have been identified, and the family members can be subcategorized into four groups denoted DOCK-A, -B, -C, and -D, and DOCK2 belongs to DOCK-A as well as DOCK180 (Fig. 1a) (Cote and Vuori 2007). The structural analysis revealed that DOCK2 consists of

a

Subgroups	***DOCK-A***	DOCK-B	DOCK-C	DOCK-D
Human DOCK -related proteins	DOCK180 ***DOCK2*** DOCK5	DOCK3 DOCK4	DOCK6 DOCK7 DOCK8	DOCK9 DOCK10 DOCK11
Target GTPase	Rac	Rac	Rac Cdc42	Cdc42

b



DHR: DOCK homology region, aa; amino acids.

DOCK2; Dedicator of Cytokinesis 2, Fig. 1 (**a**) The four subfamilies of human DOCK180-related proteins (DOCK-A, -B, -C, and -D) and the target small GTPases are indicated. DOCK2 belongs to DOCK-A and exclusively activates Rac, but not Cdc42. (**b**) Schematic diagram of the structure of the human DOCK180 and DOCK2. The DHR-1 domain is a unique evolutionarily conserved domain in all DOCK180-related proteins, and in the case of DOCK180, the DHR-1 domain was revealed to mediate a specific interaction with PtdIns (3, 5)P_2 and PtdIns (3, 4, 5)P_3 signaling lipids. The DHR-2 domains have been shown to interact with the GTPases of the Rho family including Rac1, 2 and Cdc42 leading to the exchange of GDP for GTP. Inactivation of the DHR-2 domain in DOCK180 has been shown to abrogate Rac activation, cell migration, and phagocytosis, highlighting the significance of this domain in the biological function of the DOCK180-related proteins. DOCK2 lacks the Crk-binding motif in the C-terminus which is an indispensable region in DOCK180 for binding to CRK, while CrkL was identified as a binding partner to DOCK2 through its SH3 domain in the N-terminus

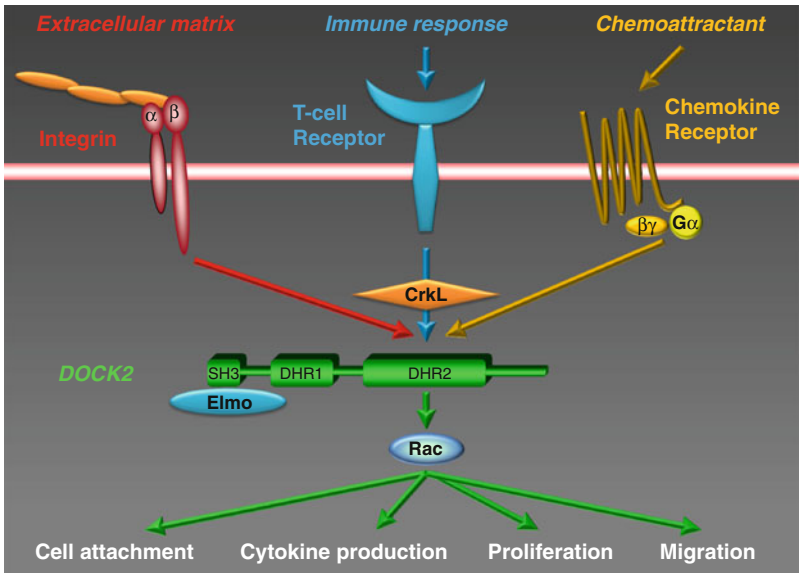
an SH3 domain in the N-terminus and DHR (DOCK homology region) -1/2 in the middle to C-terminus (Fig. 1b) (Cote and Vuori 2002). DHR-2 is highly conserved throughout DOCK180-related proteins and identified as a Rac-specific GEF in DOCK2, although DHR-2 in other family proteins, such as DOCK3, may activate Cdc-42. DOCK2 lacks the PXXP motif in the C-terminus which is an indispensable region in DOCK180 for binding to CRK (Hasegawa et al. 1996), while CrkL was identified as a binding partner to DOCK2 through its SH3 domain in the N-terminus (Fig. 2) (Nishihara et al. 2002a). Similar to DOCK180, the interaction of DOCK2 with ELMO1, which was shown to increase the catalytic activity of DOCK180 toward Rac (Lu et al. 2004), was reported (Janardhan et al. 2004; Sanui et al. 2003b).

DOCK2 in Cytoskeletal Regulation and Tumorigenesis

DOCK2 regulates the motility of lymphocytes through actin-cytoskeletal reorganization and also cell proliferation in several types of B lymphocytes through the activation of Rac (Nishihara et al. 2002a; Sanui et al. 2003b; Wang et al. 2010), although DOCK2 itself, and even Rac, seems to lack the oncogenic ability. DOCK2-knockdown of the B-cell lymphoma cell lines using shRNA represented abrogated tumor formation in nude mice, suggesting the prominent role of DOCK2 in the progression of hematopoietic malignancy through DOCK2-Rac-ERK pathway (Wang et al. 2010). In fact, the high-level expression of DOCK2 mRNA in the leukemia/lymphoma cells obtained from the patients' blood samples has been ascertained (unpublished data).

The Immunomodulatory Role of DOCK2 in Lymphocytes

Because of the exclusive expression of DOCK2 in hematopoietic cells (Nishihara et al. 1999), a hematopoietic cell-specific function such as regulation of immunity had been estimated. The analysis using DOCK2 knockout mice revealed the failure of T- and B-lymphocyte migration toward cytokines in vitro and the lack of their homing into the lymph nodes and the spleen in vivo



DOCK2; Dedicator of Cytokinesis 2, Fig. 2 The signal transduction through DOCK2. DOCK2 transmits the signals from the several types of the integrins and receptors to small GTPase Rac, and regulates the cell motility, proliferation, and immune reaction of the hematopoietic cells. The protein interaction with CrkL and Elmo has been noted in the regulation of these cellular functions

(Fukui et al. 2001). The activation of DOCK2-Rac pathway is indispensable for CXCL12 (SDF-1)-stimulated human T-lymphocyte adhesion which is mediated by alpha4beta1 integrin (Gollmer et al. 2009), and also CCL21-mediated co-stimulation in CD4 (+) T cells (Garcia-Bernal et al. 2006). In addition, the role of DOCK2 in T-cell receptor (TCR) has been clarified: The in vitro analysis using the dominant negative form of DOCK2 confirmed that DOCK2 mediates TCR-dependent activation of Rac2 leading to the regulation of IL-2 (interleukin-2) promoter activity (Nishihara et al. 2002b), and in DOCK2$^{-/-}$ T cells, antigen-induced translocation of TCR and lipid rafts was significantly impaired, resulting in a marked reduction of antigen-specific T-cell proliferation (Sanui et al. 2003a). Furthermore, DOCK2 seems to be required in T-cell precursors for development into natural killer T cells (Kunisaki et al. 2006b). Taken together, these results indicate that DOCK2 is a key regulator of immunity, and explain the fact that cardiac allografts in DOCK2 knockout mice across a complete mismatch of the major histocompatibility complex molecules were not rejected by preventing potentially alloreactive T cells from recruiting into secondary lymphoid organs (Jiang et al. 2005).

DOCK2 in Myeloid Cell Lineage

Dendritic cells (DCs), macrophages, and neutrophils are also key players in immune response. The equivalent expression of DOCK2 during the maturation from CD34 (+)-myeloid precursor cells to these cells has been observed (unpublished data); therefore, the ubiquitous cellular functions of DOCK2 in this cell lineage are anticipated. In fact, the migration of neutrophil and plasmacytoid DCs was significantly abrogated in DOCK2$^{-/-}$ mice, although myeloid DCs did not show any defects in migration, suggesting the presence of alternative molecules to activate Rac during chemotaxis in myeloid DCs (Gotoh et al. 2008; Kunisaki et al. 2006a). Furthermore, DOCK2 is essential for toll-like receptor (TLR) 7- and 9- mediated interferon-alpha induction in plasmacytoid DCs, which play a key role in antiviral immunity (Gotoh et al. 2010).

Summary

As shown above, DOCK2, i.e., activation of Rac by DOCK2, plays indispensable roles in the regulation of immune response and also in the development of hematopoietic malignancy (Fig. 2). The activation of Rac is modulated by DHR-2, and several effectors for activated Rac have been identified, although the molecular mechanism of the activation of DOCK2 is still unclear. In DOCK180, autoregulation of GEF activity by its SH3 domain, ubiquitylation, and phosphorylation associated with Elmo are regarded, although the details including the upstream molecules remain undefined (Cote and Vuori 2007).

Because deficiency of DOCK2 resulted in impairment of the immune system, pharmacological inhibition of DOCK2 could be beneficial in the treatment for autoimmune diseases (Gotoh et al. 2010) and in preventing graft rejection (Jiang et al. 2005), as well as in the regulation of the progression of hematopoietic malignancy including malignant lymphoma and leukemia (Wang et al. 2010). In addition, DOCK2+ microglia which are associated with human Alzheimer's disease have been identified in a recent report (Cimino et al. 2009), suggesting that DOCK2 could be a possible therapeutic target for neurodegenerative disorders.

References

Cimino PJ, Sokal I, Leverenz J, Fukui Y, Montine TJ. DOCK2 is a microglial specific regulator of central nervous system innate immunity found in normal and Alzheimer's disease brain. Am J Pathol. 2009;175:1622–30.

Cote JF, Vuori K. Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. J Cell Sci. 2002;115:4901–13.

Cote JF, Vuori K. GEF what? Dock180 and related proteins help Rac to polarize cells in new ways. Trends Cell Biol. 2007;17:383–93.

Fukui Y, Hashimoto O, Sanui T, Oono T, Koga H, Abe M, Inayoshi A, Noda M, Oike M, Shirai T, Sasazuki T. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature. 2001;412:826–31.

Garcia-Bernal D, Sotillo-Mallo E, Nombela-Arrieta C, Samaniego R, Fukui Y, Stein JV, Teixido J. DOCK2 is required for chemokine-promoted human T lymphocyte adhesion under shear stress mediated by the integrin alpha4beta1. J Immunol. 2006;177:5215–25.

Gollmer K, Asperti-Boursin F, Tanaka Y, Okkenhaug K, Vanhaesebroeck B, Peterson JR, Fukui Y, Donnadieu E, Stein JV. CCL21 mediates CD4+ T-cell costimulation via a DOCK2/Rac-dependent pathway. Blood. 2009;114:580–8.

Gotoh K, Tanaka Y, Nishikimi A, Inayoshi A, Enjoji M, Takayanagi R, Sasazuki T, Fukui Y. Differential requirement for DOCK2 in migration of plasmacytoid dendritic cells versus myeloid dendritic cells. Blood. 2008;111:2973–6.

Gotoh K, Tanaka Y, Nishikimi A, Nakamura R, Yamada H, Maeda N, Ishikawa T, Hoshino K, Uruno T, Cao Q, Higashi S, Kawaguchi Y, Enjoji M, Takayanagi R, Kaisho T, Yoshikai Y, Fukui Y. Selective control of type I IFN induction by the Rac activator DOCK2 during TLR-mediated plasmacytoid dendritic cell activation. J Exp Med. 2010;207:721–30.

Hasegawa H, Kiyokawa E, Tanaka S, Nagashima K, Gotoh N, Shibuya M, Kurata T, Matsuda M. DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. Mol Cell Biol. 1996;16:1770–6.

Janardhan A, Swigut T, Hill B, Myers MP, Skowronski J. HIV-1 Nef binds the DOCK2-ELMO1 complex to activate rac and inhibit lymphocyte chemotaxis. PLoS Biol. 2004;2:E6.

Jiang H, Pan F, Erickson LM, Jang MS, Sanui T, Kunisaki Y, Sasazuki T, Kobayashi M, Fukui Y. Deletion of DOCK2, a regulator of the actin cytoskeleton in lymphocytes, suppresses cardiac allograft rejection. J Exp Med. 2005;202:1121–30.

Kunisaki Y, Nishikimi A, Tanaka Y, Takii R, Noda M, Inayoshi A, Watanabe K, Sanematsu F, Sasazuki T, Sasaki T, Fukui Y. DOCK2 is a Rac activator that regulates motility and polarity during neutrophil chemotaxis. J Cell Biol. 2006a;174:647–52.

Kunisaki Y, Tanaka Y, Sanui T, Inayoshi A, Noda M, Nakayama T, Harada M, Taniguchi M, Sasazuki T, Fukui Y. DOCK2 is required in T cell precursors for development of Valpha14 NK T cells. J Immunol. 2006b;176:4640–5.

Lu M, Kinchen JM, Rossman KL, Grimsley C, deBakker C, Brugnera E, Tosello-Trampont AC, Haney LB, Klingele D, Sondek J, Hengartner MO, Ravichandran KS. PH domain of ELMO functions in trans to regulate Rac activation via Dock180. Nat Struct Mol Biol. 2004;11:756–62.

Nishihara H, Kobayashi S, Hashimoto Y, Ohba F, Mochizuki N, Kurata T, Nagashima K, Matsuda M. Non-adherent cell-specific expression of DOCK2, a member of the human CDM-family proteins. Biochim Biophys Acta. 1999;1452:179–87.

Nishihara H, Maeda M, Oda A, Tsuda M, Sawa H, Nagashima K, Tanaka S. DOCK2 associates with CrkL and regulates Rac1 in human leukemia cell lines. Blood. 2002a;100:3968–74.

Nishihara H, Maeda M, Tsuda M, Makino Y, Sawa H, Nagashima K, Tanaka S. DOCK2 mediates T cell receptor-induced activation of Rac2 and IL-2 transcription. Biochem Biophys Res Commun. 2002b;296:716–20.

Sanui T, Inayoshi A, Noda M, Iwata E, Oike M, Sasazuki T, Fukui Y. DOCK2 is essential for antigen-induced translocation of TCR and lipid rafts, but not PKC-theta and LFA-1, in T cells. Immunity. 2003a;19:119–29.

Sanui T, Inayoshi A, Noda M, Iwata E, Stein JV, Sasazuki T, Fukui Y. DOCK2 regulates Rac activation and cytoskeletal reorganization through interaction with ELMO1. Blood. 2003b;102:2948–50.

Wang L, Nishihara H, Kimura T, Kato Y, Tanino M, Nishio M, Obara M, Endo T, Koike T, Tanaka S. DOCK2 regulates cell proliferation through Rac and ERK activation in B cell lymphoma. Biochem Biophys Res Commun. 2010;395:111–5.

Domain Protein

► ARD1/TRIM23

Dopamine and Adenosine 3′,5′-Monophosphate-Regulated Phosphoprotein, 32 kDa

► DARPP

Dopamine and cAMP-Regulated Phosphoprotein

► DARPP

Dopamine- and cAMP-Regulated Phosphoprotein, Mr32 kDa

► DARPP

Double-Stranded RNA-Activated Protein Kinase

► PKR

Down Syndrome Candidate Region 1 (DSCR1)

► Regulator of Calcineurin 1 (RCAN1)

Down Syndrome Critical Region 1 (DSCR1)

► Regulator of Calcineurin 1 (RCAN1)

Drag1

► ARAP3

DRAK2

Jeniffer B. Hernandez, Ryan H. Newton, Brian M. Weist and Craig M. Walsh
Institute for Immunology and Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Synonyms

DAP kinase-related apoptotic kinase 2; STK17B

Historical Background

► DRAK2 is a serine/threonine kinase of the death associate protein kinase (DAPK) family. Of this family, DRAK2 is most similar to DRAK1, and these two kinases may represent a unique family. DRAK1 and DRAK2 were originally identified using a polymerase chain reaction (PCR) screen to identify additional DAPK members, and was first thought to be primarily involved in promoting apoptosis (Sanjo et al. 1998). While humans have genes for both DRAK1 and DRAK2, mice lack a DRAK1 gene. Although ectopic expression of DRAK2 in cell lines does induce apoptosis (Sanjo et al. 1998; Matsumoto et al. 2001), it is unlikely that apoptotic induction is its key physiologic function since DRAK2-deficient mice demonstrate no obvious defects in apoptotic signaling (McGargill et al. 2004; Friedrich et al. 2005). Instead, DRAK2 has been shown to negatively regulate calcium signaling in primary T cells. Since its catalytic activity is itself induced by calcium influx, DRAK2 may serve to maintain calcium homeostasis (Friedrich et al. 2007; Newton et al. 2011). DRAK2 (and likely its ortholog DRAK1) has been found to be an important immunomodulatory serine/threonine kinase, serving to a) set the initial threshold for thymic and peripheral T cell activation and later, to maintain the survival of effector T cells. In this capacity, mice lacking DRAK2 are resistant to organ-specific autoimmunity (see below). Thus, the development of small molecule antagonists will be of significant value to efforts aimed at combating such immune system diseases.

DRAK2 Expression

DRAK2 mRNA expression in humans and mice is fairly limited in adults. DRAK2 is highly enriched in lymphoid tissues including bone marrow, thymus, lymph nodes, and spleen. In particular, T cells and B cells express very high levels of DRAK2 mRNA and protein, and it has been demonstrated to be a critical regulator of T and B cell biology (Gatzka and Walsh 2008). During T cell development in the thymus, DRAK2 expression initiates at the single positive stage and is maintained in the periphery. Stimulation of the T cell receptor (TCR) and co-receptor CD4 leads to acute expression of DRAK2 mRNA and protein in double positive thymocytes (Friedrich et al. 2005). Similar to T cells, B cells also begin to express DRAK2 at the mature B cell stage of development, where it regulates cellular functions in these immune cells also (McGargill et al. 2004; Al-Qahtani et al. 2008).

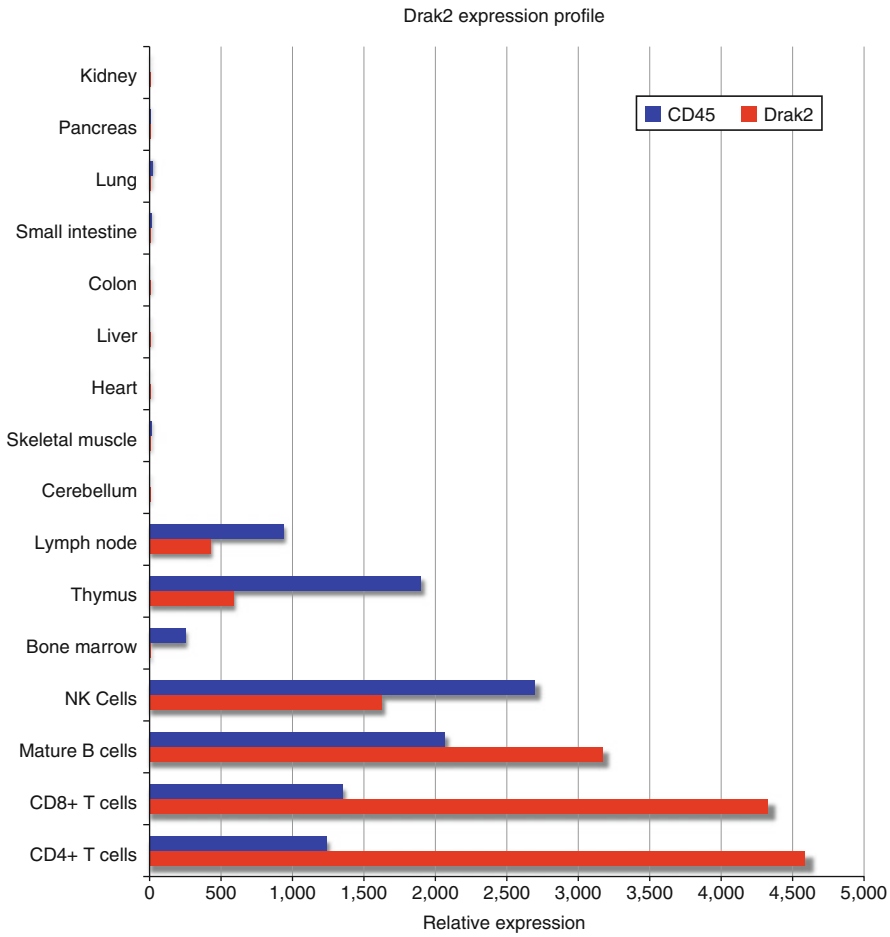
The original discovery of DRAK2 resulted from screens of human placenta and liver cDNA libraries (Sanjo et al. 1998), although the role of DRAK2 in tissues outside of the lymphoid compartment is not well understood. DRAK2 expression has been found to be very high within parts of the brain, including the Purkinje cells of the cerebellum cortex as well as the olfactory lobe, ventricular zone, pituitary, and superchiasmatic nuclei (Mao et al. 2006). During mouse development, DRAK2 mRNA expression is ubiquitous until mid-gestation stage E14, but wanes by E18 in all tissues except those of the immune system. In addition to healthy tissue expression, colorectal cancer cells have been shown to downregulate DRAK2 expression as a means to enhance transformed cell survival. Treatment with Cyclooxygenase-2 (COX-2) inhibitors resulted in enhanced expression of DRAK2, and induction of apoptosis in the HCA7 colorectal cancer cell line (Doherty et al. 2009). Evaluation of DRAK2 expression using an online expression atlas demonstrates a similar pattern of expression between DRAK2 and DRAK1, with greatest expression found in lymphoid tissues and in B cells and T cells (Wu et al. 2009). DRAK2 also has a similar expression pattern as the hemopoietically restricted phosphatase CD45 (see Fig. 1).

DRAK2's Role in Apoptosis

As a member of the ▶ DAP kinase family, DRAK2 was thought to also be important for initiation of apoptosis. Following isolation from human placental tissue, studies demonstrated that DRAK2 could, in some instances, induce apoptosis. Initial reports suggested that overexpression of DRAK2 in cells lines such as 3T3 fibroblasts, Cos7 cells, rat NRK cells, and human Caco-2 cells all led to enhanced apoptosis (Sanjo et al. 1998; Kuwahara et al. 2006). The requirements for induction of apoptosis by DRAK2 in these various cell lines is not well understood, although in some instances DRAK2 kinase activity and localization to the nucleus seems to be required (Kuwahara et al. 2006). Besides overexpression studies, DRAK2 siRNA treatment in ACL-15 and NRK cells led to diminished apoptosis following exposure to UV-irradiation, further suggesting a role for DRAK2 in apoptosis in cell lines (Kuwahara et al. 2006).

Although apoptosis can be enhanced and prevented in cell lines overexpressing or lacking DRAK2 respectively, several DRAK2 mouse models have been constructed to further elucidate the role of DRAK2 in cellular processes. First, a DRAK2 knockout mouse was created in which DRAK2 expression was abolished from all tissues. Surprisingly, DRAK2 knockout mice did not have any defects in apoptosis induction or embryonic development (McGargill et al. 2004). In particular, mice did not develop any signs of autoimmunity, cancer, or lymphadenopathy, which would be expected upon deletion of proteins involved in apoptosis. On the contrary, deletion of DRAK2 resulted in survival defects particularly in T cells and B cells. In T cells, DRAK2 has important non-apoptotic functions regulating signals transduced through the T cell receptor, and more detail as to how these processes are regulated will be covered in depth later in this entry.

Besides a DRAK2 knockout mouse, two DRAK2 transgenic mice have been studied. First, DRAK2 expression linked to a human beta actin promoter was constructed, which enhanced expression of DRAK2 roughly fivefold in all adult mouse tissues. As noted before, DRAK2 is not normally expressed in all tissues, and is generally enriched in the immune system. Nonetheless, DRAK2 transgenic mice were shown to



DRAK2, Fig. 1 DRAK2 mRNA expression profile obtained from GeneAtlas (Wu et al. 2009) (http://biogps.gnf.org/#goto=genereport&id=9262) is shown compared to CD45 expression in various mouse tissues. DRAK2 is highly enriched in tissues and cells of the immune system, similar to CD45

have enhanced T cell apoptosis following stimulation, which was dependent on high levels of cytokine exposure. Spleen size was enhanced in these mice, but no other defects were noted (Mao et al. 2006).

A second DRAK2 transgenic mouse was generated in which overexpression occurred only in the T cell compartment. Specifically, DRAK2 was linked to the lck promoter, which begins to be expressed at the double negative stage of thymocyte development. DRAK2 expression was very high in the thymus during development, and subsequently returned to wild-type levels in the periphery. In this mouse, thymocytes did not exhibit enhanced spontaneous or stimuli-induced apoptosis, and negative selection was diminished. Additionally, apoptosis of peripheral T cells was not enhanced following stimulation, although these cells were hypersensitive to stimuli (Gatzka et al. 2009).

Overall, although DRAK2 is a member of the DAP kinase family, it has controversial roles in apoptosis. Cell lines become sensitized to apoptosis when DRAK2 is overexpressed, yet DRAK2 deletion in mice does not confer resistance to apoptosis or manifest as disease pathology. Additionally, overexpression of DRAK2 in the thymus has no effect on apoptosis, while overexpression in the periphery enhanced apoptosis of stimulated T cells. It is important to note that DRAK2 seems to only enhance apoptosis in cells when overexpressed to very high levels, thus we do not expect DRAK2 to have a specific role in apoptosis under normal physiological conditions.

Structure/Function of DRAK2

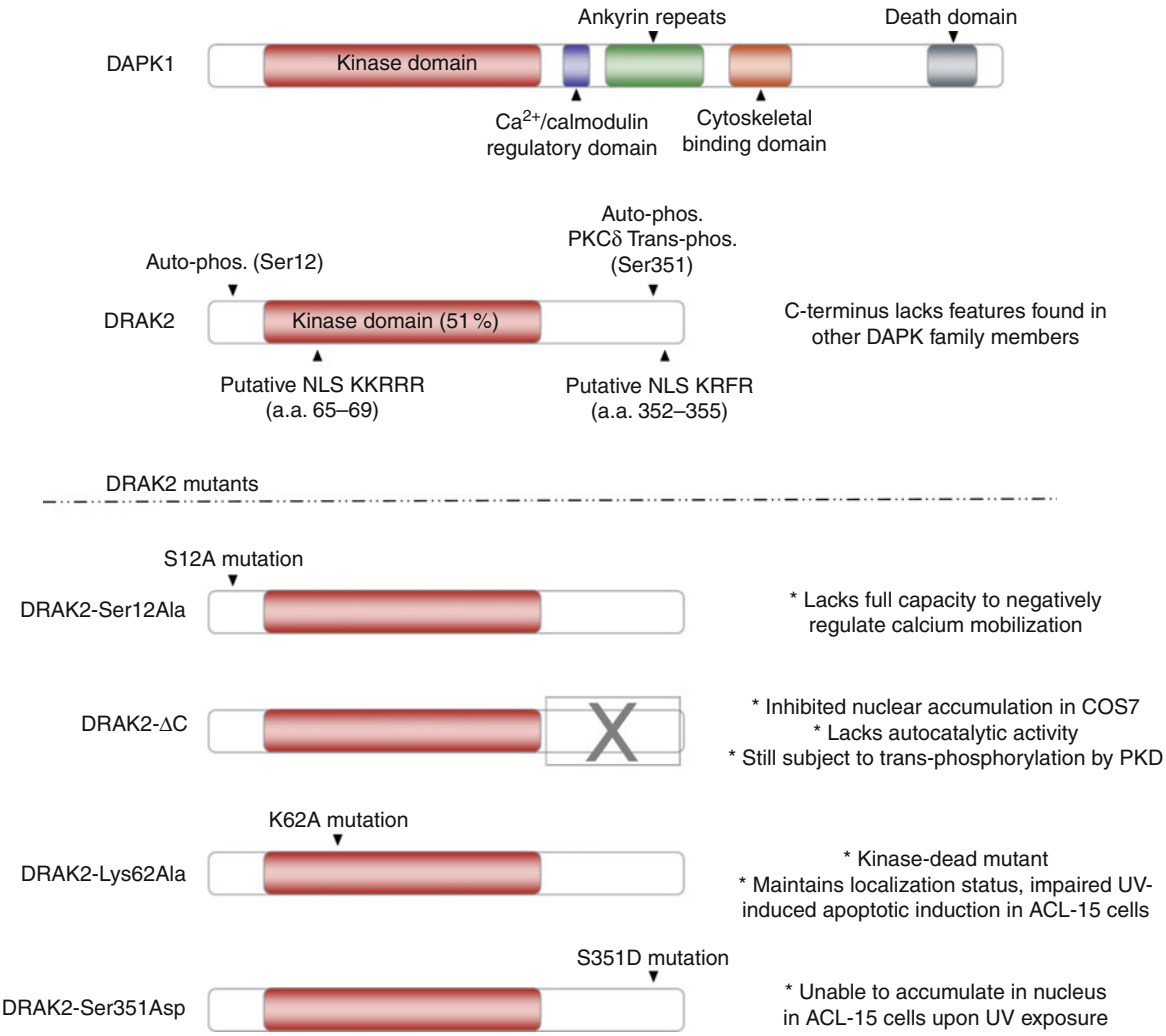
Within the DAPK family of Ca2+/calmodulin-regulated serine/threonine kinases, DAPK, DRP-1,

and Zip Kinase (ZIPK) comprise a highly homologous subfamily, whereas DRAK1 and DRAK2 represent a more distantly related group (Bialik and Kimchi 2006). DRAK2 has been the focus of intense study not only because orthologs for DRAK1 are present only in higher order primates, but because of its unique role in T cell activation. While DRP-1 and ZIPK share 80% and 83% homology, respectively, to the founding member of this family, ▶ DAPK, DRAK1 and DRAK2 share only 48% and 51%, respectively. Another important attribute that distinguishes the DRAKs from other members of this family is the complete lack of C-terminal features that command the regulation and apoptosis-promoting function of these kinases. Outside of its kinase domain, DRAK2 lacks homology with all other proteins, containing a short N-terminal region subject to autophosphorylation and a C-terminus important for its subcellular localization and its ability to induce apoptosis upon ectopic expression in various carcinoma cell lines.

In stably transfected Jurkat T cells, DRAK2 was localized primarily within the nucleus (Friedrich et al. 2005). This has also been shown to be the case in NIH3T3, NRK, and Caco-2 cell lines, whereas ACL-15, HeLa, and WI-38 cells have exhibited DRAK2 localization within the cytoplasm (Kuwahara et al. 2006). DRAK2 has been shown to contain putative nuclear-localization signals (NLS) in both its kinase domain (Friedrich et al. 2005) and C-terminal region (Kuwahara et al. 2006), the differential regulation of which potentially explaining this cell type-dependent translocation (Fig. 2). Upon stimulation of Jurkat cells with PMA plus PHA to mimic antigen receptor stimulation, DRAK2 translocated to the cytoplasm, whereas in ACL-15 cells, DRAK2 nuclear accumulation could be induced with UV-irradiation. In the latter cell type, this nuclear accumulation was not only dependent on an intact NLS within the C-terminus of DRAK2, but on phosphorylation of Ser350 mediated by protein kinase C (PKC) delta, the blockade of which prevented nuclear accumulation of DRAK2 and UV-induced apoptosis (Kuwahara et al. 2008). Interestingly, Ser350 was identified as a prominent site of autophosphorylation in studies (designating this residue as Ser351) aimed at understanding how this kinase is regulated in lymphocytes (Friedrich et al. 2007). These studies also revealed an important role for autophosphorylation of Ser12 in the ability of DRAK2 to affect T cell activation, indicating that, like other members of the DAPK family, DRAK2 autophosphorylation modulates DRAK2 activity and function.

As immature thymocytes transit through developmental stages in the thymus to become mature T cells, DRAK2 upregulation occurs and directly affects the degree of calcium mobilization elicited through antigen receptor stimulation. In the mature T cell compartment, where DRAK2 protein levels are highest, loss of DRAK2 leads to substantially enhanced calcium responses (McGargill et al. 2004). Reconstitution of DRAK2-deficient T cells with wild-type DRAK2 restored negative regulation of calcium mobilization, whereas expression of a Ser12Ala DRAK2 mutant was not sufficient to reestablish this negative regulation, indicating the importance of autophosphorylation on Ser12 for DRAK2 biological function (Friedrich et al. 2007). The generation of DRAK2-transgenic mice in which DRAK2 transgene levels are driven specifically within the immature T cell population also lends support to the role of DRAK2 in directly modulating calcium responses. Whereas immature thymocytes ectopically expressing DRAK2 exhibited dampened calcium responses to antigen receptor stimulation, restoration of normal DRAK2 levels within the peripheral T cell compartment in these mice resulted in normal calcium responses (Gatzka et al. 2009). Finally, knockdown of DRAK2 within the clonal T cell line D10 recapitulated the exacerbated calcium response phenotype seen in DRAK2-deficient T cells, arguing against a developmental defect upon loss of DRAK2, and for a role in DRAK2 signaling to regulate this aspect of T cell activation (Newton et al. 2011).

Autophosphorylation on Ser12 is itself elicited by calcium mobilization, implicating DRAK2 in a negative feedback loop whereby calcium influx is necessary for autocatalytic activity on Ser12 and is required for DRAK2 to limit calcium influx, and not ER calcium store release. This process has been shown to be dependent on protein kinase D (PKD), potentially through direct transphosphorylation of DRAK2 by ▶ PKD, suggested by in vitro kinase assays. Association of DRAK2 with PKD has been demonstrated in T cells in response to stimuli that activate PKD, and is enriched within mitochondria. Immunofluorescence images of endogenous DRAK2 in primary T cells have revealed DRAK2 punctae formation localized



DRAK2, Fig. 2 Comparison of DRAK2 and DAPK, and various DRAK2 mutants generated that have provided functional insight. The percentage within the kinase domain indicates the degree of amino acid identity to the kinase domain of DAPK. *NLS* nuclear localization signal

to staining of mitochondria in response to thapsigargin to provoke calcium mobilization, and autophosphorylation on Ser12 was induced directly through generation of mitochondrial reactive oxygen species. Together with data showing that association of PKD and DRAK2 is disrupted by molecules that scavenge reactive oxygen species, activation of DRAK2 by PKD is thought to be dependent on calcium-induced mitochondrial reactive oxygen generation in response to antigen receptor stimulation (Newton et al. 2011).

How DRAK2 down-modulates calcium influx is currently unknown, and few substrates have been identified. In vitro, DRAK2 targets myosin light chain (MLC), a result that was anticipated based on its high level of homology in its kinase domain with ▶ MLCK, and based on DAPK's ability to target MLC in vivo. DRAK2 has been shown to interact with calcineurin homologous protein (CHP) in a manner that negatively regulates its autocatalytic activity and its activity toward MLC (Matsumoto et al. 2001). Although this interaction of CHP was not shown to be dependent on calcium, the suppression of DRAK2 catalytic activity by CHP was. S6K1 has been shown to be targeted by DRAK2 on the same residue (Thr389) as targeted by ▶ mTOR, leaving a role for DRAK2 in S6K1 signaling in T cells to be determined (Mao et al. 2009).

An incredibly important tool for understanding not only what lies downstream of DRAK2, but for the selective targeting of T cell responses given the unique function of DRAK2 and its role in immune system, will be the development of specific inhibitors to target this kinase. The solved crystal structure with 2.8 Å resolution will undoubtedly aid in the discovery of novel inhibitors that offer the possibility to disrupt a pathway central to the exquisite control of T cell activation and tolerance yet dispensable for key immunological events that maintain resistance toward a multitude of pathological threats.

Role in Immune System

Since DRAK2 is highly expressed in lymphoid tissues, its role in the immune system has been extensively studied. DRAK2 expression is developmentally regulated during thymocyte maturation and its expression is increased following activation of the T cell receptor (TCR) of double positive thymocytes (Friedrich et al. 2005). DRAK2 is involved in setting the threshold for TCR signaling during thymocyte selection as evidenced by the increased calcium flux of DRAK2−/− thymocytes following the double positive stage (Friedrich et al. 2005). To study if DRAK2 plays a role in positive or negative selection, DRAK2-deficient mice have been bred with various TCR transgenic mice (McGargill et al. 2004). DRAK2-deficient mice crossed to OT-II and AND mice have slight increases in CD4 single positive T cells and slight decreases in double positive T cells. Interestingly, there was no effect on CD8 single positive distribution when DRAK2-deficient mice were crossed to OT-I or P14 mice. Although DRAK2-deficiency led to enhanced positive selection, DRAK2 does not seem to play a role in negative selection as DRAK2 deficiency did not affect the loss of self-reactive T cells under the AND or H-Y TCR transgenic backgrounds. In addition, double positive DRAK2−/− T cells from OT-I and OT-II backgrounds had slight increases in activation markers. Therefore, DRAK2 is necessary for proper TCR activation during thymocyte selection.

In peripheral tissues, CD4+ and CD8+ T cells express similar levels of DRAK2 protein. Not surprisingly, DRAK2−/− peripheral T cells also have an increased calcium flux following TCR stimulation (McGargill et al. 2004; Friedrich et al. 2005). In addition, DRAK2−/− T cells have been observed to hyperproliferate to suboptimal stimulation and to a greater rate with weak agonist. The increased calcium flux and hypersensitivity of DRAK2−/− T cells is characteristic of T cells deficient in a negative regulator. In accord with the increase in proliferation, DRAK2−/− T cells produce higher amounts of IL-2. In addition, activated DRAK2−/− T cells produce higher amounts of IFN-γ and IL-4. Suboptimal stimulation results in higher surface expression levels of the costimulatory markers CD25, IL-7R, ICOS, CD27, OX40, and 41BB in DRAK2−/− T cells compared to wild-type T cells. Interestingly, the addition of exogenous anti-CD28 restores the levels of costimulatory markers and the observed hyper-proliferation of activated DRAK2−/− T cells back to wild-type levels (McGargill et al. 2004; Ramos et al. 2008). These studies support the hypothesis that DRAK2 is a negative regulator of T cell activation.

B cells express the highest level of DRAK2 protein and similar to T cells, DRAK2 expression increases following B cell maturation (McGargill et al. 2004; Friedrich et al. 2005). Following immunization of mice with a T-dependent antigen, the loss of DRAK2 in B cells results in up to a fivefold decrease in germinal centers and, consequently, a decrease in high affinity antibodies (Al-Qahtani et al. 2008). DRAK2−/− B cells proliferate similar to wild-type B cells and have no defects in somatic hypermutation and class switch DNA recombination. Further analysis indicates that the defects in DRAK2−/− B cells is a direct consequence of a loss of DRAK2 in T cells since a T-dependent antigen was used to cause the germinal center reaction. To study any B cell intrinsic defects due to the loss of DRAK2, T-independent antigen immunizations should be conducted.

Deletion of negative regulators of T cell activation often leads to increased sensitivity to autoimmune diseases (Pentcheva-Hoang et al. 2009). Based on studies on T cell negative regulators, it was predicted that DRAK2-deficient mice would also be more vulnerable to autoimmune disease. To the contrary, DRAK2-deficient mice were less susceptible to autoimmunity than wild-type mice. Aged DRAK2-deficient mice were not predisposed to spontaneous autoimmunity since there were no differences in the levels of cellular infiltrates in major organs and autoantibodies compared to wild-type (McGargill et al. 2004).

DRAK2-deficient mice were also resistant to MOG-induced experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (McGargill et al. 2004, 2008; Ramos et al. 2008, 2007). Overexpression of DRAK2 in mice via the LCK promoter results in spontaneous autoimmunity and increased susceptibility to EAE (Gatzka et al. 2009). However, this is most likely due to ectopically expressing DRAK2 in double positive thymocytes, which may result in altered thymic selection. In addition, DRAK2−/− mice were resistant to type 1-diabetes when bred to the NOD strain of mice that spontaneously develop autoimmune diabetes (McGargill et al. 2008). The resistance is not due to developmental defects in Th17 or antigen-specific effector T cells (Ramos et al. 2008; McGargill et al. 2008). DRAK2−/− mice were susceptible to collagen-induced arthritis and systemic lupus erythematosus, both of which are mediated by autoantibodies (McGargill et al. 2008). DRAK2−/− mice were also susceptible to autoimmune diseases that depend on mast cells and neutrophils (McGargill et al. 2008). Hence, DRAK2−/− mice remain susceptible to autoimmune diseases caused by autoantibodies or cells of the innate immune system but are resistant to autoimmune diseases where pathogenesis is primarily mediated by T cells.

The response to virus has also been studied in DRAK2-deficient mice. DRAK2-deficient mice had antiviral responses to Lymphocytic Choriomeningitis Virus (LCMV) (McGargill et al. 2004) and Murine Hepatitis Virus (MHV) (Ramos et al. 2007) that were indistinguishable from wild-type mice. Interestingly, DRAK2−/− mice were also capable of efficiently eliminating West Nile Virus, but did not succumb to the lethal encephalomyelitis, suggesting that while DRAK2 is not required for antiviral responses, it does promote entry of antiviral T cells into the brain. As stated above, activated DRAK2−/− T cells respond similar to activated wild-type T cells following addition of exogenous costimulation. During a viral infection the amount of costimulation is probably maximal and this may explain why DRAK2-deficient mice mount a normal immune response to viral infection. In addition, studies with MHV showed that DRAK2-deficient mice have enhanced memory T cell function on a per cell basis (Schaumburg et al. 2007). Hence, the absence of DRAK2 does not result in generalized suppression of the immune system and blockade of DRAK2 may be useful in treating T cell–dependent autoimmune diseases and enhance antiviral responses. The role DRAK2 plays in T cell survival likely explains the mechanism behind the restoration of DRAK2−/− T cells to a wild-type phenotype with the addition of costimulation (Ramos et al. 2008).

Summary

While the catalytic targets of DRAK2 (and DRAK1) remain to be fully clarified, this serine/threonine kinase offers a unique opportunity to control autoimmunity and potentially cancer. The elucidation of the targets of the kinase, as well as the structural features of the kinase, will be of great value for understanding how DRAK2 controls cellular physiology. While DRAK2 and DRAK1 clearly share significant homology with other members of the DAPK family, ongoing studies should help to determine the functional roles these serine/threonine kinases serve in distinct organ systems. Given the significant autoimmune resistance, but overtly normal antiviral immunity that DRAK2-deficient mice possess, it is likely that small molecule antagonists of the DRAK kinases will be valuable weapons in the arsenal to control autoimmune and other autoinflammatory diseases.

Acknowledgments This work was supported by the National Institutes of Health (AI63419); the Arthritis National Research Foundation; the National Multiple Sclerosis Society; and the Juvenile Diabetes Research Foundation. R.H.N. was supported by National Institutes of Health Immunology Research Training Grant T32 AI-060573.

References

Al-Qahtani A, Xu Z, Zan H, Walsh CM, Casali P. A role for DRAK2 in the germinal center reaction and the antibody response. Autoimmunity. 2008;41:341–52.

Bialik S, Kimchi A. The death-associated protein kinases: structure, function, and beyond. Annu Rev Biochem. 2006;75:189–210.

Doherty GA, Byrne SM, Austin SC, Scully GM, Sadlier DM, Neilan TG, Kay EW, Murray FE, Fitzgerald DJ. Regulation of the apoptosis-inducing kinase DRAK2 by cyclooxygenase-2 in colorectal cancer. Br J Cancer. 2009;101:483–91.

Friedrich ML, Wen BG, Bain G, Kee BL, Katayama C, Murre C, Hedrick SM, Walsh CM. DRAK2, a lymphoid-enriched DAP

kinase, regulates the TCR activation threshold during thymocyte selection. Int Immunol. 2005;17:1379–90.
Friedrich ML, Cui M, Hernandez JB, Weist BM, Andersen HM, Zhang X, Huang L, Walsh CM. Modulation of DRAK2 autophosphorylation by antigen receptor signaling in primary lymphocytes. J Biol Chem. 2007;282:4573–84.
Gatzka M, Newton RH, Walsh CM. Negative regulation of TCR signaling in immunological tolerance: taming good and evil. Curr Immunol Rev. J Immunol. 2009;183(1):285–97.
Gatzka M, Newton RH, Walsh CM. Altered thymic selection and increased autoimmunity caused by ectopic expression of DRAK2 during T cell development. J Immunol. 2009;183:285–97.
Kuwahara H, Nakamura N, Kanazawa H. Nuclear localization of the serine/threonine kinase DRAK2 is involved in UV-induced apoptosis. Biol Pharm Bull. 2006;29:225–33.
Kuwahara H, Nishizaki M, Kanazawa H. Nuclear localization signal and phosphorylation of Serine350 specify intracellular localization of DRAK2. J Biochem. 2008;143:349–58.
Mao J, Qiao X, Luo H, Wu J. Transgenic drak2 overexpression in mice leads to increased T cell apoptosis and compromised memory T cell development. J Biol Chem. 2006;281:12587–95.
Mao J, Luo H, Han B, Bertrand R, Wu J. Drak2 is upstream of p70S6 kinase: its implication in cytokine-induced islet apoptosis, diabetes, and islet transplantation. J Immunol. 2009;182:4762–70.
Matsumoto M, Miyake Y, Nagita M, Inoue H, Shitakubo D, Takemoto K, Ohtsuka C, Murakami H, Nakamura N, Kanazawa H. A serine/threonine kinase which causes apoptosis-like cell death interacts with a calcineurin B-like protein capable of binding Na(+)/H(+) exchanger. J Biochem. 2001;130:217–25.
McGargill MA, Wen BG, Walsh CM, Hedrick SM. A deficiency in Drak2 results in a T cell hypersensitivity and an unexpected resistance to autoimmunity. Immunity. 2004;21:781–91.
McGargill MA, Choy C, Wen BG, Hedrick SM. Drak2 regulates the survival of activated T cells and is required for organ-specific autoimmune disease. J Immunol. 2008;181:7593–605.
Newton RH, Leverrier S, Srikanth S, Gwack Y, Cahalan MD, Walsh CM. Protein kinase D orchestrates the activation of DRAK2 in response to TCR-induced Ca2+ influx and mitochondrial reactive oxygen generation. J Immunol. 2011;186(2):940–50.
Pentcheva-Hoang T, Corse E, Allison JP. Negative regulators of T-cell activation: potential targets for therapeutic intervention in cancer, autoimmune disease, and persistent infections. Immunol Rev. 2009;229:67–87.
Ramos SJ, Hardison JL, Stiles LN, Lane TE, Walsh CM. Anti-viral effector T cell responses and trafficking are not dependent upon DRAK2 signaling following viral infection of the central nervous system. Autoimmunity. 2007;40:54–65.
Ramos SJ, Hernandez JB, Gatzka M, Walsh CM. Enhanced T cell apoptosis within Drak2-deficient mice promotes resistance to autoimmunity. J Immunol. 2008;181:7606–16.
Sanjo H, Kawai T, Akira S. DRAKs, novel serine/threonine kinases related to death-associated protein kinase that trigger apoptosis. J Biol Chem. 1998;273:29066–71.
Schaumburg CS, Gatzka M, Walsh CM, Lane TE. DRAK2 regulates memory T cell responses following murine coronavirus infection. Autoimmunity. 2007;40:483–8.
Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, Hodge CL, Haase J, Janes J, Huss 3rd JW, Su AI. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. Genome Biol. 2009;10:R130.

Drf1/ASKL1 (a Second Activator of Cdc7 in Human and *Xenopus*)

▶ Dbf4

DT1P1B11

▶ PHLDA1 (Pleckstrin Homology-like Domain, Family A, Member): Alias: PHRIP; TDAG51; DT1P1B11; MGC131738

DTEF-1

▶ Tead

DTR

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

DTS

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

DTSF

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

Dual Leucine Zipper Bearing Kinase

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

Dual Leucine Zipper-Bearing Kinase

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

DUSP24

▶ MK-STYX

DYNLT1/Tctex-1 (AGS2)

▶ Activators of G-Protein Signaling (AGS)

E

E3 Ubiquitin-Protein Ligase, Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6)

► TRAF6

EBP50 (Ezrin-Radixin-Moesin-Binding Phosphoprotein 50 kDa)

► NHERF

EC 3.4.22.52

► Calpain

EC 3.4.22.53

► Calpain

Ect2 (Epithelial Cell Transforming 2 Oncogene)

Toru Miki
Laboratory of Cellular Signaling, Nagaoka University of Technology, Niigata, Japan

Synonyms

Pebble (PBL) in *Drosophila melanogaster*

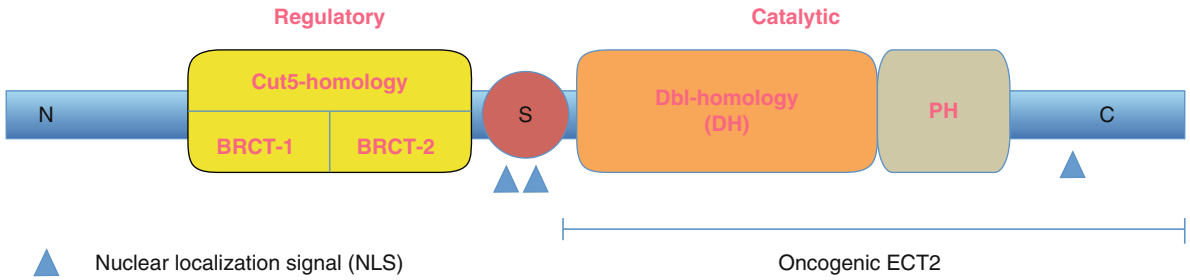
Historical Background

Ect2 is a guanine nucleotide exchange factor (GEF) of the Rho GTPases (Tatsumoto et al. 1999). It was isolated as a cDNA clone with capability of converting mouse fibroblasts to malignantly transformed cells (Miki et al. 1991, 1993). Transforming Ect2 variants lacked the N-terminal regulatory domain, and efficiently induced focus formation, anchorage-independent cell growth, and cell invasiveness in mouse fibroblasts. Injection of athymic nude mice with Ect2 transformants resulted in tumor formation. While full-length Ect2 is localized only in the nucleus, its oncogenic variants are detected in the cytoplasm as well. In M phase, Ect2 distributes in the entire cells after nuclear membrane breakdown, and is concentrated on mitotic spindles and then central spindles. In the last stage of M phase, Ect2 translocates to the midbody, which is formed between two emerging daughter cells. Perturbation of Ect2 function in M phase resulted in the inhibition of cytokinesis (cellular division) without major effects on mitosis (Tatsumoto et al. 1999). These findings established that Ect2 is a critical regulator of cytokinesis. Subsequent studies on Ect2 as well as the Rho negative regulator MgcRacGAP clarified the molecular mechanisms of cytokinesis (Yuce et al. 2005; Kamijo et al. 2006); chromosome separation initiates the formation of central spindles where MgcRacGAP and MKLP1 (mitosis-specific motor protein) form a tight complex. Recruitment of Ect2 to this complex activates Rho at the cell equator leading to cytokinesis.

Structural Properties

The full-length Ect2 contains several structural motifs (Fig. 1). The C-terminal half of the molecule contains the

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



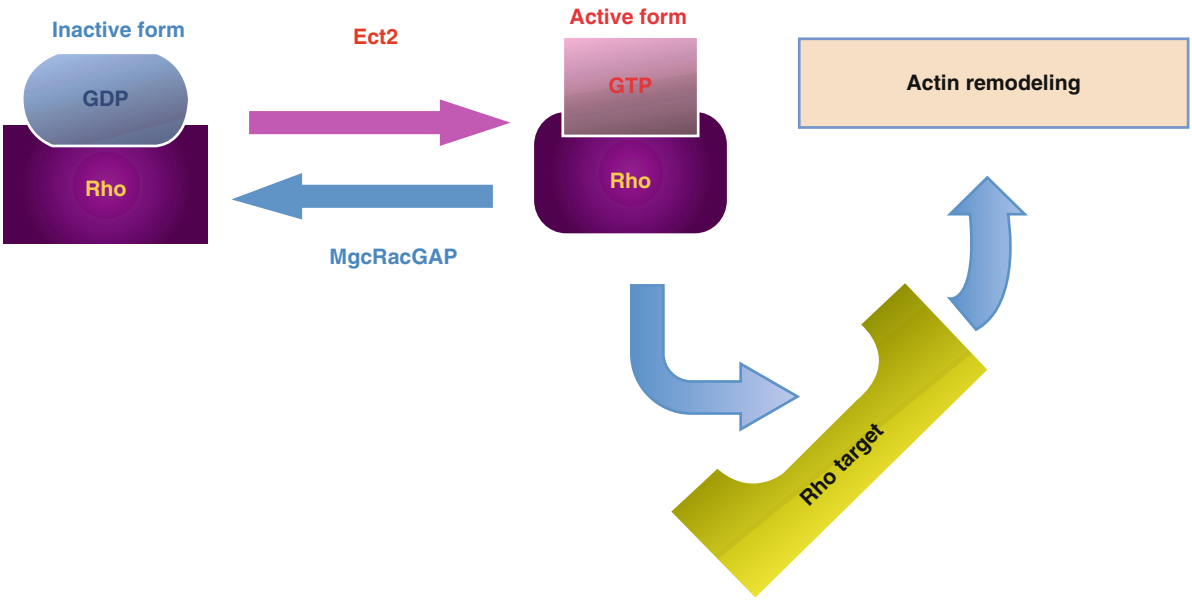
Ect2 (Epithelial Cell Transforming 2 Oncogene), Fig. 1 Schematic view of Ect2 protein: Full-length Ect2 molecule constitutes of the N-terminal regulatory and C-terminal catalytic domains as well as the central S domain. The major motifs are shown. *Triangles* denote nuclear localization signals. The region carried by oncogenic Ect2 is shown by a *line*

Dbl (diffuse B-lymphoma oncogene)-homology (DH) and Pleckstrin-homology (PH) domains, which are motifs of the guanine nucleotide factor (GEF) of the Rho GTPases. Most of Rho GEFs contain each of the domains, and usually the DH domain is located at the N-terminus of the PH domain. While the Rho GEF motif is the combination of the DH and PH domains, there are a number of proteins containing the PH domain alone, most of which function as a phosphatidylinositol binding site as well as a membrane anchoring site. The very C-terminal region of Ect2 is a serine- and threonine-rich domain and functions as a regulator of cell transformation. Transforming Ect2, which was identified as cDNA clone capable of transforming NIH 3T3 fibroblasts, lacks the N-terminal half and thus contains only the C-terminal half of the full-length Ect2 (Miki et al. 1993).

The N-terminal half of Ect2 contains the BRCA1 terminal repeat (BRCT) domains (Tatsumoto et al. 1999). Identification of the fission yeast *cut5* gene revealed a new homology region between Cut5 and the N-terminal half of Ect2. This region consists of two BRCT domains. Cut5 contains four repeats, whereas Ect2 has two. Fission yeast *cut5* mutants show a very peculiar phenotype: no coordination between mitosis and cytokinesis, which results in the induction of cytokinesis during mitosis. The BRCT domains of Ect2 have at least two functions. First, they associate with the catalytic domain of Ect2, which renders the molecule inactive. Both the BRCT domains are considered essential to this function, as the expression of both the repeats, but not the either single repeat blocks cytokinesis as a dominant-negative mutant of Ect2. The central S domain may function as a joint of the N- and C-terminal halves, and phosphorylation of the S domain is known to affect the configuration (Hara et al. 2006). Since the BRCT sequence is known to function as a phosphospecific protein-binding motif, the Ect2 BRCT domains may function after the C-terminal domain is phosphorylated. A second function of the Ect2 BRCT domains is to interact with the Rho regulator MgcRacGAP (Yuce et al. 2005). Presumably, the conversion of the closed Ect2 structure to the open form stimulates the binding to MgcRacGAP. Phosphorylation of MgcRacGAP by Plk1 also stimulates Ect2-MgcRacGAP association (Wolfe et al. 2009).

Biochemical Functions

Ect2 functions as an activator of the Rho family of small GTPases, which are represented by RhoA, Rac1, and Cdc42 (Miki et al. 2002). Rho GTPases function as molecular switches in cells and alternate between two states: an active GTP-bound state and an inactive GDP-bound state (Fig. 2). Ect2 converts inactive Rho to active Rho by replacing bound GDP by GTP. The GTP-bound form of Rho is specifically recognized by target/effector proteins to transmit the signals further downstream. As the biological functions of RhoA, Rac1, and Cdc42 are regulation of the formation of actin stress fibers, membrane ruffling, and filopodia, respectively, most of the Rho-mediated signals involve actin remodeling. Among the known Rho targets, Rho kinase (ROCK), Citron kinase, and mDia are involved in Ect2-mediated signaling. Inactivation of a Rho GTPase is carried out through the enhancement of its intrinsic GTPase activity by a cognate GTPase activating protein (GAP). A Rho GAP, designated MgcRacGAP, is known to function together with Ect2 to regulate cytokinesis and chromosome separation (Minoshima et al. 2003).



Ect2 (Epithelial Cell Transforming 2 Oncogene), Fig. 2 Biochemical function of Ect2. Ect2 replaces bound GDP by GTP on Rho GTPases, and therefore activates Rho to specifically associate with Rho targets, which in turn transmits the signal further downstream to regulate actin remodeling

Biological Functions

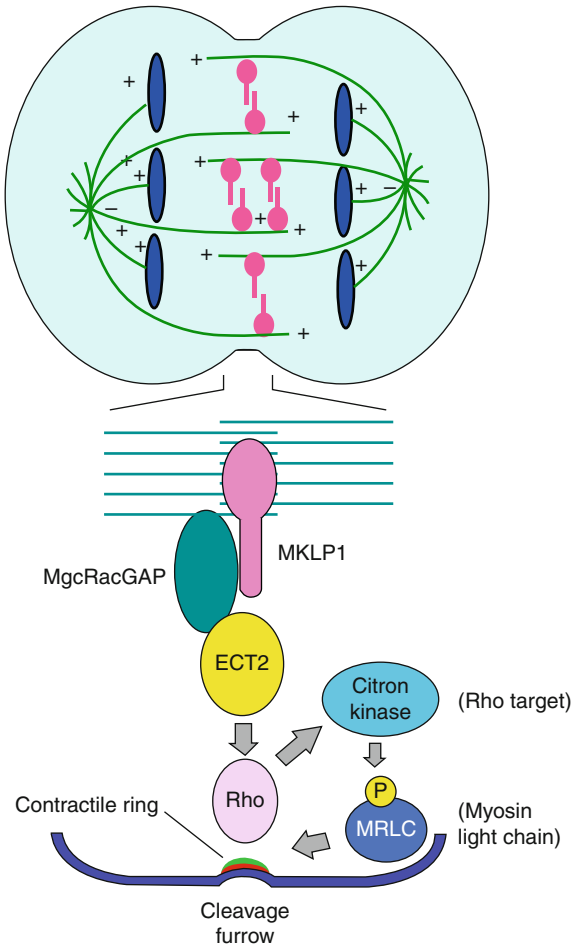
Inhibition of Ect2 function in cultured cells strongly inhibits cytokinesis without visible effects on mitosis, generating binucleate cells. Therefore, Ect2 is a critical regulator of cytokinesis (Miki 2005). In *Drosophila*, a known gene (*pebble*), whose mutation results in cytokinesis defects, was shown to encode the fly ortholog of the human *ECT2* (Prokopenko et al. 2000). Ect2 is localized in the nucleus in interphase. Upon nuclear membrane breakdown in M phase, Ect2 is found in the entire cells and then concentrated at the central spindle (Fig. 3). The Rho negative regulator MgcRacGAP and the plus-end directed motor protein MKLP1 form a stable complex and associate with the central spindle. As described below, a set of complex phosphorylation/dephosphorylation events on both Ect2 and MgcRacGAP appear to induce the association of Ect2 to MgcRacGAP on the central spindle (Yuce et al. 2005; Hara et al. 2006). The Ect2 protein that has been recruited to the central spindle then activates Rho GTPases by guanine nucleotide exchange, and activated Rho translocates to the cell equator, where it activates the Rho target Citron kinase. Citron kinase then phosphorylates the regulatory subunit of myosin heavy chain kinase. These biochemical events induce contractile ring formation and contraction (Kamijo et al. 2006).

Although Ect2 knockdown mainly inhibits cytokinesis, it also impairs the attachment of mitotic spindles to the kinetochores, leading to a delay of prometaphase and abnormal chromosomal separation (Oceguera-Yanez et al. 2005). MgcRacGAP is involved in this function as well. While RhoA is a critical molecular switch in cytokinesis regulation, Cdc42 mainly functions in chromosome alignment in this mitotic role of Ect2.

Ect2 might play a role in epithelial cell polarity. The polarity regulator Par6 was identified as an Ect2-interacting protein (Liu et al. 2004). Ect2 also associates with the other polarity complex components, consisting of Par3, atypical protein kinase (PKCiota or PKCzeta), and Cdc42 (see Fig. 5). Coexpression of Ect2 and Par6 efficiently activates Cdc42, and overexpression of Ect2 activates aPKC activity. In epithelial cells, Ect2 localization was detected at the cell–cell junction as well as the nucleus. Thus, Ect2 appears to involve epithelial cell polarity, although the other Rho GEF Tiam-1 plays a major role.

Regulation of Ect2 by Phosphorylation and Protein Associations

The central domain of Ect2, designated the S domain, functions as a joint of the catalytic C-terminal and the regulatory N-terminal halves. It contains several phosphorylation sites, which may function as switches for



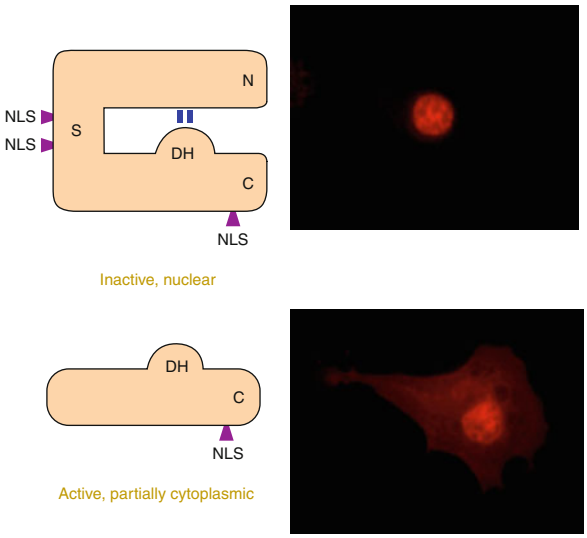
Ect2 (Epithelial Cell Transforming 2 Oncogene), Fig. 3 Biological function of Ect2: At the metaphase–anaphase transition, Ect2 associates with MgcRacGAP, which forms a tight complex with MKLP1 at the central spindle. Subsequent activation of Rho, Citron kinase, and MRLC (myosin regulatory light chain) causes the contractile ring formation, leading to cytokinesis

configuration changes (Miki et al. 2009). Phosphorylation at T341 of Ect2 by Cdk1 in M phase affects configuration of the molecule (open and closed forms) and may control the accessibility of Ect2-binding proteins (Hara et al. 2006). Phosphorylation of T412 by Cdk1 also induces binding of another M phase kinase Plk1, leading to phosphorylation of Ect2 by Plk1 (Niiya et al. 2006). It is known that this phosphorylation is required for the exchange activity of Ect2. Plk1 appears to have multiple functions in M phase. Its N-terminal half contains the regulatory Polo-box domain (PBD), which can associate with phosphorylated peptides. Cdk1 functions as a "priming kinase" in this case: Cdk1 phosphorylation stimulates Plk1 binding through the PBD domain, and the kinase domain of Plk1 subsequently phosphorylates the substrate that the Plk1 has bound. Through this mechanism, Plk1 is recruited to various cellular compartments during M phase. Plk1 phosphorylation of Ect2 and MgcRacGAP is critical for cytokinesis regulation. The N-terminal domain of Ect2 mainly consists of two BRCT repeats, which are capable of binding to phosphorylated proteins. This domain functions to localize Ect2 to the central spindle. On the other hand, Ect2 is in a closed conformation in its inactive state through the interaction of the N- and C-terminal halves. The phosphorylation status of T431 regulates the configuration. Inhibition of Plk1 by a chemical inhibitor prevents Ect2 association to the central spindle as well as binding to MgcRacGAP (Wolfe et al. 2009). Thus, Plk1 phosphorylates MgcRacGAP, which may help Ect2 to associate with MgcRacGAP. On the other hand, Ect2 is also phosphorylated by Plk1, which is required for its activity. Complex phosphorylation events might regulate cytokinesis through these protein kinases.

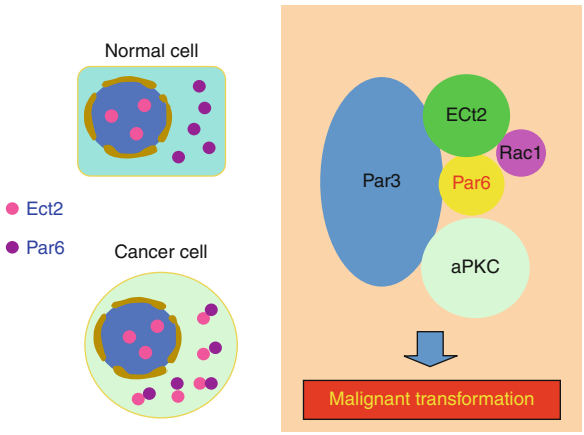
Involvement of Tumor Formation

Although full-length Ect2 is a nuclear protein, its transforming variants display various degrees of cytoplasmic localization (Fig. 4). Subsequent analysis revealed that main nuclear localization signals located at the S domain are lost in these transforming variants (Saito et al. 2004; Justilien and Fields 2009). Whereas the possible nuclear functions of Ect2 should be clarified, Ect2 might be sequestered in the nucleus not to activate Rho GTPases in interphase. Transforming variants of Ect2 that are partially located in the cytoplasm can activate Rho GTPases leading to malignant transformation. Detailed analyses indicate that deletion of the main nuclear localization signals induces only a fraction of transforming activity of Ect2. Other factors, including phosphorylation status and configuration changes, are also important for the transforming activity of Ect2. Nonetheless, nuclear localization of Ect2 is a critical factor of Ect2 transforming activity.

Despite the cytoplasmic mislocalization of transforming variants of Ect2, deletion or rearrangement of the *ECT2* gene in human cancers has not been reported. Recent analyses of huge number of human cancers using microarray analyses revealed that Ect2 is a prognostic marker of several cancers



Ect2 (Epithelial Cell Transforming 2 Oncogene), Fig. 4 Cytoplasmic mislocalization of Ect2 induces its transforming activity: (*upper*) Inactive Ect2 forms a closed structure and it is localized at the nucleus (*red*). (*Lower*) Oncogenic Ect2 (see Fig. 1) displays cytoplasmic localization although it also localizes to the nucleus. NLS, nuclear localization signal



Ect2 (Epithelial Cell Transforming 2 Oncogene), Fig. 5 Association of Ect2 with the polarity complex activates Rac1, leading to malignant transformation. In normal cells, Ect2 is localized to the nucleus, whereas it associates with Par6 in the cytoplasm of some cancer cells

including breast cancer. It is also reported that Ect2 expression inversely correlates to prognosis of glioblastomas (Sano et al. 2006). As described above, Ect2 associates with the tertiary complex consisting of the polarity determinant protein Par6, an atypical protein kinase C, and a scaffold protein Par3 (Liu et al. 2004, 2006) (Fig. 5). The gene for an aPKC, PKCiota, is located at the proximity of the *ECT2* gene in human chromosome 3. Both the loci are co-amplified in several cancers including non-small cell lung carcinomas (NSLCs). Interestingly, Ect2 is mislocalized to the cytoplasm in these cancer cell lines, reminiscent of the phenotype of transforming Ect2. When either Par6 or PKCiota is knocked down in these cells, however, Ect2 localization returns to the nucleus. Thus, upregulation of the polarity complex appears to retain Ect2 in the cytoplasm in these cancer cell lines. It is well established that the Rho family GTPases, Cdc42 and Rac1, are involved in cell polarity. Ect2 knockdown in these cells inhibits tumorigenicity and invasiveness, indicating that Ect2 activates the Rac1 pathway through the polarity complex in these cells leading to malignant transformation.

Space limitations preclude comprehensive referencing. See also references therein.

Summary

Ect2 was originally isolated as a cDNA clone with an ability to morphologically transform mouse fibroblasts. The catalytic domain (C-terminal half) catalyzes guanine nucleotide exchange on Rho GTPases, whereas the regulatory domain (N-terminal half) contains two BRCT domains, which regulate the catalytic activity and also function to interact with other signaling molecules such as MgcRacGAP. Ect2 is a critical regulator of cytokinesis. Ect2 also interacts with the polarity determinants containing Par3, Par6, aPKC, and Rac1. In some cancer cells, amplification of a chromosomal region containing the genes for Ect2 and aPKC results in overproduction of these proteins. Cytoplasmic localization of Ect2/Par complex appears to cause malignant transformation of the cells.

References

Hara T, Abe M, Inoue H, Yu LR, Veenstra TD, Kang YH, et al. Cytokinesis regulator ECT2 changes its conformation through phosphorylation at Thr-341 in G2/M phase. Oncogene. 2006;25(4):566–78.

Justilien V, Fields AP. Ect2 links the PKCiota-Par6alpha complex to Rac1 activation and cellular transformation. Oncogene. 2009;28(41):3597–607.

Kamijo K, Ohara N, Abe M, Uchimura T, Hosoya H, Lee JS, et al. Dissecting the role of Rho-mediated signaling in contractile ring formation. Mol Biol Cell. 2006;17(1):43–55.

Liu XF, Ishida H, Raziuddin R, Miki T. Nucleotide exchange factor ECT2 interacts with the polarity protein complex Par6/Par3/protein kinase Czeta (PKCzeta) and regulates PKCzeta activity. Mol Cell Biol. 2004;24(15): 6665–75.
Liu XF, Ohno S, Miki T. Nucleotide exchange factor ECT2 regulates epithelial cell polarity. Cell Signal. 2006;18(10): 1604–15.
Miki T. Involvement of Rho GTPases in cell division. In: Miki T, editor. Signal transduction of cell division. Kerala: Research Signpost; 2005. p. 263–84.
Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science. 1991; 251(4989):72–5.
Miki T, Smith C, Long J, Eva A, Fleming T. Oncogene *ect*2 is related to regulators of small GTP-binding proteins. Nature. 1993;362(6419):462–5.
Miki T, Hara T, Kamijo K, Mahoney T, Lee J-S. Rho GTPases and cell division. In: Pandalai S, editor. Recent research developments in biological chemistry. Kerala: Research Signpost; 2002. p. 255–72.
Miki T, Kim H, Senju Y.: Ect2. http://www.signaling-gateway.org/molecule/query?afcsid=A000812 (2009), ID: A000812
Minoshima Y, Kawashima T, Hirose K, Tonozuka Y, Kawajiri A, Bao YC, et al. Phosphorylation by Aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. Dev Cell. 2003;4(4):549–60.
Niiya F, Tatsumoto T, Lee KS, Miki T. Phosphorylation of the cytokinesis regulator ECT2 at G2/M phase stimulates association of the mitotic kinase Plk1 and accumulation of GTP-bound RhoA. Oncogene. 2006;25(6): 827–37.
Oceguera-Yanez F, Kimura K, Yasuda S, Higashida C, Kitamura T, Hiraoka Y, et al. Ect2 and MgcRacGAP regulate the activation and function of Cdc42 in mitosis. J Cell Biol. 2005;168(2): 221–32.
Prokopenko SN, Saint R, Bellen HJ. Untying the Gordian knot of cytokinesis. Role of small G proteins and their regulators. J Cell Biol. 2000;148(5):843–8.
Saito S, Liu XF, Kamijo K, Raziuddin R, Tatsumoto T, Okamoto I, et al. Deregulation and mislocalization of the cytokinesis regulator ECT2 activate the Rho signaling pathways leading to malignant transformation. J Biol Chem. 2004; 279(8):7169–79.
Sano M, Genkai N, Yajima N, Tsuchiya N, Homma J, Tanaka R, et al. Expression level of ECT2 proto-oncogene correlates with prognosis in glioma patients. Oncol Rep. 2006;16(5): 1093–8.
Tatsumoto T, Xie X, Blumenthal R, Okamoto I, Miki T. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. J Cell Biol. 1999;147(5):921–7.
Wolfe BA, Takaki T, Petronczki M, Glotzer M. Polo-like kinase 1 directs assembly of the HsCyk-4 RhoGAP/Ect2 RhoGEF complex to initiate cleavage furrow formation. PLoS Biol. 2009;7(5):e1000110.
Yuce O, Piekny A, Glotzer M. An ECT2-centralspindlin complex regulates the localization and function of RhoA. J Cell Biol. 2005;170(4):571–82.

Ecto-ADPase

▶ E-NTPDase Family

Ecto-apyrase

▶ E-NTPDase Family

Ecto-ATPase

▶ E-NTPDase Family

EDNRA

▶ Endothelin A Receptor (ETAR)

EFEMP1

▶ Fibulins

EFEMP2

▶ Fibulins

EIF2A Protein Kinase 2

▶ PKR

EMT

▶ Glycogen Synthase Kinase-3

Endo180

▶ MRC2

Endocytic Receptor 180

▶ MRC2

Endoplasmin

▶ Grp94 (HSP90B1)

Endothelin A Receptor (ETAR)

Randa Hilal-Dandan and Laurence L. Brunton
Pharmacology, University of California San Diego, San Diego, CA, USA

Synonyms

A-type endothelin receptor; EDNRA; ETA; ETRA; ETR; Endothelin receptor eta; Endothelin receptor type A; Endothelin type A receptor; Endothelin-A receptor; ETA receptor; ETA type endothelin receptor; ETA-R; ETAR; ETR ETA; Type A endothelin receptor

Historical Background

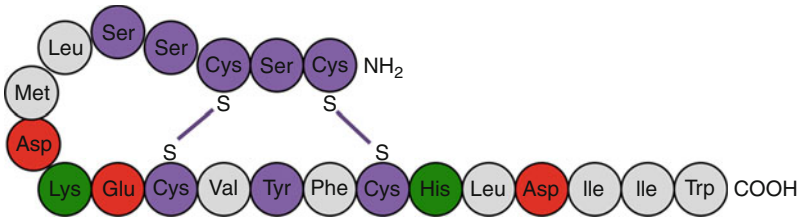
Endothelin-1 (ET-1) is a 21-amino-acid vasoactive peptide (see structure in Table 1) that was first isolated and identified in 1988 from porcine aortic endothelial cells. The endothelin family consists of three isoforms: ET-1, ET-2, and ET-3. ET-1 is the principal isoform and is a very potent vasoconstrictor and growth promoter. The physiological effects of the ET system are many and include regulation of vascular tone, renal function, and cell proliferation. ET exerts its effects through two types of endothelin receptors: type A (ETAR) and type B (ETBR). ETAR and ETBR are G-protein-coupled receptors (GPCRs) that share homology with the rhodopsin family. ETAR is pharmacologically distinguished from ETBR by ligand selectivity to the ET isoforms and cardiotoxic peptides isolated from snake venoms sarafotoxins 6b and 6c (S6b, S6c). ETAR binds ET-1 and ET-2 isoforms with much greater affinity than ET-3 (ET-1 $\geq$ ET-2 $>$ S6b $>$ ET-3), while ETBR binds ET-3 and the venoms S6b and S6c with the same affinity as ET-1 and ET-2 (ET-1 $=$ ET-2 $=$ ET-3). A third receptor subtype specific for ET-3, termed the endothelin C receptor (ETCR), has been cloned and characterized from *Xenopus laevis* but is absent in mammalian species (Barton and Yanagisawa 2008; Davenport 2002).

ETAR is expressed in many tissues and organs. It is the principal receptor subtype in the cardiovascular system mediating vasoconstriction, vascular cell proliferation, and the hypertrophic effects and positive inotropic effects of ET-1 in cardiac myocytes. In contrast, ETBR is the predominant receptor on endothelial cells and its activation opposes many of the effects mediated by ETAR stimulation; thus ETBR stimulation results in vasodilation (NO and prostacyclin release from endothelial cells), natriuresis, and clearance of ET-1 from the circulation. Endothelin binds irreversibly to ETAR and, depending on cell type, couples to multiple G proteins ($G_{q/11}$, G_i, $G_{12/13}$) activating a network of signaling pathways. Activation of ETAR is important in wound healing and is implicated in many of the pathophysiological effects of ET-1, including cardiovascular disease, pulmonary and systemic hypertension, atherosclerosis, fibrotic diseases, renal disease, diabetes, cancer, inflammation, pain, and hyperalgesia (Schneider et al. 2007; Barton and Yanagisawa 2008; Khimji and Rockey 2010; Bagnato et al. 2011).

Localization of the ETA Receptor

ETAR is the predominant subtype expressed in vascular smooth muscle cells and cardiac myocytes, but is absent in endothelial cells. ETAR is present in cells and tissues of the lungs, kidneys, liver, CNS, adrenal glands, eyes, and ovaries. Confocal microscopy and immunohistochemical analysis demonstrate that ETAR is largely localized on cell surface on plasma

Endothelin A Receptor (ETAR), Table 1 Agonists and antagonists acting at ETAR

Agonists	Antagonists
ET-1 ≥ ET-2 > S6b > ET-3	***Specific***
	*Peptide antagonists*
	BQ-123
	JKC-301
	FR139317
	Non-peptide antagonists
	PD164333
	Darusentan (LU-135252)
	Atrasentan (ABT-627)
	Clazosentan (RO 61–7790)
	Sitaxentan (TBC-11251)
	Edonentan (BMS-207940)
	Zibotentan (ZD4054)
	Less specific
	Bosentan (RO 47–0203)
	Ambrisentan (LU-208075)
	Enrasentan (SB-217242)
	Tezosentan (61–0612)

ET-1 is a 21-amino acid peptide composed of nonpolar(●), polar (●), negatively charged (●), and positively charged (●) amino acids, and stabilized by two disculfied bonds.

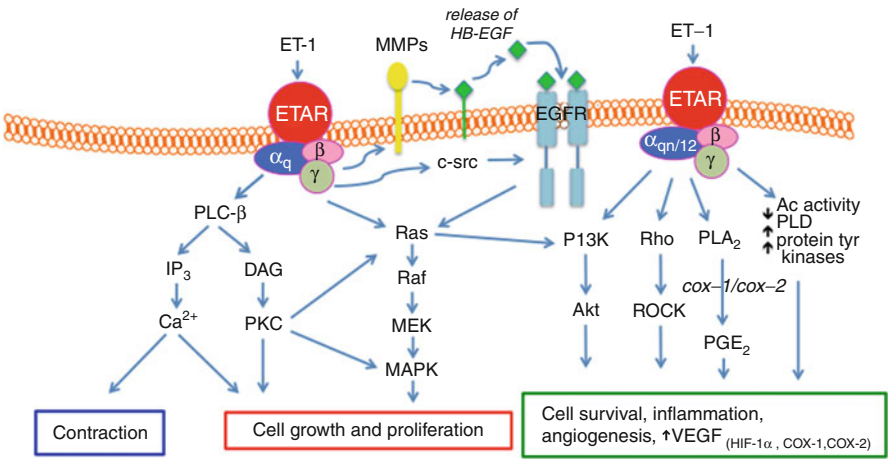
ET, endothelin; S6, sarafotoxin; ETAR, endothelin receptor type A

membranes, and the cytoplasmic tail of ETAR is important for proper localization. In cardiac myocytes, ETAR is present on the sarcolemma, transverse-tubules and, when internalized, is located in the nuclear envelope of ventricular myocytes (Kuc et al. 2006; Bkaily et al. 2011; Hilal-Dandan and Brunton 2010).

ETAR-Activated Signaling Pathways

ET-1 stimulation of ETAR may induce a variety of physiological responses, depending on the differentiated properties of the target cells. ETAR can couple to multiple G proteins, principally G_q, G_i, and likely G_{12} to modulate a large array of signaling pathways (Hilal-Dandan et al. 1997; Takigawa et al. 1995; Ivey et al. 2008). Takigawa and colleagues (1995) co-expressed ETAR with different G-protein α subunits in COS-7 cells and found that ETAR coupled to $G_{q/11}$, $G_{12/13}$, and G_s. There are a few reports of physiological coupling of ETAR activation to elevation of cyclic AMP, but this is likely not direct but mediated by paracrine mechanisms such as ET-stimulated eicosanoid production (Ivey et al. 2008).

Vasoconstrictor and growth-promoting effects of ET-1, mediated via G_q, result in the activation of phospholipase C-β and generation of inositol triphosphate (IP_3) and diacylglycerol. These molecules induce Ca^{++} release and activate protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and small G proteins (Ivey et al. 2008; Rosano et al. 2009; Khimji and Rockey 2010). In smooth muscle, IP_3 induces Ca^{++} release from the sarcoplasmic reticulum, triggering further elevation of intracellular Ca^{++} and contraction. Cell growth and proliferation are induced through coupling to G_q signaling; G_i and G_{12} signaling can also regulate activation of MAPKs and induction of immediate early genes (Fig. 1). For example, in cardiac myocytes, coupling of ETAR to G_q and pertussis-sensitive G_i pathways contribute to the hypertrophic response (Hilal-Dandan et al. 1997). ETAR mediates the activation of ▶ phospholipase D (PLD), phospholipase A_2 (PLA_2), and the release of arachidonic acid and production of prostaglandins. Depending on the cell type, ETAR may couple to G_i to inhibit ▶ adenylyl cyclase activity, resulting in a decrease in cyclic AMP production. Signaling through ETAR also induces activation of Rho, Ras, Rac, MAPK, JNK, p38 MAPK, PI-3-K-dependent Akt activation, and stimulation of several protein tyrosine kinases. The effects vary with the cell type and may contribute to inflammation, cell growth, survival and proliferation, cell invasion and migration, and inflammation (Ivey et al. 2008; Rosano et al. 2009).



Endothelin A Receptor (ETAR), Fig. 1 *ET-1 signaling via the ETAR*. ETAR couples principally to G_q and G_i, with coupling to G_{12} also possible. Cellular responses are complex due to multiplicity of G protein coupling in a single cell, cross talk amongst pathways, transactivation of the EGFR, and influence of paracrine signaling in response to activation of phospholipases and subsequent eicosanoid production

The mitogenic effects of ET-1, mediated through ETAR, may be amplified by cross talk and activation of EGFR. Stimulated ETAR can induce translocation of β-arrestin to the plasma membrane to form ETAR/β-arrestin complex. β-arrestin acts as a scaffold that recruits intracellular signaling molecules. The ETAR/β-arrestin complex interacts with c-►Src to stimulate transactivation of EGFR and tyrosine phosphorylation of β-catenin, and with axin (a negative regulator of the Wnt signaling pathway) to inactivate glycogen synthase kinase (GSK)-3β and stabilize ►β-catenin. The pathways that link ETAR with β-catenin induce Ras-dependent MAPK activation and are associated with cell migration and plaque formation in atherosclerosis, as well as the progression and invasiveness of ovarian cancer (Rosano et al. 2009).

Ligand Binding, Internalization, and Regulation of Activity

Human ETAR is located on chromosome 4, is 427 amino acids long, and shares ~60% sequence homology with ETBR. The intracellular third loop and carboxyl-terminal domains are required for binding and signaling. Based on chimeric experiments between human ETAR and ETBR, the transmembrane (TM) helices 4–6 seem to determine isoform selectivity, whereas portions of TM 1–3 and 7 plus adjacent extracellular loop regions bind the ETAR-selective antagonist BQ-123 (reviewed by Davenport 2002; Hilal-Dandan and Brunton 2010). The selectivity of ETAR binding to ET isoforms and the snake venoms S6b and S6c pharmacologically distinguishes ETAR from the nonselective ETBR. The order of potency for ETAR binding and activation is ET-1 ≥ ET-2 > S6b > ET-3; S6c is inactive (Hilal-Dandan et al. 1997). Numerous ETAR-selective and nonselective antagonists have been developed, including both peptide and non-peptide ligands (see Table 1).

The binding of ET-1 to ETAR is quasi-irreversible and the primary effects on G_q and G_i pathways are sustained and cannot by removed by washing cells exposed to ET-1 (Hilal-Dandan et al. 1997). Thus, attempts to reverse ET-1 action at ETAR using competitive inhibitors are not successful, whereas simultaneous addition of receptor antagonists and ET-1 is effective at reducing ET-1's subsequent effects. Following ligand binding, conformational changes induce receptor internalization and recycling. Ligand-bound ETAR remains intact for up to 2 h following internalization, and maintains G-protein-coupled signaling. Internalization of ligand-bound ETAR follows the β-arrestin–dynamin–caveolae and clathrin-coated pit pathways. The internalized receptors co-localize with transferrin and are recycled to the surface. Internalized ET-1/ETAR complexes can localize in perinuclear structures. The cytoplasmic C-terminal tail of ETAR determines its intracellular trafficking through a pericentriolar pathway for recycling. Truncation of the C-terminal tail causes ETAR to be directed toward lysosomal degradation (Paasche et al. 2005; Bkaily et al. 2011; see review Hilal-Dandan and Brunton 2010).

Modifications of ETAR include glycosylation, phosphorylation, and palmitoylation. Palmitoylation of the cysteine residues in the cytoplasmic tail modulates ETAR's activity in coupling to G_q signaling, ERK activation, and G_i signaling, but has no effect on ligand binding. Homologous desensitization of ETAR can be induced by GRK2- and GRK3-mediated phosphorylation in vascular smooth muscle cells and cardiac myocytes; the specific residues that are phosphorylated have not been identified (for review see Hilal-Dandan and Brunton 2010).

ETAR can form homodimers or heterodimers with ETBR but the functional significance is not clear. ETAR–ETBR heterodimers in certain cell types have been reported to modify ligand binding and transmembrane signaling, which may explain nontypical receptor behavior. Pharmacologically, selective antagonists to both ETAR and ETBR may be required to inhibit the function of heterodimers (Evans and Walker 2008). For example, there is an increased sensitivity to the contractile actions of ET-1 in rat and human ischemic cerebral arteries following subarachnoid hemorrhage (Edvinsson and Povlsen 2011). In these ischemic arteries, ETBR but not ETAR is upregulated. While the ETBR agonist S6c does not elicit any contractile responses in these arteries, antagonists to ETBR attenuate ET-1-induced contraction; these results suggest possible interaction or dimerization between ETAR and ETBR that enhances sensitivity to ET-1 (Edvinsson and Povlsen 2011). ETAR can form a complex with β-arrestin that interacts with c-Src to stimulate transactivation of EGFR (Rosano et al. 2009).

Genetic Variants

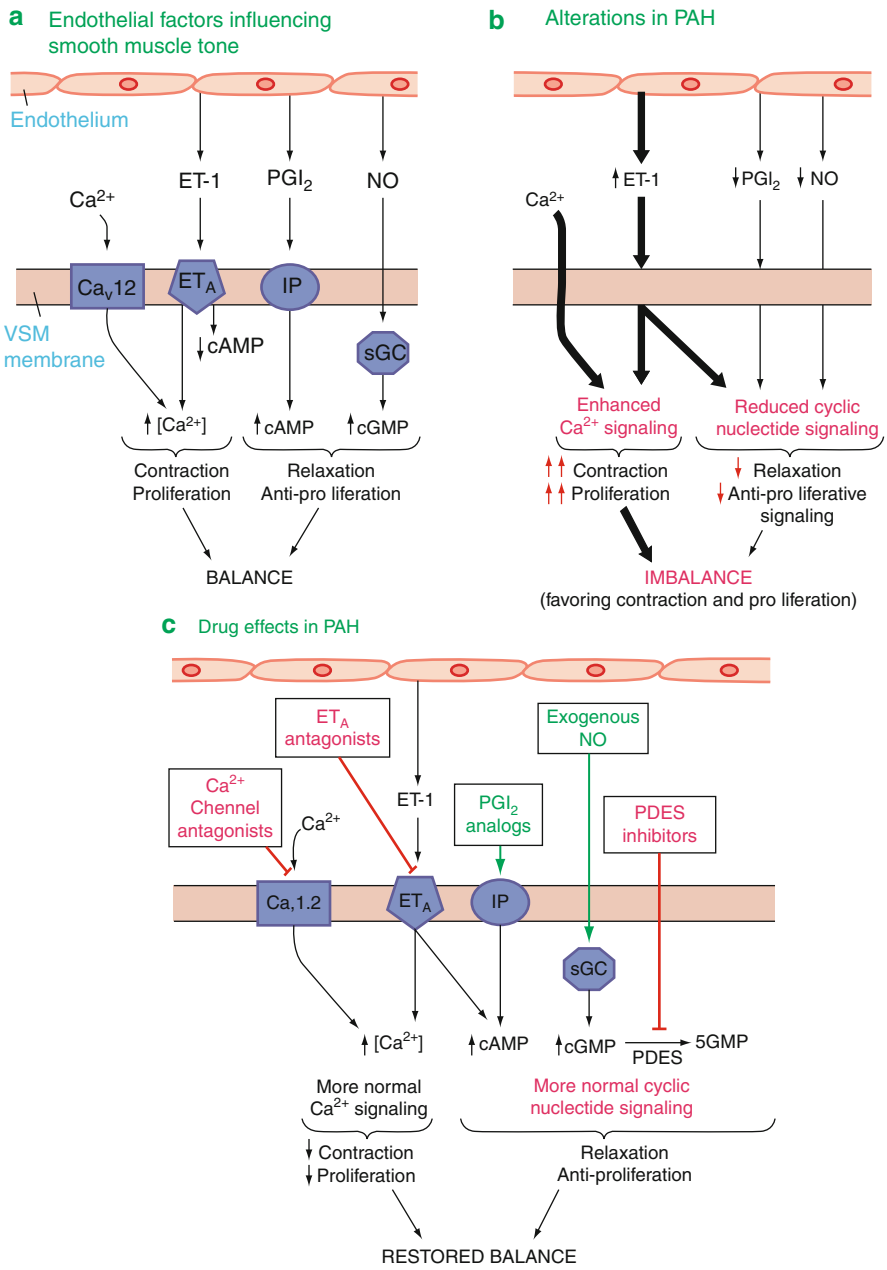
The human ETAR gene has eight exons and seven introns. Alternative mRNA transcripts of ETAR translate into truncated and nonfunctional receptors. Splice variants of ETAR isolated from human tissue include two alternative transcripts designated ETARΔ4 and ETARΔ3,4 (with deletions corresponding to exon 4 and exons 3 and 4, respectively) that result in nonfunctional receptors; a third mRNA transcript designated ETARΔ3 (deletion corresponding to exon 3), identified in human placental tissue, results in a truncated and nonfunctional ETAR. The function of these splice variants is not clear and may involve regulation of ETAR expression and function (reviewed by Davenport 2002; Hilal-Dandan and Brunton 2010).

Genetically engineered homozygous ETAR knockout mice are not viable and resemble ET-1-knockout mice. $ETAR^{-/-}$ mice have defects in cephalic neural crest cell derivatives. Abnormalities include craniofacial deformities, a poorly formed mandible, abnormal development of the middle ear structures, and tracheal narrowing. Cardiovascular defects include a defective aortic septum and defects in cardiac outflow tract with abnormalities in the aortic arch and great vessels. Signaling through ETAR is important for proper development of pharyngeal arches and aortic arch patterning. ET-1/ETAR signaling is responsible for a choice of morphogenetic program that determines maxillary and mandibular structures. The loss of ET-1/ETAR signaling affects homeotic genes, causing transformation of lower jaw into upper jaw structures, whereas constitutive activation of ETAR has the opposite effect, conversion of maxillary structures into mandibular structures (Sato et al. 2008).

ETAR in Disease

Signaling through ETAR is anti-apoptotic and induces cell growth, hypertrophy and proliferation, cell invasiveness and migration, fibrosis, and inflammation; thus, endothelin and the activation of ETAR are implicated in numerous pathological conditions.

Elevated tissue and plasma immunoreactive levels of ET-1 are associated with the severity and prognosis of congestive heart failure following myocardial infarction. In addition, upregulation of ETAR reportedly occurs in human and animal experimental models of heart failure (Schneider et al. 2007). In animal models, ETAR antagonists are beneficial in protecting against hypertensive heart failure and myocardial ischemia. Antagonism of ET_A receptor reduces cardiac hypertrophy induced by aortic stenosis in rats and improves survival in rat models with chronic heart failure by improving cardiac output and decreasing left ventricular hypertrophy. In humans, however, clinical studies have been inconclusive with regard to any long-term advantages of ETAR blockade in treating patients with hypertension and heart failure (Schneider et al. 2007). On the other hand, ET-1 levels and ETAR are upregulated in experimental and human pulmonary



Endothelin A Receptor (ETAR), Fig. 2 *Rationale for use of ET-1 antagonists in treating PAH*. (**a**) In healthy pulmonary artery, there is an appropriate balance between contractile/proliferative and relaxant/anti-proliferative influences. (**b**) In PAH, vascular tone is altered to favor contraction and proliferation. Excess ET-1 signaling via the ETAR receptor may be one of the pathogenic factors. (**c**) Pharmacotherapy to restore appropriate balance may include the use of ETAR antagonists such as ambrisentan (This diagram is reproduced with permission from *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 12th edition; see Barnes 2011. For details, consult the original reference

arterial hypertension (PAH), and blockade of ETAR is clinically beneficial in reducing pulmonary arterial pressure and reversing arterial remodeling (Schneider et al. 2007; Abman 2009). Figure 2 shows the cell-cell and paracrine interactions involved in therapy of PAH and suggests how ETAR antagonists form one facet of a multi-agent approach. Bosentan and ambrisentan are currently approved for treatment of PAH in the USA (Motte et al. 2006; Abman 2009). Sitaxsentan, approved in Europe since 2006, was withdrawn in 2010 by Pfizer due to toxic liver damage.

Downregulation of ETAR may also occur as a consequence of elevated ET-1 plasma levels. Downregulation of ETAR in the heart, aorta, and pulmonary arteries is reported in hypertension induced by adenovirus-mediated gene transfer of ET-1, and in $ETBR^{-/-}$ deficient mice that have elevated circulating ET-1 levels due to reduced clearance by ETBR

(Kuc et al. 2006). About 20–50% downregulation in ETAR levels has been observed in the aorta and pulmonary arteries of some human diseased hearts, and this has been suggested to be a compensation for elevated plasma ET-1 levels (Kuc et al. 2006).

Both ET-1 levels and ETAR are elevated in chronic kidney disease (CKD) and acute renal failure (Kohan 2010). The ET system is involved in regulation of renal blood flow, reabsorption of Na^+ and water, cell proliferation, and extracellular matrix accumulation. Preclinical studies indicate that selective blockade of the ETAR increases renal blood flow and reduces proteinuria, blood pressure, and arterial stiffness in CKD (Dhaun et al. 2011). Increased ET-1 levels are reported in dysfunctional podocytes that will cause an increase in kidney damage and protein filtration. Blockade of ETAR reverses glomerulosclerosis and inhibits release of ET-1 and the shedding of podocyte-specific protein nephrin that is elevated in CKD. Clinical studies are currently assessing the long-term effects of ETAR blockade on morbidity and mortality of patients with CKD and diabetic nephropathy (Kohan 2010).

Upregulation of the ET system and activation of ETAR are associated with proliferation of VSMC. The mitogenic effects of ET-1 are amplified by synergistic interactions with growth factors such as epidermal growth factor (EFG), insulin, transforming growth factor-β (TGF-β), basic fibroblast growth factor (b-► FGF), platelet-derived growth factor (PDGF), and release of cytokines, which contribute to the development of atherosclerotic plaques (Schneider et al. 2007; Ivey et al. 2008; Khimji and Rockey 2010). In animal models, ETAR blockade inhibits formation of atherosclerotic plaques and improves NO-mediated vasodilation (Barton and Yanagisawa 2008).

In many cancers, the ET system is upregulated and the levels correlate with the severity and progression of the disease (Bagnato et al. 2011). Increased expression of ET-1 and ETAR is associated with tumor growth and progression, neovascularizaion, and metastasis in several cancers including ovarian, prostate, breast, cervical, colon, and lung cancers (Bagnato et al. 2011). The mitogenic effects, mediated through ETAR, are enhanced by transactivation of the EGF receptor (EGFR) through activation of matrix-metalloproteinases (MMPs) that release precursor ligands of EGFR such as the heparin binding EGF-like growth factor (HB-EGF), or through direct phosphorylation by c-Src. Transactivation of EGFR potentiates cell proliferation, cell survival, and tumorigenesis by activating protein tyrosine kinases, PI3-kinase-dependent Akt activation, and Ras–MAPK pathways (Ivey et al. 2008; Rosano et al. 2009). ZD4054, an ETAR-specific antagonist, reportedly inhibits EGFR transactivation and metastasis and enhances tumor regression in ovarian cancer cell xenografts (Rosano et al. 2009). Also, silencing of β-arrestins 1 and 2 prevents ETAR-induced Src activation, transactivation of EFGR, and metastasis (Rosano et al. 2009). Neovascularization and tumor progression correlate with vascular endothelial growth factor (VEGF) levels. ETAR stimulation enhances VEGF levels by increasing the protein levels of hypoxia-inducible factor 1α (HIF-1α) and increasing expression of cyclooxygenase (COX)-1 and COX-2 and the production of PGE_2 (Bagnato et al. 2011). Clinical trials using the ETAR antagonist ZD4054 in patients with prostate cancer have reported improved overall survival (Bagnato et al. 2011). More studies are under way to evaluate the therapeutic value of ETAR antagonism in cancer treatment.

Injection of ET-1 in both human and animals induces spontaneous pain and hyperalgesia (increased sensitivity to pain). The nociceptive and hyperalgesic effects of ET-1 are predominantly mediated through ETAR activation, while ETBR may elicit either pain responses or analgesic effects through release of endorphins (Khodorova et al. 2009). The ETAR is localized on the cell bodies of small diameter sensory neurons of the dorsal root ganglia (DRG) which are associated with pain impulses. ETAR antagonists can ameliorate ET-1-induced acute pain in humans and animal models (Khodorova et al. 2009). Antagonists of ETAR are being tested for their potential analgesic effects in cancer-related pain (Bagnato et al. 2011).

Summary

Endothelin works through ETAR and ETBR to regulate multiple physiological events such as vascular tone, renal function, and cell growth and proliferation. Most of the studied pathophysiological effects of ET-1 are mediated through ETAR. Activation of ETAR is implicated in cardiac hypertrophy and congestive heart failure, ischemic heart disease, pulmonary and systemic hypertension, atherosclerosis, and renal disease. ETAR antagonists are being clinically evaluated in

a number of diseases. Both ETAR-selective and nonselective blockers have proved to be beneficial and are currently in use for treatment of pulmonary hypertension. Given the significant pathophysiological role that has emerged of ET-1/ETAR activation in nociception, inflammation, and the progression and metastasis of many cancers, ETAR-selective blockade may provide a promising target of intervention in analgesia and cancer therapy.

References

Abman SH. Role of endothelin receptor antagonists in the treatment of pulmonary arterial hypertension. Annu Rev Med. 2009;60:13–23.

Bagnato A, Loizidou M, Pflug BR, Curwen J, Growcott J. Role of the endothelin axis and its antagonists in the treatment of cancer. Br J Pharmacol. 2011;163:220–33.

Barnes PJ. Pulmonary pharmacology. In: Brunton L, Chabner B, Knollmann B, editors. The pharmacological basis of therapeutics. 12th ed. New York: McGraw-Hill; 2011. p. 1031–66.

Barton M, Yanagisawa M. Endothelin: 20 years from discovery to therapy. Can J Physiol Pharmacol. 2008;86:485–98.

Bkaily G, Avedanian L, Al-Khoury J, Provost C, Nader M, D'Orléans-Juste P, Jacques D. Nuclear membrane receptors for ET-1 in cardiovascular function. Am J Physiol. 2011;300:R251–63.

Davenport AP. International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. Pharmacol Rev. 2002;54:219–26.

Dhaun N, Macintyre IM, Kerr D, Melville V, Johnston NR, Haughie S, et al. Selective endothelin-a receptor antagonism reduces proteinuria, blood pressure, and arterial stiffness in chronic proteinuric kidney disease. Hypertension. 2011;57:772–9.

Edvinsson L, Povlsen GK. Late cerebral ischaemia after subarachnoid haemorrhage: is cerebrovascular receptor upregulation the mechanism behind? Acta Physiol (Oxf). 2011;203:209–24.

Evans NJ, Walker JW. Endothelin receptor dimers evaluated by FRET, ligand binding, and calcium mobilization. Biophys J. 2008;95:483–92.

Hilal-Dandan R, Brunton LL. Endothelin type A receptor. 2010. http://www.signaling-gateway.org/molecule/query?afcsid=A000836. Accessed 30 Mar 2011.

Hilal-Dandan R, Ramirez MT, Villegas S, Gonzalez A, Endo-Mochizuki Y, Brown JH, et al. Endothelin ETA receptor regulates signaling and *ANF* gene expression via multiple G protein-linked pathways. Am J Physiol. 1997;272:H130–7.

Ivey ME, Osman N, Little PJ. Endothelin-1 signalling in vascular smooth muscle: pathways controlling cellular functions associated with atherosclerosis. Atherosclerosis. 2008;199:237–47.

Khimji AK, Rockey DC. Endothelin – biology and disease. Cell Signal. 2010;22:1615–25.

Khodorova A, Montmayeur JP, Strichartz G. Endothelin receptors and pain. J Pain. 2009;10:4–28.

Kohan DE. Endothelin, hypertension and chronic kidney disease: new insights. Curr Opin Nephrol Hypertens. 2010;19:134–9.

Kuc RE, Maguire JJ, Davenport AP. Quantification of endothelin receptor subtypes in peripheral tissues reveals downregulation of ET(A) receptors in ET(B)-deficient mice. Exp Biol Med (Maywood). 2006;231:741–5.

Motte S, McEntee K, Naeije R. Endothelin receptor antagonists. Pharmacol Ther. 2006;110:386–414.

Paasche JD, Attramadal T, Kristiansen K, Oksvold MP, Johansen HK, Huitfeldt HS, et al. Subtype-specific sorting of the ETA endothelin receptor by a novel endocytic recycling signal for G protein-coupled receptors. Mol Pharmacol. 2005;67:1581–90.

Rosano L, Cianfrocca R, Masi S, Spinella F, Di Castro V, Biroccio A, et al. Beta-arrestin links endothelin A receptor to beta-catenin signaling to induce ovarian cancer cell invasion and metastasis. Proc Natl Acad Sci USA. 2009;106:2806–11.

Sato T, Kurihara Y, Asai R, Kawamura Y, Tonami K, Uchijima Y, et al. An endothelin-1 switch specifies maxillomandibular identity. Proc Natl Acad Sci USA. 2008;105:18806–11.

Schneider MP, Boesen EI, Pollock DM. Contrasting actions of endothelin ET(A) and ET(B) receptors in cardiovascular disease. Annu Rev Pharmacol Toxicol. 2007;47:731–59.

Takigawa M, Sakurai T, Kasuya Y, Abe Y, Masaki T, Goto K. Molecular identification of guanine-nucleotide-binding regulatory proteins which couple to endothelin receptors. Eur J Biochem. 1995;228:102–8.

Endothelin Receptor ETA

► Endothelin A Receptor (ETAR)

Endothelin Receptor Type A

► Endothelin A Receptor (ETAR)

Endothelin Type A Receptor

► Endothelin A Receptor (ETAR)

Endothelin-A Receptor

► Endothelin A Receptor (ETAR)

E-NTPDase

▶ E-NTPDase Family

E-NTPDase Family

Jean Sévigny
Department of Microbiology-Infectiology and Immunology, Centre de Recherche en Rhumatologie et Immunologie, CHUQ Research Centre, Université Laval, Quebec, QC, Canada

Synonyms

ATP diphosphohydrolase; ATPDase; CD39; Ecto-ADPase; Ecto-apyrase; Ecto-ATPase; E-NTPDase; GDA1; NTPDase; Nucleoside triphosphate diphosphohydrolase

Historical Background

Nucleoside triphosphate diphosphohydrolases (NTPDases), originally ATP diphosphohydrolases (ATPDases) with the common name apyrase, are by definition enzymes which split the γ- and β-phosphate residues of triphospho- and diphosphonucleosides such as ATP and ADP, respectively. Before the cloning of the gene, the common name apyrase was generally used for enzymes that exhibit this activity in plants and invertebrates while the terms ecto-ATPase and ATP diphosphohydrolase (ATPDase) were rather used in vertebrates. Shortly after cloning the first gene encoding such an enzyme, the nomenclature was unified in mammals to NTPDase, which better reflects the ability of these enzymes to convert not only ATP and ADP, but also other triphospho- and diphosphonucleosides.

A first apyrase was discovered in potato tubers in 1944, and was extensively characterized in the following years, although its function was unknown. Apyrases were later reported in other plant tissues such as in various peas and in cabbage leaves. Beginning in 1979, and mostly during the 1980s, apyrases were found in the salivary glands and saliva of bloodsucking animals such as ticks, mosquitoes, fleas, sand flies, and in the medicinal leech. The function of apyrase in these hematophagous animals was suggested to relate to feeding while preventing coagulation. Indeed, ADP is a major platelet aggregation agent, and the hydrolysis of this nucleotide by apyrase therefore has an anticoagulating effect.

Ectonucleotidases have long been known in vertebrate tissues, but the true identification of an ATPDase first happened in 1980 by A.R. Beaudoin's group (Sherbrooke, QC, Canada). They demonstrated the presence of this enzyme in the pig pancreas from which they purified the enzyme to a high degree and studied its kinetics in detail. Cytochemical and biochemical observations showed that this enzyme is associated with the zymogen granule and plasma membrane of acinar cells (Beaudoin et al. 1996). In certain conditions, one could find the enzyme associated with microvesicles secreted from the gland. Until the cloning of ATPDase genes, several papers reported the presence of enzymes with ATPDase activity in virtually all vertebrate tissues. During that period, whether one or two enzymes were responsible for the hydrolysis of ATP and ADP was intensely debated. The definitive identification of these proteins came with the cloning.

Handa and Guidotti in 1996 (Boston, MA, USA) cloned a soluble ATP diphosphohydrolase (apyrase) from potato tubers and noted that similar genes exist in some protozoans, in yeast and in mammals which were related to a human gene called CD39 cloned 2 years earlier. Following this work, Wang and Guidotti again in 1996 reported that CD39 encodes a protein with ecto-apyrase activity (Wang and Guidotti 1996). Independently and at the same time, several peptide sequences of purified ATPDases were obtained which also showed homology to CD39: an ATPDase from human umbilical vessels (S. Christoforidis and coll., Ioannina, Greece), and ATPDases from porcine pancreas and bovine aorta (A.R. Beaudoin's group). Then the groups of Drs. Beaudoin and Robson (Boston) joined their efforts to clone and characterize the gene responsible for encoding this enzyme with ATPDase activity. They came up with the conclusion that CD39 and ATPDase were identical (Kaczmarek et al. 1996). The enzyme is now known as NTPDase1. Shortly after this discovery, H. Zimmermann's group (Frankfurt am Main, Germany) and, independently, T.L. Kirley's group (Cincinnati, OH, USA) cloned

another related gene (Kirley1997; Kegel et al. 1997), now known as NTPDase2. Then, by analyzing expressed sequence tags (ESTs), Chadwick and Frischauf showed three more related genes in humans. We now know that this family comprises eight members in mammals, which are referred to as NTPDase1–8 since 2000 (Zimmermann et al. 2000; Bigonnesse et al. 2004; Robson et al. 2006).

Owing to the cloning of the NTPDase genes, homologous genes that encode enzymes with similar biochemical properties were found throughout the evolutionary tree. Apyrases have been found so far in vertebrates, plants, insects, protozoa, yeasts as well as in bacteria. A more detailed history of apyrase and NTPDases from different species can be consulted in excellent reviews (Beaudoin et al. 1996; Robson et al. 2006; Plesner 1995; Knowles 2011). This entry will henceforth focus exclusively on the mammalian enzymes, using the accepted nomenclature as NTPDases. The genes corresponding to human NTPDase1-8 are *ENTPD1-8*.

General Characteristics

Members of the E-NTPDase family, also called the CD39 family and sometimes, GDA1 family, are major enzymes that dephosphorylate nucleotides such as ATP and ADP. The "E-" stands for "ecto," which may be a little confusing since of the eight members of the E-NTPDase family, four, namely, NTPDase1, -2, -3, and -8, are specifically expressed on the cell surface and hydrolyze extracellular nucleotides. These enzymes are therefore called ectonucleotidases. On the other hand, NTPDase4-7 are attached to intracellular organelles, via one or two transmembrane domains, with their active site facing the intraorganellar lumen. Therefore, these enzymes, if not ecto, are at least "exo" for exocytoplasmic (or extracytoplasmic), as the catalytic site of NTPDases has never been found to face the cytosol, that is, they always face the extracellular medium or a compartment topologically identical to the extracellular medium.

Although heterologous expression showed that NTPDase5 and -6 undergo secretion via proteolytic cleavage, they have a weak activity and a low affinity toward nucleotides compared to the plasma membrane–bound NTPDases, which raises the question as to whether these enzymes significantly contribute to the hydrolysis of extracellular nucleotides in vivo. Other ectonucleotidases also involved in the conversion of nucleotides in the extracellular milieu include nucleotide pyrophosphatases/phosphodiesterases, acid and alkaline phosphatases, as well as ecto-5'-nucleotidase/CD73 (Kukulski et al. 2011; Yegutkin 2008). These enzymes differ by their biochemical properties but most importantly, by their cellular localization. This entry will focus on the four E-NTPDase members that have been clearly demonstrated to contribute to the hydrolysis of extracellular nucleotides and which appear as the dominant ectonucleotidases in physiological conditions (i.e., at pH ~7.4).

NTPDase1, -2, -3, and -8 (EC 3.6.1.5) are type II transmembrane proteins of about 500 amino acid residues. These NTPDases are firmly anchored to the plasma membrane via two transmembrane domains, one near each end of the protein, which delineate a large extracellular loop containing the active site and 10 conserved Cys residues that are obviously important for the tertiary and quaternary structure of these proteins. These four enzymes also encompass about 6–10 *N*-linked glycosylation sites, depending on the protein and species. Members of this enzyme family share five apyrase-conserved regions (ACRs). NTPDase1, -2, -3, and -8 readily form homo-oligomeric assemblies. They have indeed been observed as homodimers to tetramers, whereas hetero-oligomeric complexes between NTPDases have not been reported thus far. Homo-oligomeric assemblies appear to be important for the biochemical activity of the enzymes (Robson et al. 2006).

NTPDases catalyze the hydrolysis of the γ- and β-phosphate residues of triphosphonucleosides (e.g., ATP, UTP) and diphosphonucleosides (e.g., ADP, UDP). Optimal NTPDase activity requires low millimolar concentrations of divalent cations (Ca^{2+} and/or Mg^{2+}). NTPDase1, -2, -3, and -8 are all active at physiological pH, and a few of them are active in a wider set of pH values (see below) (Kukulski et al. 2005). The most important difference between these four NTPDases is their ability to hydrolyze diphosphonucleosides, NTPDase1 and NTPDase2 being the most and least efficient forms, respectively. NTPDases are distinct from other ATPases and are insensitive to inhibitors of F-, P-, and V-type ATPases, and of alkaline phosphatases.

The presumptive major role of plasma membrane–bound NTPDases, and more specifically of

NTPDase1-3 and -8, is the regulation of the concentration of extracellular nucleotides that are released by cells in a regulated manner as well as during cell death and cell lysis (Yegutkin 2008). Through such an action, NTPDases modulate the biological effects triggered by these nucleotides via the activation of several nucleotide receptors, namely, $P2Y_{1,2,4,6,11\text{-}14}$, P2X1-7, and three other receptors that are also activated by nucleotides, namely, cysteinyl leukotriene receptor-1 and -2 (CysLT1 and CysLT2) and GRP17. Indeed, micromolar concentrations of UDP have been shown to activate the last three receptors.

Like other ectonucleotidases, NTPDases are involved in multiple aspects of nucleotide (P2) and adenosine (P1) receptor signaling which include termination of P2 receptor activation, protection of susceptible P2 receptors such as $P2Y_1$ and P2X1 from desensitization, and promote the activation of some P2 receptors as well as of P1 receptors. More specifically, by hydrolyzing ATP, NTPDases terminate P2X1-7 activation as well as $P2Y_2$ and $P2Y_{11}$ activation. Moreover, by hydrolyzing UTP, NTPDases end $P2Y_2$ and $P2Y_4$ receptor activation. These enzymes, and more specifically NTPDase2, can also generate the agonist of some receptors from the hydrolysis of ATP and UTP. Indeed, ADP is the ligand of $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, while UDP is the agonist of $P2Y_6$. In addition, the final product of NTPDase activity on ATP, AMP, is converted to adenosine by ecto-5'-nucleotidase and/or alkaline phosphatases. The latter process is of high biological significance, since adenosine is the ligand of the four P1 receptors (A_1, A_{2A}, A_{2B}, A_3). By contributing to the generation of adenosine, NTPDases not only participate in the regulation of P1 receptor activation but they also contribute to replenish ATP stores. Indeed, once released from the cells, nucleotides cannot be re-accumulated as long as they bear a negative charge. In other words, all phosphate groups must be removed before the nucleoside core of the molecule can reintegrate into the cell. Then, nucleosides such as adenosine can reenter the cell by active or passive transport across the membrane via specific permeases, namely, concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs), respectively. These nucleotide salvage pathways are especially important for tissues and cells such as brain, bone marrow, erythrocytes, and intestinal mucosa, which cannot synthesize these valuable molecules de novo.

As almost every cell expresses more than a single type of P1 and P2 receptors, nucleotides and adenosine have functions in all organs. Therefore, the regulation of the identity and concentration of the receptor ligands by enzymes which metabolize these agonists is critical and should be appropriate for each tissue or cell (Kukulski et al. 2011; Yegutkin 2008; Deaglio and Robson 2011).

Specific Properties

NTPDases differ from each other by their subtle biochemical properties and their cellular localization. Although PCR experiments, which detect low levels of mRNAs, often display the expression of multiple NTPDases by the same tissue/cell, immunohistochemistry with specific antibodies (http://ectonucleotidases-ab.com) most often show that NTPDase1, -2, -3, and -8 are expressed by different cells, with the expression of two of these NTPDases by the same cell being the exception.

A great deal of effort is currently dedicated to the development of specific NTPDase inhibitors. Although some progress has been made, much work remains to be done. The inhibitors that have been developed and utilized thus far have been summarized in a recent review (Kukulski et al. 2011). It is also noteworthy that monoclonal antibodies have recently been developed and two of them specifically inhibit human NTPDase3 and human NTPDase2, respectively (http://ectonucleotidases-ab.com).

Key information toward the design of novel inhibitors is expected from the current work of Dr Sträter's team (Universität Leipzig, Germany). They recently crystallized the extracellular and soluble portion of NTPDase2 (Zebisch and Strater 2008). This already confirmed the importance and localization of the 10 conserved Cys residues in the extracellular domain of plasma membrane–bound NTPDases (members 1, 2, 3, and 8) (Ivanenkov et al. 2005) and the contribution of all 5 ACRs to the catalytic activity of the enzyme. These data represent only a prelude to the actual crystallization of NTPDases as this original work was performed on an extracellular domain produced by *E. coli* which does not glycosylate proteins, and because the transmembrane domains also appear to be important for the activity of NTPDase1, -2, -3, and -8 (Knowles 2011; Grinthal et al. 2006).

This section presents the distinct biochemical properties of the plasma membrane–bound form of the E-NTPDase family members, their general cellular localization, and their demonstrated and/or presumed functions. NTPDase functions involve the regulation of the concentration of P2 receptor ligands. In agreement, all the functions so far reported for these enzymes have been directly, or at least indirectly, linked to the regulation of nucleotide and nucleoside receptor activation. Therefore, the functions described below will be in line with the assumption that they implicate P2 receptors. This by no means excludes the possibility that NTPDases might have functions unrelated to ATP receptors, but due to the lack of clear evidence at the moment, I will not dwell on such hypothetical roles in this entry.

Therefore, the identity and localization of the NTPDase(s), together with the identity of the neighboring P2 and P1 receptors, the signaling pathway coupled with the activation of this (these) receptor(s) in a given cell type, as well as the identity and concentration of the nucleotide released will dictate a precise response and function in a given tissue. The current state of knowledge on the various cellular sources of nucleotides has been very well covered by Yegutkin et al. (2008). The signaling and various functions played by P1 and P2 receptors have also been extensively covered in a number of reviews.

NTPDase1

Characterization

NTPDase1 (aka CD39, ATPDase, ecto-apyrase, ecto-ATPase, or ecto-ADPase) is the best characterized NTPDase thus far, and its involvement in the regulation of several distinct biological processes has been clearly demonstrated (see below). The cDNA encoding NTPDase1 was originally cloned from human tissue. In 1994, Maliszewski et al cloned what was known at the time as CD39, a lymphocyte activation antigen with unknown function. Partial protein sequences obtained at about the same time by different groups from apyrases/ATPDases purified from different tissues finally allowed two groups to independently clone and express CD39 cDNA as a functional ATPDase (Wang and Guidotti 1996; Kaczmarek et al. 1996). This protein was renamed NTPDase1 in the following years. This was the first identification of a gene encoding a protein with true ecto-ATPase activity. The gene encoding human NTPDase1 is now named *ENTPD1* (or *CD39*) and maps to chromosome 10q24 (GeneBank access. no. U87967) (Robson et al. 2006).

Two variants, most probably originating from alternative splicing, have been observed in human placenta. One of them bearing a small modification at the N-terminus was shown to be active. The second isoform is probably inactive. Although NTPDase1 expression often appears to be constitutive, it was shown to be modulated by inflammatory cytokines, oxidative stress, hypoxia, cAMP response elements, glucocorticoids as well as by nucleotides (via $P2Y_1$ receptors).

NTPDase1 hydrolyzes all nucleotides with a similar efficacy. These include the P2 receptor ligands, namely, ATP, ADP, UTP, and UDP. NTPDase1 converts ATP to ADP, and then to AMP. In contrast to the other NTPDases, there is only a minimal transient release of ADP, as the processing step to AMP is favored. Importantly, this property of NTPDase1 makes it the most efficient to convert ATP to adenosine as it not only rapidly generates AMP, the substrate for ecto-5'-nucleotidase, but it also allows the concentration of two inhibitors of the latter enzyme, namely, ATP and ADP, to rapidly decrease. It is noteworthy that, in contrast to adenine nucleotides, NTPDase1 hydrolyzes UTP to UMP with a transient accumulation of UDP, which may favor the transient activation of the UDP receptor ($P2Y_6$). Finally, NTPDase1 has the narrowest range of pH preference for activity, being active between pH 7 and 10 (Kukulski et al. 2005).

The N-terminal intracytoplasmic domain of NTPDase1 is subject to palmitoylation, which targets a subpopulation of enzyme molecules to cholesterol-rich lipid rafts/caveolae. The presence of NTPDase1 in these structures might be of physiological importance due to their association with G-protein-coupled receptor signaling and to their colocalization with some P2 receptors and ecto-5'-nucleotidase. Although its association with caveolae does not appear to be essential for enzyme activity, cholesterol depletion results in a strong inhibition of NTPDase1. Oxidative stress has also been reported to inhibit NTPDase1 activity in vivo, which affects its normal antiplatelet function such as that observed in transplantation models (Deaglio and Robson 2011).

The N-terminus of human NTPDase1 has been shown to interact with truncated Ran-binding protein

M (RanBPM = RanBP9). RanBPM contains conserved SPRY (repeats in splA and RyR) domains which appear to be crucial for its interaction with NTPDase1. RanBPM is known to interact with Sos and to regulate ERK/Ras signaling. Such functions might therefore be altered or regulated by NTPDase1, which may therefore represent a novel, albeit hypothetical function of the enzyme (Robson et al. 2006).

Localization and Function

NTPDase1 is the predominant ectonucleotidase in blood. It is expressed in endothelial cells, smooth muscle cells as well as in leukocytes, including neutrophils, monocytes, and lymphocytes (e.g., B lymphocytes, Tregs, memory lymphocytes, natural killer T cells, and natural killer cells) (Kukulski et al. 2011). NTPDase1 has also been immunodetected in macrophages and resident macrophages such as microglia and Kupffer cells, in Langerhans and dendritic cells (Mizumoto et al. 2002), as well as in some epithelial cells, for example, in the zymogen granule membrane of pancreatic acini where it was originally demonstrated in mammals. This protein is also secreted in an active form into the pancreatic juice together with a particulate fraction (shed from the membrane) (Beaudoin et al. 1996; Robson et al. 2006).

As a result of its nucleotide hydrolysis activity, NTPDase1 has been associated with several functions through its regulation of P2 receptor activation and desensitization. For example, NTPDase1 protects P2X1 and $P2Y_1$ receptors from desensitization. *Entpd1*$^{-/-}$ mice, which are deficient in NTPDase1 expression, display abnormal platelet activation due to $P2Y_1$ receptor desensitization in platelets (Enjyoji et al. 1999). However, it must be noted that this phenotype was observed in NTPDase1-deficient animals which subsisted despite this anomaly. Such protection against P2X1 and $P2Y_1$ receptor desensitization contrasts with another important function of NTPDase1, namely, the prevention of platelet aggregation by removal of ADP, the agonist for $P2Y_1$ and $P2Y_{12}$ receptors in platelets (Robson et al. 2006; Deaglio and Robson 2011). NTPDase1 probably also contributes to this effect by hydrolyzing ATP, the ligand of the P2X1 receptor, which is also expressed in platelets and is also important for their activation in vivo. Therefore, the regulation of the level and duration of NTPDase1 activity should play a crucial role in the overall regulation of P2 receptor activity (activation vs. desensitization).

The function of the endothelial NTPDase1 is not limited to platelet aggregation. It also influences inflammatory processes (see below) as well as vascular tone. Although *Entpd1*$^{-/-}$ mice do not show a global defect in vasomotion, in vitro experiments with arteries from these mice showed that the effects of both exogenous and endogenous nucleotides on vasodilation were dramatically potentiated when NTPDase1 activity is lacking. This regulation was shown to involve the regulation of endothelial $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors by NTPDase1. Interestingly, the absence of NTPDase1 in vascular smooth muscle cells made the mice more susceptible to vasoconstriction via $P2Y_6$ receptors expressed on smooth muscle cells (Kukulski et al. 2011). These data clearly show the importance of the compartmentalization of nucleotide release as well as the fine regulation of their levels by NTPDase1, which dictates their final output on the biological system, namely, the vascular tone in the latter example.

Illustrations of the role(s) of NTPDase1 in immune responses have grown exponentially in recent years since the enzyme has been shown to be the major ectonucleotidase in leukocytes, including lymphocytes such as Tregs and natural killer cells, as well as macrophages, dendritic cells, and Langerhans cells (Mizumoto et al. 2002). In at least a subset of these cell types, NTPDase1 expression protects from ATP-induced cell death, and affects angiogenesis, leukocyte trafficking as well as the expression and release of several cytokines (Kukulski et al. 2011). NTPDase1-null mutant mice have revealed various functions of the enzyme in immune responses. *Entpd1*$^{-/-}$ animals show amplified inflammatory responses to irritant chemicals due to the lack of NTPDase1 suppressive properties, and present defective stimulation of hapten-reactive T cells (Mizumoto et al. 2002). Also in mouse, NTPDase1 is a Treg cell surface marker which appears to affect cellular immunoregulation (Deaglio and Robson 2011). Modulated nucleotide signaling was also shown to impact NKT-mediated mechanisms that result in liver immune injury. The few observations presented above on NTPDase1-deficient animals clearly suggest that the role of the enzyme in immune reactions is complex. In addition, NTPDase1 has been shown to affect cancer progression, an effect which could be attributed, at least in some models, to NTPDase1 activity in Tregs. Note that NTPDase1 activity detected in some cancers may also

be the result of NTPDase1 expression by cancer cells. Altogether, the inhibition of NTPDase1 enzymatic activity has been proposed as an adjunct therapy for primary and secondary malignancies. However, the underlying mechanism is likely very complex, as spontaneous tumors develop in old *Entpd1*$^{-/-}$ mice, in contrast to the data obtained above with models of more malignant cancers.

Not only NTPDase1 deficiency is linked to distinct anomalies, but the full range of expression and activity for the enzyme also appears to be important for the normal regulation of biological processes, as suggested above. An interesting example of this is an *ENTPD1* polymorphism that was reported in human which is associated with reduced NTPDase1 mRNA expression and which correlates with increased susceptibility to Crohn's disease. In agreement with the latter, severe colitis has been observed in NTPDase1-deficient mice in an experimental model of inflammatory bowel disease (Friedman et al. 2009). The functions of NTPDase1 have been extensively reviewed in recent papers (Kukulski et al. 2011; Deaglio and Robson 2011).

NTPDase2

Characterization

NTPDase2 (= CD39L1 or ecto-ATPase) was originally cloned from chicken and rat (Kirley 1997; Kegel et al. 1997). The *ENTPD2* human gene is located on chromosome 9q34 (AF144748). Two inactive splice variants have been observed so far in human and one such form in rat which has the main characteristics of the original NTPDase2 transcript species, with some differences. Fluctuations in NTPDase2 expression have often been noted but these may be related, as for NTPDase1, to the cell type involved. For example, in mouse hepatoma cells, transcription of NTPDase2 can be induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an environmental contaminant. Interestingly, the NTPDase2 core promoter reveals constitutive transcriptional activity that is independent of TCDD. Moreover, TCDD failed to induce NTPDase2 in a variety of other cell lines derived from various species. In rat Sertoli cells, NTPDase2 is upregulated by follicle-stimulating hormone and cAMP. In the liver, NTPDase2 is selectively downregulated in biliary cirrhosis. In portal fibroblasts, the expression of NTPDase2 is downregulated by IL-6 at the transcriptional level (Robson et al. 2006; Kukulski et al. 2011).

NTPDase2 is a preferential nucleoside triphosphatase. It converts very efficiently ATP and UTP to the respective diphosphate derivative. Although NTPDase2 can further dephosphorylate these diphosphates to their monophosphate derivatives, the latter's activity is relatively inefficient. Therefore, NTPDase2 facilitates the termination of ATP- and UTP-specific receptor activation in favor of ADP- and UDP-specific receptor activation, respectively. This enzyme is mostly active in the physiological range (as for other NTPDases) as well as in more acidic conditions, but is inactive at pH > 9 (Kukulski et al. 2005).

Localization and Function

The major distribution of NTPDase2 is on the adventitial surface of blood vessels and different types of glial cells in both central and peripheral nervous systems (e.g., astroglia, Schwann cells). NTPDase2 has also been detected in the Bowman's capsule and in some pericytes as in the heart. Interestingly, NTPDase2 is expressed by "glial-like" type I cells of taste buds, and antibodies to this protein have already become a most efficient way to identify these cells (http://ectonucleotidases-ab.com) (Kukulski et al. 2011).

NTPDase2 expression on the outer surface of blood vessels has been suggested to preserve vascular integrity by favoring the activation of ADP receptors expressed in platelets, namely, $P2Y_1$ and $P2Y_{12}$ (Sévigny et al. 2002). Indeed, upon vascular tissue disruption, ATP is released from damaged cells and platelets, which are normally found inside vessels, then come in contact with the released nucleotides as well as with NTPDase2, which does not normally face the luminal side where platelets are found under physiological conditions (Deaglio and Robson 2011).

A most fascinating observation was made by Massé et al. who showed the short but key participation of NTPDase2 in eye development in *Xenopus laevis* (Massé et al. 2007). Hydrolysis of ATP to ADP resulting from the overexpression of NTPDase2 caused the development of ectopic eye-like structures and, accordingly, increased the expression of eye field transcription factors. Neither NTPDase1 nor NTPDase3 could replace NTPDase2 for normal eye formation.

Zimmermann's group reported the expression of NTPDase2 in the subventricular zone and rostral migratory stream of adult rat brain, and suggested that together with an alkaline phosphatase, the enzyme played a role in neural development and differentiation. In the liver, NTPDase2 has also been associated with the regulation of cell proliferation, albeit at a different level. Thus, bile duct cell proliferation was prevented by NTPDase2 expressed in portal fibroblasts in coculture and, conversely, increased when NTPDase2 expression was downregulated. As mentioned above, NTPDase2 is selectively downregulated in biliary cirrhosis and accordingly, IL-6 downregulates NTPDase2 transcription. Interestingly, the latter phenomenon might represent a mechanism accounting for the aberrant proliferation of bile duct cells observed in biliary cirrhosis, where IL-6 is indeed markedly upregulated. NTPDase2 has also been shown to affect IL-8 secretion in HT29 colon carcinoma cells via the regulation of $P2Y_{11}$ receptor activation, and its reconstituted expression in a glioma cell line dramatically increased tumor growth in vivo (Robson et al. 2006; Kukulski et al. 2011).

NTPDase3

Characterization

NTPDase3, previously named in different studies as CD39L3 and HB6, was originally cloned from human brain (Smith and Kirley 1998). The *ENTPD3* gene is located on chromosome 3p21.3 (AF034840). An inactive splice variant has been identified in a human lung cDNA library. When coexpressed by heterologous transfection with the normal form in the same cells, the inactive splice variant could reduce the biochemical activity of the original form (Robson et al. 2006; Knowles 2011).

NTPDase3 converts both ATP and UTP to the monophosphate derivative with a transient accumulation of the diphospho derivative, leading to the transient activation of ADP- and UDP-specific receptors. NTPDase3 is the E-NTPDase family member which exhibits the broadest spectrum of pH for activity (from 5 to 11) (Kukulski et al. 2005).

Although much less information is available on NTPDase3 than on the two previous isoforms, Kirley's group (Cincinnati, OH) generated a large number of enzyme mutant forms in order to characterize the biochemistry of the enzyme. These studies have also been helpful to understand the general structure of the plasma membrane–bound NTPDases, which share a common general tridimensional conformation (Knowles 2011).

Localization and Function

Kirley's group also reported the expression of NTPDase3 in brain neurons, in which they suggested that the enzyme might be involved in the modulation of feeding and sleep-wake behaviors. NTPDase3 was also reported in peripheral neurons such as along rodents' bowels. In addition, it was reported in rodents in certain epithelial cells of the digestive, reproductive, renal, and respiratory systems. In the kidney, NTPDase3 is expressed in thick ascending limb, distal tubules, and in cortical and outer medullary collecting ducts. In the pancreas, NTPDase3 is expressed in all Langerhans islet cells. The pharmacological inhibition of NTPDase3 was shown to increase insulin secretion in low glycemia in the rat β-INS-1 (832/13) insulinoma cell line. NTPDase3 has also been immunolocalized in some enteroendocrine cells of the gastric antrum (Kukulski et al. 2011).

NTPDase8

Characterization

NTPDase8, (= liver canalicular ecto-ATPase or hATPDase), was originally cloned from mouse liver (Bigonnesse et al. 2004). The cDNA clone obtained had a homology with a gene previously cloned from chicken oviduct (Knowles 2011). The *ENTPD8* gene is located in proximity of *ENTPD2*, mapping also to chromosome 9q34 (AY430414) (Robson et al. 2006).

Like NTPDase3, NTPDase8 converts both ATP and UTP to the corresponding monophosphates, with transient accumulation of the intermediary, diphospho derivative. An interesting characteristic of NTPDase8 is its ability to hydrolyze nucleotides both at physiological and acidic pH (Kukulski et al. 2005).

Localization and Function

Mammalian NTPDase8 is expressed in liver canaliculi, and in the apical membrane of some epithelial cells such as in the brush border membranes (presumably on proximal tubules) of the kidney (Kukulski et al. 2011). The presence of NTPDase8 mRNA was also detected in the organ of Corti of the inner ear. Although no specific function has yet been demonstrated for this

last discovered E-NTPDase family member, its localization in the apical surface of the above structures suggests that NTPDase8 might be important for nucleotide salvaging. Indeed, the liver is likely the main source of purines for tissues incapable of de novo synthesis as mentioned in the section "General Characteristics." The presence of NTPDase8 in parallel with ecto-5'-nucleotidase and nucleoside permeases in the canalicular domain of hepatocytes is in support of a role for NTPDase8 in nucleotide salvaging in the liver.

Summary and Perspectives

The E-NTPDase members -1, -2, -3, and -8 are major enzymes responsible for the hydrolysis of nucleoside triphosphates and diphosphates at the cell surface, which regulate P2 receptors and, by extension, adenosine receptor activation as well as the recycling of purines for tissues and cells deficient in de novo synthesis. The study of the functions of these enzymes will be facilitated with the availability of inhibitors for these enzymes, thanks to the current efforts made in several drug discovery laboratories. Although some progress has been made in the last few years, more potent and specific inhibitors are still very much needed. The recent cloning, production of antibodies, as well as generation of gene-deficient animals recently achieved for each of these enzymes should prove very useful in the near future to help in defining the exact functions of these enzymes, and especially for NTPDase2, -3, and -8. Indeed, a great deal of information about NTPDase1 function has come from mice deficient in the protein which were generated about 10 years ago. In addition, the current intense research activity on nucleotide release modes, on nucleotide and adenosine receptors and on nucleoside transporters that are all part of the *purinome* system, should be very fruitful for NTPDase research as well.

References

Beaudoin AR, Sévigny J, Picher M. ATP-diphosphohydrolases, apyrases, and nucleotide phosphohydrolases: biochemical properties and functions. In: Lee AG, editor. ATPases. Greenwich: JAI Press; 1996. p. 369–401.

Bigonnesse F, Lévesque SA, Kukulski F, Lecka J, Robson SC, Fernandes MJG, et al. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. Biochemistry. 2004;43(18):5511–9.

Deaglio S, Robson SC. Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. Adv Pharmacol. 2011;61:301–32.

Enjyoji K, Sévigny J, Lin Y, Frenette PS, Christie PD, Schulte Am Esch IIJ, et al. Targeted disruption of CD39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med. 1999;5(9):1010–7.

Friedman DJ, Kunzli BM, Yi AR, Sévigny J, Berberat PO, Enjyoji K, et al. From the Cover: CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. Proc Nat Acad Sci USA. 2009;106(39):16788–93.

Grinthal A, Guidotti G. CD39, NTPDase 1, is attached to the plasma membrane by two transmembrane domains. Why? Purinergic Signal. 2006;2(2):391–8.

Ivanenkov VV, Meller J, Kirley TL. Characterization of disulfide bonds in human nucleoside triphosphate diphosphohydrolase 3 (NTPDase3): implications for NTPDase structural modeling. Biochemistry. 2005;44(25): 8998–9012.

Kaczmarek E, Koziak K, Sévigny J, Siegel JB, Anrather J, Beaudoin AR, et al. Identification and characterization of CD39 vascular ATP diphosphohydrolase. J Biol Chem. 1996;271(51):33116–22.

Kegel B, Braun N, Heine P, Maliszewski CR, Zimmermann H. An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain. Neuropharmacology. 1997;36(9): 1189–200.

Kirley TL. Complementery DNA cloning and sequencing of the chicken muscle ecto-ATPase – homology with the lymphoid cell activation antigen CD39. J Biol Chem. 1997;272(2): 1076–81.

Knowles AF. The GDA1_CD39 superfamily: NTPDases with diverse functions. Purinergic Signal. 2011;7(1):21–45.

Kukulski F, Lévesque SA, Lavoie EG, Lecka J, Bigonnesse F, Knowles AF, et al. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. Purinergic Signal. 2005;1(2):193–204.

Kukulski F, Lévesque SA, Sévigny J. Impact of ectoenzymes on P2 and P1 receptor signaling. Adv Pharmacol. 2011;61: 263–99.

Massé K, Bhamra S, Eason R, Dale N, Jones EA. Purine-mediated signalling triggers eye development. Nature. 2007;449(7165):1058–62.

Mizumoto N, Kumamoto T, Robson SC, Sévigny J, Matsue H, Enjyoji K, et al. CD39 is the dominant langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. Nat Med. 2002;8(4):358–65.

Plesner L. Ecto-ATPases: identities and functions. Int Rev Cytol. 1995;158:141–214.

Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal. 2006;2(2):409–30.

Sévigny J, Sundberg C, Braun N, Guckelberger O, Csizmadia E, Qawi I, et al. Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. Blood. 2002;99(8):2801–9.

Smith TM, Kirley TL. Cloning, sequencing, and expression of a human brain ecto-apyrase related to both the ecto-ATPases and CD39 ecto-apyrases. Biochim Biophys Acta Protein Struct Mol Enzymol. 1998;1386(1):65–78.
Wang T-F, Guidotti G. CD39 is an ecto-(Ca^{2+}, Mg^{2+})-apyrase. J Biol Chem. 1996;271:9898–901.
Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. Biochim Biophys Acta. 2008;1783(5):673–94.
Zebisch M, Strater N. Structural insight into signal conversion and inactivation by NTPDase2 in purinergic signaling. Proc Natl Acad Sci USA. 2008;105(19):6882–7.
Zimmermann H, Beaudoin AR, Bollen M, W. GJ, Guidotti G, Kirley TL, et al., editors. Proposed nomenclature for two novel nucleotide hydrolyzing enzyme families expressed on the cell surface. Proceedings of the Second International Workshop on Ecto-ATPases and Related Ectonucleotidases; 1999/2000; Diepenbeek, Belgium \Maastricht: Shaker; 2000

Eph Receptor

Kwok-On Lai and Nancy Y. Ip
Division of Life Science, Molecular Neuroscience Center and State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science and Technology, Kowloon, Hong Kong

Historical Background

The first Eph receptor, which was found to be overexpressed in *e*rythropoietin-*p*roducing *h*epatocellular carcinoma cell line (hence, the name Eph), was identified by homology cloning using the kinase domain of the viral oncogene v-fps as a probe for low-stringency hybridization (Hirai et al. 1987). Since then, other homologous members were cloned by low-stringency cross-hybridization or PCR with primers based on conserved sequences in the kinase domain. There are a total of fourteen homologous Eph receptors identified in mammals to date, comprising the largest family of RTK. They are generally categorized as either EphA or EphB receptor based on ligand specificity (see below).

During the early days when their ligands had not yet been identified, Eph receptors were often described as "orphan receptors" with unknown function. The first Eph receptor ligand, named at that time B61, was cloned as a cytokine-inducible gene (Holzman et al. 1990), although it was not recognized as a ligand of Eph receptor until several years later (Bartley et al. 1994). Using Fc-tagged soluble Eph receptors to screen expression libraries, other Eph receptor ligands have been cloned and collectively called ephrins (derived from *Eph*-inter*a*ct*i*n*g* proteins; Eph Nomenclature Committee 1997). Although B61 was first described as a secreted protein, it turns out that all ephrins are membrane proteins. The eight different ephrins can be subdivided into two families based on the type of membrane anchorage: ephrinAs (ephrinA1 to A5) are linked to the plasma membrane via a glycosyl-phosphatidyl inositol (GPI) moiety, and ephrinBs (ephrinB1 to B3) anchor to the plasma membrane through the presence of a transmembrane domain. In general, ephrinAs bind to EphA receptors (EphA1 to A8 and A10), while EphB receptors (EphB1 to B4 and B6) are preferentially activated by ephrinBs. The notable exceptions are EphA4, which binds to both the A- and B-class ephrins with high affinity, and EphB2, which also binds to ephrinA5 in addition to the transmembrane ephrins.

Multiple Eph receptors and ephrins have also been identified in other vertebrates such as zebrafish and *Xenopus*. However, there is only a single Eph receptor being identified from the invertebrates *C. elegans* and *Drosophila* (Drescher 2002), and a partial sequence of Eph receptor has been cloned from the sea slug *Aplysia californica*. It therefore appears that Eph receptors and ephrins are ancient and evolutionarily conserved, but the divergence of the *eph* ancestor gene into multiple genes might occur during the evolution of vertebrates.

Characteristics of Ephrins and Eph Receptors

Full-length Eph receptors are ~120 kD. The extracellular domain of Eph receptors can be subdivided into the amino-terminal ephrin-binding domain, the cysteine-rich region, and two fibronectin-type III repeats. This is followed by a single transmembrane domain rich in hydrophobic amino acids for membrane anchorage and the intracellular domain that can be functionally segregated into the juxtamembrane region, the kinase domain, the sterile alpha motif (SAM) protein-protein interaction domain, and the PDZ-binding motif. Almost all Eph

receptors have tyrosine kinase activity, except EphB6 which lacks certain conserved amino acid residues in the kinase domain and therefore does not possess catalytic activity. However, it is possible that EphB6 can still function in a kinase- dependent manner, probably through heterodimerization with other EphB receptors or interaction with other tyrosine kinases such as Cbl.

The full-length ephrins contain ~120 amino acids and anchor to the plasma membrane through the GPI-linkage (ephrinAs) or transmembrane domain (ephrinBs). The membrane attachment of ephrins is believed to be essential for mediating the cell contact-dependent functions of the Eph receptors (see below), although there are soluble isoforms of ephrins as a result of alternative splicing. Another unique feature of ephrins is their ability to transduce reverse signaling: upon interaction between ephrins and Eph receptors from opposing cells, not only the Eph receptors can trigger signal transduction via its catalytic kinase domain ("forward signaling"), but at the same time, both the ephrinA and ephrinB can also induce signal transduction in the ephrin-expressing cell, and this "reverse signaling" of ephrin is crucial in nervous system development and functioning, including axon guidance, synapse formation, and synaptic plasticity. The capability of transmembrane ephrinB to transduce reverse signaling is largely attributed to the presence of conserved tyrosine residues in the intracellular domain, which are phosphorylated after binding to Eph receptor by the Src family of tyrosine kinases. The phosphorylated tyrosine residues in ephrinB intracellular domain in turn recruit specific signaling molecules such as Grb4 via interaction with SH2 domain, leading to the propagation of signaling that regulates actin cytoskeleton dynamics (Cowan and Henkemeyer 2002). In addition to tyrosine phosphorylation, the carboxyl-terminal of ephrinB also contains PDZ-binding motif that is critically involved in synapse formation (Klein 2009; Lai and Ip 2009). Compared to the B-class ephrins, much less is known about how the GPI-anchored ephrinA transduces reverse signaling after binding to EphA receptor. The tyrosine kinases Fyn and ▶ Src can be activated by ephrinA5 reverse signaling to regulate cell adhesion, but the detailed mechanisms remain to be elucidated. EphrinA might also recruit the neurotrophin receptors p75 and TrkB as coreceptors for reverse signaling.

Function of Eph Receptors in the Nervous System

Ephrins and Eph receptors are crucial mediators of cell-cell communication in the nervous system, where they play indispensible roles both in neural development (e.g., axon guidance and formation of topographic mapping, synapse formation) and functioning of the matured brain (e.g., synaptic plasticity for learning and memory). Ephrin/Eph are also involved in a wide range of functions outside the nervous system, including cell sorting during embryonic segmentation, formation of blood vessels, maturation of the immune system, and bone formation (Pasquale 2008). Moreover, increasing evidence indicates that Eph receptors are critical determinants of cancer progression (Pasquale 2010). This chapter will focus on the function of ephrin/Eph in the nervous system.

Axon Guidance and Topographic Mapping

It has been known for long time that neurons from the retina connect to the optic tectum (or the superior colliculus in mammals) in a highly precise manner, such that retinal axons from the nasal side of the retina selectively project to the posterior side of the tectum, while the temporal retinal axons specifically innervate the anterior tectum. The identity of the molecules responsible to establish this precise retinotectal map remained obscure for many years until the discovery of the ephrin and Eph receptor. Three important lines of evidence indicate that ephrin represents the major repulsive guiding cue during retinotectal mapping. First, ephrins and Eph receptors are expressed in complementary gradients, with ephrinA2 and ephrinA5 expressed in a gradient from low (anterior) to high (posterior) across the tectum, while the expression of EphA3 is high in the temporal retinal and low in nasal retina. Second, in an in vitro stripe assay, ephrinA5 induces growth cone collapse of temporal retinal neurons, but not nasal retinal neurons. These observations therefore suggest that the countergradient of ephrinA and EphA in the tectum and retina is responsible for establishing the characteristic retinotectal map in a repulsive manner, such that the low expression of EphA3 in the nasal retina allows the projection of the axons into the posterior tectum where the expression of ephrinA2 and A5 is high, whereas the high expression of EphA3 in the temporal retina limits its axons to innervate the anterior tectum

where the level of ephrinAs is low. Finally, knockout mice lacking ephrinA2 or ephrinA5 show disrupted retinotectal connectivity, thus indicating the essential role of ephrin/Eph in topographic mapping in the retina.

In addition to retinotectal mapping, ephrin/Eph also participates in axon guidance and topographic mapping in other brain areas, including the hippocampus-septum, and midline crossing by the commissural axons that connect the two halves of the hemisphere. It is noteworthy that in the latter situation, it is the ephrinB reverse signaling rather than Eph receptor forward signaling that mediates the axonal repulsion.

Synapse Formation and Synaptic Plasticity

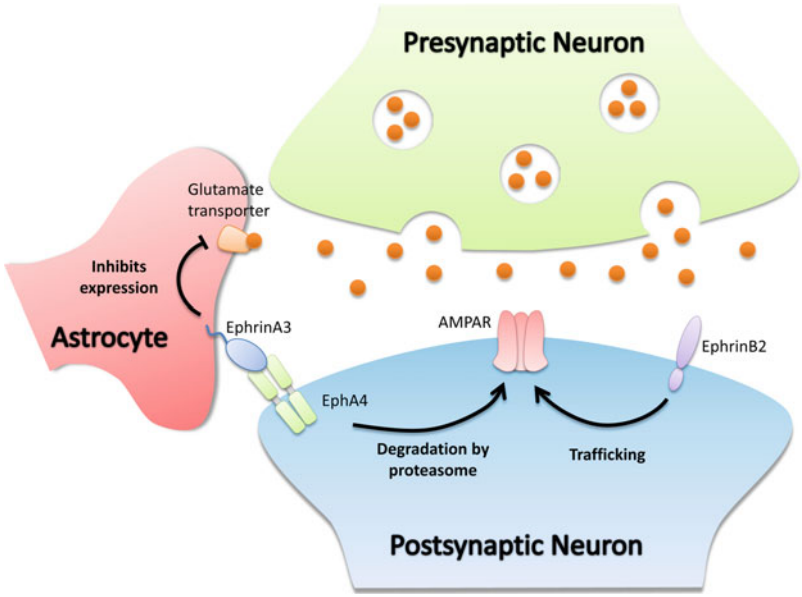
Both ephrinA and ephrinB regulate synapse formation and synaptic function, but their effects and the underlying mechanisms appear to be very different (Klein 2009; Lai and Ip 2009). The importance of EphB in synapse formation is illustrated by the observation that triple knockout mice lacking EphB1/B2/B3 neurons in the hippocampus fail to form dendritic spines, the tiny specialized dendritic protrusions where majority of excitatory neurotransmission takes place. EphrinB-EphB interaction promotes synapse formation by means of several mechanisms: (1) their high-affinity binding provides adhesion to stabilize the initial axon/dendrite contact during synapse formation; (2) both ephrinB and EphB receptor contain PDZ-binding motif at their C-termini that allows simultaneous clustering of PDZ domain-containing scaffold proteins at nascent pre- and postsynaptic specialization; (3) EphB forward signaling increases the motility of immature spines (filopodia), which in turn facilitates the axon-dendrite contact during synapse formation; (4) EphB enhances the function of NMDA receptor, the subtype of ionotropic ▶ glutamate receptor that mediates activity-dependent synapse formation during development; and (5) transsynaptic activation of ephrinB reverse signaling also promotes maturation of dendritic spines as well as formation of presynaptic termini.

In contrast to EphB forward signaling which promotes the formation and maturation of dendritic spines, activation of EphA forward signaling results in spine retraction and loss of functional synapses in cultured hippocampal neurons (Fu et al. 2007). It has been proposed that ephrinA3 localized on astrocytic processes that are in close proximity of dendritic spines activates EphA4 in the postsynaptic neuron to organize dendritic spines and prevent their unlimited growth (Murai et al. 2003). This was supported by the observations that hippocampal neurons in EphA4 knockout organotypic slices display disorganized dendritic spines. Activation of EphA4 forward signaling also leads to loss of functional synapses by triggering the polyubiquitination of AMPA receptors, the major subtype of glutamate receptors for excitatory neurotransmission. This results in degradation of AMPA receptors by the proteasome and subsequent reduction of glutamatergic neurotransmission (Fu et al. 2011).

Activity-dependent changes in synaptic strength (synaptic plasticity) form the cellular basis for learning and memory in adult animals. In particular, synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus is closely correlated with the formation of spatial memory. Ephrin/Eph interaction is required for synaptic plasticity of hippocampal neurons (Fig. 1). Studies with different transgenic mice have indicated that EphB2 and EphA4 are crucial for hippocampal LTP and LTD, but intriguingly, they regulate activity-dependent synaptic plasticity in a kinase-independent manner. This suggests a possible involvement of ephrinB reverse signaling, a notion that is supported by the findings that transgenic mice lacking the intracellular domain or tyrosine phosphorylation sites of ephirnB2 display defects in LTP and LTD (Grunwald et al. 2004). Indeed, ephrinB2 reverse signaling can directly regulate the trafficking of AMPA receptors, whose abundance is critically regulated during LTP and LTD. Another mechanism that underlies the regulation of synaptic transmission by ephrin/Eph involves glutamate reuptake by transporters expressed in glial cells. Activation of ephrinA3 in astrocytes by EphA4 in postsynaptic neurons controls the expression level of glial glutamate transporters. Notably, hyperexpression of glutamate transporters in EphA4 knockout hippocampus leads to LTP deficit that can be rescued by inhibition of glutamate transporter, indicating the essential role of EphA4 in synaptic plasticity by regulating glutamate reuptake (Filosa et al. 2009).

Adhesion Versus Repulsion: Modulation of Ephrin/Eph Interaction and Function

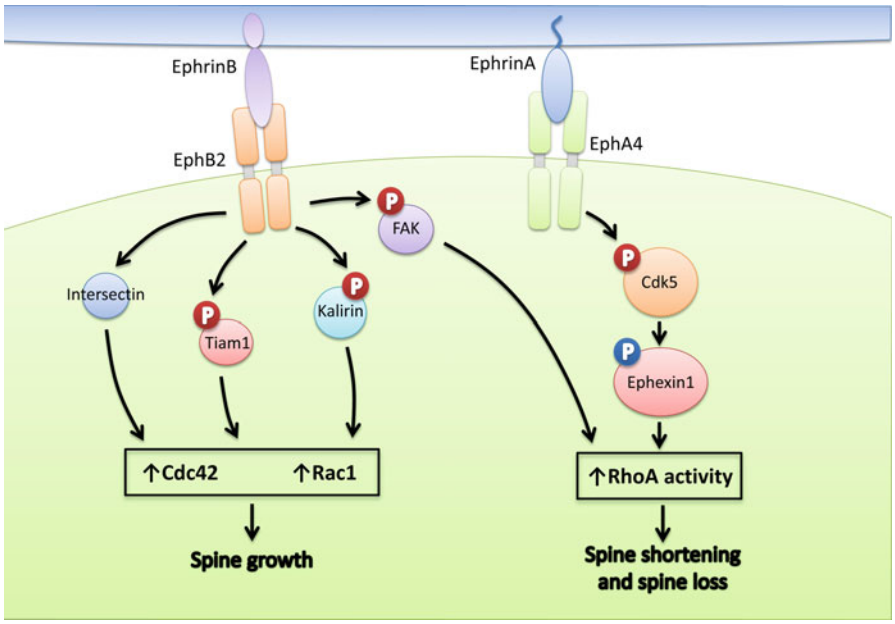
Most of the functions performed by ephrin/Eph within or outside the nervous system involve repulsive cell-cell



Eph Receptor, Fig. 1 Ephrin/Eph regulates synaptic transmission through multiple mechanisms. Excitatory neurotransmission is mediated by the neurotransmitter glutamate, which is released from presynaptic terminal and binds to the ionotropic AMPA receptor (*AMPAR*) that leads to membrane depolarization of the postsynaptic neuron via influx of Na^+. The abundance of AMPAR is regulated by (1) EphA4 forward signaling, which promotes degradation of the GluA1 subunit of AMPAR by the proteasome, and (2) ephrinB2 reverse signaling, which reduces internalization of AMPAR by increasing their interaction with synaptic scaffold protein such as GRIP. In addition, the reuptake of glutamate in the synaptic cleft is mediated by glutamate transporter, whose expression level is reduced by ephrinA3 reverse signaling in astrocytes

interaction (e.g., growth cone collapse, spine retraction, segmentation). However, ephrin/Eph interaction can also be adhesive in specific cellular context (e.g., synaptogenesis, endothelial cell attachment, and capillary assembly). One interesting topic on the study of ephrin/Eph signaling therefore involves understanding how the same family of proteins can generate both adhesive and repulsive signals in a context-dependent manner and how the initial high-affinity trans-interaction between ephrin/Eph on opposing cells switch to become repulsive. Two major mechanisms that modulate the ephrin/Eph interaction have been proposed to potentially explain the switch from adhesion to repulsion. One is internalization of the intact ephrin/Eph complex into their expressing cells by trans-endocytosis. Another mechanism involves extracellular cleavage of ephrin by the metalloprotease ADAM10/Kuzbanian. Both endocytosis and proteolytic cleavage are expected to terminate the interaction between ephrin and Eph receptor and hence allow cell retraction subsequent to the initial cell-cell contact.

Besides endocytosis and proteolytic cleavage, ephrin/Eph interaction can also be modulated by several other mechanisms that can fine-tune the adhesive and repulsive effect. For example, cis-binding between EphA3 and ephrinA5 expressed on the same cell can inhibit transactivation of the receptor by ephrinA5 expressed in neighboring cells, and this modulatory effect of ephrinA5 *cis*-binding is involved in retinotectal mapping. In addition, it has been shown that alternatively spliced isoforms of EphA7 that lack kinase domain can determine the outcome of adhesion and repulsion during neural tube formation in early development. Similarly, soluble forms of ephrin due to alternative splicing also exist, although it is not clear if they can compete with membrane-bound ephrin and inhibit the transactivation of Eph receptor. Taken together, ephrin/Eph cis-binding and the generation of inhibitory isoforms of ephrin/Eph by alternative splicing can potentially increase the diversity of interactions between ephrin and Eph receptor and hence fine-tune the adhesive and repulsive effect of ephrin. This property might be particularly advantageous for their function as positional cues (e.g., during topographic mapping) to guide cell position and connections in a highly precise manner.



Eph Receptor, Fig. 2 Regulation of actin cytoskeleton by Eph receptor signaling through Rho-GTPases. Stimulation of EphB receptor increases phosphorylation and hence activity of the Rac-GEFs TIAM1 and Kalirin, leading to Rac1 activation and formation of dendritic spines. EphB activation also promotes spine growth via the GEF intersectin and the corresponding GTPase Cdc42. On the other hand, activation of EphA4 phosphorylates and activates Cdk5, which then enhances activity of the Rho-GEF ephexin1, leading to spine loss through increased RhoA activity. EphB forward signaling also increases RhoA activity by FAK, which might be involved in shortening of dendritic spines during spine maturation

Signal Transduction of Eph Receptor

Regulation of Actin Cytoskeleton

Substantial evidence indicates that Eph receptor signaling is related to the regulation of actin cytoskeleton, which mostly involves modulating activities of the Rho family of small GTPases such as Rac1, RhoA, and Cdc42 (Fig. 2). Activation of Rac1 and Cdc42 leads to increased actin polymerization that underlies the growth and maturation of dendritic spines and increased motility of filopodia in response to EphB activation. Upon stimulation by ephrinB, tyrosine phosphorylation and clustering of the Rac1-specific guanine nucleotide exchange factors (GEF) Kalirin and Tiam1 are induced, which promote the exchange of GDP for GTP and thereby activate Rac1. The active Rac1 in turn acts on the serine/threonine kinase PAK in EphB-mediated spine and synapse formation. EphB2 also interacts with the Cdc42-specific GEF intersectin, which works together with ▶ N-WASP to promote spine formation. In addition to its effect on GEFs, EphB forward signaling also regulates activity of the Rho-GTPase RhoA through activation of focal adhesion kinase (FAK).

On the other hand, activation of EphA forward signaling leads to inhibition of Rac1 and activation of RhoA, both of which are central to the effect of ephrinA on growth cone collapse and dendritic spine loss. Activation of EphA4 reduces Rac1 activity via the GTPase-activating protein (GAP) α2-chimaerin, which is phosphorylated by EphA4 and becomes activated, leading to the increased GAP activity toward Rac1 and hence the reduction of Rac1 activity during EphA4-dependent growth cone collapse (Shi et al. 2007). In addition, stimulation by ephrinA1 triggers tyrosine phosphorylation and activation of the proline-directed serine/threonine kinase Cdk5, which then phosphorylates the RhoA-specific GEF ephexin1, leading to increased RhoA activity. The significance of Cdk5 and ephexin1 in mediating EphA4 signaling is verified by the observation that ephrinA1 stimulation fails to induce spine loss in neurons lacking Cdk5 or ephexin1 (Fu et al. 2007). EphA4 forward signaling also regulates actin cytoskeleton through phospholipase Cγ1, which

becomes activated after tyrosine phosphorylation and induces membrane dissociation of the actin depolymerization factor cofilin during EphA4-mediated loss of dendritic spines.

Cross Talk with Adhesion Molecules

Besides actin cytoskeleton, another major target of ephrin/Eph signaling is adhesion molecules. Cross talk between Eph signaling and adhesion molecules has been implicated in the control of cell migration, cell sorting, and spine morphology (Arvanitis and Davy 2008). Depending on cellular context and modes of stimulation, both enhancement and inhibition of integrin-mediated cell adhesion by ephrin/Eph signaling have been reported. Signaling molecules that link ephrin or Eph receptor activation to the integrin pathway have also been identified. For example, reverse signaling of ephrinA2 or ephrinA5 increases cell adhesion to laminin that requires Src tyrosine kinase. EphA8 also enhances integrin-mediated cell adhesion via phosphatidylinositol-3 kinase. Moreover, EphA4 interacts directly with integrin in platelets to enhance cell attachment. Intriguingly, another study showed that integrin signaling is inhibited upon EphA4 activation during dendritic spine remodeling in neurons and involves reduced phosphorylation of downstream targets of integrin signaling such as Crk-associated substrate (Cas) and the tyrosine kinases FAK and Pyk2.

Ephrin/Eph also regulates cell sorting through the adhesion molecules cadherin and claudins. EphB activation regulates membrane localization of E-cadherin, which is required for the compartmentalization of colorectal cancer cells by ephrinB1 expressed on the intestinal cells. On the other hand, claudins present at the tight junction interact directly with EphA2 and ephrinB1, and EphA2-mediated tyrosine phosphorylation of claudin-4 regulates its localization and affects paracellular permeability.

Cross Talk with NMDA Receptor and Regulation of AMPA Receptor Trafficking

Glutamate is the major neurotransmitter of excitatory neurons in the brain. There are multiple subtypes of glutamate receptors in neurons, including the ionotropic NMDA and AMPA receptors, as well as the G-protein-coupled metabotropic glutamate receptor. One major mechanism underlying how Ephrin/Eph regulates synapse formation and synaptic function involves regulating the function of NMDA receptor and localization of AMPA receptor. EphB receptor physically interacts with NMDA receptor via the extracellular domain, and this interaction is crucial to mediate the enhancing effect of ephrinB on synapse formation. Activation of EphB also facilitates the function of NMDA receptor, as indicated by the increased Ca^{2+} influx and resulting induction of gene expression. In addition, ephrinB reverse signaling triggers Src-mediated tyrosine phosphorylation of NMDA receptors during LTP of hippocampal neurons.

Ephrin/Eph signaling directly modulates neurotransmission by regulating the localization and trafficking of AMPA receptor. Exogenous expression of EphB2 induces clustering of AMPA receptors in primary cortical neurons, which requires the PDZ-binding motif in the carboxyl-terminus of EphB2. Furthermore, ephrinB2 reverse signaling also regulates AMPA receptors trafficking by two different mechanisms: (1) increasing AMPA receptor interaction with the synaptic scaffold protein GRIP and thus preventing their internalization and (2) counteracting the internalization of AMPA receptors by reducing Ser880 phosphorylation of GluR2 subunit of AMPA receptor.

Regulation of Protein Expression Via Transcription, Protein Synthesis, and Degradation

Emerging evidence suggests that ephrin/Eph also controls the abundance of specific proteins by regulating gene transcription, protein synthesis, and protein degradation. For example, both EphA forward and ephrinB reverse signaling can activate the transcription factor ▶ STAT3, which might have an implication in cancer progression (Lai et al. 2004). EphrinA also regulates the expression of acetylcholinesterase in muscle fibers, which hydrolyses the neurotransmitter acetylcholine at the neuromuscular junction. On the other hand, ephrinA-induced growth cone collapse involves inhibition of local translation of mRNA such as β-actin in the growth cone via phosphorylation of the GTPase-activating protein Tsc2, which leads to depression of the protein kinases ERK and ▶ mTOR (Nie et al. 2010). Finally, recent studies revealed that Eph receptor forward signaling activates specific ubiquitin ligases such as APC2/Cdh1 and Ube3A. This results in the ubiquitination and proteasome-dependent degradation of specific proteins such as AMPA receptor and the Rho-GEF ephexin5, which is essential for homeostatic plasticity and excitatory synapse formation, respectively (Fu et al. 2011; Margolis et al. 2010).

Future Perspectives

Since their identification in the early 1990s, there has been substantial interest in studying this unusual class of receptor tyrosine kinases (RTK) (Lackmann and Boyd 2008). As a result, significant progress has been made on elucidating their molecular properties and how they participate in different biological functions. The studies on ephrin/Eph have undoubtedly generated many surprises and greatly increased the horizon of our understanding of cell-cell communication in a wide range of biological systems. It is also becoming clear that our knowledge on ephrin/Eph interaction and signaling might prove to be useful in the design of therapeutic agents against various diseases. For example, abnormal expression of ephrin/Eph has been reported in different types of tumors. Given their capability of regulating cell adhesion, cell migration, and vascular formation, it is generally believed that ephrin/Eph plays important role in tumor formation and invasion. Their role in tumor formation, however, appears to be highly complicated: they can either promote or suppress tumorigenesis, depending on the types of tumor (Pasquale 2008). Nonetheless, inhibition of ephrin/Eph function might be useful for antiangiogenic therapies for cancer, and small molecules that inhibit Eph receptor kinase activity or ephrin-Eph interaction have been designed and shown to reduce tumor growth in animal models (Pasquale 2010).

In addition to tumor growth and malignancy, recent study also raises the exciting possibility of targeting EphB receptor in Alzheimer's disease therapy. EphB2 interacts with NMDA receptor and regulates its function in synapse formation and plasticity. It was recently reported that β-amyloid binds to the fibronectin-type III repeat of EphB2 and promotes its degradation by the proteasome, and EphB2 expression in the hippocampus is reduced in hAPP transgenic mice, an animal model of Alzheimer's disease. Remarkably, reversing EphB2 depletion by exogenous expression of EphB2 using lentivirus recues the synaptic and cognitive deficits of hAPP mice (Cisse et al. 2011). This study therefore suggests that small peptide that blocks the interaction between β-amyloid and EphB2 might represent a potential pharmaceutical agent for the disease.

References

Arvanitis D, Davy A. Eph/ephrin signaling: networks. Gene Dev. 2008;22:416–29.

Bartley TD, Hunt RW, Welcher AA, Boyle WJ, Parker VP, Lindberg RA, Lu HS, Colombero AM, Elliott RL, Guthrie BA, et al. B61 is a ligand for the ECK receptor protein-tyrosine kinase. Nature. 1994;368:558–60.

Cisse M, Halabisky B, Harris J, Devidze N, Dubal DB, Sun B, Orr A, Lotz G, Kim DH, Hamto P, et al. Reversing EphB2 depletion rescues cognitive functions in Alzheimer model. Nature. 2011;469:47–52.

Cowan CA, Henkemeyer M. Ephrins in reverse, park and drive. Trends Cell Biol. 2002;12:339–46.

Drescher U. Eph family functions from an evolutionary perspective. Curr Opin Genet Dev. 2002;12:397–402.

Eph Nomenclature Committee. Unified nomenclature for Eph family receptors and their ligands, the ephrins. Cell. 1997;90:403–4.

Filosa A, Paixao S, Honsek SD, Carmona MA, Becker L, Feddersen B, Gaitanos L, Rudhard Y, Schoepfer R, Klopstock T, et al. Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport. Nat Neurosci. 2009;12:1285–92.

Fu AK, Hung KW, Fu WY, Shen C, Chen Y, Xia J, Lai KO, IpNY.APC(Cdh1) mediates EphA4-dependent downregulation of AMPA receptors in homeostatic plasticity. Nat Neurosci. 2011;14:181–9.

Fu WY, Chen Y, Sahin M, Zhao XS, Shi L, Bikoff JB, Lai KO, Yung WH, Fu AK, Greenberg ME, Ip NY. Cdk5 regulates EphA4-mediated dendritic spine retraction through an ephexin1-dependent mechanism. Nat Neurosci. 2007;10:67–76.

Grunwald IC, Korte M, Adelmann G, Plueck A, Kullander K, Adams RH, Frotscher M, Bonhoeffer T, Klein R. Hippocampal plasticity requires postsynaptic ephrinBs. Nat Neurosci. 2004;7:33–40.

Hirai H, Maru Y, Hagiwara K, Nishida J, Takaku F. A novel putative tyrosine kinase receptor encoded by the eph gene. Science. 1987;238:1717–20.

Holzman LB, Marks RM, Dixit VM. A novel immediate-early response gene of endothelium is induced by cytokines and encodes a secreted protein. Mol Cell Biol. 1990;10:5830–8.

Klein R. Bidirectional modulation of synaptic functions by Eph/ephrin signaling. Nat Neurosci. 2009;12:15–20.

Lackmann M, Boyd AW. Eph, a protein family coming of age: more confusion, insight, or complexity? Sci Signal. 2008;1:re2.

Lai KO, Chen Y, Po HM, Lok KC, Gong K, Ip NY. Identification of the Jak/Stat proteins as novel downstream targets of EphA4 signaling in muscle: implications in the regulation of acetylcholinesterase expression. J Biol Chem. 2004;279:13383–92.

Lai KO, Ip NY. Synapse development and plasticity: roles of ephrin/Eph receptor signaling. Curr Opin Neurobiol. 2009;19:275–83.

Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP, Mardinly AR, Hu L, Greer PL, Bikoff JB, Ho HY, et al. EphB-mediated degradation of the RhoA

GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. Cell. 2010;143:442–55.
Murai KK, Nguyen LN, Irie F, Yamaguchi Y, Pasquale EB. Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling. Nat Neurosci. 2003;6:153–60.
Nie D, Di Nardo A, Han JM, Baharanyi H, Kramvis I, Huynh T, Dabora S, Codeluppi S, Pandolfi PP, Pasquale EB, Sahin M. Tsc2-Rheb signaling regulates EphA-mediated axon guidance. Nat Neurosci. 2010;13:163–72.
Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev. 2010;10:165–80.
Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell. 2008;133:38–52.
Shi L, Fu WY, Hung KW, Porchetta C, Hall C, Fu AK, Ip NY. Alpha2-chimaerin interacts with EphA4 and regulates EphA4-dependent growth cone collapse. Proc Natl Acad Sci USA. 2007;104:16347–52.

EphA3

▶ EphA3, Erythropoietin-Producing Hepatocellular Carcinoma Cell Receptor A3

EphA3, Erythropoietin-Producing Hepatocellular Carcinoma Cell Receptor A3

Renping Zhou
Department of Chemical Biology, Susan Lehman Cullman Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA

Synonyms

Cek4 (chicken); EphA3; Hek (human); Mek4 (mouse); Tyro4 (rat)

Historical Background

A fragment of EphA3 was first cloned in 1991 by Sajjadi et al., in an expression-cloning screen of a chicken embryonic cDNA library using antibodies against phosphotyrosine (Sajjadi et al. 1991). Using this cDNA fragment as a probe, full-length cDNA from both chicken and mouse embryos were identified (Sajjadi et al. 1991). Human EphA3 was independently isolated as a cell surface antigen of a pre-B-cell leukemia cell line (Boyd et al. 1992; Wicks et al. 1992). EphA3 is a member of a large family of tyrosine kinase receptors, which plays many critical roles in both physiological and pathological conditions (Pasquale 2008).

EphA3 Protein

The full-length human EphA3 protein consists of 983 amino acids, organized into the typical extracellular, transmembrane, and intracellular domains as that of other Eph family tyrosine kinase receptors (Wicks et al. 1992). A splice variant, which contains only the extracellular domain, was also identified in the mouse (Sajjadi et al. 1991). The relative abundance and difference in function between the full-length and truncated receptors have not been analyzed at present. Since the truncated form lacks intracellular signaling capacity but is capable of binding to Eph ligands (see the Ligands section for details), it may serve to down modulate EphA3 receptor function. Due to promiscuity of ligand–receptor interactions in the Eph family, it is possible that this truncated form may regulate functions of many other Eph receptors. Similar truncated form has been identified for TrkB, the receptor for the neurotrophic factor brain-derived neurotrophic factor, and has been shown to inhibit full-length TrkB receptor function in developing mouse brain (Carim-Todd et al. 2009).

EphA3 Expression

EphA3 is expressed both in the nervous system and in nonneural tissues. In the developing mouse brain, EphA3 expression is detectable as early as E12, but is undetectable after P10 (Kilpatrick et al. 1996; Kudo et al. 2005; Lai and Lemke 1991). Both mRNA and protein were detected in the developing cerebral cortex, ganglionic eminence, and presumptive caudate-putamen at E12. Expression was also found in the dorsal thalamus and in spinal motor neurons (Iwamasa et al. 1999; Kilpatrick et al. 1996; Kudo et al. 2005; Mackarehtschian et al. 1999). High levels of protein expression were found in the thalamocortical axons.

What is intriguing is that there is an extensive overlap with the adhesion molecule L1 expression, suggesting that EphA3 and L1 interact or at least regulate similar targets. Indeed it has been shown that EphA3 is physically associated with L1, and requires L1 protein for ephrin-A5-induced growth cone collapse (Demyanenko et al. 2011). It will be extremely interesting to examine how EphA3 and L1 interact to regulate axon guidance.

The most striking expression patterns of EphA3 were found in the developing chick and wallaby retina with a nasal (low) to temporal (high) gradient (Cheng et al. 1995; Stubbs et al. 2000). This gradient plays a critical role in the specification of retinotectal topographic axon projection map as discussed in the *Biological Functions* section. How this graded expression is regulated is not entirely clear at present. There is evidence suggesting that two homeobox transcription factors, SOHo1 and GH6, expressed in opposing gradients as that of EphA3 in the retina, inhibit EphA3 transcription, defining the expression in the temporal region (Schulte and Cepko 2000). Indeed, ecotopic expression of either transcription factor resulted in inhibition of EphA3 expression and a partial disruption of retinal axon guidance to the tectum. Two members of the winged-helix (WH) transcription factors, chick brain factor (CBF) 1 and 2, have also been shown to influence EphA3 expression in the chick retina, with CBF1 inhibiting and CBF2 inducing EphA3 expression (Takahashi et al. 2003; Takahashi et al. 2009). Extracellular signals have also been shown to regulate EphA3 expression. For example, the T-cell co-stimulatory signal CD28 and the type 1 insulin-like growth factor can enhance both EphA3 protein levels (Smith et al. 2004b). In contrast, interleukin-1β reduces the expression (Li et al. 1998). However, the regulation of EphA3 expression is incompletely understood, and it remains unclear how different signals coordinate the expression important for its biological functions.

EphA3 mRNA was also detected in a number of nonneural tissues during embryogenesis including axial muscles, heart, cartilage, and bones (Iwamasa et al. 1999; Kilpatrick et al. 1996).

Ligands and Mechanisms of Interaction with EphA3

Two ephrins, ephrin-A1 (initially named B61) and ephrin-A2 (Elf-1), were identified as ligands of EphA3 in 1994 using EphA3 extracellular domain fusion proteins as affinity probes in expression screening experiments (Beckmann et al. 1994; Cheng and Flanagan 1994). Two new ligands, ephrin-A3 and ephrin-A4, were identified by Kozlosky and colleagues using similar methods in the following year (Kozlosky et al. 1995). An additional ligand, ephrin-A5, was identified as a retinal axon guidance molecule (RAGS) (Drescher et al. 1995), and as an expressed sequence tag with homology to ephrin-B1 (Cerretti et al. 1995). Ephrin-A5 was also independently isolated by Lackmann and colleagues from the conditioned medium of human placenta (Lackmann et al. 1996). This ligand was found to be identical to AL-1, a ligand purified with affinity chromatography using the extracellular domain of the related receptor EphA5 from the conditioned medium of a human breast cancer cell line BT20 (Winslow et al. 1995). It is now known that the ligand–receptor interactions of the Eph family are highly promiscuous, and thus EphA3 receptor can interact with all the A-subclass ephrins. A careful study by Lackmann and colleagues in 1997 showed that while different bivalent ephrin-A-Fc fusion proteins interact with EphA3 with similar high affinity, the monomeric form of ephrin-A5 can form a much more stable complex with EphA3 than that of ephrin-A3 (Lackmann et al. 1997), suggesting that ephrin-A5 is the preferred high affinity ligand. Whether ephrins interact with Eph receptors in dimeric/oligomeric forms or monomeric form in vivo is not clear at present. Although nearly all functional assays reported have used multimeric forms of ephrins, monomeric ephrins may indeed have important functions in development or adult, since both ephrin-A1 and ephrin-A5 have been isolated initially from conditioned media as released monomeric form (Holzman et al. 1990; Lackmann et al. 1996; Winslow et al. 1995), it would not be surprising if novel functions were discovered for released monomer ephrins in the future.

The interaction of EphA3 with the ligand ephrin-A5 has been extensively analyzed (Lackmann et al. 1998; Smith et al. 2004a). The ligand-binding domain of EphA3 is located to the N-terminal region to the first IgG domain, encoded by exon III (Lackmann et al. 1998). This is consistent with findings in other Eph receptors (Himanen and Nikolov 2003; Labrador et al. 1997). Ligand–receptor interaction involves a high affinity and a low-affinity binding site. The former is responsible for ligand–receptor dimerization, and the

latter facilitates ligand–receptor tetramer formation (Himanen et al. 2001; Himanen and Nikolov 2003; Smith et al. 2004a). In addition, a ligand independent receptor dimerization domain has also been identified using both protein domain deletion and functional mutagenesis approaches in the cysteine-rich hinge region of the receptor (Lackmann et al. 1998; Smith et al. 2004a). Similar analysis also identified three domains in ephrin-A5 that play key roles in interaction with EphA3 receptor (Day et al. 2005). Two of the sites interact with the high- and low-affinity interaction domains identified in EphA3 receptor, and a third novel interface, which was proposed to bind to the cysteine-rich receptor domain of Eph receptors in adjacent ligand–receptor tetramers, allowing polymerization of large number of the ligand–receptor complexes and effective functional activation.

What is interesting about ephrin–Eph interaction is that simple ligand–receptor binding is not sufficient to fully activate the receptors (Pabbisetty et al. 2007; Stein et al. 1998; Vearing and Lackmann 2005). It has been shown that addition of monomeric ephrin-A5 failed to induce EphA3 kinase activation as assayed by autophosphorylation on tyrosine (Vearing et al. 2005). In contrast, dimeric ephrin-A5-Fc was found to be sufficient to induce EphA3 autophosphorylation. This difference is in part explained by the observation that monomeric ligands have a much lower binding affinity than the dimeric ligands (Pabbisetty et al. 2007). However, ephrin-A5 dimer-induced partial EphA3 activation is not enough to induce a cellular response as demonstrated by actin cytoskeleton contraction, which required additional receptor clustering (Vearing et al. 2005). Similar observations have been reported for ephrin-B1 (Stein et al. 1998). Correlated with the ability to affect cellular functions, only the fully activated Eph receptors are capable of recruiting downstream signaling molecules, although much remains uncharacterized (Stein et al. 1998; Vearing and Lackmann 2005). However, it is not known whether highly clustered ephrins are necessary to induce the full range of biological functions of all Eph receptors. For example, it has been reported that no differences were observed in EphA2 receptor autophosphorylation or endothelial cell migration by dimer or further cross-linked ephrin-A1-Fc (Brantley-Sieders et al. 2004). This issue is complicated by the possibility that different Eph receptors may respond differently to ephrin dimers. In addition, few studies have addressed this issue carefully since ephrin-Fc preparations may contain a mixture of dimers and larger aggregates. Adding to the complexity of ephrin–Eph interactions, it has been shown that interaction of ephrin-A5 and EphA3 in cis silences EphA3 receptor activation, possibly modulating concentration of actively signaling EphA3 receptor molecules (Carvalho et al. 2006).

Since both the EphA3 receptor and its ephrin ligands are membrane anchored, the question of how the ligand–receptor complex can be internalized by endocytosis becomes an interesting question. Elegant mutational and crystallographic studies by Nikolov and colleagues (Janes et al. 2005) showed that although the protease ADAM10 (A disintegrin and metalloprotease 10) is constitutively associated with EphA3, association of ephrin-A5 to EphA3 receptor creates a new binding site for ADAM10, and allows the cleavage of ephrin-A5 releasing the EphA3/ephrin-A5 complex for internalization. In addition, only ephrin-A5 ligand expressed in trans to ADAM10 can be cleaved after EphA3 binding. Such a mechanism ensures only ligands interacting with the receptor are cleaved.

Activation and Signal Transduction

Similar to other receptor tyrosine kinases, EphA3 is activated by ligand binding. Upon ligand binding, the two tyrosine residues located at the juxtamembrane segment of EphA3 become phosphorylated. Crystallographic analysis showed that the unphosphorylated juxtamembrane domain tyrosine residues interact with the active site to prevent adoption of a catalytically active confirmation of the catalytic site (Davis et al. 2008). Mutagenesis studies showed that mutation of Y596F but not Y602F leads to the loss of EphA3 kinase activity as well as the ability to inhibit cell migration and induce cellular process and neuronal growth cone retraction (Davis et al. 2008; Hu et al. 2009; Shi et al. 2010). In contrast, restoration of the negative charge of phospho-Y596 with an Y596E mutation restores EphA3 kinase activity, suggesting that phosphorylation of Y596 is necessary to release inhibition of EphA3 kinase activity. The activation mechanism of EphA3 kinase is likely to be complex and involve many other amino acid residues in both the juxtamembrane domain and the kinase domain. Significantly, both Y742 and S768 also contribute to the regulation of EphA3 kinase activity (Davis et al. 2008).

Although phospho-Y602 is not required for EphA3 kinase activity, it is necessary for its biological functions as measured with either morphological changes (membrane blebbing and cellular process retraction) or cell migration (Hu et al. 2009; Lawrenson et al. 2002; Shi et al. 2010). In fact, Y779 phosphorylation is also important for these biological activities. Both Y602F and Y779F mutations cause a partial loss of biological activity, while the double mutant Y602/779F resulted in a complete loss of biological activity while maintaining its kinase activity (Shi et al. 2010). These observations are consistent with the notion that phosphorylated Y602 and Y779 serve as docking sites for downstream signaling molecules that mediate EphA3 biological effects, and that different tyrosine residues together with their respective signaling pathways collaborate to achieve full biological activity for the receptor.

Downstream of the receptor, activation of EphA3 in T cells by ephrin-A1 results in tyrosine phosphorylation of c-cbl proto-oncogene (Sharfe et al. 2003). The process is dependent on the ▶ Src family kinases, indicating that Src mediates effects of EphA3 activation on c-cbl phosphorylation. c-cbl may serve as a regulator of receptor ubiquitination and degradation, thus downregulating EphA3 protein levels upon activation by ephrins (Sharfe et al. 2003).

It has also been shown that the adaptor protein Nck binds to the phosphotyrosine residue Y602 through its Src Homology domain 2 (SH2), and blocking Nck signaling also reduces EphA3-mediated inhibition of cell migration and process retraction (Hu et al. 2009). Nck is known to interact with multiple signals that regulate cytoskeleton dynamics, including p21-activated kinase (▶ PAK1), and the Wiskott–Aldrich syndrome family proteins that control actin reorganization (Li et al. 2001).

Biological Functions

EphA3 is widely expressed in both neural and nonneural tissues during development and plays multiple roles during embryogenesis.

Substrate detachment and adhesion. Binding of EphA3 by clustered ephrin-A5 has been shown to induce detachment and rounding of HEK293 cells and melanoma cells, but promote adhesion of pre-B and T-cell leukemic cells (Lawrenson et al. 2002; Wimmer-Kleikamp et al. 2008). The opposing effects in these two different types of cells are due to the presence of a protein tyrosine phosphatase activity in the leukemic cells, which prevents autophosphorylation of EphA3. The emerging model is that when EphA3 is fully activated and autophosphorylated on the tyrosine residues, it triggers the activation of Rho-GTPase and leads to cell detachment and repulsion (Lawrenson et al. 2002). In contrast, when receptor activity is down modulated by phosphatase activity, interactions between EphA3 and its ligands lead to cell adhesion. Although the identity of the phosphatase that dephosphorylates EphA3 in pre-B leukemia cells has yet to be identified, evidence indicates that PTP1B may play a similar role in EphA3 dephosphorylation in HEK293 cells and in glioblastoma cells by interacting with the activated receptor at the plasma membrane as well as in endosomal vesicles (Nievergall et al. 2010).

Axon guidance. The earliest hint for a critical function of EphA3 and its ligands in embryonic development came when ephrin-A5 was found to be the repulsive axon guidance signal (RAGS) responsible for generating the topographic retinal axon termination map in the optical tectum in the brain (Cheng et al. 1995; Drescher et al. 1995). The landmark study by Drescher and colleagues, together with the studies by Cheng and colleagues, which demonstrated the expression of EphA3 and ephrin-A2, another ligand, are expressed in opposing gradients in the projecting retina and the target tectum, unraveled an age-old mystery of how axon terminals are topographically mapped onto target brain tissues. Further studies demonstrated that ephrin-A2 and ephrin-A5 together serve as repulsive axon guidance signals for the formation of the retinotectal project map (Ciossek et al. 1998; Feldheim et al. 2000), and that EphA3 as well as other EphA receptors mediate the effects of these ephrins (Brown et al. 2000; Feldheim et al. 2004). Further studies now show that ephrin–Eph interactions play a general role in the specification of topographic maps in several other axon pathways including hippocamposeptal projections (Yue et al. 2002), thalamocortical projections (Dufour et al. 2003; Uziel et al. 2002, 2006), the dopaminergic pathways (Cooper et al. 2008; Passante et al. 2008; Sieber et al. 2004; Yue et al. 1999) and possibly throughout the nervous system. In addition to guiding axons, EphA3 has been shown to play a role in the segregation of axial motor and sensory axon tracks during development through interaction with ephrin-A–EphA transaxonal interaction (Gallarda et al. 2008).

Heart development. EphA3 plays critical roles in development of extraneuronal tissues as well during embryogenesis. One clearly defined function is heart development (Stephen et al. 2007). Inactivation of EphA3 resulted in perinatal heart failure and death in about 75% of newborn mice. Postmortem examinations showed that the EphA3-null mice had enlarged atria and presence of blood in the lung and liver due to capillary disruption caused by exceedingly high cardiac filling pressure. The EphA3-deficient heart showed defects in the atrioventricular valves and in the atrioventricular septum, which separates the right atrium from the left ventricle. Analysis of EphA3 expression in early embryos revealed that the receptor is transcribed in the developing atrioventricular and outflow tract endocardial cushions at E10.5, and in the mesenchymal cap of the developing septum primum at E12.5. The endocardial cushions later generate the atrioventricular valves and septa. A ligand, ephrin-A1, was found to be expressed in the neighboring cells. In the EphA3-null embryos, the endocardial cushions appear to be smaller, possibly due to cell migration defects from an altered cytoskeletal structure of these cells. Consistent with ephrin-A1 expression in the developing heart, deletion of the gene also results in heart defects (Frieden et al. 2010). However, ephrin-A1 KO hearts showed thickened aortic and mitral valves and elevated mesenchymal marker expression, a different phenotype than that of EphA3-null mice, suggesting distinct functions. These observations, together with results from studies in the nervous system, indicate that EphA3 receptor and the ephrin-A ligands, may participate in the development and function of many different tissues and organs during embryogenesis and in adult.

Cancer. In addition to functions in normal animals, EphA3 mutations have been associated with tumor formation. EphA3 has been identified as a melanoma tumor antigen, and is overexpressed in several other tumors including lung cancer, kidney tumors, and brain tumors (Chiari et al. 2000). EphA3 mutations have been identified in primary lung and colon cancer in tumor genome sequencing projects (Wood et al. 2006; Ding et al. 2008). Mutational profiling of kinases in cancer also identified an EphA3 mutation in the cys-rich extracellular domain involved in ligand binding and receptor tetramerization in human pancreatic cancer (Corbo et al. 2010). EphA3 mutations have also been reported in glioblastoma, melanoma, and liver tumor samples (Bae et al. 2009; Balakrishnan et al. 2007). Mutations were identified in both the extracellular and kinase domains, with no hotspot preferences. Although the exact nature of these mutations are currently unknown, it has also been shown that EphA3 is among genes mutated or lost in the loss of heterozygocity in head and neck squamous cell carcinoma suggesting that this receptor serves as a tumor suppressor (Lee et al. 2010).

Summary

EphA3, a tyrosine kinase receptor of the Eph family, plays critical roles in the regulation of both physiological and pathological processes. The receptor is required for axon guidance to proper targets in the nervous system, and for heart development. EphA3 mutations have been detected in many different types of tumors. Since EphA3 expression is found in many different tissues during embryonic development, it is within reason that new functions will be attributed to this receptor in the future. However, understanding of EphA3 function and how it conveys the signals in the cells are at their infant stage. For example, the role EphA3 plays in carcinogenesis remains undefined, since both overexpression and mutations have been found in tumors. In addition, downstream signaling pathways for EphA3 are poorly understood at present. It is not clear whether EphA3 uses similar signaling strategies as that of other Eph receptors or uses unique pathways. Furthermore, roles of the truncated receptor in development and disease remain to be elucidated.

References

Bae HJ, Song JH, Noh JH, Kim JK, Jung KH, Eun JW, et al. Low frequency mutation of the Ephrin receptor A3 gene in hepatocellular carcinoma. Neoplasma. 2009;56:331–4.

Balakrishnan A, Bleeker FE, Lamba S, Rodolfo M, Daniotti M, Scarpa A, et al. Novel somatic and germline mutations in cancer candidate genes in glioblastoma, melanoma, and pancreatic carcinoma. Cancer Res. 2007;67:3545–50.

Beckmann MP, Cerretti DP, Baum P, Vanden Bos T, James L, Farrah T, et al. Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors. EMBO J. 1994;13:3757–62.

Boyd AW, Ward LD, Wicks IP, Simpson RJ, Salvaris E, Wilks A, et al. Isolation and characterization of a novel receptor-type protein tyrosine kinase (hek) from a human pre-B cell line. J Biol Chem. 1992;267:3262–7.

Brantley-Sieders DM, Caughron J, Hicks D, Pozzi A, Ruiz JC, Chen J. EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation. J Cell Sci. 2004;117:2037–49.

Brown A, Yates PA, Burrola P, Ortuno D, Vaidya A, Jessell TM, et al. Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling. Cell. 2000;102:77–88.

Carim-Todd L, Bath KG, Fulgenzi G, Yanpallewar S, Jing D, Barrick CA, et al. Endogenous truncated TrkB.T1 receptor regulates neuronal complexity and TrkB kinase receptor function in vivo. J Neurosci. 2009;29:678–85.

Carvalho RF, Beutler M, Marler KJ, Knoll B, Becker-Barroso E, Heintzmann R, et al. Silencing of EphA3 through a cis interaction with ephrinA5. Nat Neurosci. 2006;9:322–30.

Cerretti DP, Vanden Bos T, Nelson N, Kozlosky CJ, Reddy P, Maraskovsky E, et al. Isolation of LERK-5: a ligand of the eph-related receptor tyrosine kinases. Mol Immunol. 1995; 32:1197–205.

Cheng HJ, Flanagan JG. Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. Cell. 1994;79:157–68.

Cheng HJ, Nakamoto M, Bergemann AD, Flanagan JG. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell. 1995;82:371–81.

Chiari R, Hames G, Stroobant V, Texier C, Maillere B, Boon T, et al. Identification of a tumor-specific shared antigen derived from an Eph receptor and presented to CD4 T cells on HLA class II molecules. Cancer Res. 2000;60:4855–63.

Ciossek T, Monschau B, Kremoser C, Loschinger J, Lang S, Muller BK, et al. Eph receptor-ligand interactions are necessary for guidance of retinal ganglion cell axons in vitro. Eur J Neurosci. 1998;10:1574–80.

Cooper MA, Son AI, Komlos D, Sun Y, Kleiman NJ, Zhou R. Loss of ephrin-A5 function disrupts lens fiber cell packing and leads to cataract. Proc Natl Acad Sci USA. 2008;105: 16620–5.

Corbo V, Ritelli R, Barbi S, Funel N, Campani D, Bardelli A, et al. Mutational profiling of kinases in human tumours of pancreatic origin identifies candidate cancer genes in ductal and ampulla of vater carcinomas. PLoS One. 2010;5: e12653.

Davis TL, Walker JR, Loppnau P, Butler-Cole C, Allali-Hassani A, Dhe-Paganon S. Autoregulation by the juxtamembrane region of the human ephrin receptor tyrosine kinase A3 (EphA3). Structure. 2008;16:873–84.

Day B, To C, Himanen JP, Smith FM, Nikolov DB, Boyd AW, et al. Three distinct molecular surfaces in ephrin-A5 are essential for a functional interaction with EphA3. J Biol Chem. 2005;280:26526–32.

Demyanenko GP, Siesser PF, Wright AG, Brennaman LH, Bartsch U, Schachner M, et al. L1 and CHL1 cooperate in thalamocortical axon targeting. Cereb Cortex. 2011;21: 401–12.

Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature. 2008;455:1069–75.

Drescher U, Kremoser C, Handwerker C, Loschinger J, Noda M, Bonhoeffer F. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell. 1995;82:359–70.

Dufour A, Seibt J, Passante L, Depaepe V, Ciossek T, Frisen J, et al. Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes. Neuron. 2003;39:453–65.

Feldheim DA, Kim YI, Bergemann AD, Frisen J, Barbacid M, Flanagan JG. Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron. 2000;25:563–74.

Feldheim DA, Nakamoto M, Osterfield M, Gale NW, DeChiara TM, Rohatgi R, et al. Loss-of-function analysis of EphA receptors in retinotectal mapping. J Neurosci. 2004;24:2542–50.

Frieden LA, Townsend TA, Vaught DB, Delaughter DM, Hwang Y, Barnett JV, et al. Regulation of heart valve morphogenesis by Eph receptor ligand, ephrin-A1. Dev Dyn. 2010;239:3226–34.

Gallarda BW, Bonanomi D, Muller D, Brown A, Alaynick WA, Andrews SE, et al. Segregation of axial motor and sensory pathways via heterotypic trans-axonal signaling. Science (New York NY). 2008;320:233–6.

Himanen JP, Nikolov DB. Eph signaling: a structural view. Trends Neurosci. 2003;26:46–51.

Himanen JP, Rajashankar KR, Lackmann M, Cowan CA, Henkemeyer M, Nikolov DB. Crystal structure of an Eph receptor-ephrin complex. Nature. 2001;414:933–8.

Holzman LB, Marks RM, Dixit VM. A novel immediate-early response gene of endothelium is induced by cytokines and encodes a secreted protein. Mol Cell Biol. 1990;10:5830–8.

Hu T, Shi G, Larose L, Rivera GM, Mayer BJ, Zhou R. Regulation of process retraction and cell migration by EphA3 is mediated by the adaptor protein Nck1. Biochemistry. 2009;48:6369–78.

Iwamasa H, Ohta K, Yamada T, Ushijima K, Terasaki H, Tanaka H. Expression of Eph receptor tyrosine kinases and their ligands in chick embryonic motor neurons and hindlimb muscles. Dev Growth Differ. 1999;41:685–98.

Janes PW, Saha N, Barton WA, Kolev MV, Wimmer-Kleikamp SH, Nievergall E, et al. Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell. 2005;123:291–304.

Kilpatrick TJ, Brown A, Lai C, Gassmann M, Goulding M, Lemke G. Expression of the Tyro4/Mek4/Cek4 gene specifically marks a subset of embryonic motor neurons and their muscle targets. Mol Cell Neurosci. 1996;7:62–74.

Kozlosky CJ, Maraskovsky E, McGrew JT, VandenBos T, Teepe M, Lyman SD, et al. Ligands for the receptor tyrosine kinases hek and elk: isolation of cDNAs encoding a family of proteins. Oncogene. 1995;10:299–306.

Kudo C, Ajioka I, Hirata Y, Nakajima K. Expression profiles of EphA3 at both the RNA and protein level in the developing mammalian forebrain. J Comp Neurol. 2005;487:255–69.

Labrador JP, Brambilla R, Klein R. The N-terminal globular domain of Eph receptors is sufficient for ligand binding and receptor signaling. EMBO J. 1997;16:3889–97.

Lackmann M, Bucci T, Mann RJ, Kravets LA, Viney E, Smith F, et al. Purification of a ligand for the EPH-like receptor HEK using a biosensor-based affinity detection approach. Proc Natl Acad Sci USA. 1996;93:2523–7.

Lackmann M, Mann RJ, Kravets L, Smith FM, Bucci TA, Maxwell KF, et al. Ligand for EPH-related kinase (LERK) 7 is the preferred high affinity ligand for the HEK receptor. J Biol Chem. 1997;272:16521–30.

Lackmann M, Oates AC, Dottori M, Smith FM, Do C, Power M, et al. Distinct subdomains of the EphA3 receptor mediate ligand binding and receptor dimerization. J Biol Chem. 1998;273:20228–37.

Lai C, Lemke G. An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. Neuron. 1991;6:691–704.

Lawrenson ID, Wimmer-Kleikamp SH, Lock P, Schoenwaelder SM, Down M, Boyd AW, et al. Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3-expressing 293 T and melanoma cells by CrkII and Rho-mediated signalling. J Cell Sci. 2002;115:1059–72.

Lee DJ, Schonleben F, Banuchi VE, Qiu W, Close LG, Assaad AM, et al. Multiple tumor-suppressor genes on chromosome 3p contribute to head and neck squamous cell carcinoma tumorigenesis. Cancer Biol Ther. 2010;10:689–93.

Li YY, McTiernan CF, Feldman AM. IL-1 beta alters the expression of the receptor tyrosine kinase gene r-EphA3 in neonatal rat cardiomyocytes. Am J Physiol. 1998;274:H331–41.

Li W, Fan J, Woodley DT. Nck/Dock: an adapter between cell surface receptors and the actin cytoskeleton. Oncogene. 2001;20:6403–17.

Mackarehtschian K, Lau CK, Caras I, McConnell SK. Regional differences in the developing cerebral cortex revealed by ephrin-A5 expression. Cereb Cortex. 1999;9:601–10.

Nievergall E, Janes PW, Stegmayer C, Vail ME, Haj FG, Teng SW, et al. PTP1B regulates Eph receptor function and trafficking. J Cell Biol. 2010;191:1189–203.

Pabbisetty KB, Yue X, Li C, Himanen JP, Zhou R, Nikolov DB, et al. Kinetic analysis of the binding of monomeric and dimeric ephrins to Eph receptors: correlation to function in a growth cone collapse assay. Protein Sci. 2007;16:355–61.

Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell. 2008;133:38–52.

Passante L, Gaspard N, Degraeve M, Frisen J, Kullander K, De Maertelaer V, et al. Temporal regulation of ephrin/Eph signalling is required for the spatial patterning of the mammalian striatum. Development(Cambridge, England). 2008;135:3281–90.

Sajjadi FG, Pasquale EB, Subramani S. Identification of a new eph-related receptor tyrosine kinase gene from mouse and chicken that is developmentally regulated and encodes at least two forms of the receptor. New Biol. 1991;3:769–78.

Schulte D, Cepko CL. Two homeobox genes define the domain of EphA3 expression in the developing chick retina. Development (Cambridge, England). 2000;127:5033–45.

Sharfe N, Freywald A, Toro A, Roifman CM. Ephrin-A1 induces c-Cbl phosphorylation and EphA receptor down-regulation in T cells. J Immunol. 2003;170:6024–32.

Shi G, Yue G, Zhou R. EphA3 functions are regulated by collaborating phosphotyrosine residues. Cell Res. 2010;20:1263–75.

Sieber BA, Kuzmin A, Canals JM, Danielsson A, Paratcha G, Arenas E, et al. Disruption of EphA/ephrin-a signaling in the nigrostriatal system reduces dopaminergic innervation and dissociates behavioral responses to amphetamine and cocaine. Mol Cell Neurosci. 2004;26:418–28.

Smith FM, Vearing C, Lackmann M, Treutlein H, Himanen J, Chen K, et al. Dissecting the EphA3/Ephrin-A5 interactions using a novel functional mutagenesis screen. J Biol Chem. 2004a;279:9522–31.

Smith LM, Walsh PT, Rudiger T, Cotter TG, Mc Carthy TV, Marx A, et al. EphA3 is induced by CD28 and IGF-1 and regulates cell adhesion. Exp Cell Res. 2004b;292:295–303.

Stein E, Lane AA, Cerretti DP, Schoecklmann HO, Schroff AD, Van Etten RL, et al. Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. Genes Dev. 1998;12:667–78.

Stephen LJ, Fawkes AL, Verhoeve A, Lemke G, Brown A. A critical role for the EphA3 receptor tyrosine kinase in heart development. Dev Biol. 2007;302:66–79.

Stubbs J, Palmer A, Vidovic M, Marotte LR. Graded expression of EphA3 in the retina and ephrin-A2 in the superior colliculus during initial development of coarse topography in the wallaby retinocollicular projection. Eur J Neurosci. 2000;12:3626–36.

Takahashi H, Shintani T, Sakuta H, Noda M. CBF1 controls the retinotectal topographical map along the anteroposterior axis through multiple mechanisms. Development (Cambridge, England). 2003;130:5203–15.

Takahashi H, Sakuta H, Shintani T, Noda M. Functional mode of FoxD1/CBF2 for the establishment of temporal retinal specificity in the developing chick retina. Dev Biol. 2009;331:300–10.

Uziel D, Muhlfriedel S, Zarbalis K, Wurst W, Levitt P, Bolz J. Miswiring of limbic thalamocortical projections in the absence of ephrin-A5. J Neurosci. 2002;22:9352–7.

Uziel D, Garcez P, Lent R, Peuckert C, Niehage R, Weth F, et al. Connecting thalamus and cortex: the role of ephrins. Anat Rec. 2006;288:135–42.

Vearing CJ, Lackmann M. Eph receptor signalling; dimerisation just isn't enough. Growth factors (Chur, Switzerland). 2005;23:67–76.

Vearing C, Lee FT, Wimmer-Kleikamp S, Spirkoska V, To C, Stylianou C, et al. Concurrent binding of anti-EphA3 antibody and ephrin-A5 amplifies EphA3 signaling and downstream responses: potential as EphA3-specific tumor-targeting reagents. Cancer Res. 2005;65:6745–54.

Wicks IP, Wilkinson D, Salvaris E, Boyd AW. Molecular cloning of HEK, the gene encoding a receptor tyrosine kinase expressed by human lymphoid tumor cell lines. Proc Natl Acad Sci USA. 1992;89:1611–5.

Wimmer-Kleikamp SH, Nievergall E, Gegenbauer K, Adikari S, Mansour M, Yeadon T, et al. Elevated protein tyrosine phosphatase activity provokes Eph/ephrin-facilitated adhesion of pre-B leukemia cells. Blood. 2008;112:721–32.

Winslow JW, Moran P, Valverde J, Shih A, Yuan JQ, Wong SC, et al. Cloning of AL-1, a ligand for an Eph-related tyrosine kinase receptor involved in axon bundle formation. Neuron. 1995;14:973–81.

Wood LD, Calhoun ES, Silliman N, Ptak J, Szabo S, Powell SM, et al. Somatic mutations of GUCY2F, EPHA3, and NTRK3 in human cancers. Hum Mutat. 2006;27:1060–1.

Yue Y, Widmer DA, Halladay AK, Cerretti DP, Wagner GC, Dreyer JL, et al. Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. J Neurosci. 1999;19:2090–101.

Yue Y, Chen ZY, Gale NW, Blair-Flynn J, Hu TJ, Yue X, et al. Mistargeting hippocampal axons by expression of a truncated Eph receptor. Proc Natl Acad Sci USA. 2002;99:10777–82.

Epidermal Growth Factor Receptor Kinase Substrate 8

▶ Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8)

Epidermal Growth Factor Receptor Pathway Substrate 8

▶ Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8)

Epidermal Surface Antigen (ESA)

▶ Flotillin-2 (FLOT2)

Epithelial to Mesenchymal Transition

▶ Glycogen Synthase Kinase-3

EPM2A

▶ Laforin: Function and Action of a Glucan Phosphatase

Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8)

Francesca Milanesi[1], Niels Volkmann[2], Giorgio Scita[1,3] and Dorit Hanein[2]
[1]IFOM, Milan, Italy
[2]Bioinformatics and Systems Biology Program, Sanford-Burnham Medical Research Institute, La Jolla, CA, USA
[3]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Universita' degli Studi di Milano, Milan, Italy

Synonyms

Epidermal growth factor receptor kinase substrate 8; Epidermal growth factor receptor pathway substrate 8

Historical Background

Eps8 (EGFR pathway substrate #8) is an actin-binding and signaling molecule with a molecular weight of 97KDa encoded by a gene comprising 21 exons located on the human chromosome 12p12. It was originally identified through an expression cloning approach designed to isolate intracellular substrates for the tyrosine kinase of the Epidermal Growth Factor Receptor (EGFR) (Fazioli et al. 1993). Eps8 is efficiently phosphorylated on tyrosine residues by a variety of both receptor and non-receptor tyrosine kinases (Fazioli et al. 1993). Following stimulation with neurotrophic factor BDNF, which critically controls growth and differentiation processes in the brain during development, through the activation of Trk tyrosine kinase receptors, Eps8 can also be phosphorylated, in a MAPK-dependent manner, on serine and threonine residues. Notably, while the physiological and functional implication of Eps8 tyrosine phosphorylation remains to be identified, BDNF induced, MAPK-dependent phosphorylation of S624 and T628 of mouse Eps8 are critical for regulation of its localization and functions (Menna et al. 2009) (see below).

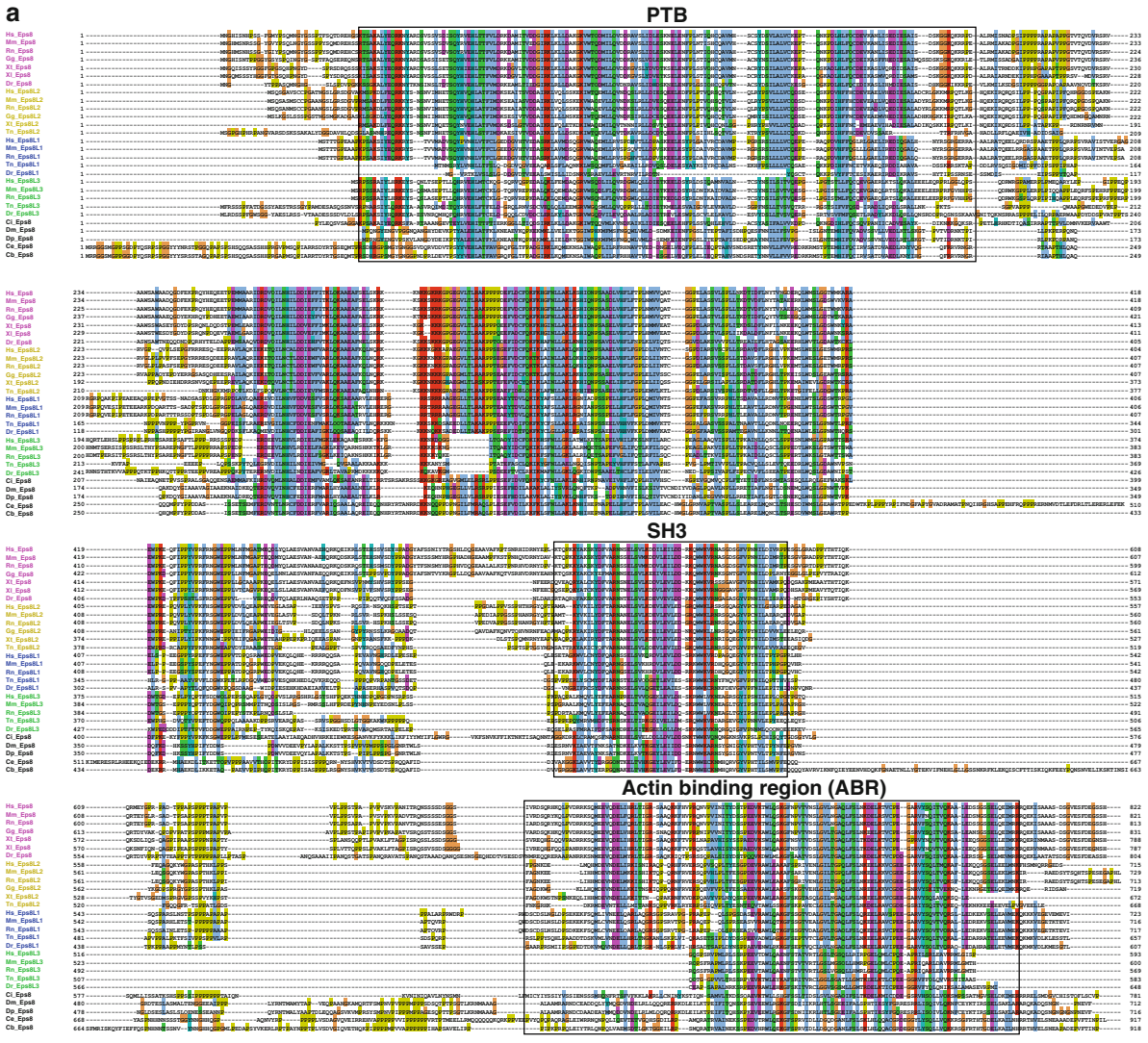
Eps8 is the founding member of a family of eps8-related proteins, comprising three additional gene products in vertebrates, named eps8L1, eps8L2, and eps8L3 (Fig. 1a-b). All Eps8Ls display collinear topology and 27–42% identity to Eps8, sharing a similar modular organization into distinct structural and functional domains that comprise an N-terminal Phosphotyrosine Binding domain (PTB), a central Src homology-3 domain (SH3), two Proline rich regions, and a C-terminal "effector actin-binding region" (ABR) that mediates Eps8 interactions with actin filaments (Offenhauser et al. 2004). EPS8-like orthologues are also present in nonmammalian species (Fig. 1a-b). In *Drosophila melanogaster* there are two like genes called *arouser* and LP01469p. A single *eps8* gene is, instead, present in *Caernorabditis elegans* that gives rise to two splicing isoform *eps-8A* and *eps-8b*, differing at their carboxyl termini. While the function of *eps8* in the fly is unknown, in the nematode, *eps-8* is essential for embryonic development (Croce et al. 2004). Furthermore, EPS-8A, but not EPS-8B protein, is specifically required for proper morphogenesis of intestinal cells. This latter phenotype could be precisely correlated with the evolutionarily conserved actin-binding

activity, which is present in the C terminus of the EPS-8A isoform, providing genetic evidence that the actin-related properties of EPS-8 are critical for its function in the whole organism.

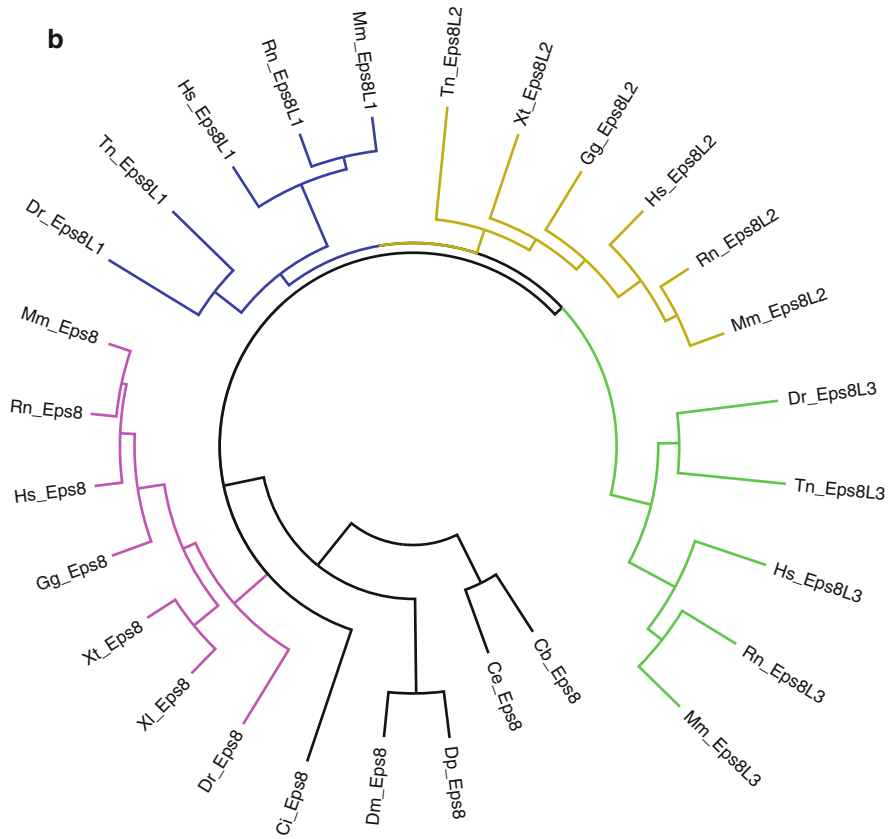
Domain Organization and Interacting Partners

The modular organization of Eps8 and its family members supports the notion that Eps8L family proteins generally act as adaptors for the assembly of macromolecular complexes required for the transduction of signals from receptor tyrosine kinases (RTKs) leading to regulation of actin remodeling and cell migration (Fig. 1a). This section will describe what is currently known about the PTB and SH3 domain of Eps8, while more detailed information on the effector ABR will be provided in the section entitled "Eps8 and Actin Dynamics."

Among the distinctive protein:protein interaction domains, the least functionally characterized is the N-terminal PTB domain. Originally, PTB domains were identified as protein–protein interaction modules, binding to a variety of NPXY containing motifs with high affinity when the tyrosine residue is



Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8), Fig. 1 (continued)



Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8), Fig. 1 The C-terminal region of EPS8 is highly conserved among metazoan homologues. (**a**) Multiple sequence alignment of EPS8 homologues. Protein sequences were identified by BLAST searches against all known protein sequences at NCBI or predicted from the genomic sequences of the specific organism using the GeneWise software (http://www.ebi.ac.uk/Wise2/). Sequences were aligned using the ClustalW program and the picture was produced using Jalview. Domains are boxed and indicated. PTB, phosphotyrosine binding domain; SH3, Src homology domain 3; ABR, Actin-binding region, EPS8 possesses a barbed end capping activity encoded by its evolutionarily conserved, C-terminal region (residues 648–821 for murine EPS8) (Fig. 1a). This region of EPS8 displays no sequence similarity with other known capping proteins or actin-binding motifs, but displays a similarity (similarity, E = 60.8; confidence E = 2.4) with the profile of the SAM Pointed (SAM_PNT) domain (Fig. 1a). SAM_PNT domains, which belong to the larger family of sterile alpha motif (SAM) (Slupsky et al. 1998), are present in a subset of the ETS-like transcription factors, including mammalian Ets1, Ets2, Erg, Fli1, GABPa, and Tel, and *Drosophila* Pnt and Yan (Slupsky et al. 1998). The C-terminal portion of EPS8 and its family members can be aligned with known SAM_PNT domain sequences, and its predicted secondary structure is superimposable to that determined for Ets1 (not shown). (**b**) Phylogenetic tree was produced with ClustalW and illustrated with FigTree (software by Andrew Rambaut, http://evolve.zoo.ox.ac.uk/software.html/id=figtree). GenBank accession numbers for each protein or coordinates of the genomic sequences from where they were reconstructed are provided in Table 1

phosphorylated, such as in response to RTK stimulation. This latter feature, however, is not shared by the majority of PTB domains that bind NPXY motifs irrespective of ligand phosphorylation or preferentially recognize unphosphorylated ligands, such as the PTB of the endocytic adaptor NUMB (Forman-Kay and Pawson 1999). The high structural homology between Eps8 and the latter PTB domain suggests that also Eps8 PTB may preferentially recognize unphosporylated NPXY motifs (Smith et al. 2006). In vitro evidence in this direction has, indeed, been provided; however, the lack of physiological validation of any of the putative binding partners of Eps8 PTB domain has prevented not only to validate the mode of binding of Eps8 PTB domain, but also to elucidate the functional consequence of this interaction module.

A canonical SH3 fold consists of two antiparallel beta sheets packed against each other at an approximate right angle. The first crystal structure of the SH3 domain of Eps8 revealed an intertwined dimer,

Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8), Table 1 Accession numbers for the protein sequences used in Fig. 1

Name in alignment	GenBank accession	Description	Organism
Hs_EPS8	Q12929	Epidermal growth factor receptor kinase substrate Eps8	*Homo sapiens*
Hs_EPS8L1	AAL76117.1	Epidermal growth factor receptor pathway substrate 8 related protein 1	*Homo sapiens*
Hs_EPS8L2	AAL76118.1	Epidermal growth factor receptor pathway substrate 8 related protein 2	*Homo sapiens*
Hs_EPS8L3	AAL76119.1	Epidermal growth factor receptor pathway substrate 8 related protein 3	*Homo sapiens*
Mm_EPS8	Q08509	Epidermal growth factor receptor kinase substrate eps8	*Mus musculus*
Mm_EPS8L1	AAL76120.1	Epidermal growth factor receptor pathway substrate 8 related protein 1	*Mus musculus*
Mm_EPS8L2	AAH05492.1	Similar to hypothetical protein FLJ21935	*Mus musculus*
Mm_EPS8L3	AAL76121.1	Epidermal growth factor receptor pathway substrate 8 related protein 3	*Mus musculus*
Rn_EPS8	Predicted	ENSEMBL gene prediction ENSRNOT00000009328.3	*Rattus norvegicus*
Rn_EPS8L1	XP_341783.1	Similar to epidermal growth factor receptor pathway substrate 8-like protein 1	*Rattus norvegicus*
Rn_EPS8L2	Predicted	ENSEMBL gene prediction ENSRNOT00000024725.3	*Rattus norvegicus*
Rn_EPS8L3	XP_215677.4	Similar to epidermal growth factor receptor pathway substrate 8_Elike protein 3	*Rattus norvegicus*
Gg_EPS8	XP_416405.2	Similar to Epidermal growth factor receptor pathway substrate 8	*Gallus gallus*
Gg_EPS8L2	XP_001232098.1	Hypothetical protein	*Gallus gallus*
Xl_EPS8	AAH68768.1	MGC81285 protein	*Xenopus laevis*
Xt_EPS8	NP_001072508.1	Epidermal growth factor receptor pathway substrate 8	*Xenopus tropicalis*
Xt_EPS8L2	Predicted	Genewise gene prediction from xenopus genome scaffold_296:324,626–357,189	*Xenopus tropicalis*
Dr_EPS8	NP_956536.1	Similar to epidermal growth factor receptor pathway substrate 8	*Danio rerio*
Dr_EPS8L1	Predicted	N-SCAN gene predictions (chr2.1002.1)	*Danio rerio*
Dr_EPS8L3	XP_691442.2	Hypothetical protein	*Danio rerio*
Tn_EPS8L1	Predicted	Geneid gene prediction (chr2_362.1)	*Tetraodon nigroviridis*
Tn_EPS8L2	Predicted	Geneid gene prediction (chr13_663.1)	*Tetraodon nigroviridis*
Tn_EPS8L3	CAG06824.1	Unnamed protein product	*Tetraodon nigroviridis*
Ci_EPS8	Predicted	Genewise gene prediction from ciona genome chr05q:2,271,763–2,296,713	*Ciona intestinalis*
Dm_EPS8	AAL13963.1	Lp01469p	*Drosophila melanogaster*
Dp_EPS8	XP_001359077.1	GA21402-PA	*Drosophila pseudoobscura*
Cb_EPS8	CAE68196.1	Hypothetical protein CBG13858	*Caenorhabditis briggsae*
Ce_EPS8	NP_001041047.1	EPS (human endocytosis) related family member, eps-8	*Caenorhabditis elegans*

characterized by "strand exchange," in which the two antiparallel beta sheets are contributed by different polypeptide chains (Kishan et al. 1997). Surprisingly, this results in half-dimers whose folds are superimposable to that of a canonical SH3 module. An important consequence of the dimeric configuration is that the proline helix-binding groove is partially occluded in the context of the hybrid-dimer, impeding

binding to ligands (Kishan et al. 1997). More recently, a monomeric structure of the SH3 domain of Eps8 has been reported, as obtained from crystals grown at low pH. In this case, the SH3 domain of Eps8 displays a canonical SH3 fold (Kishan et al. 2001). While this latter configuration is likely the one adopted under physiological conditions, dimerization of Eps8 SH3 domain may occur under some circumstances and might function as an "OFF" signal, which can be switched "ON" as the molecule becomes monomeric thereby allowing protein–protein interactions at the sites occluded in the dimer to occur. It should be noted, however, that the formation of a strand-exchanged dimeric Eps8–SH3 domain results in an extensive dimerization interface, much greater than usually observed for reversible regulated protein–protein associations in signal transduction. Thus, the physiological relevance of a dimer–monomer equilibrium in vivo remains to be established.

The identification of the optimal binding peptides for the SH3 domain of Eps8 provided another unexpected result. In spite of the overall conservation of the primary structure and the similarity to the canonical SH3 fold, the SH3 domain of Eps8 binds preferentially to peptides containing a PXXDY, instead of the XPXXP, consensus sequence (Mongiovi et al. 1999). This binding specificity is conserved among the three related genes (Mongiovi et al. 1999). Solution by NMR of the Eps8L1 SH3 domain in complex with the PPVPNPDYEPIR peptide from the CD3epsilon cytoplasmic tail further highlighted the specific molecular requirements at the basis of this unusual mode of binding (Aitio et al. 2008). Indeed, the polyproline peptide binds Eps8L1 SH3 in a class II orientation, but neither adopts a polyproline II helical conformation nor engages the first proline-binding pocket of the SH3 ligand-binding interface. Critical conserved residues in the SH3 domain of Eps8L family members, that are unique with respect to the other SH3 domains, account for this specificity. Most notably, an Eps8L-conserved isoleucine (I531 in EpsL1) is present instead of Y or F in the hydrophobic pocket rendering it nonoptimal for binding to conventional PxxP peptides. Furthermore, specific electrostatic and hydrogen bonding between the D and Y residue of the ligand peptide with positive charge of R and carboxyl group of E in the SH3 domain of Eps8L1, respectively, account for the critical role of the PxxDY motif tyrosine residue in binding to Eps8 family SH3 (Aitio et al. 2008). Thus, the SH3 of Eps8 represents the prototype of a unique family of SH3 modules, which do not bind to canonical XPXXP-containing peptides, and contract specific and distinct interactions in the signaling network. This contention is further supported by the isolation of two physiological interactors of the SH3 domain of Eps8, Abi1 (Abelson Binding Interactor 1) and RN-tre (a GAP for the endocytic Rab5) which display the PXXDY motif and, as it will be discussed later on, establish a novel network involved in the regulation of RTK-dependent signaling.

Recently, the human lanthionine synthetase C-like protein 1 (LanCL1), a eukaryotic homologue to prokaryotic lanthionine cyclases, has been identified as a novel interactor of the SH3 domain of Eps8 (Zhang et al. 2009). Lanthionine cyclase in prokaryotic cells is a peptide-modifier enzyme frequently required to generate antibiotic peptides. However, the function of LanCL1 in mammalian cells remains ill defined. Remarkably, the interaction with Eps8 appears to be required for LanCL1-mediated neuritogenesis (Zhang et al. 2009). Furthermore, the biochemical basis of the association is unusual since it is not mediated by a linear PXXDY motif of LanCL1, but presumably requires an extensive molecular surface interaction from both of these molecules, and is regulated by redox signaling, thus establishing a novel paradigm in the binding and regulation of SH3-mediated protein:protein interactions (Zhang et al. 2009).

Multiple Functions of Eps8 in the Small GTPase Pathways Control Actin Dynamics–Based Processes

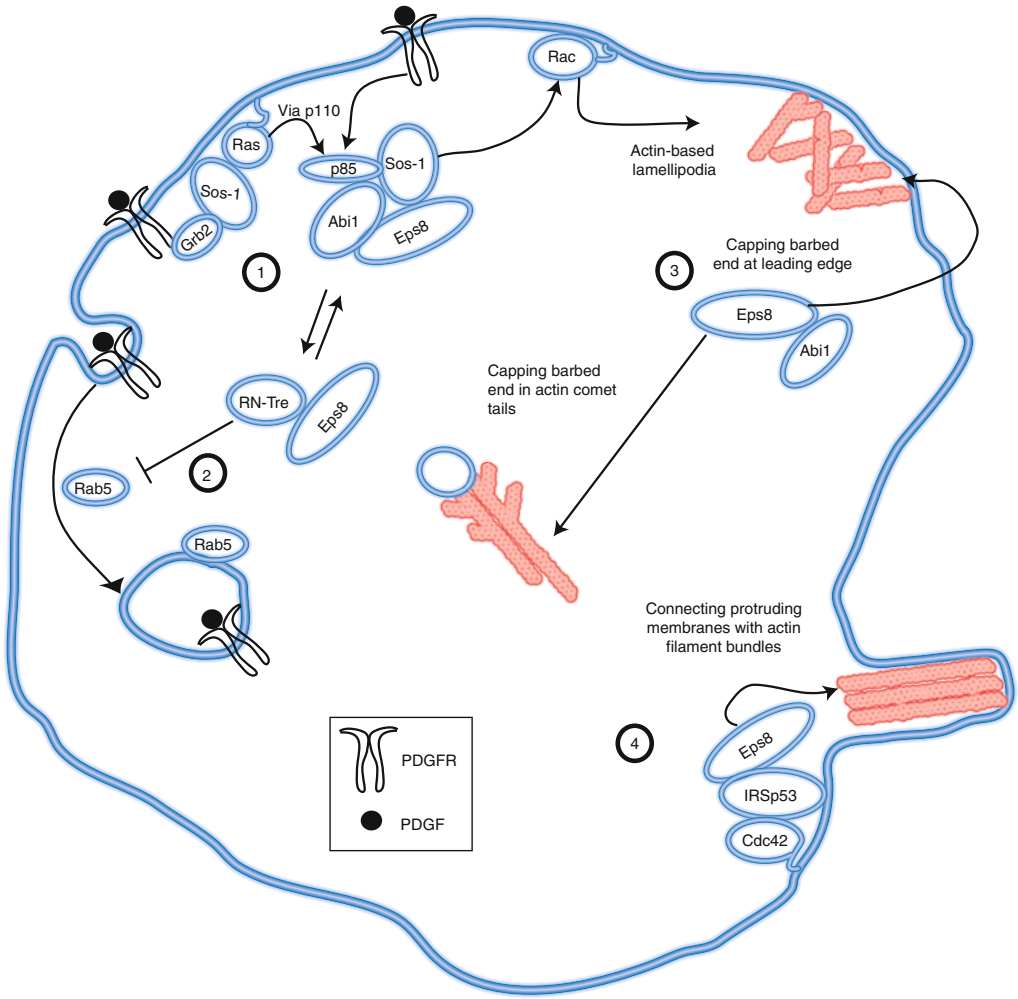
Eps8 has emerged as a key regulator in controlling actin cytoskeleton remodeling. The structural organization of Eps8 in multiple protein–protein interaction domains suggests that it acts both by participating in signaling cascades downstream RTKs receptors, through its interaction with Abi1, Sos-1, and RN-tre, and by directly controlling the actin cytoskeleton dynamics and structural organization through its capping and a bundling activity. It is noteworthy that virtually all these functions are mediated by Eps8 C-terminal domain (aa 648–821), where both the Sos-1 and the actin-binding surfaces are located.

Eps8 in the Rho GTPase signaling cascade. Eps8 as a molecular adaptor participates in the RTKs signaling

cascade leading to Rac-dependent actin cytoskeleton remodeling. At the molecular level, Eps8 was shown to enter into a multimolecular complex with Abi1, p85, and Sos-1 (for a review of this pathway see Di Fiore and Scita (2002)). Abi1 is the non-catalytic subunit of PI3K, and Sos-1 is the dual GEF for Ras and Rac (Fig. 2). Within this complex, Abi1 acts as a scaffold facilitating the interaction between Eps8, p85, and Sos1 (Di Fiore and Scita 2002). The assembly of this signaling unit is critical to unmask the otherwise silent Rac-specific GEF activity of Sos-1, through mechanisms that remain to be fully elucidated, mediating actin cytoskeleton remodeling in response to growth factors stimulation (Di Fiore and Scita 2002). Notably, the C-terminal region of Eps8 mediates in vitro a direct, low-affinity interaction with Sos-1, sufficient to activate the Rac-specific GEF of the latter protein in cells, suggesting that this region acts as a bona fide effector region within Eps8, mediating all the functions so far ascribed to this protein both as signal adaptors and actin regulatory binding interactors (Di Fiore and Scita 2002).

The above scenario became more complex with the identification of another Eps8 interactor, IRSp53, which primarily acts as downstream effector of Cdc42 (Fig. 2). IRSp53 was originally discovered as a substrate of the insulin receptor. Subsequently, IRSp53 was shown to participate in the formation of various Cdc42- and Rac-dependent, actin-based protrusions, including filopodia and lamellipodia, neurites and dendritic spines, through a mechanism that remains to be fully elucidated. The role of IRSp53 in filopodia is more clearly established. Filopodia are actin-rich, finger-like structures that protrude from the cell membrane of a variety of cell types and play important roles in cell migration, neurite outgrowth, and wound healing (Scita et al. 2008). They are characterized by a small number of long and parallel actin filaments that deform the cell membrane, giving rise to protrusions. Within this context, IRSp53 is thought to connect actin regulatory complexes with the extending membranes. In keeping with this notion, IRSp53 possesses an N-terminal helical domain, which belongs to the BAR (Bin-amphipysin-Rvs) family of domains and folds into zeppelin-shaped dimers. BAR domains themselves are banana-shaped structures that induce curvature in membranes via their concave face promoting membrane invagination and the generation of tubules and vesicles important in endocytic and intracellular trafficking processes. In contrast to these standard BAR domains, the I(inverted)-BAR domain of IRSp53 induces negative curvature in membranes, thus inducing protrusions rather than invagination and contributing to the formation of filopodia (Scita et al. 2008). In this latter process, IRSp53 is thought to act as an effector of Cdc42, which in its activated GTP-bound form can bind to a Cdc42-specific CRIB-like sequence of IRSp53. This interaction leads to relocalization of IRSp53 to sites where filopodia are initiated. At this location, IRSp53 can physically link the protruding membrane to the underlying actin filaments by binding, through its SH3 domain, a number of regulators of actin dynamics and architectural organization, including Dia1, Mena, WAVE2, and Eps8 (Scita et al. 2008). It is noteworthy that, among these interactors, Eps8 is the only protein that can form a stable complex with IRSp53 both in vitro and in vivo (Hertzog et al. 2010). The assembly of a Eps8::IRSp53 complex endows this unit with actin bundling activity in vitro, that may initiate, in vivo, the formation of actin bundles, which would subsequently become tightly cross-linked by other more efficient bundlers, such as fascin. Thus, Eps8 together with IRSp53 may act at the interface of membranes and the actin cytoskeleton, initiating in a Cdc42-dependent fashion the bundling of filaments, which may subsequently elongate and become tightly bundled supporting filopodia extension.

Eps8 and Rab5 endocytic networks. Eps8 involvement in endocytic processes derives from its ability to bind yet another SH3 interactor, RN-tre. RN-tre is a GAP specific for Rab family proteins, including Rab5 (Lanzetti et al. 2000), indicating its involvement in trafficking processes. Most notably, RN-tre was originally demonstrated to function both in vitro and in vivo on Rab5 (Lanzetti et al. 2000), which is in turn essential for regulating multiple steps of internalization of various membrane receptors, including early endocytic events and early endosomal formation, maturation, and trafficking. In particularly, Rab5 is essential for EGF receptor endocytosis upon ligand binding. Interaction between Eps8 and RN-tre has been shown to be required for the activation of the Rab5-GAP specific activity of the latter protein. This leads to the inhibition of the small GTPase accompanied by increased EGFR at the cell surface due to impaired receptor internalization and increased signaling (Lanzetti et al. 2000) (Fig. 2).



Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8), Fig. 2 Eps8-based complexes in the midst of GTPases signaling controlling actin dynamics–based processes. The presence of multiple protein–protein interaction domains enables Eps8 to form diverse multimolecular complexes that act on various small GTPases-dependent pathways. (1) Through its unique SH3 domain, Eps8 can bind to Abi1, which forms the scaffold for a larger complex together with Eps8, Sos-1, and p85 the regulatory subunit of phosphatidyl-inositol-3- kinase (PI3K) mediating the propagation of signaling leading to Rac activation and Rac-dependent actin remodeling from activated PDGFR either directly or through the Grb2-Sos-1-Ras pathways (Di Fiore and Scita 2002). (2) The SH3 domain of Eps8 mediates also the interaction with RN-tre, a GAP for the small G protein Rab5 (Lanzetti et al. 2000), which, in turn, controls RTK internalization and trafficking, thus regulating the duration and cellular localization of RTK signaling, resulting in the formation of specialized actin-rich membrane protrusion (not shown) (Lanzetti et al. 2004). (3) Through its C-terminal actin-binding region, Eps8, which is localized at the very leading edge of actin-rich lamellipodial protrusions as well as in rocketing tails propelling endosomal vesicles and intracellular pathogens (Disanza et al. 2004), binds and caps actin filaments thereby controlling their dynamic and architectural organization. Remarkably, the capping activity of full-length Eps8 is inhibited and can be induced, at least in vitro, through binding to Abi1, which is also localized at the very leading edges of lamellipodia. (4) Eps8 can also cross-link actin filament when it is bound to IRSp53 (Hertzog et al. 2010; Disanza et al. 2004). IRSp53 dimerizes, thus enabling the formation of Eps8 clusters that may act to enhance the cross-linking activity of the complex, which can be recruited by activated Cdc42 to plasma membrane sites for filopodia initiation resulting from the combined membrane deformation ability of IRSp53 and actin-binding property of Eps8. It is of note that the actin-related activities of Eps8 are conserved throughout evolution and have been shown to mediate the morphogenesis of intestinal microvilli in nematodes (Hertzog et al. 2010) and mice (Tocchetti et al. 2010) as well as the development of stereocilia (not shown). Both microvilli and stereocilia are formed by parallel array of bundled filaments whose elongation is tightly controlled by a variety of actin regulatory proteins

Thus, Eps8 may regulate Rac activity either directly through assembly of a plasma-membrane-localized signaling complex that promotes optimal transmission of motogenic RTK signaling, and indirectly through endocytsosis that confers a spatial dimension to signaling that is ultimately required for the regulation of polarized function, first and foremost actin dynamics–based directional migration.

Eps8 and Actin Dynamics

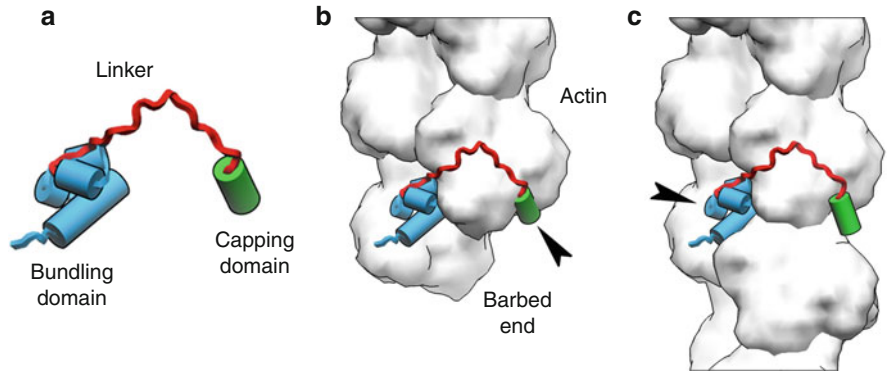
Eps8 is able to directly control actin cytoskeleton remodeling through inherent capping and bundling activities. Eps8 can accelerate actin treadmilling by blocking polymerization at filaments ends, acting as a capping protein. Eps8 can also regulate the architectural organization of the actin cytoskeleton by cross-linking actin filaments, in this case acting as a bundling protein. These properties in conjunction with in vivo localization data suggest that Eps8 critically participates both in the regulation of cell motility and in the architectural organization of specific actin-based structures, including cell protrusions at the leading edge of migrating cells, microvilli of intestinal cells, and stereocilia. Both capping and bundling activities are mediated and retained by the C-terminal region of the protein (amino acids 648–821) but the full-length protein is tightly regulated by protein–protein interactions. The bundling activity can be isolated and attributed to the C-terminal four helices of the region (Hertzog et al. 2010). The capping activity can also be isolated and can be attributed to the first helix of the region that is tethered to the C-terminal four helices by a flexible linker (Hertzog et al. 2010). Thus, the C-terminal effector region of Eps8 contains two structurally distinct actin-binding modules, one responsible for capping and one responsible for bundling (Fig. 3a).

Eps8 capping activity. In vitro, the conserved C-terminal region of Eps8, in substoichiometric amounts with respect to actin, inhibits barbed end growth by up to 90% and enables propulsion of N-WASP-coated beads in the absence of gelsolin or other cappers (Disanza et al. 2004). In vivo, Eps8 localizes in rocketing tails propelling phosphatidylinositol-4,5-phosphate-(PI45P) enriched vesicles or intracellular pathogens such as *Listeria monocytogenes* or *Shighella flexneri* (Disanza et al. 2004). Structurally, the Eps8 capping activity is mediated by binding of the amphipathic first helix of the Eps8 C-terminal region within the hydrophobic pocket at the barbed ends of actin (Hertzog et al. 2010). Occupation of this site on actin filaments by the helix blocks further addition of actin monomers (Fig. 3b). The capping activity of full-length Eps8 is regulated by interactions with Abi1 (Disanza et al. 2004). While the exact mechanism of this regulation remains obscure, it is likely that the binding of Abi1 induces a conformational change in full-length Eps8 that exposes the amphipathic first helix within the C-terminal region and makes it available for interactions with the filament barbed ends.

Eps8 bundling activity. The C-terminal four-helix region of Eps8 binds to the sides of actin filaments as a compact helix bundle (Hertzog et al. 2010) and possesses bundling activity in vitro when dimerization domains such as GST are attached to the construct (Disanza et al. 2006). The four-helix bundle interacts with three adjacent subunits along the short-pitch helix of the actin filament (Fig. 3c). Full-length Eps8 can form a constitutive, stable complex with IRSp53 that bundles actin filaments (Disanza et al. 2006). While Eps8 expression alone has no effect on filopodia formation in vivo, the concomitant expression of the two proteins leads to a dramatic increase in filopodia number and length (Disanza et al. 2006). The Eps8:IRSp53 interaction mediates the bundling activity of the side-bound full-length Eps8 most likely through dimerization of IRSp53, with a possible contribution from the inherent, weak actin-binding activity of the N-terminal IMD domain of IRSp53, which is auto-inhibited in full-length IRSp53 but may be released by Eps8 binding.

Physiological Roles of Eps8: From Cells to Multicellular Organisms

The dissection of the biochemical and signaling properties of Eps8 together with its ability to participate in various, distinct macromolecular complexes has provided a rational framework to account for a number of apparently unrelated and diverse cell biological phenotypes associated with this protein. Thus, for example, the presence of Eps8 in actin dynamics–based motile structures (Hertzog et al. 2010; Disanza et al. 2004) reflects the ability of this protein to cap the barbed ends of actin filament. Conversely, Eps8 localization to microspikes and filopodia is accounted for, at least in part, by the ability to form a complex with



Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8), Fig. 3 Eps8 C-terminal actin-binding region. (**a**) Structure of the conserved C-terminal region of Eps8 shown in cartoon representation. The region contains two distinct actin-binding sites responsible for bundling (blue) and capping (green) respectively. These regions are connected by a flexible linker (red). (**b**) Low resolution representation of an actin filament barbed end (white surface) with the Eps8 C-terminal domain bound and the capping domain occupying the hydrophobic groove of the barbed end actin subunit (arrowhead). This interaction prevents the addition of new filament subunits. (**c**) Low resolution representation of an actin filament (white surface) with the Eps8 C-terminal domain bound to the side (arrowhead), contacting three successive actin subunits. In conjunction with dimerization, this interaction is responsible for the bundling activity of Eps8. The capping domain does not occupy the hydrophobic groove of actin in the configuration

IRSp53 at the plasma membrane where Eps8 may both "protect" plus ends from stronger cappers, such as CP, while promoting the convergent association and bundling of filament ends, a presumably limiting step in filopodia initiation (Hertzog et al. 2010). This context allows to begin rationalizing how the actin-related activities of Eps8, controlled by distinct molecular partners in different cellular contexts, may account for seemingly paradoxical and opposite effects on filopodia whereby genetic removal of Eps8 reduced filopodia in HeLa cells (Disanza et al. 2004), but increased them in hippocampal neurons (Menna et al. 2009). It is likely that the dynamic and context-dependent interactions established by Eps8, IRSp53, and Abi1 with actin filaments generate a flexible signaling network that governs the multifunctional activities of its components in the formation of diverse actin-based structures: a notion that is being supported by computational modeling of this network (GS personal communication). Whatever the case, Eps8 capping and bundling activities are emerging as critical in diverse phenotypes both at the cellular and organism levels, revealing the involvement of actin dynamics in a variety of diverse and frequently unexpected physiological processes. Specific impairment of bundling activity, for example, of the only EPS-8 family member in nematodes leads to a severely altered intestinal morphogenesis that correlates with a disruption of the stereotypical orderly organization of actin-bundle-rich microvilli (Croce et al. 2004). This specific function appears evolutionary conserved since also 8 null mice display shorter intestinal microvilli (Tocchetti et al. 2010). Notably, this alteration is accompanied by a defective intestinal fat absorption that results in calorie-restricted metabolism and increased life span, providing genetic evidence of actin dynamics as a novel variable in the determination of longevity (Tocchetti et al. 2010). It is also noteworthy that the three other Eps8L family members do not compensate for the lack of Eps8 in the intestine suggesting that differential tissues expression and cellular localization (in this case to microvilli) contribute to functional specificity within the Eps8L family. A similar situation may also account for the recent discovery that eps8 null mice are deaf. Hearing impairment is a consequence of disrupted stereocilia morphology, where Eps8 localizes together with the Myosin XVa and the adaptor protein whirlin (Manor et al. 2011). While the specific contribution of Eps8 actin-related activities to proper stereocilia elongation has not yet been established, it is reasonable to assume that, in analogy to intestinal microvilli, which share a similar architectural organization in parallel actin bundles with stereocilia, the actin cross-linking property of Eps8 is

responsible for the phenotype. However, a role of Eps8 capping in fine-tuning filament elongation at the basis of the precise staircase pattern of stereocilia cannot be ruled out at the present stage.

Finally, a specific functional role of Eps8 with unexpected physiopathological consequences has emerged when the function of this protein was analyzed in the central nervous system. Eps8 is uniquely expressed in the hippocampus as well as the cerebellum (Offenhauser et al. 2006). In cerebellar neurons, Eps8 is significantly more abundant. Consistently, genetic removal of 8 alters actin dynamics, particularly at postsynaptic sites, where Eps8 is part of the N-methyl-D-aspartate (NMDA) receptor complex. Moreover, in Eps8 null mice, NMDA receptor currents and their sensitivity to inhibition by ethanol are abnormal. In addition, 8 null cerebellar neurons are resistant to the actin-remodeling activities of NMDA and ethanol (Offenhauser et al. 2006). These alterations presumably explain why 8 null mice are resistant to acute intoxicating effects of ethanol and show increased ethanol consumption (Offenhauser et al. 2006), further supporting the notion that proper regulation of the actin cytoskeleton by Eps8 is a key determinant of cellular and behavioral responses to ethanol.

In cortical neurons, the capping activity of Eps8 is, instead, required downstream of Brain Derived Neurotrophic Factor (BDNF) for the regulation of axonal filopodia: a process with crucial impacts on neuronal development and synapse formation (Menna et al. 2009). In this cell system, Eps8 capping activity is negatively regulated by BDNF-mediated, MAPK-dependent Eps8 phosphorylation, which reduces the affinity of the Eps8:Abi1 complex for barbed ends, thus promoting the elongation of uncapped actin filament and the generation of axonal filopodia (Menna et al. 2009). Notably, filopodia and short axonal branches are frequently extended and retracted to form boutons onto postsynaptic structures, or to originate en passant boutons, which are added and eliminated along the axonal shaft: all processes that characterize synaptic plasticity. Consistent with this possibility, *eps8* null brains display a significantly higher number of presynaptic boutons (Scita 2008) that might originate from the increased axonal filopodia during neuronal development, thus suggesting a role of the protein in controlling synapse formation and presynaptic plasticity phenomena in the developing brain and, possibly, in the adult brain.

Eps8 and Cancer

While the involvement of Eps8 in cytoskeletal reorganization and its molecular modes of action are gradually being elucidated, experimental evidence has also accumulated supporting the implication of Eps8 in tumorigenesis. Since its discovery, Eps8 was shown to enhance mitogenesis and, in some circumstances, also cell transformation in response to growth factor treatment (reviewed in (Di Fiore and Scita 2002)). Furthermore, constitutive tyrosine phosphorylation of Eps8, frequently accompanied by the elevated phosphotyrosine levels of a number of RTK signaling molecules, was detected in a large variety of tumor cells (reviewed in (Di Fiore and Scita 2002)). These initial observations supported the notion that Eps8, presumably through its signal-transducing adaptor functions, may be relevant in promoting mitogenic signaling particularly in those tumors where RTKs signaling is elevated. This latter contention was subsequently extended by the finding that constitutive tyrosine phosphorylation of Eps8 was also frequently coupled to elevated protein expression, such as in cells transformed by non-receptor tyrosine kinases, v-SRC (reviewed in (Di Fiore and Scita 2002)), and in a variety of human tumors, including colorectal (Maa et al. 2001), cervical (Chen et al. 2008; Wang et al. 2009), pituitary (Xu et al. 2008), pancreatic (Welsch et al. 2007), and oral cancers (Yap et al. 2009). Consistent with this, the overexpression of Eps8 confers the ability of fibroblasts to form foci in culture dishes and to grow tumors in mice (Maa et al. 2001). These findings confirm the initial observations and further corroborate the oncogenic potential of Eps8. In agreement with this notion, Eps8 attenuation retarded cellular growth in v-SRC transformed cells and human cancer cells (Maa et al. 2007). Frequently, Eps8 overexpression in tumors correlates with increased MAPK and/or AKT signaling. While the mechanisms and pathways through which Eps8 mediates these effects remain ill defined, it is reasonable to propose that it is the ability of Eps8 to form diverse multimolecular signaling complexes acting in the Ras-PI3K-Rac (Di Fiore and Scita 2002) or RTK-p66 shc (Bashir et al. 2010) transduction cascades that accounts for its role in promoting mitogenesis or increased resistance to apoptosis. It should be noted, however, that an additional, much less explored possibility, is that deregulated levels of Eps8 may disturb

the endocytosis or intracellular trafficking of RTKs, in turn, affecting the duration and spatial localization of RTK-dependent signaling, ultimately resulting in enhanced tumorigenesis. In this latter respect, it is noteworthy that in *C. elegans* elevated and basolaterally localized EPS-8 in vulval precursor cells (VPCs) blocks EGFR internalization, leading to enhanced signaling. Conversely, low levels of EPS-8 in the neighboring secondary VPCs result in the rapid degradation of the EGFR, allowing these cells to adopt a secondary cell fate (Stetak et al. 2006). Thus, Eps8 regulation of EGFR trafficking may be an evolutionary conserved mechanism to control signaling in pattern formation during normal organogenesis or to promote enhanced mitogenic signaling, when its levels are deregulated, such as in tumor development.

Finally, it is important to point out that at least in specific tumor types, EPS8 expression levels correlate with an increase in their capacity to migrate and invade surrounding tissues. This is the case for head and neck squamous cell carcinomas (HNSCCs) (Yap et al. 2009) and for a subset of colon carcinoma (Maa et al. 2007). In HNSCC, Eps8 is upregulated in 32% of primary tumors compared with normal oral mucosa, and its expression significantly correlated with lymph node metastasis, suggesting a disease-promoting effect. Consistent with this notion, the ablation of Eps8 from high-expressing HNSCC derived from tumor patients impaired cell migration and invasion both in in vitro organotypic and in mouse model systems (Wang et al. 2009; Yap et al. 2009). Some of these effects of Eps8 were attributed to elevated Rac activity (Yap et al. 2009; Bashir et al. 2010) or to indirect regulation of transcription factors, such as FOXM1, in turn controlling signaling proteins important for cell cycle progression and cell migration (Wang et al. 2010). However, it is tempting to speculate that the actin regulatory activities of Eps8 may directly contribute to the acquisition of highly migratory and invasive properties of advanced stage of squamous cell carcinoma. The possibility to dissect at the molecular levels the diverse actin regulatory functions of Eps8 will be instrumental in the years to come to test this hypothesis.

Summary

Eps8, originally identified as a substrate of the EGFR, is the founding member of a family of proteins that includes three additional members in mammals. They all display a modular organization into various protein–protein interaction domains that suggest the involvement of this protein family in the transduction of receptor tyrosine kinases (RTKs) signals. Consistently, Eps8 can participate in various, distinct macromolecular complexes that either mediate the activation of small GTPases Rac and Rac-dependent actin remodeling or regulate RTK signal duration by controlling receptor internalization and trafficking. Remarkably, Eps8 is able to bind to actin filaments and to control actin dynamics, through its ability to associate with and block the plus growing ends of filaments. Eps8 can also regulate the architectural organization of actin meshworks and bundles by cross-linking adjacent filaments. These two properties can be dissected in vitro and are differentially regulated by Eps8 interactors: Abi1 activates actin capping, and IRSp53 promotes actin bundling. Thus, Eps8 by participating in multiple and distinct molecular complexes plays roles in a variety of actin-based processes, including motile cellular protrusions such as lamellipodia, rocketing tail, and filopodia, as well as sensory structures such as microvilli and stereocilia. The cellular roles of Eps8 are mirrored at the organism level by the requirement of Eps8 in diverse and specific physiological actin-based processes. Thus, Eps8 null mice display a range of actin-dependent phenotypes ranging from defective extension of neuronal filopodia and synaptogenesis that can result in deregulation of behavioral responses to ethanol or altered morphogenesis of intestinal microvilli and stereocilia that can cause fat malabsorption and deafness, respectively. Finally, emerging evidence implicates Eps8 in tumourigenesis whereby elevated levels of this protein either favors cell proliferation or enhances cell migration and invasion of various cancer cells. Thus, Eps8 represent a unique actin regulatory protein endowed with signal-transducing properties accounting for its pleiotropic functional roles.

Acknowledgment This study was supported by the NIH Cell Migration Consortium grant U54 GM064346 from the National Institute of General Medical Sciences (NIGMS) to DH and NV; from the Associazione Italiana per la Ricerca sul Cancro (AIRC), the Italian Ministries of Education-University-Research (MIUR-PRIN) and of Health, AICR (International Association For Cancer Research – AICR), and from IFOM Foundation to GS; FM is supported by a fellowship from Fondazione Italiana Ricerca sul Cancro (FIRC).

References

Aitio O, Hellman M, Kesti T, Kleino I, Samuilova O, Paakkonen K, et al. Structural basis of PxxDY motif recognition in SH3 binding. J Mol Biol. 2008;382(1):167–78.

Bashir M, Kirmani D, Bhat HF, Baba RA, Hamza R, Naqash S, et al. P66shc and its downstream Eps8 and Rac1 proteins are upregulated in esophageal cancers. Cell Commun Signal. 2010;8:13.

Chen YJ, Shen MR, Maa MC, Leu TH. Eps8 decreases chemosensitivity and affects survival of cervical cancer patients. Mol Cancer Ther. 2008;7(6):1376–85.

Croce A, Cassata G, Disanza A, Gagliani MC, Tacchetti C, Malabarba MG, et al. A novel actin barbed-end-capping activity in EPS-8 regulates apical morphogenesis in intestinal cells of *Caenorhabditis elegans*. Nat Cell Biol. 2004;6(12):1173–9.

Di Fiore PP, Scita G. Eps8 in the midst of GTPases. Int J Biochem Cell Biol. 2002;34(10):1178–83.

Disanza A, Carlier MF, Stradal TE, Didry D, Frittoli E, Confalonieri S, et al. Eps8 controls actin-based motility by capping the barbed ends of actin filaments. Nat Cell Biol. 2004;6(12):1180–8.

Disanza A, Mantoani S, Hertzog M, Gerboth S, Frittoli E, Steffen A, et al. Regulation of cell shape by Cdc42 is mediated by the synergic actin-bundling activity of the Eps8-IRSp53 complex. Nat Cell Biol. 2006;8(12):1337–47.

Fazioli F, Minichiello L, Matoska V, Castagnino P, Miki T, Wong WT, et al. Eps8, a substrate for the epidermal growth factor receptor kinase, enhances EGF-dependent mitogenic signals. EMBO J. 1993;12(10):3799–808.

Forman-Kay JD, Pawson T. Diversity in protein recognition by PTB domains. Curr Opin Struct Biol. 1999;9(6):690–5.

Hertzog M, Milanesi F, Hazelwood L, Disanza A, Liu H, Perlade E, et al. Molecular basis for the dual function of Eps8 on actin dynamics: bundling and capping. PLoS Biol. 2010;8(6):e1000387.

Kishan KV, Scita G, Wong WT, Di Fiore PP, Newcomer ME. The SH3 domain of Eps8 exists as a novel intertwined dimer. Nat Struct Biol. 1997;4(9):739–43.

Kishan KV, Newcomer ME, Rhodes TH, Guilliot SD. Effect of pH and salt bridges on structural assembly: molecular structures of the monomer and intertwined dimer of the Eps8 SH3 domain. Protein Sci. 2001;10(5):1046–55.

Lanzetti L, Rybin V, Malabarba MG, Christoforidis S, Scita G, Zerial M, et al. The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5. Nature. 2000;408(6810):374–7.

Lanzetti L, Palamidessi A, Areces L, Scita G, Di Fiore PP. Rab5 is a signalling GTPase involved in actin remodelling by receptor tyrosine kinases. Nature. 2004;429(6989):309–14.

Maa MC, Hsieh CY, Leu TH. Overexpression of p97Eps8 leads to cellular transformation: implication of pleckstrin homology domain in p97Eps8-mediated ERK activation. Oncogene. 2001;20(1):106–12.

Maa MC, Lee JC, Chen YJ, Chen YJ, Lee YC, Wang ST, et al. Eps8 facilitates cellular growth and motility of colon cancer cells by increasing the expression and activity of focal adhesion kinase. J Biol Chem. 2007;282(27):19399–409.

Manor U, Disanza A, Grati M, DeAndrade L, Lin H, Di Fiore P, et al. Regulation of stereocilia length by myosin XVa and whirlin depends on the actin regulatory protein Eps8. Curr Bio. 2011;21(2):167–72.

Menna E, Disanza A, Cagnoli C, Schenk U, Gelsomino G, Frittoli E, et al. Eps8 regulates axonal filopodia in hippocampal neurons in response to brain-derived neurotrophic factor (BDNF). PLoS Biol. 2009;7(6):e1000138.

Mongiovi AM, Romano PR, Panni S, Mendoza M, Wong WT, Musacchio A, et al. A novel peptide-SH3 interaction. EMBO J. 1999;18(19):5300–9.

Offenhauser N, Borgonovo A, Disanza A, Romano P, Ponzanelli I, Iannolo G, et al. The eps8 family of proteins links growth factor stimulation to actin reorganization generating functional redundancy in the Ras/Rac pathway. Mol Biol Cell. 2004;15(1):91–8.

Offenhauser N, Castelletti D, Mapelli L, Soppo BE, Regondi MC, Rossi P, et al. Increased ethanol resistance and consumption in Eps8 knockout mice correlates with altered actin dynamics. Cell. 2006;127(1):213–26.

Scita G, Confalonieri S, Lappalainen P, Suetsugu S. IRSp53: crossing the road of membrane and actin dynamics in the formation of membrane protrusions. Trends Cell Biol. 2008;18(2):52–60.

Slupsky CM, Gentile LN, Donaldson LW, Mackereth CD, Seidel JJ, Graves BJ, et al. Structure of the Ets-1 pointed domain and mitogen-activated protein kinase phosphorylation site. Proc Natl Acad Sci USA. 1998;95(21):12129–34.

Smith MJ, Hardy WR, Murphy JM, Jones N, Pawson T. Screening for PTB domain binding partners and ligand specificity using proteome-derived NPXY peptide arrays. Mol Cell Biol. 2006;26(22):8461–74.

Stetak A, Hoier EF, Croce A, Cassata G, Di Fiore PP, Hajnal A. Cell fate-specific regulation of EGF receptor trafficking during Caenorhabditis elegans vulval development. EMBO J. 2006;25(11):2347–57.

Tocchetti A, Soppo CB, Zani F, Bianchi F, Gagliani MC, Pozzi B, et al. Loss of the actin remodeler Eps8 causes intestinal defects and improved metabolic status in mice. PLoS ONE. 2010;5(3):e9468.

Wang H, Patel V, Miyazaki H, Gutkind JS, Yeudall WA. Role for EPS8 in squamous carcinogenesis. Carcinogenesis. 2009;30(1):165–74.

Wang H, Teh MT, Ji Y, Patel V, Firouzabadian S, Patel AA, et al. EPS8 upregulates FOXM1 expression, enhancing cell growth and motility. Carcinogenesis. 2010;31(6):1132–41.

Welsch T, Endlich K, Giese T, Buchler MW, Schmidt J. Eps8 is increased in pancreatic cancer and required for dynamic actin-based cell protrusions and intercellular cytoskeletal organization. Cancer Lett. 2007;255(2):205–18.

Xu M, Shorts-Cary L, Knox AJ, Kleinsmidt-Demasters B, Lillehei K, Wierman ME. Epidermal growth factor receptor pathway substrate 8 (Eps8) is overexpressed in human pituitary tumors: role in proliferation and survival. Endocrinology. 2008. doi:10.1210/en.2008-1265.

Yap LF, Jenei V, Robinson CM, Moutasim K, Benn TM, Threadgold SP, et al. Upregulation of Eps8 in oral squamous

cell carcinoma promotes cell migration and invasion through integrin-dependent Rac1 activation. Oncogene. 2009;28(27):2524–34.
Zhang W, Wang L, Liu Y, Xu J, Zhu G, Cang H, et al. Structure of human lanthionine synthetase C-like protein 1 and its interaction with Eps8 and glutathione. Genes Dev. 2009;23(12):1387–92.

ERK1/ERK2

Jacques Pouysségur and Philippe Lenormand
Institute of Developmental Biology and Cancer, CNRS UMR6543, Université de Nice, Nice, France

Synonyms

DERK (*Drosophila melanogaster*); Extracellular regulated kinase 1 and 2; Fus3 (*Saccaromyces cerevisiae*); Kss1 (*Saccaromyces cerevisiae*); Mitogen-activated protein kinase 1 and 2; MPK-1 (*Caenorhabditis elegans*); p42/p44 MAPK; Spk1 (yeast *Saccaromyces pombe*)

Historical Background

Several years prior to ERK1 and ERK2 cloning, respectively, in 1990 and 1991 (Boulton et al. 1991), the close correlation between mitogen action and the increased double phosphorylation of two proteins of 41 and 43 kDa on a phosphotyrosine residue and a phosphothreonine/phosphoserine was revealed by two-dimensional polyacrylamide-gel electrophoresis (reviewed in Chambard et al. [2007]). Because of the sustained phosphorylation during the critical part of G0/G1 phase of the cell cycle, these two proteins known as p41 and p43 were suspected of playing a key role in cell cycle entry. Finally ERK1 was purified and cloned as a protein that phosphorylates microtubules-associated protein2 (MAPK2) during the global hunt of substrates of tyrosine kinase receptors in the late 1980s. Just after the discovery of ERK it was demonstrated in fibroblasts that ERK activation occurred in two phases, a rapid one in response to many stimuli, and a late phase lasting several hours induced by mitogenic agonists; moreover blocking ERK expression was shown to block cell cycle entry (Pages et al. 1991). ERK activation requires double phosphorylation on the TEY sequence, hence ERK1 is the protein that was called p43 (44 kD) and ERK2 is the protein that was called p41 (42 kD).

As soon as ERK1 was cloned, its similarity with two yeast proteins, Fus3 and Kss1 was revealed (cited in Boulton et al. [1991]), hence ERK is a kinase that is conserved from yeasts to humans. Interestingly, the two proteins in yeast are not linked one to one with ERK1 and ERK2 since Fuss3 and Kss1 appeared consequently to a genome duplication in the yeast phylla. This is evidenced by the fact that the yeast saccharomyces pombe which did not undergo the yeast genome duplication has only one ERK (Spk1). As for most isoforms in human genome, ERK1 and ERK2 appeared during two genome duplications in paleovertebrates when cartilaginous fish appeared. Incidentally, the most primitive living vertebrate, the lamprey and also invertebrates such as flies and worms, have only one ERK since their genomes did not undergo these two duplications.

By a combination of genetic studies in flies and worms, and biochemical studies in xenopus and mammalian cells, it was established that ERK is one element in the signaling cascade Ras/Raf/▶ MEK/ERK. Due to space limitations, this presentation will focus only on mammalian ERKs, excellent reviews are available to learn more on yeast, plants, and invertebrate ERKs. ERK plays a central role in this cascade of activation because all upstream members have few substrates or effectors, to the point that ERK is the only known substrate of MEK. On the contrary, ERK can phosphorylate hundred of substrates, furthermore ERK is the first molecule of this cascade able to remain in the nucleus, hence ERK has substrates in all cell compartments unlike all other upstream components of this cascade.

Structure of ERK Activation

ERK is a member of the CMGC Ser/Thr protein kinase family, which encompasses the **C**DKs, **M**APK, **G**SK, and **C**DK-like (respectively, cyclin-dependent kinases, mitogen-activated kinases, glycogen synthase kinase, and cyclin-dependent-like kinase). ERK is a member of the MAPK cluster, the closest kinase to ERK1/2 is ERK5 which lies on a distinct signaling cascade.

ERK can phosphorylate substrates on a serine or a threonine residue, the consensus phosphorylation site was defined by phosphorylated peptide banks, it is: PXS/TP (X can be any amino acid). ERK is a proline-directed kinase since there is an absolute requirement for +1proline for the phosphorylation site of substrates (reviewed in Lee et al. [2004]). This requirement was closely analyzed: it results from a close association of Ala-187 of ERK2 after remodeling of ERK2 to convert a small depression on the surface to a deep pocket where the proline of the substrate interacts. Interestingly ERKs are resistant to activating point mutations, and there are no known associated oncogenes. Replacement of either or both phosphorylation site residues by negatively charged amino acids does not lead to activation unlike many kinases. It is thought that the interaction of proline +1 of substrates is key to prevent undesired activation of ERK, since this step requires the formation of an energetically unfavorable structure. Similarly to all proline-directed kinases, ERK phosphorylation sites can integrate information from different signaling cascades via isomerization of the proline close to the phosphorylation site as it is the case for ▶ c-Myc. C-myc is phosphorylated on serine 62 by ERK then it can be phosphorylated on threonine 58 by GSK-3β. The doubly phosphorylated c-Myc is then recognized by Pin-1 which isomerizes the serine 62-proline 63 bond. This isomerization increases the activity of the phosphatase PP2A to dephosphorylate serine 62, leading ultimately to poly-ubiquitinylation and degradation of c-Myc.

ERK is a compact protein encompassing two lobes. The active site of the kinase is located within the cleft between the two lobes. In the inactive state, the two lobes are rotated in comparison with the two lobes of PKA (constitutively active subunit), which is probably key for inactive ERK to display very low specific activity unless the kinase is doubly phosphorylated (cited in Lee et al. [2004]). Activation of ERK is a combination of rotation of the two lobes upon double phosphorylation by MEK of the activation lip on threonine 193 and thyrosine 195, and remodeling of ERK by binding of the +1proline of the substrate.

Many kinases are proline directed, such as the CDKs, hence the sequence of the phosphorylation site is not sufficient to provide substrate specificity. Indeed substrates must harbor at least one of two types of docking motifs to be specifically phosphorylated by ERK. Firstly, substrates containing the FXFP site (or DEF domain, for Docking site for ERK, FXF) bind to a hydrophobic pocket formed by residues located close to the small depression where the +1proline of the substrates binds (Lee et al. 2004). As a consequence of the proximity between the FXFP/DEF binding site and the small depression for docking to the +1Proline on substrates, the phosphorylation site is usually located close to the FXFP docking site (on its C-terminal side). When ERK is inactive (not doubly phosphorylated) this hydrophobic pocket is occluded, hence substrates cannot bind to inactive ERK via this site. On the contrary, the secondly binding site to ERK, the D docking motif (also called DEJL or KIM for Kinase Interacting Motif), binds to ERK when it is active or inactive (cited in Ebisuya et al. [2005] and Murphy and Blenis [2006]). Furthermore, the D docking motif is found in all types of ERK partners: substrates, activator (MEK), and inhibitors (MAP kinase phosphatases). The D motif consists of a cluster of basic amino acids at the N-term of a hydrophobic motif on substrates and partners. This D motif binds to the CD domain (Common Docking), a docking groove located on the back of ERK (the front of the kinase being the active site) (reviewed in Ebisuya et al. [2005]; Murphy and Blenis [2006]). This docking groove is found in all MAP kinases, the binding specificity for ERK is provided by the sequence of amino acids that constitute this groove and few adjacent amino acids. The fact that the CD domain is located on the back of the kinase allows greater flexibility for the localization of the D domain on substrates (respectively to the phosphorylation site).

Subcellular Localization of ERK

ERK accumulates in the nucleus after acute stimulation of the cell. Nuclear translocation of ERK is required for cell cycle entry. This was demonstrated upon retaining ERK in the cytoplasm by expression of inactive MKP3/DUSP6 that binds tightly to ERK and remains strictly located in the cytoplasm. Retention of ERK in the cytoplasm was shown to alter neither ERK kinase activity nor phosphorylation of cytoplasmic substrates while ERK-dependent transcription and cell proliferation were blocked (reviewed in Chambard et al. [2007]).

MEK behaves as the cytoplasmic anchor for ERK such that MEK co-overexpression maintains the cytoplasmic localization of overexpressed ERK, whereas saturating levels of the ERK-binding site of MEK abrogates ERK export from the nucleus (reviewed in Ebisuya et al. [2005]). MEK is sequestered in the cytoplasm as a consequence of active export out of the nucleus mediated by its NES sequence (Nuclear Export Sequence). MEK binds to inactive ERK in resting cells, hence inactive ERK localization in the cytoplasm is mainly driven by MEK. The natural regulation of ERK translocation has also been demonstrated by differential expression of cytoplasmic anchors such as PEA15 and Sef or nuclear anchors such as DUSP5 and Vanishing (reviewed in Ebisuya et al. [2005]). It has been shown that the stimulation-induced nuclear accumulation of ERK requires the synthesis of short-lived nuclear anchors (reviewed in Chambard et al. [2007]). If protein synthesis is blocked during stimulation, ERK enters inside the nucleus but cannot accumulate in it. Regulation of ERK nuclear translocation is an essential feature of the Raf/MEK/ERK signaling cascade. As an example, cytoplasmic retention of ERK by ▶ PEA-15 has been linked to Ras-induced senescence in fibroblasts (reviewed in Ebisuya et al. [2005]).

How does ERK enter into the nucleus? ERK lacks a nuclear localization sequence (NLS), leading to the suggestion that ERK may enter by a piggyback mechanism via binding to NLS-containing proteins. NLS-dependent mechanisms require energy for Ran-dependent cycling of importins. However, reconstituted import assays have shown that ERK can bind directly to FxFG-repeated sequences of nucleoporin in the lumen of the NPC (Nuclear Pore Complex), indicating that it may enter the nucleus in the absence of energy sources or cytosolic factors (reviewed in Ebisuya et al. [2005] and Lidke et al. [2010]). Point mutations of ERK revealed that inactive and active ERK interact with nucleoporins via different domains; thus both active and inactive ERK can be transported across the nuclear pore in an energy-independent fashion (Yazicioglu et al. 2007). However, in a reconstituted import assay, thiophosphorylated ERK2 import increased in the presence of energy. Overall, these observations suggest a role for both an energy-dependent and an energy-independent mechanism (via direct binding to nucleoporins) in ERK cytoplasmic-nuclear translocation. Since ERK was shown to form dimers when crystallized and when purified in vitro, the possibility that ERK dimerization could play a role in nuclear entry was scrutinized. Several independent studies have failed to detect existence of ERK dimers in vivo, either by lack of FRET between GFP-ERKs of different colors (Burack and Shaw 2005) or by advanced microscopic techniques such as emFRET and fluorescence correlation spectroscopy (Lidke et al. 2010). An ERK "dimerization-mutant" that failed to dimerise in vitro was shown to be activated more slowly than wild-type ERK and to enter more slowly in the nucleus, but this mutant accumulated normally in the nucleus (Lidke et al. 2010). These experiments confirm that the activation step is central for ERK nuclear entry, not dimerization. Half-maximal nuclear accumulation of ERK is reached within 3 min (movie in supplemental data of Lidke et al. [2010]).

Interestingly, ERK has been shown to phosphorylate several nucleoporins such as NUP50 which was shown to reduced its affinity for importin-beta family proteins, importin-beta and transportin, hence ERK activation can regulate nucleocytoplasmic transport (Kosako et al. 2009). These interactions can have a mutual relationship since it was shown that the nuclear pore complex protein Tpr is both a substrate and a scaffold for activated ERKs, hence Tpr is facilitating ERK transport across the nucleus while ERK regulates global nucleocytoplasmic transport via phosphorylation of Tpr (Vomastek et al. 2008).

Concerning cell motility, active ERK is targeted to newly forming focal adhesion after integrin engagement or activation of v-Scr, where it could phosphorylate substrates specifically located in focal adhesion (cited in Pullikuth and Catling [2010]). Several studies have demonstrated a requirement for ERK signaling in the disassembly of focal adhesions of migrating cells, in part through the activation of calpain proteases that can downregulate focal adhesion kinase signaling (cited in Pullikuth and Catling [2010]). On the contrary, ERK activity was shown to be required for fibronectin-stimulated Rho-GTP loading, Rho-kinase function, and the maturation of focal adhesions in spreading cells via phosphorylation of the C-terminus of p190A RhoGAP via regulation of its localization and activity (Pullikuth and Catling 2010).

Functions and Substrates of ERK

ERK can phosphorylate many substrates, it was demonstrated that ERK activation is required for cell

proliferation, cell growth, cell differentiation, development, memory formation, senescence, and apoptosis. Antiproliferative mechanisms triggered by ERK activation are reviewed in Cagnol and Chambard (2010) and proliferative roles of ERK are reviewed in Chambard et al. (2007) and Torii et al. (2006). Instead of listing the many proven substrates of ERK, the consequences of ERK activation to trigger biological responses will be illustrated. A special emphasis will be put on how to convert a gradual ERK signal to an on/off switch, such as the decision for an individual cell to proliferate or not.

In fibroblasts ERK activation occurs in two phases: an initial rapid phase lasting about 30 min in response to many stimuli and a late phase lasting several hours that is induced only by the persistent presence of mitogenic agonists. Whereas mitogenic and non-mitogenic agonists can activate ERK efficiently during the rapid phase, only long-lasting stimulation of ERK induces the robust accumulation of immediate-early gene products (IEG products), such as c-Fos protein that ultimately leads to cyclin D1 expression and cell cycle entry (reviewed in Murphy and Blenis [2006]). Typically, in quiescent cells, activation of surface receptors leads to ERK activation and immediate entry of ERK in the nucleus to phosphorylate preexisting transcription factors, such as Elk-1, which in turn induce transcription of IEGs, such as c-fos. When ERK stimulation is transient, the c-Fos protein is not accumulated enough prior to the decline of ERK activation. On the contrary, sustained ERK activity can phosphorylate C-terminal phosphorylation sites on c-Fos, which in turn unmasks an FXFP site/DEF domain which increases c-Fos binding to ERK to ensure phosphorylation of other sites for full stabilization of the c-Fos protein. Consequently, persistent activation of ERK leads to persistent expression of c-Fos in the nucleus versus almost no c-Fos protein expressed during transient activation of ERK, converting the duration of ERK activation to an on/off switch.

In order to increase markedly transcription of IEGs, ERK also induces chromatin remodeling and increases protein translation (directly or indirectly by activating downstream kinases such as RSK, MSK, and MNK) (substrates reviewed in Chambard et al. [2007] and Yoon and Seger [2006]).

Another way for ERK to "sense" the duration of activation occurs via the downregulation of antiproliferative genes (Yamamoto et al. 2006). As soon as ERK activation decreases, these genes are reexpressed and block cell cycle progression, only persistent ERK activation and persistent downregulation of these genes allow proliferation.

Interestingly, in PC12 cells, a transient ERK stimulation leads to cell division (contrarily to fibroblasts) whereas long-lasting ERK activation is required for differentiation. Hence for a given cell type, a given cell fate is driven by a unique combination of duration, localization, and strength of ERK activation (reviewed in Chambard et al. [2007], Ebisuya et al. [2005], and Murphy and Blenis [2006]).

When scrutinizing the role of ERK in differentiation, up to 284 proteins were identified by quantitative proteomics to bind to ERK (von Kriegsheim et al. 2009). Furthermore, 60 of these proteins were shown to change their binding to ERK during differentiation. Considering that several downstream kinases increase markedly the repertoire of ERK targets, this work illustrates why ERK is central in the cascade of activation to drive cell-surface receptor activation (on the contrary, upstream of ERK, MEK has only one proven substrate, for example).

It is important to mention that ERK2 was shown, in 2009, to bind directly to DNA on the sequence G/CAAAG/C in a manner independent of kinase activity. ERK2 can behave as a transcriptional repressor of interferon signaling, which increases markedly the multiplicity of ERK functions (Hu et al. 2009).

ERK in Human Pathologies

As discussed earlier, so far no activating mutations of ERK have been found. However, very often the signaling cascade leading to ERK activation is constitutively activated in human diseases at the level of cell surface receptors (such as the EGF-R2 receptor), at the level of Ras (e.g., K12V mutation), at the level of Raf (e.g., V600E mutation), and even when ERK phosphatases such as DUSP6 are deleted. There are also indices that modulators of ERK cascade such as Sprouty and Sef may play a role in tumorigenesis (Torii et al. 2006).

Around 70% of human melanomas display activating mutations of Raf, hence ERK cascade is constitutively active (Pratilas et al. 2009; Dhomen et al. 2009; Dankort et al. 2009), in many other melanomas the ERK cascade is also constitutively activated via Ras mutations. However, measuring the activation level of

ERK has not been demonstrated to correlate with cancer progression (Pratilas et al. 2009). This is likely due to the fact that feedback inhibitions can normalize ERK activation even when the signaling cascade is pathologically active. Surprisingly, it was shown that extremely elevated and long-lasting ERK activation blocks cell proliferation by increasing the levels of the cell cycle inhibitor p21 (reviewed in Chambard et al. [2007], Murphy and Blenis [2006] and Torii et al. [2006]). As a consequence, when a cell has to cope with constitutive activation of the Ras/Raf/MEK/ERK pathway, the cell must evolve to overcome this cell cycle block to become oncogenic. At the level of ERK, retro-inhibition can be done by phosphorylation of Sos or Raf or MEK by ERK to reduce the input signal. Furthermore, ERK has been shown to induce transcriptionally MKPs/DUSPs phosphatases that dephosphorylate and inactivate ERK directly (Keyse 2008). Indeed it was demonstrated recently that the transcriptional output of tumors with mutant BRaf and those with receptor tyrosine kinase (RTK) activation is very different, despite the fact that these tumors have similar levels of phosphorylated ERK (Pratilas et al. 2009). In fact, only V600E-BRaf cells display very high transcriptional activity of many genes, including negative feedback inhibitors of the pathway such as MKPs/DUSPs phosphatases and Sprouty gene family members. On the other hand, physiologic feedback inhibition of Raf/MEK signaling down-regulates ERK output in cells with mutated RTK without resorting to massive transcriptional induction of ERK regulators.

On the opposite, diminution of ERK activation during development has also negative consequences on human health. For example, several children with a 1 Mb microdeletion on chromosome 22q11.2 encompassing the ERK2 gene were identified (Samuels et al. 2008). These children have reduced ERK2 levels and exhibit microcephaly, impaired cognition, and developmental delay, which is expected considering the multiple roles played by ERK on synaptic plasticity and memory (Kelleher et al. 2004). Blocking ERK activity also plays a role in the pathogenicity of Bacillus anthracis infection. This bacterium secretes lethal factor (a component of anthrax lethal toxin) that inactivates MEK family members through proteolysis of their amino termini (Duesbery et al. 1998). Proteolysis of MEK1/2 reduces interaction of MEK with ERK and also the phosphorylation of ERK by MEK, which leads to total loss of ERK activity in the cell during infection.

Specific Roles of ERK1 and ERK2

ERK activation is carried by ERK1 and ERK2 that are 84% identical at the amino acid level in humans. No agonist is known to more specifically activate ERK1 over ERK2, and both ERK1 and ERK2 were shown to translocate to the nucleus upon stimulation. MEK1/2 can indiscriminately phosphorylate ERK1 and ERK2 on their identical TEY sequence. Both ERK1 and ERK2 phosphorylate substrates on the consensus PXS/TP sites with similar specific activities in vitro, measured with bacterially expressed ERKs and with immuno-precipitated ERKs (reviewed in Lefloch et al. [2009] and Voisin et al. [2010]). The CD docking domain diverge only for a leucine instead of an isoleucine between ERK1 and ERK2, and the FXFP/DEF docking is fully conserved between ERK1 and ERK2. More recently, 284 partners of ERKs that were identified by quantitative proteomics following immunoprecipitation were shown to associate as well with ERK1 and ERK2 (von Kriegsheim et al. 2009).

Therefore, why mammals express ubiquitously ERK1 and ERK2 if they appear redundant? In fact it is unlikely that each ERK performs an essential function in vertebrates since it was found that frogs and birds have only one ERK after loosing one isoform during evolution (Lefloch et al. 2009). However, invalidation of ERKs in mice gave strikingly opposite results, lack of ERK2 led to early embryonic cell death whereas mice lacking ERK1 live and reproduce normally. Reproducibly, mice lacking ERK1 display minor phenotypes such as behavioral excitement; they also present abnormal responses to stresses such as resistance to obesity when fed a fat-rich diet or reduced occurrence of skin papilloma triggered by DMBA and TPA treatment (reviewed in Lefloch et al. [2009] and Voisin et al. [2010]). However, one allele of either Erk1 or Erk2 in endothelial cells was necessary and sufficient for embryonic development (Srinivasan et al. 2009), and similarly one allele of ERK1 or ERK2 is sufficient but necessary in granulosa cells for maturation of oocytes (Fan et al. 2009). In most cells, ERK2 is more expressed than ERK1, and usually deletion of ERK2 has more pronounced effects than deletion of ERK1. In fact ERK activation is concentrated on the remaining isoform as a compensatory effect. When these compensatory effects were taken into account it was concluded that both ERK isoforms convey proliferative signals according to their relative expression

level (Lefloch et al. 2009). This was confirmed genetically recently in mouse embryo fibroblasts that can proliferate in the absence of one or the other ERKs, but stop growing without ERK (Voisin et al. 2010). A direct correlation between the rate of proliferation and the extent of total ERK activation after 5 min stimulation was established in this study.

At this point, the data tend to indicate that for major functions such as proliferation and differentiation, ERK1 and ERK2 are redundant in a given cell; however one cannot exclude that ERK isoforms possess a unique role and interact with a few isoform-specific substrates. A good ERK1-specific candidate could be p62, signaling adapter p62/sequestosome1, that plays a role in mature-onset obesity and insulin resistance. P62 was demonstrated recently to bind specifically to ERK1 but not to ERK2 and genetic invalidation of ERK1 in the context of p62 knockout, reversed the increased adiposity and adipogenesis (Lee et al. 2010). These data are in accordance with the observation that mice lacking ERK1 have decreased adiposity and fewer adipocytes than wild-type animals (cited in Lee et al. [2010]).

Summary-Perspectives

ERK is a central player in the Ras/Raf/MEK/ERK cascade from its unique ability to phosphorylate a number of substrates in all cell compartments. The efforts to study ERK stemmed initially from the recognition that it is a key downstream target of tyrosine kinase receptors activated during G1 phase of cell cycle; interest for ERK remained elevated when this signaling cascade was found to be abnormally activated in about 30% of all cancers. Strikingly, it was recently demonstrated that a single activating point mutation of one upstream activator or ERK (BRaf V600E) is sufficient to initiate melanoma in mice (Dhomen et al. 2009; Dankort et al. 2009).

Concerning the specific roles of ERK isoforms, progress needs to be made to identify isoform-specific substrates and measure precisely the phosphorylation kinetics and binding affinities of ERK isoforms to these unique substrates (if they exist). How does ERK2 binding to DNA act to repress transcription? Can ERK1 bind to DNA? Does ERK1 bind to the same DNA sequence as ERK2? Why do ERK1 and ERK2 have these unique N-terminal ends, almost consisting of stretches of alanine and glycine residues: MAAAAAQGGGGG- for human ERK1 and MAAAAAAGAG- for human ERK2?

ERK plays ambivalent roles in different cells: how to reconcile that long-term activation of ERK triggers proliferation in some cells while it switches other cells to stop proliferating and enter differentiation? Moreover ERK can play ambivalent roles in the same cells types: how to understand the role of ERK in brain since it is implicated both in promoting neuronal cell death and neuronal plasticity? Hopefully advances in "omics" technologies will allow us to grasp a wider picture by knowing at the same time: the level of regulators of ERK, the level and phosphorylation status of ERK substrates and the level of proteins whose genes are induced by ERK activation. For many aspects of ERK regulation, modeling will be required to take into account the multiplicity of players. For example, ERK can be inactivated by many phosphatases: threonine phosphatases such as PP2A, tyrosine phosphatases such as STEP, and MKPs/DUSPs (MAPK phosphatases that dephosphorylate both the tyrosine and threonine residues). At least 11 DUSPs can bind specifically to MAPKs and most of them can inactivate ERK in vitro. When these phosphatases were invalidated individually in the whole animal unexpected results/specificities were observed such as during DUSP2/PAC1 invalidation in mice (reviewed in Keyse [2008]).

Several mathematical models of ERK activation have been published. Some are elaborated since they have measured the concentration of cascade members, localization, and dissociation kinetics with fluorescent probes (Fujioka et al. 2006); however, all models are still simple solely because none take into account the widespread multiplicity of cascade members, multiplicity of feedback inhibitors, and multiplicity of localization partners; hopefully models will be refined and will suggest experimental settings to validate them.

During the reviewing process of this manuscript, it was reported the first success of the ERK pathway inhibitor vemurafenib to increase overall and progression-free survival of patients with metastatic melanoma (Chapman et al. 2011). Vemurafenib inhibits BRaf mutant V600E specifically. Unfortunately, very often melanoma becomes resistant to treatment by vemurafenib via mutation of MEK1 (C121S) that renders the ERK cascade insensitive to Raf and MEK inhibitors (Wagle et al. 2011). Combinatorial

therapy may prove to be the solution, for example synergistic effects were reported in tumor xenografts by combining MEK inhibition with microtubule-destabilizing agents (Watanabe et al. 2011). It would be invaluable to dispose of ERK inhibitors as efficient as these MEK inhibitors. Indeed by directly targeting ERK kinase activity, it may be possible to avoid resetting the basal ERK activation level during long-term treatment (consequent to the decrease of ERK-driven retro-inhibitions). Hopefully better modeling of ERK actions will allow us to design new strategies to tackle cancer more efficiently by combining existing drugs.

Due to extreme space limitations we were unable to cite directly many excellent contributions to this field. We would like to apologize to all authors whose work was only cited indirectly from more recent publications.

References

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, et al. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell. 1991;65(4):663–75.

Burack WR, Shaw AS. Live cell imaging of ERK and MEK: simple binding equilibrium explains the regulated nucleocytoplasmic distribution of ERK. J Biol Chem. 2005;280(5):3832–7.

Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death–apoptosis, autophagy and senescence. FEBS J. 2010;277(1):2–21.

Chambard JC, Lefloch R, Pouyssegur J, Lenormand P. ERK implication in cell cycle regulation. Biochim Biophys Acta. 2007;1773(8):1299–310.

Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA. Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation. N Engl J Med. 2011 Jun 5. [Epub ahead of print].

Dankort D, Curley DP, Cartlidge RA, Nelson B, Karnezis AN, Damsky Jr WE, et al. Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. Nat Genet. 2009;41(5):544–52.

Dhomen N, Reis-Filho JS, da Rocha Dias S, Hayward R, Savage K, Delmas V, et al. Oncogenic Braf induces melanocyte senescence and melanoma in mice. Cancer Cell. 2009;15(4):294–303.

Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, Copeland TD, et al. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor [see comments]. Science. 1998;280(5364):734–7.

Ebisuya M, Kondoh K, Nishida E. The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. J Cell Sci. 2005;118(14):2997–3002.

Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, et al. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. Science. 2009;324(5929):938–41.

Fujioka A, Terai K, Itoh RE, Aoki K, Nakamura T, Kuroda S, et al. Dynamics of the Ras/ERK MAPK cascade as monitored by fluorescent probes. J Biol Chem. 2006;281(13):8917–26.

Hu S, Xie Z, Onishi A, Yu X, Jiang L, Lin J, et al. Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. Cell. 2009;139(3):610–22.

Kelleher 3rd RJ, Govindarajan A, Jung HY, Kang H, Tonegawa S. Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell. 2004;116(3):467–79.

Keyse SM. kinase phosphatases (MKPs) and cancer. Cancer Metastasis Rev. 2008;27(2):253–61.

Kosako H, Yamaguchi N, Aranami C, Ushiyama M, Kose S, Imamoto N, et al. Phosphoproteomics reveals new ERK MAP kinase targets and links ERK to nucleoporin-mediated nuclear transport. Nat Struct Mol Biol. 2009;16(10):1026–35.

Lee T, Hoofnagle AN, Kabuyama Y, Stroud J, Min X, Goldsmith EJ, et al. Docking motif interactions in MAP kinases revealed by hydrogen exchange mass spectrometry. Mol Cell. 2004;14(1):43–55.

Lee SJ, Pfluger PT, Kim JY, Nogueiras R, Duran A, Pages G, et al. A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis. EMBO Rep. 2010;11(3):226–32.

Lefloch R, Pouyssegur J, Lenormand P. Total ERK1/2 activity regulates cell proliferation. Cell Cycle. 2009;8(5):705–11.

Lidke DS, Huang F, Post JN, Rieger B, Wilsbacher J, Thomas JL, et al. ERK nuclear translocation is dimerization-independent but controlled by the rate of phosphorylation. J Biol Chem. 2010;285(5):3092–4102.

Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci. 2006;31(5):268–75.

Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, Pouyssegur J. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci U S A 1993;90(18):8319–23.

Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB, et al. (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. Proc Natl Acad Sci USA. 2009;106(11):4519–24.

Pullikuth AK, Catling AD. Extracellular signal-regulated kinase promotes Rho-dependent focal adhesion formation by suppressing p190A RhoGAP. Mol Cell Biol. 2010;30(13):3233–48.

Samuels IS, Karlo JC, Faruzzi AN, Pickering K, Herrup K, Sweatt JD, et al. Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. J Neurosci. 2008;28(27):6983–95.

Srinivasan R, Zabuawala T, Huang H, Zhang J, Gulati P, Fernandez S, et al. Erk1 and Erk2 regulate endothelial cell proliferation and migration during mouse embryonic angiogenesis. PLoS ONE. 2009;4(12):e8283.

Torii S, Yamamoto T, Tsuchiya Y, Nishida E. ERK MAP kinase in G cell cycle progression and cancer. Cancer Sci. 2006;97(8):697–702.
Voisin L, Saba-El-Leil MK, Julien C, Fremin C, Meloche S. Genetic demonstration of a redundant role of extracellular signal-regulated kinase 1 (ERK1) and ERK2 mitogen-activated protein kinases in promoting fibroblast proliferation. Mol Cell Biol. 2010;30(12):2918–32.
Vomastek T, Iwanicki MP, Burack WR, Tiwari D, Kumar D, Parsons JT, et al. Extracellular signal-regulated kinase 2 (ERK2) phosphorylation sites and docking domain on the nuclear pore complex protein Tpr cooperatively regulate ERK2-Tpr interaction. Mol Cell Biol. 2008;28(22):6954–66.
von Kriegsheim A, Baiocchi D, Birtwistle M, Sumpton D, Bienvenut W, Morrice N, et al. Cell fate decisions are specified by the dynamic ERK interactome. Nat Cell Biol. 2009;11(12):1458–64.
Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, Kehoe SM, Johannessen CM, Macconaill LE, Hahn WC, Meyerson M, Garraway LA. Dissecting Therapeutic Resistance to RAF Inhibition in Melanoma by Tumor Genomic Profiling. J Clin Oncol 2011 Mar 7. [Epub ahead of print].
Watanabe K, Tanimura S, Uchiyama A, Sakamoto T, Kawabata T, Ozaki K, Kohno M. Blockade of the extracellular signal-regulated kinase pathway enhances the therapeutic efficacy of microtubule-destabilizing agents in human tumor xenograft models. Clin Cancer Res 2011;16(4):1170–8.
Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S, Nishida E. Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. Curr Biol. 2006;16(12):1171–82.
Yazicioglu MN, Goad DL, Ranganathan A, Whitehurst AW, Goldsmith EJ, Cobb MH. Mutations in ERK2 binding sites affect nuclear entry. J Biol Chem. 2007;282(39):28759–67.
Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors. 2006;24(1):21–44.

Erk3 and Erk4

Sonia Klinger[1] and Sylvain Meloche[2,3]
[1]Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada
[2]Department of Pharmacology, Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada
[3]Program of Molecular Biology, Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada

Synonyms

Mapk4 (Erk4); Mapk6 (Erk3)

Historical Background

Extracellular signal-regulated kinase 3 (Erk3) and Erk4 are atypical members of the mitogen-activated protein (MAP) kinase family of serine/threonine kinases. The *Erk3* and *Erk4* genes were originally identified in 1991 and 1992, by homology cloning with probes derived from the MAP kinase Erk1 (Boulton et al. 1991; Gonzalez et al. 1992). In human, Erk3 is encoded by the *MAPK6* gene located on chromosome 15q21.2. The *MAPK4* gene present on chromosome 18q21.1 encodes Erk4. The high sequence identity of Erk3 and Erk4 proteins and the similar organization of their genes indicate that the two proteins are true paralogs. It is noteworthy that *MAPK6* and *MAPK4* are the only ▶ MAP kinase genes that are restricted to the vertebrate lineage.

Structure of Erk3 and Erk4

Erk3 and Erk4 are related protein kinases of 100 and 70 kDa, respectively, that define a distinct subfamily of ▶ MAP kinases (Coulombe and Meloche 2007). They are characterized by the presence of a catalytic kinase domain at the N-terminal end, which is 73% amino acid identical, and a long C-terminal extension. Although highly conserved in vertebrate evolution, the function of the C-terminal domain remains to be defined. By contrast to classical MAP kinases like ▶ Erk1/Erk2, which are activated by dual phosphorylation of the threonine and tyrosine residues in the conserved Thr-Xxx-Tyr motif of the activation loop, Erk3 and Erk4 contain a single phospho-acceptor site (Ser-Glu-Gly sequence) in their activation loop. In addition, they bear the sequence Ser-Pro-Arg instead of Ala-Pro-Glu in kinase subdomain VIII, and are the only protein kinases in the human genome to have an arginine residue at this position. The impact of these structural features on the regulation of Erk3 and Erk4 is unknown.

Expression of Erk3 and Erk4

Erk3 is expressed ubiquitously in adult mammalian tissues, whereas Erk4 shows a more restricted expression profile. The level of expression of the two kinases varies considerably between tissues, but the highest

expression is found in the brain (Boulton et al. 1991; Kant et al. 2006; Turgeon et al. 2000). In the mouse embryo, they share a similar temporal pattern of regulation with a peak of expression at embryonic day 11, coincident with the time of early organogenesis (Rousseau et al. 2010). In vitro studies have shown that Erk3 mRNA is upregulated upon differentiation of P19 embryonal carcinoma cells to the neuronal or muscle lineage (Boulton et al. 1991).

At the subcellular level, Erk3 and Erk4 exhibit distinct localization. Erk3 is found in both the cytoplasm and the nucleus of a variety of cell types, whereas Erk4 is localized mainly in the cytoplasmic compartment (Aberg et al. 2006; Julien et al. 2003; Kant et al. 2006). The cytoplasmic localization of Erk3 and Erk4 requires an active nuclear export by a Crm1-dependent mechanism. In contrast to classical MAP kinases, the cellular distribution of Erk3 and Erk4 does not change in response to common mitogenic or stress stimuli. However, Erk3 localization is regulated through its interaction with ▶ MAP kinase-activated protein kinase 5 (MK5) (Schumacher et al. 2004; Seternes et al. 2004). MK5 is a member of the MAP kinase-activated protein kinase family of protein kinases that lie downstream of ▶ MAP kinase signaling cascades. The formation of a complex between Erk3 and MK5 results in the nuclear to cytoplasmic redistribution of both proteins. The kinase activity of either protein is dispensable for this relocalization. In addition to its effect on localization, MK5 also affect endogenous Erk3 expression. Embryonic fibroblasts prepared from MK5-deficient mice or HeLa cells transfected with a si-RNA targeting MK5 exhibit a marked reduction of Erk3 protein level (Seternes et al. 2004).

Interestingly, Erk3 and Erk4 proteins display different stability. Whereas Erk4 is a relatively stable protein, Erk3 was shown to be a highly unstable protein in proliferating cells, with a half-life of about 30 min (Aberg et al. 2006; Coulombe et al. 2003; Kant et al. 2006). Erk3 is constitutively degraded by the ubiquitin-proteasome pathway, and two regions in the N-terminal lobe of the kinase domain are both necessary and sufficient to target Erk3 for proteolysis (Coulombe et al. 2003). Thus, Erk3 biological activity is regulated at the level of cellular abundance. The short half-life of Erk3 has a physiological significance since the protein is stabilized and accumulates to high levels in the course of cellular differentiation and during mitosis. Indeed, Erk3 stability increases with time during the neurogenic and myogenic differentiation of PC12 and C2C12 cells, respectively, leading to protein accumulation (Coulombe et al. 2003). Upregulation of Erk3 during muscle differentiation is concomitant to accumulation of the cell cycle inhibitor $p21^{Cip1}$ and cell cycle exit. Recent findings have shown that phosphorylation of four residues located in the extreme C-terminal extension stabilizes Erk3 protein, leading to its accumulation in mitosis (Tanguay et al. 2010).

Regulation of Erk3 and Erk4 Activity and Substrates

Little is known about the regulation of Erk3 and Erk4 biological activity. In intact cells, Erk3 and Erk4 are phosphorylated on Ser189 and Ser186, respectively, in the Ser-Glu-Gly motif of their activation loop (Cheng et al. 1996; Coulombe et al. 2003; Deleris et al. 2008; Perander et al. 2008). Unlike classical ▶ MAP kinases, this phosphorylation event is detected in resting cells and is not modulated by common mitogenic or stress stimuli.

Classical ▶ MAP kinases like Erk1 are multifunctional kinases that phosphorylate a vast array of substrates. However, Erk3 and Erk4 do not phosphorylate generic MAP kinase substrates such as myelin basic protein, microtubule-associated protein-2, c-Jun, or Elk-1 (Cheng et al. 1996), suggesting that they have a more restricted substrate specificity. The identification of MK5 as specific interaction partner and substrate of both Erk3 and Erk4 was an important step toward a better characterization of these kinases. Binding of Erk3 or Erk4 to MK5 results in the phosphorylation of MK5 on Thr182, and its resulting enzymatic activation (Aberg et al. 2006; Kant et al. 2006; Schumacher et al. 2004; Seternes et al. 2004). While the catalytic activity of Erk4 is required to activate MK5, its activation by Erk3 appears to depend both on the kinase activity of Erk3 as well as on a cytoplasmic scaffolding role of Erk3 with subsequent MK5 autophosphorylation. Importantly, Erk3 and Erk4 are bonafide physiological regulators of MK5 activity. The endogenous activity of MK5 is partially reduced in cells deprived in either Erk3 or Erk4, and the combined knockdown of the two kinases results in a greater reduction of MK5 activity (Aberg et al. 2006; Seternes et al. 2004).

Recently, a more complex interaction between Erk3/Erk4 and MK5 has been proposed (Deleris et al. 2008; Perander et al. 2008). According to these studies, the interaction between Erk3/Erk4 and MK5 recruits and/or contributes to the activation of Erk3/Erk4 activation loop kinase, resulting in the enhancement of Erk3/Erk4 phosphorylation on Ser189/Ser186. This, in turn, leads to the stabilization of Erk3/Erk4-MK5 complexes, the full enzymatic activation of Erk3/Erk4, and the phosphorylation of MK5 on activation loop Thr182. Activated MK5 reciprocally phosphorylates Erk3 and Erk4 on unidentified sites located outside the activation loop. The finding that the interaction between Erk3/Erk4 and MK5 requires activation loop phosphorylation of Erk3/Erk4 indicates that the mechanism by which these atypical MAP kinases bind to effector kinases is distinct to that of classical MAP kinases. Indeed, ▶ Erk1/Erk2 and p38 interact with downstream MAP kinase-activated protein kinases through the common docking (CD) domain, a cluster of negatively charged amino acids located near the kinase domain of MAP kinases. The conserved CD domain within Erk3 and Erk4 is dispensable for MK5 interaction.

Physiological Functions of Erk3 and Erk4

Much remain to be learned about the cellular functions of Erk3 and Erk4. However, in vitro studies in different model cell lines and in vivo analysis of mice deficient for Erk3 or Erk4 have started to shed light on the potential roles of these kinases. All these studies suggest that Erk3 and Erk4 are likely to be involved in the control of cell proliferation and differentiation. Erk3 expression is upregulated during differentiation of P19 and PC12 cells into neurons and of C2C12 cells into myotubes, in association with proliferation arrest (Boulton et al. 1991; Coulombe et al. 2003). It has been reported that the overexpression of stable forms of Erk3 in fibroblasts inhibits S-phase entry (Coulombe et al. 2003). Moreover, Erk3 was shown to interact with the cell cycle regulatory proteins cyclin D3, cell-division cycle 14A (Cdc14A) and Cdc14B through its C-terminal extension (Hansen et al. 2008; Sun et al. 2006; Tanguay et al. 2010). The phosphorylation of Erk3 also varies during the cell cycle. Erk3 is phosphorylated in the C-terminal extension during entry into mitosis and dephosphorylated at the M- to G1-phase transition. Cdc14A and Cdc14B phosphatases reverse the C-terminal phosphorylation of Erk3, and the mitotic kinase cyclin B1/cyclin-dependent kinase 1 (Cdk1) is most likely responsible for the phosphorylation of these sites. A potential role of Erk3 in insulin secretion has also been reported in another study (Anhe et al. 2006). Prolactin treatment of isolated rat pancreatic islets was found to increase Erk3 expression. This hormone is involved in the adaptive response of pancreatic β-cells toward peripheral insulin resistance during pregnancy. Silencing of Erk3 in isolated pancreatic islets prevents glucose-stimulated insulin secretion.

The study of mice deficient for Erk3, Erk4, and both Erk3 and Erk4 has provided important clues about their physiological roles. Loss of Erk3 leads to intrauterine growth restriction, delayed lung maturation associated with decreased sacculation and defective type II pneumocyte differentiation, and neonatal lethality (Klinger et al. 2009). Whereas the lung maturation defect can be overcome by in utero glucocorticoid administration, the newborn mice cannot be rescued from neonatal death. This indicates that additional physiological alterations contribute to the neonatal lethality. Erk3-deficient mice have reduced levels of insulin-like growth factor 2 (IGF-2) in the serum, suggesting that Erk3 might be a regulator of IGF-2 levels. Erk4-deficient mice are viable, develop normally, and show no gross physiological anomalies (Rousseau et al. 2010). Interestingly, behavioral analysis revealed that Erk4 mutant mice manifest depression-like behavior. In these mice, the loss of Erk4 is not compensated by increased activity or level of Erk3. Also, additional deletion of Erk4 in Erk3-deficient mice does not aggravate the fetal growth restriction and pulmonary immaturity phenotypes. This indicates that Erk3 and Erk4 protein kinases have acquired specific nonredundant functions through evolutionary diversification.

To date, MK5 remains the only identified substrate of Erk3 and Erk4, but the function of MK5 is still elusive. Of note, a recent study reported that mice deficient for MK5 display enhanced skin carcinogenesis after dimethylbenzanthracene (mutagen causing activating Ras mutations) application and compromised senescence induction (Sun et al. 2007). It remains to be determined whether Erk3 and Erk4 play a role in Ras-induced senescence and tumor suppression. The identification of additional substrates of Erk3 and Erk4 will help to better characterize the biological functions of these kinases and the signaling pathways in which they are involved.

Summary

Erk3 and Erk4 were among the first MAP kinases to be identified in the early 1990s. However, the characterization of these protein kinases has progressed much slower than that of classical MAP kinase family members such as ▶ Erk1/Erk2. Much still remains to be learned about their regulation, the identity, and spectrum of their substrates, their physiological roles, and their putative involvement in human diseases.

References

Aberg E, Perander M, Johansen B, Julien C, Meloche S, Keyse SM, Seternes OM. Regulation of MAPK-activated protein kinase 5 activity and subcellular localization by the atypical MAPK ERK4/MAPK4. J Biol Chem. 2006;281:35499–510.

Anhe GF, Torrao AS, Nogueira TC, Caperuto LC, Amaral ME, Medina MC, Azevedo-Martins AK, Carpinelli AR, Carvalho CR, Curi R, Boschero AC, Bordin S. ERK3 associates with MAP2 and is involved in glucose-induced insulin secretion. Mol Cell Endocrinol. 2006;251:33–41.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell. 1991;65:663–75.

Cheng M, Boulton TG, Cobb MH. ERK3 is a constitutively nuclear protein kinase. J Biol Chem. 1996;271:8951–8.

Coulombe P, Meloche S. Atypical mitogen-activated protein kinases: structure, regulation and functions. Biochim Biophys Acta. 2007;1773:1376–87.

Coulombe P, Rodier G, Pelletier S, Pellerin J, Meloche S. Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation. Mol Cell Biol. 2003;23:4542–58.

Deleris P, Rousseau J, Coulombe P, Rodier G, Tanguay PL, Meloche S. Activation loop phosphorylation of the atypical MAP kinases ERK3 and ERK4 is required for binding, activation and cytoplasmic relocalization of MK5. J Cell Physiol. 2008;217:778–88.

Gonzalez FA, Raden DL, Rigby MR, Davis RJ. Heterogenous expression of four MAP kinase isoforms in human tissues. FEBS Lett. 1992;304:170–8.

Hansen CA, Bartek J, Jensen S. A functional link between the human cell cycle-regulatory phosphatase Cdc14A and the atypical mitogen-activated kinase Erk3. Cell Cycle. 2008;7:325–34.

Julien C, Coulombe P, Meloche S. Nuclear export of ERK3 by a CRM1-dependent mechanism regulates its inhibitory action on cell cycle progression. J Biol Chem. 2003;278:42615–24.

Kant S, Schumacher S, Singh MK, Kispert A, Kotlyarov A, Gaestel M. Characterization of the atypical MAPK ERK4 and its activation of the MAPK-activated protein kinase MK5. J Biol Chem. 2006;281:35511–19.

Klinger S, Turgeon B, Levesque K, Wood GA, Aagaard-Tillery KM, Meloche S. Loss of Erk3 function in mice leads to intrauterine growth restriction, pulmonary immaturity, and neonatal lethality. Proc Natl Acad Sci USA. 2009;106:16710–15.

Perander M, Aberg E, Johansen B, Dreyer B, Guldvik IJ, Outzen H, Keyse SM, Seternes OM. The Ser(186) phosphoacceptor site within ERK4 is essential for its ability to interact with and activate PRAK/MK5. Biochem J. 2008;411:613–22.

Rousseau J, Klinger S, Rachalski A, Turgeon B, Deleris P, Vigneault E, Poirier-Heon JF, Davoli MA, Mechawar N, El Mestikawy S, Cermakian N, Meloche S. Targeted inactivation of Mapk4 in mice reveals specific non-redundant unctions of Erk3/Erk4 subfamily mitogen-activated protein kinases. Mol Cell Biol. 2010;30:5752–63.

Schumacher S, Laass K, Kant S, Shi Y, Visel A, Gruber AD, Kotlyarov A, Gaestel M. Scaffolding by ERK3 regulates MK5 in development. EMBO J. 2004;23:4770–9.

Seternes OM, Mikalsen T, Johansen B, Michaelsen E, Armstrong CG, Morrice NA, Turgeon B, Meloche S, Moens U, Keyse SM. Activation of MK5/PRAK by the atypical MAP kinase ERK3 defines a novel signal transduction pathway. EMBO J. 2004;23:4780–91.

Sun M, Wei Y, Yao L, Xie J, Chen X, Wang H, Jiang J, Gu J. Identification of extracellular signal-regulated kinase 3 as a new interaction partner of cyclin D3. Biochem Biophys Res Commun. 2006;340:209–14.

Sun P, Yoshizuka N, New L, Moser BA, Li Y, Liao R, Xie C, Chen J, Deng Q, Yamout M, Dong MQ, Frangou CG, Yates 3rd JR, Wright PE, Han J. PRAK is essential for ras-induced senescence and tumor suppression. Cell. 2007;128:295–308.

Tanguay PL, Rodier G, Meloche S. C-terminal domain phosphorylation of ERK3 controlled by Cdk1 and Cdc14 regulates its stability in mitosis. Biochem J. 2010;428:103–11.

Turgeon B, Saba-El-Leil MK, Meloche S. Cloning and characterization of mouse extracellular-signal-regulated protein kinase 3 as a unique gene product of 100 kDa. Biochem J. 2000;346(Pt 1):169–75.

Erp99

▶ Grp94 (HSP90B1)

ESA1

▶ Flotillin-2 (FLOT2)

Ese

▶ ITSN

ESR1, Estrogen Receptor Alpha, ERalpha

▶ Estrogen Receptor

ESR2, Estrogen Receptor Beta, ERbeta

▶ Estrogen Receptor

Estrogen Receptor

Karin Dahlman-Wright and Chunyan Zhao
Department of Biosciences and Nutrition, Novum, Karolinska Institutet, Huddinge, Sweden

Synonyms

ESR1, estrogen receptor alpha, ERalpha; ESR2, estrogen receptor beta, ERbeta

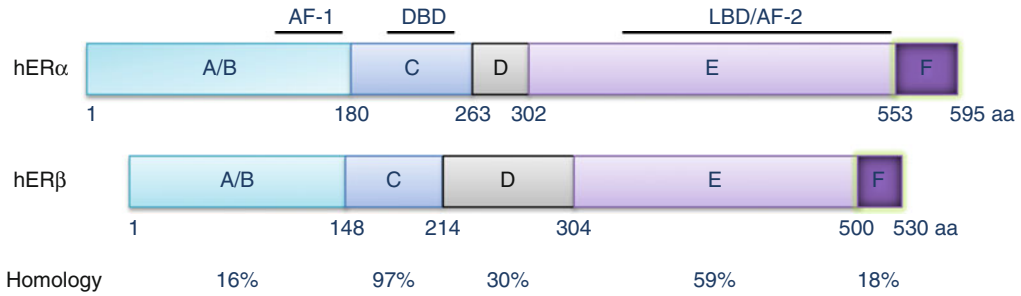
Historical Background

In 1896, physician George Beatson observed that removal of the ovaries caused regression of mammary tumors in women. His experiments were the first to establish a link between ovarian secretions and breast cancer. Later in the early 1930s, the "female" sex hormones, estrone and estriol, could be isolated from human pregnancy urine, followed by isolation of a third estrogen, estradiol (E2), from pig follicular fluid. Shortly thereafter, a number of estrogenic compounds were synthesized and used therapeutically, but the mechanisms of estrogen action remained obscure. However, in 1958 Elwood Jensen produced a major breakthrough when he used a radioactive marker to demonstrate that only estrogen-responsive tissues were able to concentrate injected estrogen from the blood, suggesting the existence of estrogen-binding components, which were called "estrogen receptors (ERs)." Further on, Noteboom and Gorski reported that the estrogen receptor is stereospecific and probably a protein. In 1966, the cytosolic estrogen-binding component was isolated from rat uterus. By treating the estrogen receptor with proteolytic enzymes, binding of estrogen was abolished, indicating that this component was a protein. Later in 1968, O'Malley observed changes in ovalbumin mRNA levels upon estrogen stimulation of the chick oviduct, indicating that ER functions as a transcription factor. In 1986, an ER was cloned from the uterus and its role as a ligand-dependent transcription factor was established (Welboren et al. 2007). This receptor, now known as ERα (NR3A1), was long believed to be the only existing ER that mediates estrogenic effects, until a second ER, now denoted ERβ (NR3A2), was cloned from rat prostate in 1996 (Kuiper et al. 1996). The first ERα knockout mouse was created in 1993, while the ERβ knockout mice became available in 1998. It is known today that ERs are members of the superfamily of nuclear receptors and specifically the family of steroid receptors that act as ligand-regulated transcription factors.

ER Gene and Protein Structure

ERα and ERβ are transcribed from different genes located on distinct chromosomes. The human ERα gene is located on chromosome 6q25.1 spanning a total of 140 kb, while the human ERβ gene is located on chromosome 14q23.2 spanning 60 kb. Both ERs consist of eight coding exons. In humans, ERα is expressed in the reproductive tissues (e.g., uterus, testis, breast), kidney, bone, white adipose tissue, and liver. ERβ has been found to be expressed in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems (Matthews and Gustafsson 2003).

ERs share common structural characteristics with other members of the nuclear receptor family including five distinguishable domains, named the A/B, C, D, E, and F domains, respectively (Fig. 1). The N-terminal A/B domain is the most variable region and the human ERα and ERβ share less than 20% amino acid identity in this region, indicating that this domain may contribute to ER subtype specific actions on target genes. This region harbors an activation function (AF-1) that is ligand-independent and shows promoter- and cell-specific activity. The central C-domain is the DNA-binding domain (DBD), which is involved in specific DNA binding and receptor dimerization. This domain is highly conserved between ERα and ERβ and shares 95% amino acid identity. The D-domain works as a flexible hinge between the DBD and the



Estrogen Receptor, Fig. 1 Schematic structural comparison of ERα and ERβ. The domains A–F are made up of activation function domains (AF-1 and AF-2), DNA-binding domain (DBD), and ligand-binding domain (LBD). Full-length ERα is 595 amino acids long whereas ERβ is 530 amino acids long

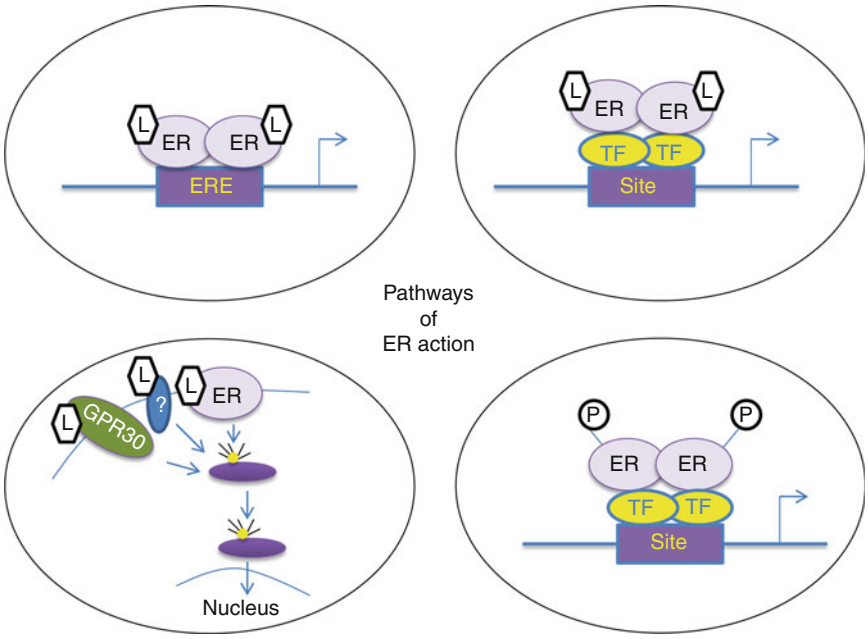
ligand-binding domain (LBD), and is thus referred to as the hinge domain. This domain, which is not well conserved between ERα and ERβ (30%), appears to be important for nuclear translocation and has been reported to contain a nuclear localization signal. The E-domain is referred to as the LBD, and the ERα and ERβ share approximately 59% amino acid identity in this region. The LBD contains a hormone-dependent activation function (AF-2) and is responsible for ligand binding and receptor dimerization. The LBDs of ERα and ERβ have very similar three-dimensional structures. However, the amino acids lining the ligand-binding cavities of ERα and ERβ differ in two positions. Furthermore, the ligand-binding cavity of ERβ is significantly smaller (∼20%) than that of ERα, and this may have implications for the selective affinity and pharmacology of ligands. The F-domain has less than 20% amino acid identity between the two ER subtypes, and the functions of this domain remain undefined (Zhao et al. 2008).

Mechanisms of ER Signaling

Estrogen action is exerted in target tissues via binding to one or both of the two ERs, ERα and ERβ. Like other steroid hormone receptors, ERs act as dimers to regulate transcriptional activation. Full transcriptional activity of the ERs is thought to proceed through a synergism between two activation domains, AF-1 at the N terminus and AF-2 in the LBD. Both ERα and ERβ contain a potent AF-2 function, but unlike ERα, ERβ seems to have a weaker corresponding AF-1 function and thus depends more on the ligand-dependent AF-2 for its transcriptional activation function (Dahlman-Wright et al. 2006). These AFs have been shown to exhibit distinct transactivation properties that depend on both cell and promoter contexts.

ERs, upon ligand activation, can regulate biological processes by divergent pathways (Fig. 2). The classical signaling occurs through direct binding of ER dimers to estrogen-responsive elements (EREs) in the regulatory regions of estrogen-responsive genes, followed by recruitment of coregulators to the transcription start site. The consensus ERE consists of a 5-bp palindrome with a 3-bp spacer: GGTCAnnnTGACC. However, many natural EREs deviate substantially from the consensus sequence (O'Lone et al. 2004). Estrogen also modulates gene expression by a second mechanism in which ERs interact with other transcription factors, such as activating protein-1 (AP-1) and stimulating protein-1 (Sp-1), through a process referred to as transcription factor cross-talk. Estrogen may also elicit effects through nongenomic mechanisms, which occur much more rapidly. This action has been shown to involve the activation of downstream cascades such as protein kinase A (PKA), protein kinase C (PKC), and ▶ MAP kinase, via membrane-localized ERs. Recently, an orphan G protein-coupled receptor (GPR)30 in the cell membrane was reported to mediate nongenomic estrogen signaling. Subsequent studies by others demonstrated that the activities of GPR30 in response to estrogen were through its ability to induce expression of ERα36, a novel variant of ERα, and ERα36, in turn, acts as an extranuclear ER to mediate nongenomic estrogen signaling (Kang et al. 2010).

In addition to these ligand-induced transcriptional activities of ERs, ligand-independent pathways to activate ERs have been described. Growth factor signaling or stimulation of other signaling pathways leads to activation of kinases that can phosphorylate and thereby activate ERs or associated coregulators in the absence of ligand. For example, it has been shown that the HER2 downstream signaling



Estrogen Receptor, Fig. 2 Four different pathways of ER action. (**a**) In the classical signaling, ER dimers directly bind to estrogen-responsive elements (EREs) following ligand activation. (**b**) ERs, upon ligand binding, interact with other transcription factors (TFs), such as activating protein-1 (AP-1) and stimulating protein-1 (Sp-1), through a process referred to as transcription factor cross-talk. (**c**) Estrogen may elicit effects through nongenomic mechanisms via ERα or GPR30 in the cell membrane, involving interactions with cytoplasmic signal transduction proteins. (**d**) ER activity can be regulated through a ligand-independent pathway in which ERs are phosphorylated by activated kinases

molecules ERK1 and ERK2 can phosphorylate ER, leading to ligand-independent receptor activation (Martin et al. 2005).

Genome-Wide Profiling of ER Gene Expression Programs

There have been a number of studies in the past few years aimed at comprehensively unraveling the complete estrogen-regulated gene expression programs in cancer cells. These reports can be attributed to the introduction of microarrays for global gene expression profiling. DNA microarray technology allows quantitative monitoring of changes in the expression of thousands of genes simultaneously and has been described in several configurations including oligonucleotide arrays and microarrays of cDNAs spotted on glass slides. During the past few years, the development of high throughput DNA sequencing (HTS) methods for global gene expression profiling, also known as "RNA-Seq," has challenged microarray technology because of its superior capability for detection of low-expressed genes, alternative splice variants, and novel transcripts (Cloonan et al. 2008). However, to our knowledge, no studies that explore HTS to assay genome-wide transcriptional regulation by estrogen have been reported.

Several reports have described the gene expression profiles in ERα-expressing breast cancer cell lines in response to E2 treatment. The available studies have reported different numbers of E2/ERα-regulated genes in MCF7 breast cancer cells, ranging from ∼200 to ∼1,500. These discrepancies can be attributed to differences in the length of the E2 treatment, application of different microarray platforms and different analysis strategies (Kininis and Kraus 2008). Two studies that aimed to identify E2/ERα-direct targets by short-term E2 treatment (3 h) in MCF7 cells identified similar numbers of E2 target genes. In one of the studies, 122 genes were identified as stimulated by E2 and 95 genes were identified as inhibited by E2 (Kininis et al. 2007). In the other study, 134 genes were up-regulated and 141 genes were down-regulated after E2 treatment (Carroll et al. 2006). However, a comparison of the E2-regulated genes between the

studies has not been reported. Overall, gene expression profiling and candidate gene analysis have identified several well-known estrogen-regulated genes in breast cancer cells such as *TFF1*, *CCND1*, *IGFBP4*, *C3*, *ADORA1*, *GREB1*, and *MYC*. Furthermore, gene expression profiling has identified categories of genes regulated by estrogen, including those that modulate the cell cycle, transcriptional regulation, morphogenesis, and apoptosis, compatible with a role of estrogen in inducing breast cancer cell proliferation and survival (Carroll and Brown 2006).

Due to the lack of high levels of endogenous ERβ in breast cancer cell lines, gene expression profiling studies aimed at revealing the genes regulated by ERβ have been performed in breast cancer cell lines stably expressing ERβ. Of the categories of genes downregulated by ERβ, the "regulation of cell proliferation" category was the most overrepresented one, consistent with the observations that ERβ expression was associated with suppression of breast cancer cell proliferation (Williams et al. 2008). Gene expression profiles for ER-subtypes showed that ERα and ERβ share some common target genes, although each receptor also appears to have distinct sets of downstream target genes.

ER and Breast Cancer

Estrogen is essential for growth and development of the mammary glands and has been associated with promotion and growth of breast cancer. ERβ is found in both ductal and lobular epithelial and stromal cells of the rodent, whereas ERα is only found in the ductal and lobular epithelial cells and not in stroma. The presence of significant amounts of ERα in breast cancer at the time of diagnosis is taken as an indication of hormone dependence. On this basis, treatment with ERα antagonistic compounds, such as tamoxifen, is first line for adjuvant therapy. ERα is also an important prognostic factor in breast cancer. ERα-negative breast cancers are associated with poor prognosis and a more aggressive phenotype (Spears and Bartlett 2009).

To date, ERβ expression in normal human breast and breast cancer specimens and the relationship between ERβ and clinicopathological features and response to endocrine treatment has been extensively investigated at both mRNA and protein levels. Overall, these studies suggest a protective role of ERβ in breast cancer development. ERβ is lost in a majority of breast tumors, which has been shown to be correlated with ERβ promoter methylation in breast cancer cells (Zhao et al. 2003). Promoter methylation is frequently observed for cancer suppressor genes. Several studies have demonstrated that ERβ is an important modulator of proliferation and invasion of breast cancer cells, thus supporting the hypothesis that loss of ERβ expression could be one of the events leading to breast cancer development. Currently, only the ERα form is measured for clinical decision-making and treatment of breast cancer patients.

SERMs in Breast Cancer Treatment

Selective estrogen receptor modulators, referred to as SERMs, are a class of compounds with mixed ER-agonist/antagonist activities. Tamoxifen is the most commonly used SERM for treating all stages of ERα-positive breast cancer. In primary breast cancer, adjuvant tamoxifen significantly decreases relapse rates and mortality in pre- and postmenopausal patients, and the therapy benefit from 5 years of adjuvant tamoxifen is maintained, even >10 years after the primary diagnosis. Tamoxifen has also been used as a chemopreventive agent in women who have high risk for breast cancer. The mechanism behind the antitumorigenic function of tamoxifen is through competing with estrogens for the LBD of ERα, thereby inhibiting ERα-mediated mitogenic estrogen signaling in the breast. However, tamoxifen has been shown to have an agonistic effect on endometrium and thus may increase the risk for endometrial cancer.

Raloxifen is a newly developed SERM and has been shown to be as effective as tamoxifen in reducing the incidence of breast cancer in postmenopausal women who are at increased risk of the disease. Because of its nonproliferative effects in the uterus and estrogenic effects on bone, this drug has also been approved for prevention of osteoporosis in high-risk women. Aromatase inhibitors, which potently suppress estrogen synthesis in postmenopausal women, are now considered to be more effective in treating metastatic breast cancer in postmenopausal women than tamoxifen. Fulvestrant (ICI 182,780) is a new class of compounds for endocrine treatment, functioning as a pure ER-antagonist with no agonist effects.

Despite substantial improvements in the treatment of breast cancer, resistance to therapy remains a major clinical problem. Approximately one third of the patients with ERα-positive tumors fail to respond to tamoxifen treatment due to intrinsic or de novo

resistance of the tumor. Furthermore, even patients who initially respond eventually acquire tamoxifen resistance, leading to tumor progression and death. Several mechanisms have been proposed to account for the observed resistance, including changes in the expression of ERα or ERβ, altered levels of co-regulatory proteins, and the influences of cellular kinase signal transduction pathways (Ring and Dowsett 2004). Today, circumvention of endocrine resistance represents a major challenge for clinicians and cancer researchers.

Summary

Estrogens are thought to exert their biological effects predominantly via ERs through their interactions with DNA. Interestingly, more evidence has indicated that estrogens, like androgen and progesterone, can exert nongenomic effects. Nongenomic steroid activity typically involves the rapid induction of secondary messengers of signal transduction cascades via membrane-localized ERs, including activation of protein kinase A (PKA), protein kinase C (PKC), and ▶ MAP kinase. Interestingly, recent studies have suggested that GPR30 functions as a novel type of extranuclear ER that mediates nongenomic estrogen signaling. It is still possible that additional membrane receptors for estrogen are involved in mediation of the nongenomic estrogen action. The mechanistic details of activation through these nongenomic pathways, such as target genes, remain to be characterized.

Gene expression profiling has furthered our understanding of the role of ERs and provided important glimpses into the molecular basis of ER-mediated estrogen action in target cells. Future studies will need to explore time courses of estrogen-regulated genes thus facilitating the identification of primary, secondary, and higher order estrogen-regulated genes, finally revealing the gene regulatory networks affected by estrogen signaling.

Currently only ERα status is an important marker for routine prognostic and predictive evaluation in breast cancer. Over the last decade, the role of ERβ in estrogen action in breast cancer involving cooperation, as well as competition with ERα, has been revealed. Future studies should include validation of ERβ as a target for breast cancer and exploration of ERβ as a marker for clinical decision-making and treatment. The discovery of clinically useful ERβ-agonists might hold great promise. Furthermore a better understanding of the biological pathways of tamoxifen resistance should allow the circumvention of tamoxifen resistance using rational therapeutic approaches.

References

Carroll JS, Brown M. Estrogen receptor target gene: an evolving concept. Mol Endocrinol. 2006;20:1707–14.

Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, et al. Genome-wide analysis of estrogen receptor binding sites. Nat Genet. 2006;38:1289–97.

Cloonan N, Forrest AR, Kolle G, Gardiner BB, Faulkner GJ, Brown MK, et al. Stem cell transcriptome profiling via massive-scale mRNA sequencing. Nat Meth. 2008;5:613–9.

Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, et al. International union of pharmacology. LXIV. Estrogen receptors. Pharmacol Rev. 2006;58:773–81.

Kang L, Zhang X, Xie Y, Tu Y, Wang D, Liu Z, et al. Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. Mol Endocrinol. 2010;24:709–21.

Kininis M, Kraus WL. A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis. Nucl Recept Signal. 2008;6:e005.

Kininis M, Chen BS, Diehl AG, Isaacs GD, Zhang T, Siepel AC, et al. Genomic analyses of transcription factor binding, histone acetylation, and gene expression reveal mechanistically distinct classes of estrogen-regulated promoters. Mol Cell Biol. 2007;27:5090–104.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA. 1996;93:5925–30.

Martin LA, Farmer I, Johnston SR, Ali S, Dowsett M. Elevated ERK1/ERK2/estrogen receptor cross-talk enhances estrogen-mediated signaling during long-term estrogen deprivation. Endocr Relat Cancer. 2005;12(Suppl 1):75–84.

Matthews J, Gustafsson JA. Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv. 2003;3:281–92.

O'Lone R, Frith MC, Karlsson EK, Hansen U. Genomic targets of nuclear estrogen receptors. Mol Endocrinol. 2004;18:1859–75.

Ring A, Dowsett M. Mechanisms of tamoxifen resistance. Endocr Relat Cancer. 2004;11:643–58.

Spears M, Bartlett J. The potential role of estrogen receptors and the SRC family as targets for the treatment of breast cancer. Expert Opin Ther Targets. 2009;13:665–74.

Welboren WJ, Stunnenberg HG, Sweep FC, Span PN. Identifying estrogen receptor target genes. Mol Oncol. 2007;1:138–43.

Williams C, Edvardsson K, Lewandowski SA, Strom A, Gustafsson JA. A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. Oncogene. 2008;27:1019–32.

Zhao C, Lam EW, Sunters A, Enmark E, De Bella MT, Coombes RC, et al. Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. Oncogene. 2003;22:7600–6.
Zhao C, Dahlman-Wright K, Gustafsson JA. Estrogen receptor beta: an overview and update. Nucl Recept Signal. 2008;6:e003.

ETA

► Endothelin A Receptor (ETAR)

ETA Receptor

► Endothelin A Receptor (ETAR)

ETA Type Endothelin Receptor

► Endothelin A Receptor (ETAR)

ETAR

► Endothelin A Receptor (ETAR)

ETA-R

► Endothelin A Receptor (ETAR)

Etdf

► Tead

ETF

► Tead

ETFR-1

► Tead

ETR

► Endothelin A Receptor (ETAR)

ETR ETA

► Endothelin A Receptor (ETAR)

ETRA

► Endothelin A Receptor (ETAR)

EVEC

► Fibulins

Exophilin

► Slp (Synaptotagmin-Like Protein)

Expressed Sequence AI851258

► ARAP3

Extracellular Matrix Receptor II (ECMR II)

► Integrin α2 (ITGA2)

Extracellular Regulated Kinase 1 and 2

► ERK1/ERK2

F

Farnesyltransferase

▶ Protein Farnesyltransferase

Fat

▶ Cadherins

FGD4

▶ Frabin

FGF

▶ FGF (Fibroblast Growth Factor)

FGF (Fibroblast Growth Factor)

Masaru Katoh
Division of Integrative Omics and Bioinformatics, National Cancer Center, Tokyo, Japan

Synonyms

Acidic FGF; aFGF; basic FGF; bFGF; FGF; FGF1; FGF10; FGF11; FGF12; FGF13; FGF14; FGF16; FGF17; FGF18; FGF19; FGF2; FGF20; FGF-20; FGF21; FGF22; FGF23; FGF3; FGF4; FGF5; FGF6; FGF7; FGF8; FGF9; int-2; K-FGF; KGF

Historical Background

Founding members of the fibroblast growth factor (FGF) family are FGF1 (acidic FGF or aFGF) and FGF2 (basic FGF or bFGF), which were initially purified from cow brain and pituitary gland as mitogens for fibroblasts (Gospodarowicz et al. 1978). Mouse Fgf3 (int-2) was cloned and characterized as a proto-oncogene aberrantly upregulated in mammary tumors due to proviral integration of mouse mammary tumor virus (MMTV) (Dickson et al. 1984). Human FGF4 (K-FGF) was cloned and characterized as a proto-oncogene following transfection of genome DNA of Kaposi sarcoma into mouse NIH 3T3 cells (Delli Bovi et al. 1987). Additional 18 FGFs were then cloned and characterized based on their homology to FGF1 ~ FGF4 (Reviewed in Katoh 2002). Because *CCND1 – ORAOV1 – FGF19 – FGF4 – FGF3* locus at human chromosome 11q13.3 is conserved in chicken and zebra fish genomes, and is syntenic to rodent *Ccnd1 – Oraov1 – Fgf15 – Fgf4 – Fgf3* locus, it was concluded that human FGF19, chicken fgf19, and zebra fish fgf19 are orthologs of rodent Fgf15 (Katoh and Katoh 2003). Therefore, 22, but not 23, *FGF* family genes are conserved in mammalian genomes.

FGF family members with conserved FGF core domain of about 120 amino acid residues are classified into the following seven subgroups (Popovici et al. 2005): subgroup A consisting of

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

FGF (Fibroblast Growth Factor), Table 1 FGF family

Subgroup	FGF	FGF receptor	Hereditary human disease
A	FGF1	FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c, FGFR4	
	FGF2	FGFR1b, FGFR1c, FGFR2c, FGFR3b, FGFR3c, FGFR4	
B	FGF3	FGFR1b, FGFR2b	Deafness associated with complete absence of inner ear structures, microtia, and microdontia
	FGF7	FGFR2b	
	FGF10	FGFR1b, FGFR2b	Autosomal-dominant lacrimo-auriculo-dento-digital syndrome
	FGF22	FGFR2b	
C	FGF4	FGFR1c, FGFR2c, FGFR3c, FGFR4	
	FGF5	FGFR1c	
	FGF6	FGFR1c, FGFR2c, FGFR4	
D	FGF8	FGFR3c, FGFR4	Hypogonadotropic hypogonadism and Kallmann syndrome
	FGF17	FGFR3c, FGFR4	
	FGF18	FGFR3c, FGFR4	
E	FGF9	FGFR2c, FGFR3b, FGFR3c	
	FGF16	FGFR2c	
	FGF20	FGFR2c, FGFR3b, FGFR3c	
F	FGF11	FGFR-independent	
	FGF12	FGFR-independent	
	FGF13	FGFR-independent	
	FGF14	FGFR-independent	Autosomal-dominant spinocerebellar ataxia 27
G	FGF19	FGFR1c, FGFR2c, FGFR3c, FGFR4	
	FGF21	FGFR1c, FGFR2c, FGFR3c, FGFR4	
	FGF23	FGFR1c, FGFR2c, FGFR3c, FGFR4	Autosomal-dominant hypophosphatemic rickets

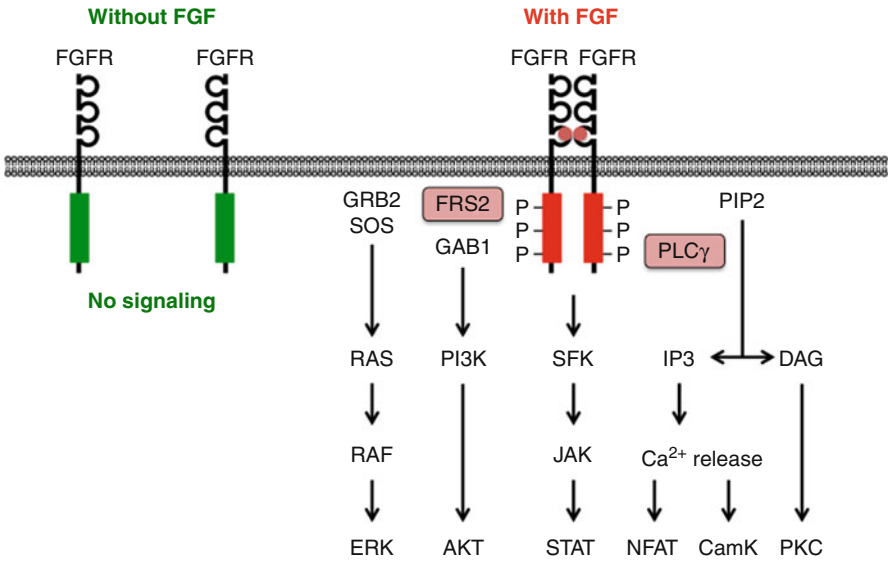
FGF family members are classified into seven subgroups. Receptors and hereditary human disease associated with each FGF are listed

FGF1 and FGF2; subgroup B consisting of FGF3, FGF7 (KGF), FGF10, and FGF22; subgroup C consisting of FGF4, FGF5, and FGF6; subgroup D consisting of FGF8, FGF17, and FGF18; subgroup E consisting of FGF9, FGF16, and FGF20 (FGF-20); subgroup F consisting of FGF11, FGF12, and FGF13, and FGF14; subgroup G consisting of FGF19, FGF21, and FGF23. Members of subgroups A, B, C, D, E, and G are extracellular FGFs transducing signals through cell-surface FGF receptors (FGFRs), whereas members of subgroup F are intracellular FGFs functioning in an FGFR-independent manner (Table 1).

FGF Signaling Cascades

Signals of 18 extracellular FGFs are transduced through FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c, or FGFR4, which share the common domain architecture consisting of extracellular immunoglobulin (Ig)-like domains and cytoplasmic tyrosine kinase domain (Eswarakumar et al. 2005). *FGFR1*, *FGFR2*, *FGFR3* genes encode two receptor isoforms with distinct ligand specificity due to alternative splicing in the third Ig-like domain, while *FGFR4* gene encodes a single receptor (Table 1). For example, FGFR2b on epithelial cells functions as the receptor for FGF1, FGF3, FGF7, FGF10, and FGF22, whereas FGFR2c on mesenchymal cells functions as the receptor for FGF1, FGF2, FGF4, FGF6, FGF9, FGF16, FGF17, FGF18, and FGF20 (Eswarakumar et al. 2005; Zhang et al. 2006).

Extracellular FGFs utilize heparan sulfate proteoglycan (HSPG) and/or Klotho-type co-receptors to fine-tune their interaction with FGFRs. FGF-mediated dimerization and stepwise autophosphorylation of FGFRs result in phosphorylation of adaptor molecule, FGFR substrate 2 (FRS2), and also recruitment and activation of phospholipase C-γ (PLCγ) (Dailey et al. 2005; Katoh 2009; Bae and Schlessinger 2010). FRS2 phosphorylation leads to GRB2 recruitment, and following activation of RAS-ERK and PI3K-AKT



FGF (Fibroblast Growth Factor), Fig. 1 FGF signaling cascades. FGF signals are transduced through FGFRs to RAS-ERK, PI3K-AKT, PKC, and JAK-STAT signaling cascades for survival, proliferation, differentiation, or migration of target cells

signaling cascades. PLCγ-mediated catalysis of phosphatidylinositol diphosphate (PIP2) gives rise to inositol triphosphate (IP3) for Ca^{2+} release from endoplasmic reticulum, and also diacylglycerol (DAG) for activation of protein kinase C (PKC). In addition, FGF signals activate JAK-STAT signaling cascade via SRC family kinases. FGF signals are transduced to RAS-ERK, PI3K-AKT, PKC, and JAK-STAT signaling cascades to induce survival, proliferation, differentiation, or migration of target cells (Fig. 1). Therefore, FGF signals are involved in a variety of cellular responses, such as self-renewal of stem cells, proliferation or differentiation of progenitor cells, angiogenesis, would healing, and tumor invasion.

Physiological Roles of FGF Signaling

Embryonic stem cells (ESCs), epiblast-derived stem cells (EpiSCs), and induced pluripotent stem cells (iPSCs) are characterized by potentials for self-renewal and differentiation of three germ layers to generate whole body. Human ESCs and mouse EpiSCs are supported by FGF2 and Activin/Nodal, whereas mouse ESCs by LIF (Katoh 2008).

Mesenchymal stem cells (MSCs) are somatic stem cells with the potential to differentiate into mesoderm-derived chondrocytes, osteoblasts, adipocytes, fibroblasts, myocytes as well as non-mesoderm-derived hepatocytes, and neurons. FGF signals are required for osteoblastic differentiation of MSCs, and adipocytic differentiation of preadipocytes (Katoh 2008).

Subgroup B members of the FGF family, including FGF3, FGF7, FGF10, and FGF22, preferentially transduce signals through FGFR2b and/or FGFR1b to function as local regulators involved in fetal morphogenesis, axon guidance, and wound repair (Katoh 2009). On the other hand, subgroup G members, including FGF19, FGF21, and FGF23, are secreted into circulation to function as endocrine hormonal factors involved in metabolism of glucose, lipid, bile acid, inorganic phosphate (Pi), and vitamin D (Kharitonenkov 2009).

Pathological Roles of FGF Signaling

Missense mutations of human *FGF* family members are involved in pathogenesis of several hereditary diseases (Table 1) (Krejci et al. 2009). For example, *FGF23* gene at human chromosome 12p13.32 is the causative gene for autosomal-dominant hypophosphatemic rickets (ADHR), featured by rickets, osteomalacia, and lower limb deformity. Missense mutations of *FGF23* around proteolytic cleavage site lead to elevation of serum FGF23 level. FGF23 is associated with Klotho to transduce signals through FGFR1c, FGFR3c, and FGFR4 to the RAS-ERK signaling branch. Because FGF23 is an osteocyte-derived

hormonal factor to downregulate reabsorption of inorganic phosphate (Pi) from the proximal convoluted tubules in the kidney, missense mutations of FGF23 in ADHR patients result in hypophosphatemia due to decreased Pi reabsorption (Marsell and Jonsson 2010).

FGF20 gene at human chromosome 8p22 is located within the susceptibility locus of Parkinson's disease. Clinical features of Parkinson's disease, such as resting tremor, cogwheel rigidity, bradykinesia, and impaired postural reflexes, are caused by the loss of dopaminergic neurons in substantia nigra. FGF20 secreted from substantia nigra activates the RAS-ERK signaling branch to induce differentiation of dopaminergic neurons. SNP rs12720208 at the 3'-UTR of *FGF20* mRNA is associated with the risk of Parkinson's disease due to the creation of miRNA-433 target sequence (Kirikoshi et al. 2000; Wang et al. 2008).

FGFR1, *FGFR2*, or *FGFR3* are the causative genes for congenital skeletal abnormalities, such as Crouzon syndrome, Jackson-Weiss syndrome, Apert syndrome, Pfeiffer syndrome, Beare-Stevenson syndrome, or Saethre-Chotzen syndrome (Wilkie et al. 2002). Missense mutations of FGFRs around the third Ig-like domain lead to ectopic FGFR2 activation due to altered ligand-binding specificity, whereas those within the tyrosine kinase domain results in ligand-independent FGFR signaling activation due to release of FGFR from autoinhibition.

Fgf3, Fgf4, and *Fgf10* are upregulated due to MMTV proviral integration during mouse mammary carcinogenesis. Interestingly, *Fgf3* and/or *Fgf4* are activated by MMTV, because *Fgf3* and *Fgf4* genes are clustered as mentioned above. *Fgf3* was demonstrated as a proto-oncogene involved in mouse mammary carcinogenesis based on tumorigenesis in the MMTV-*Fgf3* transgenic mice expressing *Fgf3* transgene under the control of MMTV promoter. Mammary carcinogenesis in the MMTV-*Fgf3* transgenic mice is accelerated due to *Wnt10b* upregulation based on additional MMTV proviral integration, and that in the MMTV-*Wnt1* transgenic mice due to *Fgf3* upregulation. FGF signals promote mouse mammary carcinogenesis in cooperation with canonical WNT signals (Katoh 2011).

In human, the *CCND1 – ORAOV1 – FGF19 – FGF4 – FGF3* locus at chromosome 11q13.3 region is amplified in breast cancer, and other types of tumors. *CCND1*, but not *FGF3* nor *FGF4*, is overexpressed in human tumors due to copy-number gain. Because *CCND1* gene encodes Cyclin D1, involved in cell-cycle progression, *CCND1* within the 11q13.3 amplicon is the driver gene of human carcinogenesis.

Instead of *FGF* genes, *FGFR* genes are involved in human carcinogenesis (Katoh 2009). *FGFR1* is activated due to overexpression associated with gene amplification in ER-positive breast cancer, and ovarian cancer, and also due to chromosomal translocation in myeloproliferative syndrome. *FGFR2* is activated due to overexpression associated with gene amplification in triple-negative breast cancer and diffuse-type gastric cancer. Single-nucleotide polymorphisms (SNPs) in an intronic region of *FGFR2* gene are associated with increased risk of breast cancer through altered *FGFR2* transcription due to SNP-based creation of transcription factor–binding sites. *FGFR3* is activated due to chromosomal translocation in multiple myeloma. In addition, SNP in an exonic region of *FGFR4* gene, resulting in Gly388Arg amino acid substitution, is associated with a poor prognosis in breast cancer and lung cancer. *FGFR* genes are aberrantly activated in human tumors due to gene amplification, chromosomal translocation, and missense mutation (summarized in Katoh 2010).

Together these facts indicate that aberrant FGF signaling is involved in a broad spectrum of pathologies, such as endocrine disease, neurodegenerative disease, skeletal abnormality, and cancer (Katoh 2009).

Summary

FGF signals are transduced through FGFRs to the RAS-ERK, PI3K-AKT, PKC, and JAK-STAT signaling cascades for survival, proliferation, differentiation, or migration of target cells (Eswarakumar et al. 2005; Dailey et al. 2005; Katoh 2009). FGF signaling cascades cross-talk with WNT, Notch, Hedgehog, and TGFß/BMP signaling cascades to constitute the stem-cell signaling network, which is involved in fetal-tissue morphogenesis, adult-tissue homeostasis, and tumorigenesis (Katoh and Katoh 2007). Because FGFs are involved in self-renewal of stem cells, proliferation or differentiation of progenitor cells, angiogenesis, would healing, and tumor invasion, FGF signaling cascades are therapeutic target in the fields of regenerative medicine, endocrinology and metabolism, and clinical oncology. Recombinant FGF7 and

FGF20 proteins are applicable for treatment of mucosal injury associated with chemotherapy or radiotherapy, whereas recombinant FGF2 protein and FGF4 expression vector are applicable for therapeutic angiogenesis (Katoh and Katoh 2006). Small-molecule compounds targeted to FGFRs and other receptors, such as Cediranib, TKI258, Ki23057, MK-2461, and brivanib, are applicable to cancer therapeutics (Katoh 2009).

References

Bae JH, Schlessinger J. Asymmetric tyrosine kinase arrangements in activation or autophosphorylation of receptor tyrosine kinases. Mol Cells. 2010;29:443–8.

Dailey L, Ambrosetti D, Mansukhani A, Basilico C. Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev. 2005;16:233–47.

Delli Bovi P, Curatola AM, Kern FG, Greco A, Ittmann M, Basilico C. An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. Cell. 1987;50:729–37.

Dickson C, Smith R, Brookes S, Peters G. Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. Cell. 1984;37:529–36.

Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 2005;16:139–49.

Gospodarowicz D, Bialecki H, Greenburg G. Purification of the fibroblast growth factor activity from bovine brain. J Biol Chem. 1978;253:3736–43.

Katoh M. *WNT* and *FGF* gene clusters. Int J Oncol. 2002;21:1269–73.

Katoh M. Dysregulation of stem cell signaling network due to germline mutation, SNP, Helicobacter pylori infection, epigenetic change, and genetic alteration in gastric cancer. Cancer Biol Ther. 2007;6:832–9.

Katoh M. WNT signaling in stem cell biology and regenerative medicine. Curr Drug Targets. 2008;9:565–70.

Katoh M. FGFR2 abnormalities underlie a spectrum of bone, skin, and cancer pathologies. J Invest Dermatol. 2009;129:1862–7.

Katoh M. Genetic alterations of FGF receptors: an emerging field in clinical cancer diagnostics and therapeutics. Expert Rev Anticancer Ther. 2010;10:1375–9.

Katoh M, Katoh M. Evolutionary conservation of *CCND1 – ORAOV1 – FGF19 – FGF4* locus from zebrafish to human. Int J Mol Med. 2003;12:45–50.

Katoh M, Katoh M. FGF signaling network in the gastrointestinal tract. Int J Oncol. 2006;29:163–8.

Katoh M, Katoh M. WNT signaling pathway and stem cell signaling network. Clin Cancer Res. 2007;13:4042–5.

Katoh M. Network of WNT and other regulatory signaling cascades in pluripotent stem cells and cancer stem cells. Curr Pharm Biotechnol. 2011;12:160–70.

Kharitonenkov A. FGFs and metabolism. Curr Opin Pharmacol. 2009;9:805–10.

Kirikoshi H, Sagara N, Saitoh T, Sekihara H, Shiokawa K, Katoh M. Molecular cloning and characterization of human FGF-20 on chromosome 8p21.3-p22. Biochem Biophys Res Commun. 2000;274:337–43.

Krejci P, Prochazkova J, Bryja V, Kozubik A, Wilcox WR. Molecular pathology of the fibroblast growth factor family. Hum Mutat. 2009;30:1245–55.

Marsell R, Jonsson KB. The phosphate regulating hormone FGF23. Acta Physiol.. 2010;200:97–106.

Popovici C, Roubin R, Coulier F, Birnbaum D. An evolutionary history of the FGF superfamily. Bioessays. 2005;27:849–57.

Wang G, van der Walt JM, Mayhew G, Li YJ, Züchner S, Scott WK, Martin ER, Vance JM. Variation in the miRNA-433 binding site of FGF20 confers risk for Parkinson disease by overexpression of a-synuclein. Am J Hum Genet. 2008;82:283–9.

Wilkie AOM, Patey SJ, Kan S, van den Ouweland AMW, Hamel BCJ. FGFs, their receptors, and human limb malformations: clinical and molecular correlations. Am J Med Genet. 2002;112:266–78.

Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM. Receptor specificity of the fibroblast growth factor family: the complete mammalian FGF family. J Biol Chem. 2006;281:15694–700.

FGF1

► FGF (Fibroblast Growth Factor)

FGF10

► FGF (Fibroblast Growth Factor)

FGF11

► FGF (Fibroblast Growth Factor)

FGF12

► FGF (Fibroblast Growth Factor)

FGF13

► FGF (Fibroblast Growth Factor)

FGF14

▶ FGF (Fibroblast Growth Factor)

FGF16

▶ FGF (Fibroblast Growth Factor)

FGF17

▶ FGF (Fibroblast Growth Factor)

FGF18

▶ FGF (Fibroblast Growth Factor)

FGF19

▶ FGF (Fibroblast Growth Factor)

FGF2

▶ FGF (Fibroblast Growth Factor)

FGF20

▶ FGF (Fibroblast Growth Factor)

FGF-20

▶ FGF (Fibroblast Growth Factor)

FGF21

▶ FGF (Fibroblast Growth Factor)

FGF22

▶ FGF (Fibroblast Growth Factor)

FGF23

▶ FGF (Fibroblast Growth Factor)

FGF3

▶ FGF (Fibroblast Growth Factor)

FGF4

▶ FGF (Fibroblast Growth Factor)

FGF5

▶ FGF (Fibroblast Growth Factor)

FGF6

▶ FGF (Fibroblast Growth Factor)

FGF7

▶ FGF (Fibroblast Growth Factor)

FGF8

▶ FGF (Fibroblast Growth Factor)

FGF9

▶ FGF (Fibroblast Growth Factor)

FGR (Gene Name)

Giorgio Berton and Anna Baruzzi
Department of Pathology and Diagnostics, Section of General Pathology, University of Verona, Verona, Italy

Synonyms

c-FGR; Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene

Historical Background

FGR is a cytoplasmic protein-tyrosine kinase belonging to the ▶ SRC family of protein tyrosine kinases. This kinase was originally described in the context of a feline sarcoma virus isolated from a spontaneous sarcoma of a domestic house cat and named GR-FeSV (Gardner-Rasheed feline sarcoma virus), whose primary translation product was shown to be a 70 kDa gag-fgr fusion peptide (Rasheed et al. 1982). Later studies identified this peptide as having a portion with amino acid homology with actin and another one related to a tyrosine kinase homologous to gene sequences present in the DNA of diverse invertebrate species (Naharro et al. 1984; Naharro et al. 1983). FGR is expressed in human and murine hematopoietic cells including granulocytes, monocytes/macrophages, natural killer cells, platelets, and erythrocytes (Berton 2006; Lowell 2004). Its function has been mainly characterized in innate immunity cells and in particular in granulocytes and monocyte/macrophages (Berton et al. 2005; Lowell 2004).

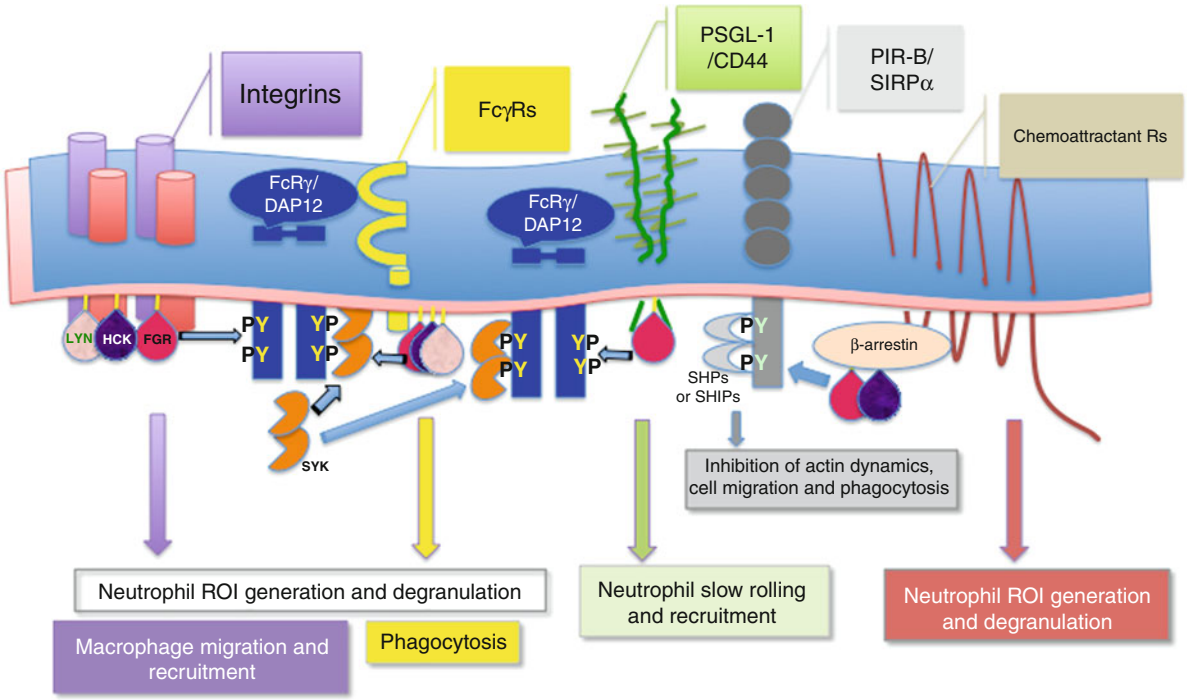
FGR Functions: An Overview

Studies in mice with the genetic deficiency of FGR established the concept that this kinase serves both specific functions and functions emerging from its combined action together with other members of the ▶ SRC family of protein tyrosine kinases (SFKs). A paradigm emerged on the role of SFKs in immune cell responses is that they are implicated in activating and inhibitory pathways (Lowell 2011). Consistent with this scenario, FGR deficiency has been reported to result either in defective or enhanced cell responses. In contrast, multiple deficiency of SFKs expressed at the highest level in innate immunity cells, i.e., FGR, HCK and/or LYN, or nonselective inhibition of SFK activities by inhibitory compounds, invariably result in a marked defect in signal transduction and cell activation by different surface receptors.

Four major signaling pathways have been identified as being regulated by FGR in innate immunity cells (Fig. 1): (1) the signaling pathway triggered by receptors belonging to the wide family of immune receptors; (2) "outside-in" integrin signaling; (3) signaling by trimeric G protein-coupled receptors for chemoattractants and chemokines; and (4) the P-selectin glycoprotein ligand-1 (PSGL-1)/CD44-dependent pathway triggered by granulocyte interaction with E-selectin or P-selectin and regulating neutrophil slow rolling and recruitment.

Immunoreceptor Signaling

The immunoreceptors represent a wide family of receptors including antigen receptors expressed by T (TCR) and B lymphocytes (BCR), receptors for the Fc portion of IgE (FcεRs) and IgG (FcγRs), and other ones (Lowell 2004, 2011). Most of them consist of one or more transmembrane proteins implicated in ligand recognition and noncovalently associated homo/heterodimeric signaling adapters such as the FcγR-associated γ chain (FcRγ) or DAP12 (ITAM-Contaning Signal Adaptors). Classical immunoreceptor engagement leads to activation of SFKs, which in turn phosphorylate a tyrosine residue present in the immunoreceptor tyrosine-based activation motif (ITAM) present in the signaling adapters or, in the case of FcγRIIA, in its cytoplasmic tale; phosphorylated ITAMs recruit and activate the



FGR (Gene Name), Fig. 1 Major signal transduction pathways implicating FGR in myeloid leukocytes. Signal transduction by integrins, immunoreceptors, and PSGL-1 requires phosphorylation of tyrosine residues of the ITAM-containing adaptors FcRγ or DAP12 by FGR or other SFKs (HCK and LYN). Binding to phosphorylated ITAMs (yellow Y), together with its tyrosine phosphorylation by SFKs, activates SYK kinase activity. SFKs may associate with the integrin β chain and be activated upon integrin interaction with the ligand and/or integrin clustering. Alternatively, fatty acid bound to the SFK N-terminus can induce colocalization of receptors and SFKs in lipid rafts (see Yago et al. 2010). In the chemoattractant receptor signal transduction pathway, SFKs (FGR and HCK) may bind to β-arrestin associated to the receptor cytoplasmic tail. Major responses triggered by the different receptors are highlighted using the same color for the receptor, the arrow pointing to a specific cell response and the cell response itself. Both integrins and FcγRs lead to neutrophil ROI generation and degranulation (*in white*). In the integrin signal transduction pathway, FGR and other SFKs are involved in regulation of migration and recruitment in macrophages and of phagocytosis in neutrophils (first two arrows from the left). In chemoattaractant receptor, but also FcγR (not illustrated in the figure) signaling, FGR, and other SFKs, can phosphorylate ITIM-containing receptors and inhibit some myeloid cell responses (see text). For SFK and SYK substrates or interacting partners, see text and (Abram and Lowell 2009; Baruzzi et al. 2008; Berton et al. 2005; Lowell 2011)

tyrosine kinase SYK (Berton 1999; Lowell 2004, 2011). FGR, HCK, LYN and, based on inhibitory studies, other SFKs, have been implicated in macrophage IgG-mediated phagocytosis (Berton 1999; Berton et al. 2005). However, studies with myeloid leukocytes with the sole deficiency of FGR demonstrated that, whereas this kinase is essential for optimal FcγR-dependent phagocytosis in neutrophils, it negatively regulates the phagocytic response in macrophages (Gresham et al. 2000). This inhibitory function involves the immunoreceptor tyrosine-based inhibitory motifs (ITIM)-containing receptor SIRPα which binds the inhibitory tyrosine phosphatase SHP-1 in a FGR-dependent manner. Phosphorylation of tyrosine residues in the ITIM sequence of surface receptors such as FcγRIIB, SIRPα, PIR − B represents the best characterized mechanism of transduction of inhibitory signals by SFKs (Lowell 2004, 2011). Mechanisms underlying a predominant stimulatory or inhibitory effect of the different SFKs, or of one specific SFK like FGR in dependence of the cell context, for example, in neutrophils compared to macrophages (see above), are still unclear (Lowell 2011; Scapini et al. 2009).

Integrin Signaling

Integrins are heterodimeric transmembrane proteins consisting of an α and a β subunit implicated in cell-extracellular matrix or cell–cell interaction. The integrin receptor family includes at least 19 α subunits and 8 β subunits which associate to form at least 25 distinct receptors, several of which are expressed at variable levels in leukocytes. Innate immunity cells express members of the β1, β2, and β3 families and these regulate essential cell responses, including recruitment into inflamed tissues, reactive oxygen intermediate (ROIs) generation, degranulation, cytokine expression and release (Berton and Lowell 1999). Following the demonstration that β2 integrin engagement activates FGR in human neutrophils (Berton et al. 1994), a great deal of information has been accumulated on the role of SFKs in integrin signal transduction in myeloid leukocytes (Abram and Lowell 2009; Berton and Lowell 1999; Berton et al. 2005; Lowell and Berton 1999; Schymeinsky et al. 2007). Studies with mice with the single or multiple genetic deficiency of SFKs established the paradigm that within the integrin signaling pathway FGR appears to work in concert with other SFKs and more specifically to play a redundant role with HCK and, to a minor extent, LYN. The double deficiency of FGR and HCK results in an almost total suppression of several integrin-mediated responses including neutrophil ROI generation and degranulation (Abram and Lowell 2009; Berton et al. 2005; Lowell and Berton 1999; Schymeinsky et al. 2007) and macrophage migration (Baruzzi et al. 2008; Berton et al. 2005). Several components of the myeloid leukocyte integrin signaling pathways that are substrates of FGR/HCK have been identified, including c-CBL, SYK, FAK/PYK2, the p85 subunit of PI3-kinase, cortactin, paxillin SLP-67, and p190RHOGAP (Abram and Lowell 2009; Berton et al. 2005). A direct association of FGR with some of these proteins (c-Cbl, cortactin, SYK, FAK/PYK2) has been reported (Berton 2006).

A major advance in the understanding of integrin signaling in myeloid leukocytes has been the recent demonstration that, similarly to classical immunoreceptors, it involves the ITAM-containing adaptors DAP12 and FcRγ, establishing the paradigm that integrins signal though a SFK/ITAM-containing adaptors/SYK module (Jakus et al. 2007; Lowell 2011).

Signaling by Trimeric G Protein-Coupled Receptors

Several reports implicated SFKs in signal transduction by trimeric G protein-coupled receptors for chemoattractants or chemokines (Berton 1999; Berton et al. 2005; Lowell 2011). Neutrophils deficient of FGR and HCK or FGR, HCK, and LYN, or treated with SFK inhibitors, manifest a marked defect in both superoxide anion generation and degranulation in response to formylated peptides. Similarly, leukocyte responses to several chemokines including CXCL1, CXCL8, CXCL12 and CCL11 were reported to require FGR, HCK, or LYN. However, in analogy with what seen in ITAM/ITIM-containing adaptor-mediated signaling, also within the trimeric G protein-coupled receptor signaling pathway a possible inhibitory role of FGR and HCK in some myeloid leukocyte responses to different chemokines was established (Lowell 2011). Notably, FGR plays a major role in this inhibitory pathway compared to other SFKs and acts via phosphorylation of the ITIM-containing adaptor PIR-B (see Fig. 1 and (Lowell 2011). These contradictory findings may be reconciled emphasizing differences in specific cell responses to trimeric G protein-coupled receptor stimulation. ROIs generation and degranulation seem to require SFKs, whereas activation of integrin adhesiveness and cell migration occur independently of or are even inhibited by SFKs.

E- and P-Selectin-Induced Signaling

The latest advance in the understanding of the role of FGR in regulation of myeloid leukocyte function has been its implication in a signaling pathway triggered by engagement of E- or P-selectin by neutrophil counter-receptors (PSGL-1 and CD44) and regulating β2 integrin LFA-1-dependent slow rolling (Kuwano et al. 2010; Yago et al. 2010; Zarbock and Ley 2009). FGR seems to play a predominant role in the context of this pathway, although, in analogy with other signaling pathways, HCK and LYN are also involved. Notably, the SFK/ITAM-containing adaptors/SYK module identified to be essential for immunoreceptor and integrin signaling is also required for PSGL-1/CD44 signal transduction. Regulation by FGR and other SFKs of selectin-mediated slow rolling represents

one of the several mechanisms by which this tyrosine kinase family plays a central role in myeloid leukocyte recruitment into inflammatory sites (Baruzzi et al. 2008; Zarbock and Ley 2009).

Summary

FGR and related SFKs have emerged as essential upstream components of several signal transduction pathways regulating myeloid cell responses. Accumulating evidence suggests that several of these pathways share a common module consisting of SFKs, ITAM-containing adaptors and SYK wherein SFKs phosphorylate ITAM tyrosine residues causing binding and activation of SYK. Due to the pleiotropy of SFKs effects, a major challenge of future research is to dissect components of distinct signaling pathways leading to a specific cell response. Additionally, a question that needs to be addressed concerns the mechanisms of activation of SFKs. In fact, although direct or indirect binding to the receptor cytoplasmic tail or codistribution within lipid rafts likely represent a prerequisite for SFK activation, mechanisms responsible for this activation are still elusive. One of the most intriguing aspects of FGR and SFK functions is certainly their capability to activate or inhibit specific cell responses on the basis of their targeting "activation" or "inhibitory" motifs. The balance between these two different actions in the context of a specific SFK or cell function is certainly an issue that deserves more efforts in the future. The present knowledge favors the view that the combined action of different SFKs is essential for activation of a series of cell responses that is critical for the recruitment and activation of myeloid leukocytes into inflammatory sites. If so, it is tempting to speculate that inhibition of this upstream signaling component would tone down responses that are essential for inflammation-based pathologies. Targeting of SFKs as a new strategy to control inflammation is a major challenge for future research.

References

Abram CL, Lowell CA. The ins and outs of leukocyte integrin signaling. Annu Rev Immunol. 2009;27:339–62.

Baruzzi A, Caveggion E, Berton G. Regulation of phagocyte migration and recruitment by Src-family kinases. Cell Mol Life Sci. 2008;65:2175–90.

Berton G. Tyrosine kinases in neutrophils. Curr Opin Hematol. 1999;6:51–8.

Berton G. Fgr. UCSD-Nature Molecule Pages [serial on the Internet]. 2006.

Berton G, Lowell CA. Integrin signalling in neutrophils and macrophages. Cell Signal. 1999;11:621–35.

Berton G, Fumagalli L, Laudanna C, Sorio C. Beta 2 integrin-dependent protein tyrosine phosphorylation and activation of the FGR protein tyrosine kinase in human neutrophils. J Cell Biol. 1994;126:1111–21.

Berton G, Mocsai A, Lowell CA. Src and Syk kinases: key regulators of phagocytic cell activation. Trends Immunol. 2005;26:208–14.

Gresham HD, Dale BM, Potter JW, Chang PW, Vines CM, Lowell CA, et al. Negative regulation of phagocytosis in murine macrophages by the Src kinase family member, Fgr. J Exp Med. 2000;191:515–28.

Jakus Z, Fodor S, Abram CL, Lowell CA, Mocsai A. Immunoreceptor-like signaling by beta 2 and beta 3 integrins. Trends Cell Biol. 2007;17:493–501.

Kuwano Y, Spelten O, Zhang H, Ley K, Zarbock A. Rolling on E- or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils. Blood. 2010;116:617–24.

Lowell CA. Src-family kinases: rheostats of immune cell signaling. Mol Immunol. 2004;41:631–43.

Lowell CA. Src-family and Syk kinases in activating and inhibitory pathways in innate immune cells: signaling cross talk. Cold Spring Harb Perspect Biol. 2011;3. doi:10.1101/cshperspect.a002352.

Lowell CA, Berton G. Integrin signal transduction in myeloid leukocytes. J Leukoc Biol. 1999;65:313–20.

Naharro G, Tronick SR, Rasheed S, Gardner MB, Aaronson SA, Robbins KC. Molecular cloning of integrated Gardner-Rasheed feline sarcoma virus: genetic structure of its cell-derived sequence differs from that of other tyrosine kinase-coding onc genes. J Virol. 1983;47:611–19.

Naharro G, Robbins KC, Reddy EP. Gene product of v-fgr onc: hybrid protein containing a portion of actin and a tyrosine-specific protein kinase. Science. 1984;223:63–6.

Rasheed S, Barbacid M, Aaronson S, Gardner MB. Origin and biological properties of a new feline sarcoma virus. Virology. 1982;117:238–44.

Scapini P, Pereira S, Zhang H, Lowell CA. Multiple roles of Lyn kinase in myeloid cell signaling and function. Immunol Rev. 2009;228:23–40.

Schymeinsky J, Mocsai A, Walzog B. Neutrophil activation via beta2 integrins (CD11/CD18): molecular mechanisms and clinical implications. Thromb Haemost. 2007;98:262–73.

Yago T, Shao B, Miner JJ, Yao L, Klopocki AG, Maeda K, et al. E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin alphaLbeta2-mediated slow leukocyte rolling. Blood. 2010;116:485–94.

Zarbock A, Ley K. Neutrophil adhesion and activation under flow. Microcirculation. 2009;16:31–42.

FHIT

Jennifer Boylston and Charles Brenner
Department of Biochemistry and Program in Molecular & Cellular Biology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Synonyms

Fragile histidine triad gene

Historical Background

To date, approximately 80 common fragile sites (CFS) have been identified in the human genome (Ruiz-Herrera et al. 2006). Unlike rare fragile sites, which map to sites that are genetically altered in individuals, CFS occur at consistent genomic locations in particular cell types, which are defined by the paucity of replication initiation events at these loci (Letessier et al. 2011). DNA breaks at CFS, referred to as expression of the fragile site, can be induced *via* treatment with inhibitors of DNA replication, carcinogens, and environmental stresses (Lukusa and Fryns 2008). Several CFS colocalize with common deletions and translocations in tumors. Early efforts to find tumor suppressor genes encoded within fragile sites focused on *FRA3B* at chromosomal location 3p14.2, the most frequently expressed human CFS. Perturbations at 3p14.2 had been noted in many cancers and the site includes the t(3;8) translocation breakpoint associated with hereditary clear cell renal carcinoma (cRCC) (Cohen et al. 1979; Dreijerink et al. 2001). Positional cloning identified the fragile histidine triad gene, *FHIT*, as the gene that spans *FRA3B* (Ohta et al. 1996). In addition to losses of *FHIT* on the short arm of chromosome 3, many cRCC malignancies have been subject to gene losses including *RASSF1A* at 3p21.3 (Dreijerink et al. 2001) and *VHL* at 3p25 (Latif et al. 1993).

FHIT is a Tumor Suppressor Gene

Numerous cancer cell lines and primary tumors exhibit both homo- and hemizygous deletions within the *FHIT* gene. These deletions are concomitant with loss of mRNA expression in esophageal, stomach, colon, kidney, breast, and lung cancer cells. Immunohistochemistry of several types of primary tumors, including esophageal cancers, colon lesions, and cervical and lung cancers show frequent loss or absence of Fhit protein expression (Saldivar et al. 2010). This evidence notwithstanding, acceptance of *FHIT* as a tumor suppressor gene initially met with resistance, as loss of genetic material at a fragile site could be attributed as a consequence of genomic instability rather than a cause of cancer. However, accumulated evidence indicates that loss of Fhit is selected in carcinogenesis.

Mouse modeling and functional analysis has been used to show that *FHIT* mutations are drivers of malignancy. The murine *Fhit* locus shares key similarities with human *FHIT*: *Fhit* encompasses a fragile site, *Fra14A2*, and *Fhit* expression is altered in several murine cancer cell lines, suggesting that a mouse model of *Fhit* inactivation would be relevant to human disease (Glover et al. 1998). Mice heterozygous and homozygous at *Fhit* alleles have been established. These mice are fertile, live normal life spans, and do not present with gross defects, although an immune deficiency related to a low granulocyte count has been noted (Zanesi et al. 2001). *Fhit* heterozygous mice possess increased susceptibility to development of carcinogen-induced tumors. Oral administration of the carcinogen *N*-nitrosomethylbenzylamine (NMBA) produces tumors in 25% of a wild-type cohort of mice, while administration of an equivalent dose produces tumors in 100% of *Fhit* heterozygotes (Fong et al. 2000). *Fhit* heterozygous and *Fhit*-deficient mice are also more susceptible to spontaneous tumor development (Zanesi et al. 2001).

Gene therapy studies established that *Fhit* re-expression not only prevents development of cancer, but also contributes to tumor regression via apoptosis. Delivery of *Fhit* via adenovirus (Ad-*Fhit*) or adeno-associated virus (AAV-*Fhit*) significantly inhibits tumor development after NMBA treatment (Dumon et al. 2001). Immunohistochemical staining of tissue resected from sacrificed animals showed opposite Bax and Bcl2 staining in control versus Fhit-expressing animals, suggesting that failure to induce apoptosis contributed to tumor development in control animals while apoptosis due to delivery of Fhit in vector-treated animals contributed to tumor regression (Ishii et al. 2003).

Fhit Encodes a Dinucleoside Polyphosphate Hydrolase

Fhit and its homologs constitute one branch of histidine triad (HIT) proteins that have been strongly conserved throughout evolution. HIT proteins encode enzymes that bind and hydrolyze or phosphorylyze unusual nucleotide substrates. The HIT motif (His-Ø-His-Ø-His-Ø-Ø, in which Ø is a hydrophobic amino acid) forms part of the active site (Brenner 2002). Fhit protein and its orthologs are dinucleoside polyphosphate hydrolases, enzymes which bind and cleave a class of dinucleotides, typically containing two adenosines joined by 5′-5′ bridges of 3–5 phosphates. The favored substrate of Fhit is ApppA (Barnes et al. 1996) and the substrate typically used in vitro is GpppBODIPY (Draganescu et al. 2000). Hydrolysis of such compounds produces a nucleoside monophosphate product (e.g., AMP or GMP) plus the other nucleotide or analog (e.g., ADP or ppBODIPY).

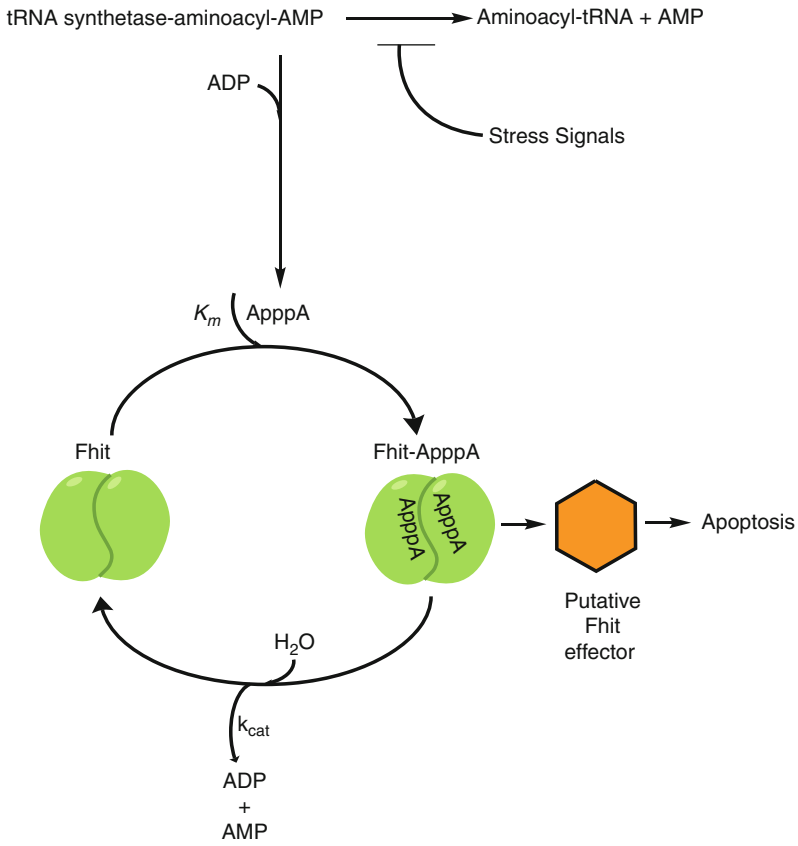
Fhit is a dimer, which contains two ApppA-binding sites per dimer. (Pace et al. 1998). ApppA molecules are by-products of reactions catalyzed by tRNA synthetases and are proposed to have several intracellular functions, including signaling stress responses (Campiglio et al. 2006). Interestingly, intracellular concentration of ApppA increases in mammalian cells exposed to the carcinogenic metal cadmium and the apoptosis-inducing topoisomerase poison etoposide (Fisher and McLennan 2008). Experiments were designed to determine whether the tumor-suppressing function of Fhit depends on ApppA-binding, hydrolysis, or both Appp-binding and hydrolysis. Re-expression of Fhit in Fhit-null cancer cells suppresses tumorigenicity in a manner that is largely insensitive to mutation of the active-site His (His96) to Asn (Siprashvili et al. 1997). This result suggested that the tumor suppressing function of Fhit might be independent of the ApppA substrate. However, a Fhit allele series was created to test the hypothesis that substrate binding but not hydrolysis is limiting for the pro-apoptotic activity of Fhit re-expression. Indeed, as mutations were created to reduce substrate binding, the pro-apoptotic activity of Fhit re-expression was reduced. In contrast, in mutations targeted to the active site His, which reduced catalytic activity by as much as 100,000-fold and reduced substrate binding by two-fold, only modest reductions in apoptotic activities were observed (Trapasso et al. 2003). As illustrated in Fig. 1, these data were interpreted to suggest that formation of a Fhit-ApppA substrate complex is limiting for interaction with a pro-aptoptic effector (Pace et al. 1998).

The Fhit Protein Interactome

Protein association studies have attempted to place Fhit into specific cellular pathways that might depend on diadenosine polyphosphates for signaling. Both wild-type Fhit and the catalytically inactive H96N mutant were shown to interact with tubulin in vitro and promote microtubule assembly in the presence of the microtubule binding proteins MAP2 and Tau (Chaudhuri et al. 1999). In addition, yeast two-hybrid data suggest that Fhit interacts with the SUMO conjugating enzyme, Ubc9 (Shi et al. 2000). Biochemical assays have confirmed an interaction in vitro with one report (Golebiowski et al. 2004) but not another (Shi et al. 2000) indicating ApppA-dependent protein binding. Another appealing model is that Fhit may bind and destablize Mdm2, an E3 ubiquitin ligase that marks p53 for degradation (Nishizaki et al. 2004).

Fhit and a member of the nitrilase superfamily are encoded as fusion proteins in worms and flies (Pekarsky et al. 1998), suggesting that Fhit and Nit1, the ortholog of the nitrilase-related domain may participate in the same pathway and associate as seen in the crystal structure of the worm NitFhit protein (Pace et al. 2000). The data suggest that like Fhit, Nit1 functions as a tumor suppressor. The *Nit1* knockout mouse shows increased susceptibility to NMBA-induced carcinogenesis and increased cell growth in culture (Semba et al. 2006). Moreover, loss of Fhit and loss of Nit1 are apparently additive for tumor suppression (Sun et al. 2009).

The Holy Grail in the molecular oncology of Fhit would be a set of protein interactions that provide a mechanism by which epithelial cells acquiring inactivating mutations in *FHIT* would obtain a survival advantage. The mechanism would also have to account for how Fhit re-expression induces apoptosis in a manner that depends on diadenosine polyphosphate binding. Though it is almost axiomatic that no unifying theory is accepted, there is one proposed mechanism notable for its far-reaching scope. According to this mechanism, Fhit transits to mitochondria *via* interaction with Hsp60 where it interacts



FHIT, Fig. 1 Stress is proposed to inhibit completion of the tRNA synthetase-catalyzed reaction, resulting in increased cytoplasmic ApppA. Fhit dimers, normally in the inactive state, now bind ApppA, forming pro-apoptotic Fhit-ApppA complexes. Fhit-ApppA complexes signal apoptosis *via* a putative Fhit effector. Cleavage of ApppA is proposed to terminate the pro-apoptotic state of Fhit

with and stabilizes ferredoxin reductase (Fdxr) (Trapasso et al. 2008). Fdxr transfers electrons from NADPH to cytochrome P450 via ferredoxin. Under stress, Fdxr levels increase, leading to depletion of NADPH, which is required to detoxify reactive oxygen species (ROS), thereby inducing ROS-mediated apoptosis. Consistent with the expectation that diadenosine polyphosphate binding is limiting for the tumor suppressing function of Fhit, the investigators have shown that mutations that reduce substrate binding also reduce mitochondrial localization, association with Hsp60 and Fdxr, and cellular responses to oxidative damage (Pichiorri et al. 2009).

Summary

Genetic analysis of Fhit has indicated that reintroduction of this protein induces apoptosis in cancer cells with *FHIT* deletions, which are a common cancer genotype. Challenges remain in defining the specific consequences of Fhit inactivation in the preneoplastic epithelial cells from which Fhit is typically lost and in defining the relationship between diadenosine polyphosphate signaling and cellular survival in cells, early in the process of carcinogenesis.

Cross-References

► p53

References

Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, et al. Fhit, a putative tumor suppressor in humans, is a dinucleoside 5′,5‴-P1, P3-triphosphate hydrolase. Biochemistry. 1996;35(36):11529–35.

Brenner C. Hint, Fhit, and GalT: function, structure, evolution, and mechanism of three branches of the histidine triad superfamily of nucleotide hydrolases and transferases. Biochemistry. 2002;41(29):9003–14.

Campiglio M, Bianchi F, Andriani F, Sozzi G, Tagliabue E, Menard S, et al. Diadenosines as FHIT-ness instructors. J Cell Physiol. 2006;208(2):274–81.

Chaudhuri AR, Khan IA, Prasad V, Robinson AK, Luduena RF, Barnes LD. The tumor suppressor protein Fhit. A novel interaction with tubulin. J Biol Chem. 1999;274(34):24378–82.
Cohen AJ, Li FP, Berg S, Marchetto DJ, Tsai S, Jacobs SC, et al. Hereditary renal-cell carcinoma associated with a chromosomal translocation. N Engl J Med. 1979;301(11):592–5.
Draganescu A, Hodawadekar SC, Gee KR, Brenner C. Fhit-nucleotide specificity probed with novel fluorescent and fluorogenic substrates. J Biol Chem. 2000;275(7):4555–60.
Dreijerink K, Braga E, Kuzmin I, Geil L, Duh FM, Angeloni D, et al. The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc Natl Acad Sci USA. 2001;98(13):7504–9.
Dumon KR, Ishii H, Fong LY, Zanesi N, Fidanza V, Mancini R, et al. FHIT gene therapy prevents tumor development in Fhit-deficient mice. Proc Natl Acad Sci USA. 2001;98(6):3346–51.
Fisher DI, McLennan AG. Correlation of intracellular diadenosine triphosphate (Ap3A) with apoptosis in Fhit-positive HEK293 cells. Cancer Lett. 2008;259(2):186–91.
Fong LY, Fidanza V, Zanesi N, Lock LF, Siracusa LD, Mancini R, et al. Muir-Torre-like syndrome in Fhit-deficient mice. Proc Natl Acad Sci USA. 2000;97(9):4742–7.
Glover TW, Hoge AW, Miller DE, Ascara-Wilke JE, Adam AN, Dagenais SL, et al. The murine Fhit gene is highly similar to its human orthologue and maps to a common fragile site region. Cancer Res. 1998;58(15):3409–14.
Golebiowski F, Szulc A, Szutowicz A, Pawelczyk T. Ubc9-induced inhibition of diadenosine triphosphate hydrolase activity of the putative tumor suppressor protein Fhit. Arch Biochem Biophys. 2004;428(2):160–4.
Ishii H, Zanesi N, Vecchione A, Trapasso F, Yendamuri S, Sarti M, et al. Regression of upper gastric cancer in mice by FHIT gene delivery. FASEB J. 2003;17(12):1768–70.
Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, et al. Identification of the von Hippel-Lindau disease tumor suppressor gene. Science. 1993;260(5112):1317–20.
Letessier A, Millot GA, Koundrioukoff S, Lachages AM, Vogt N, Hansen RS, et al. Cell-type-specific replication initiation programs set fragility of the FRA3B fragile site. Nature. 2011;470(7332):120–3.
Lukusa T, Fryns JP. Human chromosome fragility. Biochim Biophys Acta. 2008;1779(1):3–16.
Nishizaki M, Sasaki J, Fang B, Atkinson EN, Minna JD, Roth JA, et al. Synergistic tumor suppression by coexpression of FHIT and p53 coincides with FHIT-mediated MDM2 inactivation and p53 stabilization in human non-small cell lung cancer cells. Cancer Res. 2004;64(16):5745–52.
Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, et al. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell. 1996;84(4):587–97.
Pace HC, Garrison PN, Robinson AK, Barnes LD, Draganescu A, Rosler A, et al. Genetic, biochemical, and crystallographic characterization of Fhit-substrate complexes as the active signaling form of Fhit. Proc Natl Acad Sci USA. 1998;95(10):5484–9.
Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganowski P, Pekarsky Y, et al. Crystal structure of the worm NitFhit Rosetta stone protein reveals a Nit tetramer binding two Fhit dimers. Curr Biol. 2000;10(15):907–17.
Pekarsky Y, Campiglio M, Siprashvili Z, Druck T, Sedkov Y, Tillib S, et al. Nitrilase and Fhit homologs are encoded as fusion proteins in Drosophila melanogaster and Caenorhabditis elegans. Proc Natl Acad Sci USA. 1998;95(15):8744–9.
Pichiorri F, Okumura H, Nakamura T, Garrison PN, Gasparini P, Suh SS, et al. Correlation of fragile histidine triad (Fhit) protein structural features with effector interactions and biological functions. J Biol Chem. 2009;284(2):1040–9.
Ruiz-Herrera A, Castresana J, Robinson TJ. Is mammalian chromosomal evolution driven by regions of genome fragility? Genome Biol. 2006;7(12):R115.
Saldivar JC, Shibata H, Huebner K. Pathology and biology associated with the fragile FHIT gene and gene product. J Cell Biochem. 2010;109(5):858–65.
Semba S, Han SY, Qin HR, McCorkell KA, Iliopoulos D, Pekarsky Y, et al. Biological functions of mammalian Nit1, the counterpart of the invertebrate NitFhit Rosetta stone protein, a possible tumor suppressor. J Biol Chem. 2006;281(38):28244–53.
Shi Y, Zou M, Farid NR, Paterson MC. Association of FHIT (fragile histidine triad), a candidate tumour suppressor gene, with the ubiquitin-conjugating enzyme hUBC9. Biochem J. 2000;352(Pt 2):443–8.
Siprashvili Z, Sozzi G, Barnes LD, McCue P, Robinson AK, Eryomin V, et al. Replacement of Fhit in cancer cells suppresses tumorigenicity. Proc Natl Acad Sci USA. 1997;94(25):13771–6.
Sun J, Okumura H, Yearsley M, Frankel W, Fong LY, Druck T, et al. Nit1 and Fhit tumor suppressor activities are additive. J Cell Biochem. 2009;107(6):1097–106.
Trapasso F, Krakowiak A, Cesari R, Arkles J, Yendamuri S, Ishii H, et al. Designed FHIT alleles establish that Fhit-induced apoptosis in cancer cells is limited by substrate binding. Proc Natl Acad Sci USA. 2003;100(4):1592–7.
Trapasso F, Pichiorri F, Gaspari M, Palumbo T, Aqeilan RI, Gaudio E, et al. Fhit interaction with ferredoxin reductase triggers generation of reactive oxygen species and apoptosis of cancer cells. J Biol Chem. 2008;283(20):13736–44.
Zanesi N, Fidanza V, Fong LY, Mancini R, Druck T, Valtieri M, et al. The tumor spectrum in FHIT-deficient mice. Proc Natl Acad Sci USA. 2001;98(18):10250–5.

Fibulins

Yong-Hun Lee and William Schiemann
Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

Synonyms

BM-90; DANCE; EFEMP1; EFEMP2; EVEC; H411; Hemicentin; Him4; MBP1; S15; T16; TM14; UP50; UPH1

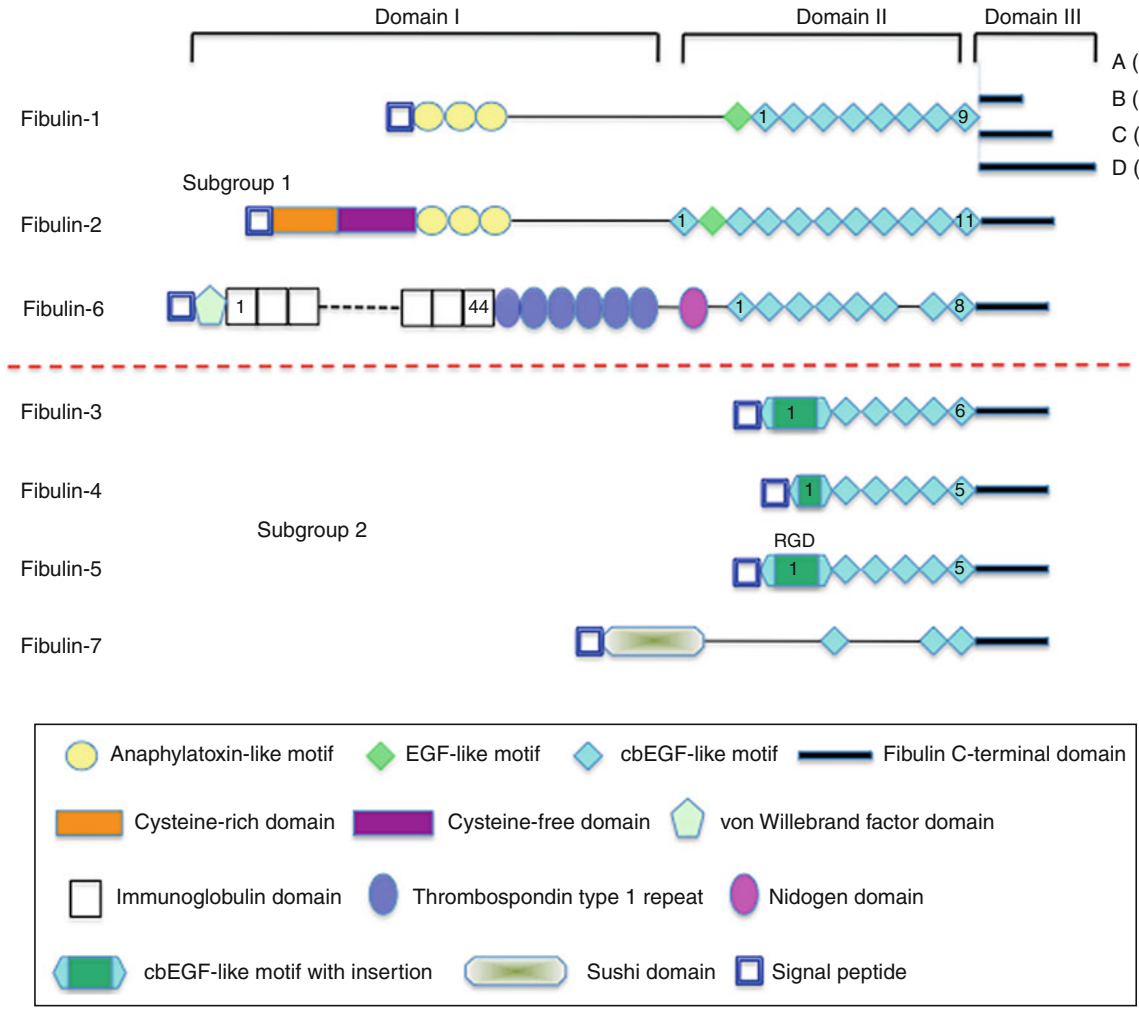
Historical Background

The extracellular matrix (ECM) is very diverse in its nature and composition in vertebrates, and is essential for normal development and maintenance of the microenvironment of embryonic and adult tissues. Many ECM proteins are secreted into extracellular milieu where they aggregate with existing matrix molecules to form supramolecular structures. The functions of the ECM include supporting cells, regulating intercellular communication, controlling cell motility, growth, and development, overseeing wound healing, and directing fibrosis (Albig and Schiemann 2005; de Vega et al. 2009; Timpl et al. 2003). These ECM proteins have been classified into several families based on their domain structures and/or functions, with fibulins now being recognized as one such family of ECM molecules. Fibulins share a common structural organization with tandem repeats of a calcium-binding epidermal growth factor (cbEGF)-like module and a unique C-terminal fibulin-type module (Albig and Schiemann 2005; Gallagher et al. 2005). The first fibulin (fibulin-1) was discovered two decades ago by affinity chromatography as an interacting molecule with the cytoplasmic tail of the β subunit of the fibronectin receptor (also known as β1 integrin receptor) (de Vega et al. 2009; Timpl et al. 2003). At first, fibulin-1 was considered to function as a bridging element between β1 integrin and intracellular molecules. Later, fibulin-1 nucleotide sequencing and immunohistochemistry showed this ECM protein to be secreted and present in the fibril matrix in fibroblasts, and in the blood. Since the discovery of fibulin-1, six additional fibulin family members have been reported. Fibulin-2 was identified from a mouse fibroblast cDNA library by comparative sequence analysis. Fibulin-3 (also known as S1-5, Efemp1) was first isolated from the screening of differentially expressed genes between senescent and quiescent human fibroblasts, and fibulin-4 (also known as Efemp2) was identified as a member of the fibulin family by sequence homology. Fibulin-5 (also known as EVEC, DANCE) was identified independently by searching for genes that regulate the transition of vascular smooth muscle cells from quiescent to proliferative states, and by searching for novel secreted molecules involved in the control of cardiovascular development and disease using signal sequence trap method. Hemicentin-1, the gene product of the him-4 locus in *Caenorhabditis elegans*, has been designated as fibulin-6 based on its cbEGF-like domain and C-terminal fibulin-type module. Recently, fibulin-7 (also known as TM14) was identified as the newest member of fibulin family by differential hybridization using tooth germ cDNA microarrays (de Vega et al. 2009; Timpl et al. 2003; Gallagher et al. 2005).

Fibulins are ECM proteins that collectively play important roles in the biology of tissue organogenesis and vasculogenesis, as well as in the pathology of fibrogenesis, tumorigenesis, and other genetic diseases. Herein we describe the multifunctional nature of fibulins in regulating the development of cancer and genetic diseases in humans, as well as their interactions with other ECM molecules and regulation of cellular signaling systems.

Fibulin Protein Structure and Their Interaction with Other Proteins

Structurally, fibulins contain a N-terminal (Domain I), which is variable among fibulin family members, and sequential repeats of cbEGF-like modules (Domain II), which is followed by a globular C-terminal fibulin-type module (Domain III) (Fig. 1). The fibulin family can be further subdivided into two distinct subgroups based on their size and the absence or presence of additional functional domains (Albig and Schiemann 2005). For instance, subgroup 1 consists of fibulin-1, fibulin-2, and fibulin-6. Fibulin-1 and fibulin-2 are larger and more structurally complex because they house more cbEGF-like modules in Domain II and three anaphylatoxin (AT) modules in domain I, which are components of the complement system involved in inflammation and defense against parasites. In addition, fibulin-2 has an extra N-terminal domain with that contains cysteine-rich and cysteine-free segments. Fibulin-6 contains largest N-terminal domain with more than 40 repeats of immunoglobulin motifs, six thrombospondin type I repeats, and a von Willebrand factor domain. Subgroup 2 contains the remaining family members, namely, fibulin-3, 4, 5 and fibulin-7, which collectively are smaller and structurally simpler. Fibulin-7 contains a sushi domain, which is known to be involved in protein–protein interactions and in the regulation of the complement system and blood coagulation (de Vega et al. 2009; Timpl et al. 2003; Gallagher et al. 2005). Although fibulins represent a small gene family, their genetic and biological diversity is increased significantly by their



Fibulins, Fig. 1 Schematic presentation of fibulin family proteins. Fibulins 1, 2, and 6 comprise subgroup 1, while fibulins 3, 4, 5, and 7 comprise subgroup 2. Although fibulins 1–4 are subject to alternative splicing, only fibulin-1 splice variants are shown. Fibulin-5 contains a unique evolutionarily conserved RGD (arginine-glycine-aspartic amino acid) sequence in the first cbEGF-like motif. The 44 immunoglobulin repeats in fibulin-6 are shown. Parenthesized number indicate amino acids present in alternatively spliced fibulin-1C-termini

propensity to undergo alternative mRNA splicing. Indeed, alternative splicing of fibulin-1 mRNA produces four splice variants (A, B, C, and D) in humans, and two splice variants (C and D) in mice, chickens, zebrafish, and nematodes. Splice variants of fibulin-1 differ exclusively in Domain III, such that fibulin-1A completely lacks this domain, while variants B, C, and D possess varying sequences throughout Domain III. In fibulin-2, the third EGF-like module is either or absent or present *via* alternative splicing; however, the functional consequences of these two variants remain unknown. Alternative splicing produces five variants of fibulin-3, all of which have a partial or complete absence of Domain I. Like fibulin-3, fibulin-4 also exhibits a splice variant that lacks a signal sequence in Domain I (de Vega et al. 2009; Gallagher et al. 2005).

As a member of the ECM, fibulins interact physically with a growing list of ECM and other secreted proteins in the matrix (Table 1). Fibulin-1 and fibulin-2 are localized in basement membranes in various tissues. Basement membranes are specialized sheet-like structures of the ECM that separates cells from the surrounding connective tissue; they also provide the

Fibulins, Table 1 Overview of fibulins: interaction, human pathologies, and cancer association

Proteins	Synonyms	Interacting proteins	Heritable disease	Cancer association
Fibulin-1	BM-90	Fibronectin, fibrinogen, nidogen	Synpolydactyly	Yes
		Laminin, aggrecan, versican	Giant platelet syndrome	
		Extracellular matrix protein1 (ECM1)	Vitroretinal dystrophy	
		Angiogenin, tropoelastin		
		ADAMTS-1(disintegrin-like and metalloproteinase with thrombospondin motifs), fibulin-7,		
		Sex-hormone binding globulin (SHBG)		
		β-amyloid precursor protein		
Fibulin-2	-	αIIβ3, αVβ3 integrin, laminin,	Unknown	Yes
		Fibrilin, fibronectin, nidogen		
		Aggrecan, versican, prelecan, SHBG		
Fibulin-3	S15	Tropoelastin	Malattia leventinese	Yes
	T16		Doyne honeycomb retinal dystrophy	
	EFEMP1		Age-related macular degeneration	
Fibulin-4	MBP1	Tropoelastin	Autosomal recessive cutis laxa	Yes
	EFEMP2	Mutant p53	Arachnodactyly	
	UPH1		Aortic aneyrysm	
	H411			
Fibulin-5	DANCE	Tropoelastin, Lox-like protein-1 (Loxl-1), Loxl-2, Loxl-4, emilin-1, fibrilin-1	Autosomal dominant cutis laxa	Yes
	EVEC	Latent TGF-β binding protein 2 (LTBP2)	Age-related macular degeneration	
	UP50	Extracellular superoxide dismutase		
		(ecSOD), Lipoprotein A,		
		αVβ1, αVβ3, αVβ5, α4β1, α9β1 integrin		
Fibulin-6	Hemicentin	Not available	Age-related macular degeneration (uncertain)	Not described
	Him4			
Fibulin-7	TM14	Fibronectin, heparin, fibulin-1	Unknown	Not described
		Dentin sialoprotein (DSP)		

essential scaffolding for cells and tissues during tissue morphogenesis that affects cell adhesion, migration, proliferation, and differentiation (Yanagisawa and Davis 2010). Basement membranes contain a vast array of proteins, such as laminin, perlecan, nidogen, and other molecules that interact with one another to form supramolecular structures (Yanagisawa and Davis 2010). Fibulin-1 and fibulin-2 bind to several basement membrane components including laminin, nidogen, perlecan, and fibronectin. The extensive network of interactions between basement membrane proteins and fibulin-1 and -2 plays an important scaffold function that supports the integrity and function of tissues. Fibulin-1 and fibulin-2 are also localized in elastic fibers, which provide the scaffold for connective tissues essential for the function of the skin, lungs, arteries, and other organs. The tropoelastins are deposited on microfibrils in an orderly manner and cross-linked by ▶ lysyl oxidases (LOXs) during the elastic fiber assembly (de Vega et al. 2009; Yanagisawa and Davis 2010). Aggrecan and versican are expressed in cartilage and provide mechanical strength to resist compression in the joints through the formation of large aggregates with hyaluronan that link proteins in the cartilage matrix (de Vega et al. 2009; Yanagisawa and Davis 2010). The binding of fibulin-1 and fibulin-2 to tropoelastin, aggrecan, and versican suggests that these fibulins play an important role in elastic fiber

assembly, as well as in the stabilization and function of cartilage matrix. While fibulin-1 and fibulin-2 interact with dozens of proteins, the other members of fibulin family typically display reduced ability to bind other proteins with the notable exception of fibulin-5, which exhibits overlapping binding affinities with fibulin-1 and fibulin-2. Fibulin-7 binds to dentin sialoprotein (Dsp), fibronectin, heparin, and fibulin-1, while no protein interactions have been reported for fibulin-6. Fibulin-3 and fibulin-4 bind to tropoelastin and play important roles in the assembly of elastic fibers during development (de Vega et al. 2009). Fibulin-5 not only binds to tropoelastin, but also interacts physically with a growing list of ECM and secreted proteins through three different mechanisms (Albig and Schiemann 2005; Yanagisawa et al. 2009). First, fibulin-5 binds αvβ3, αvβ5, α5β1, α4β1, and α9β1 integrins through an integrin-binding RGD motif that is unique amongst the fibulin family. The integrin-binding RGD motif of fibulin-5 is conserved in chicken, mouse, and rat, suggesting an important function in fibulin-5 biology. Second, cbEGF-like domains bind calcium with moderate-to-high affinity, which aids in maintaining protein stability, and in mediating protein–protein interactions. Fibulin-5 binds to LTBP-2 (latent TGF-β binding protein-2) through cbEGF-like modules, and in doing so, LTBP-2 may determine which microfibrils fibulin-5 becomes deposited on during elastic fiber assembly. The calcium-dependent binding of fibulin-5 to tropoelastin suggests that fibulin-5 structure and function also are dependent on calcium binding by its cbEGF-like domains. Fibulin-5 binds to the monomeric form of elastin through distinct N- and C-terminal elastin-binding regions, and to preexisting matrix scaffolds through cbEGF-like modules. Thus, fibulin-5 serves as an adaptor molecule that links monomeric elastin to matrix scaffolds to facilitate elastic fiber assembly. Fibulin-5 also binds the elastin-binding protein, elastin microfibril interface-located protein (EMILIN)-1, whose genetic ablation in mice induces mild elastinopathy reminiscent of that observed in fibulin-5-deficient mice. Whether fibulin-5 interacts physically with EMILIN-1 via its cbEGF-like domains remains to be determined. Finally, fibulin-5 interacts with apolipoprotein A, extracelluar superoxide dismutase (ecSOD), and lysyl oxidase-like 1, 2, and 3 (LOXL1-3) via its C-terminal fibulin-type module. The interaction between ecSOD and fibulin-5 is required for ecSOD binding to vascular tissues, thereby regulating vascular superoxide levels. The binding of fibulin-5 to LOXL1 appears essential for normal elastogenesis of the uterus, lung, skin, and vasculature. It has been reported that a fibulin-5 fragment lacking its C-terminus cannot be deposited on microfibrils and causes inactivation of the elastogenic activities of the full-length fibulin-5. Interestingly, the concentration of this fibulin-5 fragment actually increases with age, while the presence of full-length fibulin-5 is actually reduced in the skin of mouse. Since elasticity in tissue is thought be reduced with aging, C-terminal truncation of fibulin-5 by proteolysis may be involved in the deterioration of tissue elasticity during aging. Taken together, the extensive interactions of fibulins with other ECM proteins suggests that fibulins are indeed multifunctional proteins that couple the ECM to its formation of supramolecular structures, as well as its regulation of cellular processes.

Fibulins and Cancer

Oncogenic and tumor suppressive roles of fibulin family members have been proposed (Albig and Schiemann 2005; Gallagher et al. 2005; Argraves et al. 2003). A tumor suppressive role for fibulin-1 was proposed due to the fact that the overexpression of fibulin-1D in fibrosarcoma cells lowered their ability to grow in soft agar and invade reconstituted basement membranes in vitro. More importantly, expression of fibulin-1D delayed tumor formation in vivo and inhibited papillomavirus-E6 protein-mediated transformation. These findings support the conclusion that fibulin-1D acts as a tumor suppressor. However, fibulin-1 protein expression is progressively increased in the stroma adjacent to carcinoma cells during ovarian tumor progression. In addition, increased expression of fibulin-1 mRNA and protein was observed in primary breast carcinomas as compared with normal breast tissue. Furthermore, findings from DNA microarray studies of lung adenocarcinomas showed that fibulin-1 is consistently associated with matrix metalloproteinase 2 expression, a protein that promotes tumor invasion and metastasis (Gallagher et al. 2005; Argraves et al. 2003). These findings seem paradoxical to the idea that fibulin-1 is the product of a tumor suppressor gene. An explanation may come from findings that fibulin-1C and fibulin-1D splice variants are differentially expressed in ovarian carcinomas. Of the four fibulin-1 splice

variants, fibulin-1C and -1D transcripts are predominately expressed in most cell and tissue types, and it was shown that the ratio of fibulin-1C to -1D mRNA is increased in ovarian carcinomas relative to normal ovaries, suggesting that the fibulin-1C variant might play a role in carcinogenesis. In fact, it has been hypothesized that fibulin-1C variants possess oncogenic properties, while fibulin-1D variants exhibit tumor suppressive properties. Along these lines, genetic alterations of fibulin-1D have been linked to a congenital disorder that is typified by limb malformations, suggesting a functional independence of fibulin-1C and fibulin-1D variants. Precisely how fibulin-1C and fibulin-1D differentially impact cell behavior remains unknown. The splice variants of fibulin-1 all share the first 566 residues from the N-terminus, at which point they diverge to encode differing C-terminal polypeptide segments that add 0 (1A), 35 (1B), 117 (1C), and 137 (1D) residues, respectively. Thus, the difference in the C-terminal fibulin-type modules between fibulin-1 splice variants may account for the differential functions of fibulin-1C and fibulin-1D, possibly by modifying the repertoire of protein–protein interaction signatures. Likewise, a second possible mechanism to explain the imbalances that favor fibulin-1C expression over that of fibulin-1D in tumors has recently been proposed. Indeed, fibulin-1 expression is regulated by estrogen and its receptor at both the transcriptional and post-transcriptional levels in ovarian cancer cell lines. While estradiol treatment selectively decreased the half-life of fibulin-1D mRNA, the same treatment had no effect on fibulin-1C mRNA in ovarian cancer cells in vitro. This suggests that the oncogenic and tumor suppressive roles of fibulin-1 are governed by variant-specific expression in response to estrogens, which specifically destabilizes fibulin-1D mRNA. At present, the generality of this mechanism to other types of tumors, including those of breast, remains to be determined definitively.

To date, there exists little evidence that links fibulin-2, -6, and -7 with cancer; however, elevated fibulin-2 expression has been associated with the acquisition of metastatic phenotypes during adenocarcinoma progression. Fibulin-3 expression is altered in some human tumors and its constitutive expression in endothelial cells inhibited their proliferation, invasion, and angiogenic sprouting, as well as their response to vascular endothelial growth factor (Albig et al. 2006). The rat homologue of fibulin-3 associates with DA41, which interacts with the tumor suppressor protein DAN. Thus, fibulin-3 might have an indirect role in regulating cell growth through a network of molecular interactions. In addition, fibulin-3 prevented angiogenesis and vessel infiltration, as well as decreased vessel growth and density in tumors produced by MCA102 fibrosarcoma cells (Albig et al. 2006). Fibulin-4 expression is elevated in human colon tumors. Although fibulin-4 is clearly a secreted protein, recent work has identified a mutation in its signal sequence (i.e., Ala5Thr) that prevents its efficient secretion from cells, raising the possibility that aberrant subcellular localization of fibulin-4 contributes to tumorigenesis in humans. Accordingly, the intracellular form of fibulin-4 enhances cellular transformation and proliferation by interacting physically with mutant ▶ p53 proteins. Fibulin-5 was identified independently as a novel fibroblast and endothelial cell gene target of the tumor suppressor, transforming growth factor (TGF)-β (Albig and Schiemann 2005). Fibulin-5 mRNA expression is downregulated dramatically in the majority of human tumors, such as kidney, breast, ovary, and colon compared to corresponding normal tissues. Also, fibulin-5 has been established as a multifunctional signaling molecule that (1) regulates the proliferation, motility, and invasion of normal and malignant cells both in vitro and in vivo; (2) antagonizes endothelial cell activities coupled to angiogenesis both in vitro and in vivo; and (3) inhibits the growth of fibrosarcomas in mice (Lee et al. 2008). These findings suggest that the loss or inactivation of fibulin-5 may participate in cancer progression. Epithelial-mesenchymal transition (EMT) is a normal physiological process that regulates tissue development, remodeling, and repair; however, aberrant EMT also elicits disease development in humans, including lung fibrosis, rheumatoid arthritis, and cancer metastasis (Wendt et al. 2009). TGF-β is a master regulator of EMT in normal mammary epithelial cells (MECs), wherein this pleiotropic cytokine also functions as a potent suppressor of mammary tumorigenesis (Tian and Schiemann 2009). Since fibulin-5 is expressed in a developmentally regulated manner to regions of EMT during tissue development, and is a target of TGF-β, fibulin-5 has been considered an important and novel regulator of normal EMT during embryonic development, as well as an inducer of oncogenic EMT during the development and progression of

human breast cancers. Indeed, mammary epithelial cells engineered to ectopically expression fibulin-5 acquired an EMT phenotype as measured by monitoring alterations in the actin cytoskeleton, as well as by alterations in the expression of various markers of EMT (Lee et al. 2008). In addition, overexpression of fibulin-5 in 4T1 breast cancer cells increased their invasiveness by enhancing TGF-®-induced EMT and matrix metalloproteinase (MMP) expression (Lee et al. 2008). These alterations led to increased tumor growth in vivo when these cells were implanted into normal wild-type mice. Given the differential effects of fibulin-5 on cells of epithelial versus mesenchymal origin, the overall effect of fibulin-5 on tumor development must be carefully evaluated. Taken together, these findings suggest that fibulins play important roles in tumor development by functioning as tumor suppressors or tumor promoters through mechanisms that remain to be fully elucidated.

Fibulins and Human Genetic Diseases

Several human genetic diseases associated with the mutation of fibulin genes have been identified (Table 1). For instance, the fibulin-1 gene is disrupted in patients from one family with a rare dominantly inherited malformation of the distal limbs. Moreover, it has been reported that a defect in fibulin-1D expression is associated with the autosomal dominant giant platelet syndromes, which represent a group of disorders with combinations of deafness, renal disease, and eye abnormalities (de Vega et al. 2009; Gallagher et al. 2005; Argraves et al. 2003). Both genetic diseases are caused by the specific absence fibulin-1D transcripts, suggesting that the C-terminal fibulin-type motif of fibulin-1 plays an important causative role in initiating these pathologies. Along these lines, mice lacking fibulin-1 expression die shortly after birth, suggesting an essential function of this ECM in organism survival. In stark contrast, no human diseases have been linked to fibulin-2, and as such, fibulin-2 knockout mice are viable, fertile, and free of anatomic abnormalities (de Vega et al. 2009; Gallagher et al. 2005; Argraves et al. 2003). These findings suggest that fibulin-2 possesses functional redundancy with other matrix proteins that compensate for the absence of fibulin-2 during development. A fibulin-3 missense mutation (R345W) is associated with malattia leventinese (ML), which is a dominant macular degenerative disease characterized by the appearance of yellow deposits beneath the retinal pigment epithelium. The association of fibulin-3 mutation with ML was further supported by knock-in mice containing the R345W mutation, which develop early onset of macular degeneration in both heterozygous and homozygous mice. A fibulin-4 missense mutation (G169A) is associated with autosomal recessive cutis laxa, which is a connective tissue disorder characterized by cutaneous abnormalities such as loose skin. This mutation also affects elastic fiber densities in internal organs, such as the lung and the arteries. Moreover, fibulin-4 knockout mice showed lung and vascular malformations due to defects in elastic fiber formation, indicating a key role of fibulin-4 in vascular homeostasis.

Defects in the fibulin-5 gene are associated with cutis laxa and age-related macular degeneration (AMD). Two homozygous missense mutations (S227P and C217R) have been found in autosomal recessive cutis laxa families. Both fibulin-5 mutations decrease its binding to tropoelastin; they also significantly decreased its synthesis and secretion from cells, as well as impaired its association with fibrillin-1. As expected, defects in elastic fiber development were also evident under these conditions, suggesting that fibulin-5 is essential for proper elastic fiber formation. Accordingly, fibulin-5 knockout mice developed disorganized elastic fibers, which is reminiscent of the cutis laxa syndrome observed in humans that express fibulin-5 mutants. Heterozygous missense variations in fibulin-5 (G412E, G267S, I169 T, and Q124P) are associated with AMD and showed decreased fibulin-5 secretion. However, the causal relationship between heterozygous missense mutations in fibulin-5 and AMD needs to be further investigated. Fibulin-6 mutation (Q5346R) is proposed as a causal mutation for AMD pedigree; however, there is no supporting evidence for this hypothesis. At present, no human diseases have yet to be associated with the fibulin-7.

Summary

The fibulin family is comprised of matricellular proteins that clearly contribute to the structural development of elastogenic tissues, as well as mediate various cellular functions required for the maintenance of tissue homeostasis. Seven fibulin family members have been found in mammals, all of which interact with

various ECM molecules to stabilize supramolecular structures and to oversee various cellular processes, such as cell growth, differentiation, angiogenesis, and tumorigenesis. The variety of cellular activities attributed to fibulins are often cell-type specific and/or in a context-dependent manner, suggesting that the specific interactions between fibulins and ECM proteins leads to various cell behaviors and outcomes. Therefore, it remains to be determined precisely how fibulins mediate or antagonize intracellular signaling events and whether the modulation of fibulins possesses therapeutic potential to alleviate fibulins-related pathologies. Finally, new insight into the regulated steps of elastic fiber assembly from the study of fibulins may provide new opportunities to explore novel therapeutic regimens, including the regeneration of damaged elastic fibers, the prevention of elastic fiber-degenerative conditions, and the development of efficient artificial blood vessels.

References

Albig AR, Schiemann WP. Fibulin-5 function during tumorigenesis. Future Oncol. 2005;1(1):23–35.

Albig AR, Neil JR, Schiemann WP. Fibulins 3 and 5 antagonize tumor angiogenesis in vivo. Cancer Res. 2006;66(5):2621–9.

Argraves WS, Greene LM, Cooley MA, Gallagher WM. Fibulins: physiological and disease perspectives. EMBO Rep. 2003;4(12):1127–31.

de Vega S, Iwamoto T, Yamada Y. Fibulins: multiple roles in matrix structures and tissue functions. Cell Mol Life Sci. 2009;66(11–12):1890–902.

Gallagher WM, Currid CA, Whelan LC. Fibulins and cancer: friend or foe? Trends Mol Med. 2005;11(7):336–40.

Lee YH, Albig AR, Regner M, Schiemann BJ, Schiemann WP. Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-beta in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis. 2008;29(12):2243–51.

Tian M, Schiemann WP. The TGF-beta paradox in human cancer: an update. Future Oncol. 2009;5(2):259–71.

Timpl R, Sasaki T, Kostka G, Chu ML. Fibulins: a versatile family of extracellular matrix proteins. Nat Rev Mol Cell Biol. 2003;4(6):479–89.

Wendt MK, Allington TM, Schiemann WP. Mechanisms of the epithelial-mesenchymal transition by TGF-beta. Future Oncol. 2009;5(8):1145–68.

Yanagisawa H, Davis EC. Unraveling the mechanism of elastic fiber assembly: the roles of short fibulins. Int J Biochem Cell Biol. 2010;42(7):1084–93.

Yanagisawa H, Schluterman MK, Brekken RA. Fibulin-5, an integrin-binding matricellular protein: its function in development and disease. J Cell Commun Signal. 2009;3(3–4):337–47.

FKBP-Rapamycin-Associated Protein (FRAP)

► mTOR

FKHR (Forkhead in Rhabdomyosarcoma)

► FoxO1

Flamingo Cadherins

► Cadherins

FLJ17670

► ZAP-70

FLJ17679

► ZAP-70

FLJ20819

► Rab7a in Endocytosis and Signaling

FLJ38026

► LCoR

FLJ46592

► DOCK2; Dedicator of Cytokinesis 2

FLJ59152

▶ CDK11

FLJ92943

▶ p53

Flotillin-1 (*flot1*)

Rainer Prohaska and Ulrich Salzer
Max F. Perutz Laboratories (MFPL), Medical University of Vienna, Vienna, Austria

Synonyms

Reggie-2 (Reg-2)

Historical Background

Flotillin-1 was originally identified in 1997, when Bickel et al. analyzed caveolin-rich membrane fractions from mouse lung and isolated a 45 kDa protein that was microsequenced. Degenerate primers were designed from the peptide sequences and used for PCR of mouse lung cDNA. The PCR-product was then used as a probe for screening of a 3T3-L1 adipocyte cDNA library. The characterized sequence showed similarity to the ESA protein. An anti-peptide antibody identified a 47 kDa protein on Western blots. Because this protein was detected in *flo*ating, caveolin-rich, Triton X-100-insoluble membrane fractions, it was termed "*flo*tillin-1." The similar ESA protein (47% identity) was renamed as "flotillin-2." Also in 1997, Schulte et al. identified and characterized two proteins that were induced in *reg*enerating fish retinal ganglion cell axons after injury and thus were named "*reg*gie-1" and "*reg*gie-2." In 1998, Lang et al. cloned the rat reggie proteins and found that rat reggie-1 is 99% identical to human flotillin-2, while rat reggie-2 is 98% identical to human flotillin-1. Reggie-2/flotillin-1 was identified at the plasma membrane of neurons and non-neuronal cells in non-caveolar micropatches co-clustering with its homologue reggie-1/flotillin-2 and GPI-anchored cell adhesion molecules. These micropatches localize along the axon and in lamellipodia and filopodia of growth cones. In 1999, Volonté et al. presented co-immunoprecipitation data that identified flotillin-1, flotillin-2, caveolin-1, and caveolin-2 in one complex. This led to the hypothesis that flotillins are resident components of caveolae; however, this hypothesis could not be verified by several groups, as reviewed in Langhorst et al. (2005). In 2001, Stuermer et al. identified clusters of reggies/flotillins with GPI-anchored proteins and Fyn kinase in non-caveolar plasma membrane microdomains of neurons and astrocytes. These microdomains had a diameter of less than 100 nm. In Jurkat cells, cross-linking of Thy-1 induced colocalization of Thy-1, reggie/flotillin proteins, GM1, the T cell receptor, and Fyn kinase, suggesting the formation of a raft-associated signal transduction center. Reggies/flotillins were also found in lysosomes, suggesting that the signaling complex becomes subject to degradation, as reviewed in Langhorst et al. (2005). More details on the historical background can be found in Salzer and Prohaska (2011).

Protein Structure and Domain Organization

Mammalian flotillin-1 is a highly conserved, 48 kDa monotopic integral membrane protein of 427 amino acids that is divided into two large domains of similar size, an N-terminal "stomatin/prohibitin/flotillin/HflC/K" (SPFH) domain, also known as "prohibitin homology domain" (PHB) or "band 7 domain," and a C-terminal "Flotillin domain" (Morrow and Parton 2005; Rivera-Milla et al. 2006; Babuke and Tikkanen 2007). The N-terminus of flotillin-1 contains two hydrophobic regions, residues 10–35 and 134–151, respectively, that are thought to be associated with the cytoplasmic face of the bilayer but not spanning it. Both flotillins are not cleaved when proteinases are added to intact cells (Morrow and Parton 2005). It is not known if these hydrophobic regions adopt "hairpin loops" similar to caveolin-1 or the related stomatin. Such structures appear less likely in flotillin-1 due to interspersed charged and hydrophilic residues. For additional hydrophobic interaction, the N-terminal region contains three cysteine residues, Cys-5,

Cys-17, and Cys-34 that are likely to be palmitoylated (Rivera-Milla et al. 2006). In Vero cells, palmitoylation of Cys-34 was shown to be essential for plasma membrane targeting along a Golgi-independent pathway and for providing the necessary affinity for raft association (Morrow et al. 2002). In differentiated 3T3-L1 adipocytes, mutation of Cys-34 to Ala did not affect plasma membrane targeting, whereas the second hydrophobic region was found to be essential for plasma membrane localization (Liu et al. 2005). Intact Cys-34 was however suggested to play a role in lipid raft association. It is possible that cell type–specific differences are the causes of these differing results. The structure of the SPFH domain of the mouse homologue flotillin-2 was determined by NMR analysis in solution (PDB ID: 1WIN; MMDB ID: 30602) and revealed a compact ellipsoid structure built from four β-strands on one side and four α-helices on the other. The crystal structure of the SPFH domain of an archaeal stomatin is surprisingly similar and both structures can be superposed very well (PDB ID: 3BK6; MMDB ID: 62565), showing the high conservation of this domain from archaea to mammals. This evolutionary conservation suggests an important function but this is currently not clear. Because many SPFH-domain proteins are lipid raft associated, it is possible that this domain itself mediates raft association (Morrow and Parton 2005). The crystal structure shows that the membrane-facing surface of this domain contains hydrophobic patches that would favor interactions with membrane lipids. Moreover, the interaction with cortical actin of one SPFH-domain α-helix has been predicted and identified (Rivera-Milla et al. 2006). The C-terminal flotillin domain is characterized by three contiguous blocks of coiled-coil heptad motifs that contain multiple GluAla repeats as well as charged residues at the heptad positions 2, 3, and 5 (Rivera-Milla et al. 2006). This coiled-coil domain is predicted to form dimers, trimers, and oligomers. Using chemical cross-linking, it was determined that flotillins form homo- or hetero-tetramers as building blocks of larger oligomeric complexes (Solis et al. 2007). Moreover, it was shown that flotillin-1 is stabilized by flotillin-2 in hetero-oligomeric complexes. Near the C-terminal end, a conserved PDZ3-binding consensus was recognized that could be involved in regulating oligomerization of both flotillins (Rivera-Milla et al. 2006). A schematic model of the flotillin/reggie structural and functional domains (Rivera-Milla et al. 2006; Stuermer 2010) is shown in the flotillin-2 entry.

Interactions

Flotillin-1 interacts with a variety of proteins in different tissues. It appears that most interactions described for flotillin-1 actually involve the hetero-oligomers of flotillin-1 and flotillin-2; however, the relevance of this hetero-oligomerization has been recognized only recently and was ignored in the past. A list of described interaction partners is shown in Table 1.

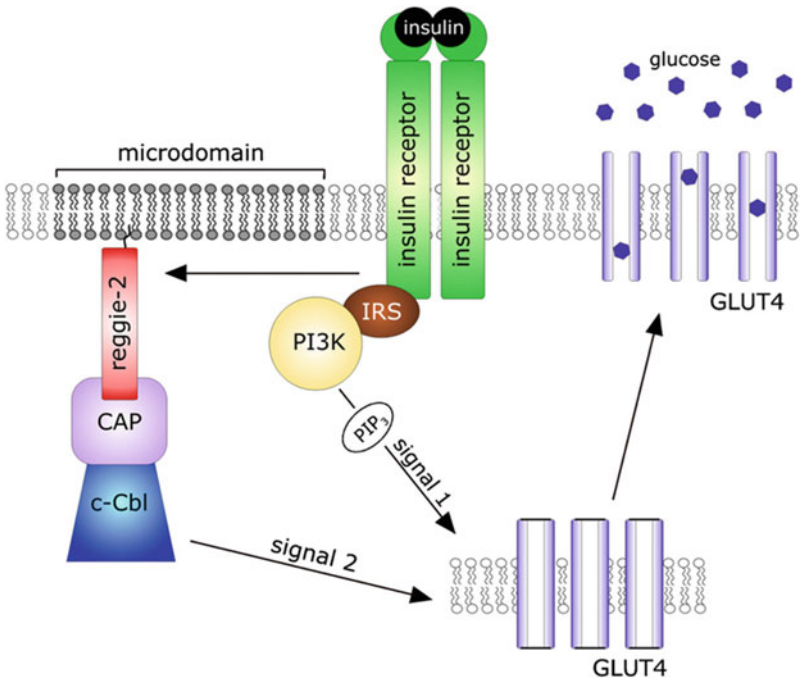
Flotillin-1 preferentially forms hetero-oligomers with flotillin-2 by interacting with the C-terminal coiled-coil domains (Solis et al. 2007). Hetero-oligomerization is essential for flotillin-1 stability, because flotillin-2 depletion induces flotillin-1 degradation by proteasomes (Solis et al. 2007). Flotillin-1 defines a specific, clathrin-independent endocytic pathway in mammalian cells; however, hetero-oligomerization with flotillin-2 is required for this clathrin- and caveolin-independent endocytosis (Frick et al. 2007). This process is regulated by Fyn kinase, which phosphorylates Tyr-160 in flotillin-1 and Tyr-163 in flotillin-2 (Riento et al. 2009). The region between the two N-terminal hydrophobic domains of flotillin-1 appears to be necessary for the interaction with Fyn (Liu et al. 2005). An important interaction of flotillin-1 with the multifunctional adapter protein c-Cbl-associated protein (CAP) and c-Cbl in a ternary complex was found to induce a second signaling pathway required for insulin-stimulated glucose transport via GLUT4 (Baumann et al. 2000). An outline of these insulin signaling pathways is shown in Fig. 1. The flotillin-1 interaction site on CAP was identified as the sorbin homology (SoHo) domain. This domain is also present in vinexin α and ArgBP2, enabling these proteins to interact with flotillin-1 in the same way. All three members of this SoHo family of adaptor proteins contain three SH3 domains within their C-termini. The functional significance of these interactions can be seen in the recruitment of signaling proteins to flotillin-specific lipid rafts (Babuke and Tikkanen 2007; Stuermer 2010).

Flotillin-1 and -2 were found to associate with Niemann-Pick C1-like 1 (NPC1L1) in a complex with clathrin heavy chain (CHC) and AP2 subunit μ2

Flotillin-1 (*flot1*), Table 1 Flotillin-1 interaction partners

Interacting protein	Location	Function	Remarks
Flotillin-2	Plasma membrane lipid rafts	Hetero-oligomerization	Flotillin-2 is essential for flotillin-1 stability
Fyn kinase	Lipid rafts	Tyr phosphorylation	Induces endocytosis
Lyn kinase	Basophilic leukemia cells	Enhances kinase activity	Involved in IgE-receptor signaling
NPC1L1	Plasma membrane lipid rafts	Cholesterol endocytosis and transport	Involves clathrin heavy chain and AP2
CAP/ponsin	Plasma membrane lipid rafts	Insulin signaling via c-Cbl, CrkII, and C3G.	Binding of flotillin-1 to the SoHo domain of CAP
	Focal adhesion sites	Regulation of actin cytoskeleton	
Vinexin α	Cell adhesion sites	Integrin-mediated cell adhesion	Binding to the SoHo domain of vinexin α
ArgBP2	Lipid rafts	Regulation of the actin cytoskeleton	Binding to the SoHo domain of ArgBP2
PTOV1 (Prostate tumor overexpressed gene 1)	Plasma membrane rafts and nucleus	Mitogenic complex	Recruitment to the nucleus in S phase
Aurora B kinase	Nucleus	Mitotic regulator	Flotillin-1 regulates Aurora B abundance and activity
Neuroglobin	Lipid rafts	Protects neurons from hypoxia	Inhibits Gα proteins
NMDAR (*N*-methyl-D-aspartate receptor)	Neuronal plasma membrane	Glutamate-binding calcium channel	NR2A/B subunits bind to SPFH domain
Gαq	Lipid rafts	Gαq-induced p38 MAPK activation	Depends on Src-family kinases
APP (amyloid precursor protein)	Lipid rafts	APP recruitment and processing in lipid rafts	APP binds to flotillin-1 C-terminus
Myocilin	Eye	Mutated in primary open-angle glaucoma	Binding to myocilin C-terminal domain
TRIM5α	Cytoplasmic bodies	Antiviral tripartite motif family	Dependent on cholesterol
LGI3 (Leucine-rich glioma inactivated 3)	Endosomes	Vesicle trafficking and endosome formation	Regulates APP trafficking
PSGL-1 (P-selectin glycoprotein ligand 1)	Plasma membrane	Involved in the polarization of neutrophils	Both flotillins and PSGL-1 accumulate in uropods
Arginase	Erythrocyte membrane	Involved in NO signaling	Upregulates arginase activity
ABCA1 (ATP-binding cassette transporter A1)	Plasma membrane rafts, phagosomes	Cellular cholesterol efflux, phagocytosis	Complex with ABCA1 and syntaxin 13
Syntaxin 13	Plasma membrane rafts, endosomes, phagosomes	Phagocytosis	Complex with ABCA1 and syntaxin 13
PrP^c (prion protein)	Plasma membrane, lipid-rich vesicles	Clustering at cell-cell contacts, focal adhesions	Complex with both flotillins
DAT (Dopamine transporter)	Lipid rafts	PKC-triggered endocytosis of DAT-flotillin-1 complex	Flotillin-1 residues Cys-34 and Ser-315 are essential

(Ge et al. 2011). The localization of this complex is cholesterol-dependent. Cholesterol depletion causes transport of flotillins and NPC1L1 to the plasma membrane, whereas cholesterol replenishment leads to endocytosis of both. Thus, flotillins play a role in NPC1L1-mediated cellular cholesterol uptake and regulation of lipid levels. Knockdown of flotillin-1, flotillin-2, or both, dramatically decreased NPC1L1 endocytosis and cholesterol uptake and reduced the association between the CHC-AP2 complex and NPC1L1. Because the knockdown of CHC had no effect on the flotillin-NPC1L1 association, it appears that flotillins are required for recruitment of the CHC-AP2 complex to NPC1L1 and thus act upstream to



Flotillin-1 (*flot1*),
Fig. 1 Role of flotillin-1/reggie-2 in insulin signaling. In adipocytes and muscle cells, insulin receptor activation triggers the classic signaling cascade through phosphatidylinositol 3-kinase, PI3K. A second signaling route proceeds through membrane rafts and involves a ternary complex c-Cbl/CAP/reggie-2. Both pathways mediate the recruitment of the glucose transporter GLUT4 to the plasma membrane and uptake of glucose into the cell (Figure reprinted from Babuke and Tikkanen (2007) with permission from Elsevier)

mediate the internalization of NPC1L1 and cholesterol (Ge et al. 2011). The NPC1L1-binding drug Ezetimibe was found to disrupt the flotillin-NPC1L1 complex. This study reveals an important functional role of the flotillins as mediators of cholesterol absorption via flotillin-NPC1L1-generated, cholesterol-enriched membrane microdomains (Ge et al. 2011). Flotillins were also found to interact with the tetrameric *N*-methyl-D-aspartate receptor (NMDAR), an ionotropic glutamate receptor, with the subunits NR1, NR2A, and NR2B. The distal C-terminus of NR2B interacted with both flotillin-1 and flotillin-2, whereas the C-terminus of NR2A bound only flotillin-1. Both flotillins were found to colocalize with NMDARs in hippocampal neurons (Swanwick et al. 2009). Flotillins also interact with the guanine nucleotide-binding protein Gq subunit alpha (Gαq), independent of the Gαq nucleotide-binding state (Sugawara et al. 2007). The N-terminal regions of both flotillins are essential for Gαq binding. Knockdown of flotillins attenuated the UTP-induced activation of ▶ p38 mitogen-activated protein kinase (MAPK). Because activation of MAPK depends on ▶ Src-family kinases and intact lipid rafts, these results suggest that flotillins mediate Gαq-induced MAPK activation through ▶ Src-kinases in lipid rafts (Sugawara et al. 2007). In leukocytes, flotillins interact with P-selectin glycoprotein ligand-1 (PSGL-1), also known as SELPLG or CD162 (Rossy et al. 2009). When neutrophils are activated, flotillins rapidly form caps and later accumulate in the uropods of the polarized cells. While PSGL-1 accumulates in the uropods concomitantly, other uropod components such as ▶ CD43 and ERM-proteins accumulate at a slower pace. Flotillins accumulate in uropods even in the absence of PSGL-1, indicating that PSGL-1 accumulation depends on flotillins (Rossy et al. 2009). This study was extended by using neutrophils from the flotillin-1 knockout mouse (Ludwig et al. 2010). Flotillin-2 was affected by the lack of flotillin-1, showing only weak, uniform staining of the plasma membrane and absence from detergent-resistant membranes. Importantly, the recruitment of flotillin-1-deficient neutrophils toward the chemoattractant fMLP was strongly reduced. Immunoisolation of tagged flotillin-2 followed by mass spectrometry of co-isolated proteins revealed the association of flotillins with cytoskeletal proteins, such as α- and β-spectrin and ▶ myosin IIa (Ludwig et al. 2010). The authors conclude that this association is important for neutrophil migration, uropod formation, and regulation of ▶ myosin IIa. Recently, flotillin-1 was found to be required for protein kinase C (PKC)-regulated internalization of the dopamine transporter (DAT) and the glial glutamate transporter EAAT2 (Cremona et al. 2011).

Flotillin-1 associates with DAT in plasma membrane lipid rafts and is required to maintain DAT in rafts. For internalization of the DAT-flotillin-1 complex, palmitoylation of flotillin-1 at Cys-34 and phosphorylation at Ser-315 is essential (Cremona et al. 2011). More details on flotillin-1 interactions are described in Salzer and Prohaska (2011).

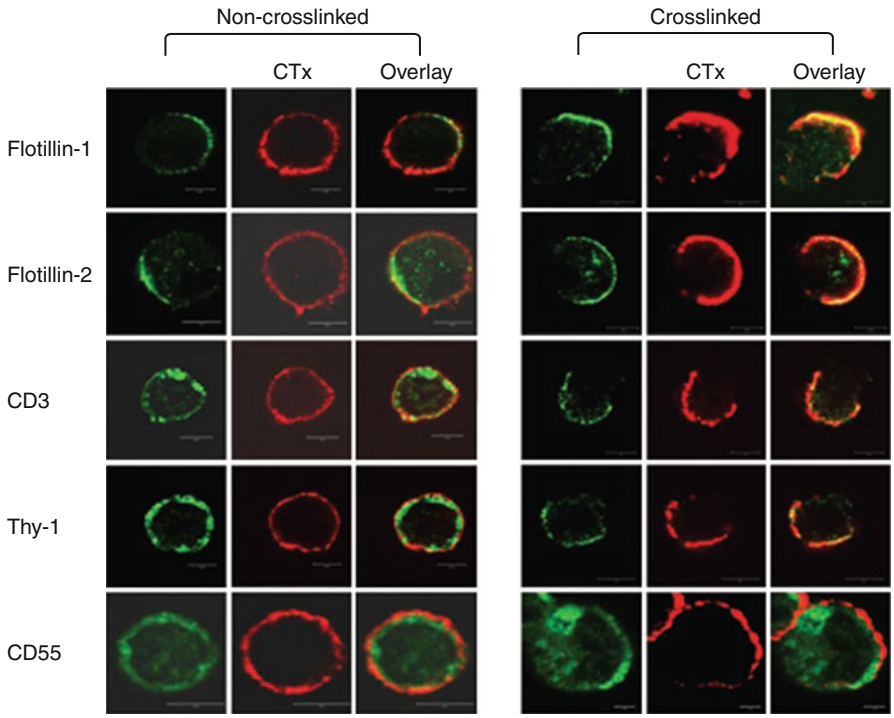
Cellular and Subcellular Localization

Both flotillins are expressed ubiquitously in virtually all tissues and cell types but have been studied particularly in neurons, hematopoietic cells, and adipocytes (Babuke and Tikkanen 2007; Stuermer 2010). Gene expression analyses by quantitative PCR of human and mouse (Mouse Genome Informatics MGI:1100500) tissues have shown flotillin-1 expression in all tissues analyzed. In cells, flotillin-1 is localized to the plasma membrane and internal vesicles such as Golgi vesicles, lipid droplets, recycling endosomes, multivesicular bodies, exosomes, late endosomes, lysosomes, phagosomes, and centrosomes, respectively (Langhorst et al. 2005). Moreover, it can translocate to the nucleus when interacting with the mitogenic protein PTOV1 (Prostate tumor overexpressed gene 1) or with the mitotic regulator Aurora B kinase. At the plasma membrane of neuronal cells, flotillin-1 is found in micropatches along the axons, in filopodia and lamellipodia of growth cones, and at synapses (Stuermer 2010). Flotillin hetero-oligomers accumulate at cell-cell contacts of many cell types (Langhorst et al. 2005; Stuermer 2010). These hetero-oligomers in plasma membrane microdomains are eventually endocytosed (Frick et al. 2007; Babuke et al. 2009; Riento et al. 2009) and apparently recycle back to the plasma membrane. Endocytosis takes place either in a clathrin- and caveolin-independent way (Riento et al. 2009) or in association with clathrin and NPC1L1 (Ge et al. 2011). In hematopoietic cells such as the B cell lines Raji and Ramos, T leukemic Jurkat cells, and the promonocytic cell line U937, both flotillins are asymmetrically localized in stable, preassembled platforms of the plasma membrane, to which signaling components are recruited on activation (Rajendran et al. 2003). An example for this extraordinary phenomenon is shown in Fig. 2.

In T lymphocytes, one stimulus for T cell receptor (TCR) complex association with the preassembled platform is the clustering of GPI-anchored proteins by antibody cross-linking. Thy-1 or cellular prion protein (PrP) cross-linking leads to their selective association with the flotillin platform. This induces the MAPK pathway and calcium signaling, resulting in recruitment of the TCR-CD3 complex. Thus, the flotillin membrane domains serve as platforms for cluster formation, signaling, cytoskeletal rearrangement, and recruitment of ▶ CD3 (Stuermer 2010). In chemoattracted neutrophils, flotillin microdomains accumulate rapidly in the uropod (Rossy et al. 2009) due to specific interaction with the cytoskeletal proteins α- and β-spectrin and ▶ myosin II (Ludwig et al. 2010). More details on flotillin-1 localizations are described in (Salzer and Prohaska 2011). The role of flotillin membrane microdomains in signaling, cytoskeletal regulation, and endocytosis, was reviewed and discussed recently (Otto and Nichols 2011).

Regulation of Expression and Activity

In fish and mammals, both flotillins are upregulated in retinal ganglion cells during axon regeneration after optic nerve section; reviewed in Langhorst et al. (2005), Stuermer (2010). Flotillin-1 stability depends on flotillin-2 expression (Solis et al. 2007). Conversely, flotillin-2 expression correlates with flotillin-1 expression (Ludwig et al. 2010). Both flotillins co-assemble at the plasma membrane in roughly equal amounts and act together to generate microdomains (Frick et al. 2007), which are endocytosed after Fyn-dependent tyrosine phosphorylation together with GPI-anchored proteins (Riento et al. 2009). Mutation of flotillin-1 residue Tyr-160 and flotillin-2 residue Tyr-163 prevents Fyn-induced internalization and leads to reduced uptake of GPI-anchored proteins. Thus, the uptake of flotillin microdomains is established as a tyrosine-kinase-regulated, endocytic process (Riento et al. 2009). Deletion of flotillin-1 apparently results in complete destruction of flotillin microdomains (Ludwig et al. 2010). The localization of the flotillin-1 and -2 complex with NPC1L1 and clathrin heavy chain and AP2 μ2 is regulated by the cholesterol level in the plasma membrane. Cholesterol depletion causes transport to the plasma membrane, whereas cholesterol replenishment leads to endocytosis. Thus, flotillins play a role in NPC1L1-mediated cellular cholesterol uptake and regulation of lipid levels (Ge et al. 2011).



Flotillin-1 (*flot1*),
Fig. 2 *Flotillin preassembled platforms*. Lipid raft clustering recruits raft-associated signaling molecules to the preassembled platforms and activates T cells. Non-cross-linked T cells (*Left*) show uniform distribution of GM1 (Cholera toxin-B, *red*), CD3, Thy-1, and CD55 (*green*) but very polarized expressions of flotillins-1 and -2. GM1 cross-linking (*Right*) by Cholera toxin-B (*red*) induces patching and recruits signaling molecules CD3 and Thy-1, but not CD55, (*green*) to preassembled flotillin platforms (Figure reprinted from Rajendran et al. (2003) with permission from PNAS)

For the PKC-triggered endocytosis of the lipid raft-associated dopamine transporter-flotillin-1 complex, palmitoylation of flotillin-1 at Cys-34 and phosphorylation at Ser-315 is essential (Cremona et al. 2011). In many biological processes, flotillins act as platforms associated with membrane receptors or transporters and the cytoskeleton to mediate signaling and endocytosis, as described above and reviewed in Morrow and Parton (2005), Langhorst et al. (2005) Babuke and Tikkanen (2007) Stuermer (2010), Salzer and Prohaska (2011).

Summary

Flotillin-1, also known as reggie-2, is a highly conserved, oligomeric, lipid-raft-associated, integral membrane protein of 48 kDa that is widely expressed in different tissues and cell types. It is associated with the cytosolic side of the plasma membrane and endosomes due to hydrophobic interaction and palmitoylation. Flotillin-1 is mainly found in a hetero-oligomeric complex with its homologue flotillin-2, also known as reggie-1. Both flotillins/reggies are members of the SPFH (stomatin/prohibitin/flotillin/HflC/K) superfamily, more recently termed PHB (prohibitin homology domain) superfamily. Hetero-oligomerization is required for endocytosis of flotillin microdomains either in a clathrin- and caveolin-independent way, such as Fyn kinase-regulated endocytosis of GPI-anchored proteins, or in a clathrin-dependent way, such as cholesterol uptake mediated by Niemann-Pick C1-like 1 (NPC1L1). In neurons, flotillins/reggies control axon growth and growth cone elongation by coordinating the assembly of signaling complexes that regulate cortical cytoskeleton remodeling. In neutrophils, flotillin interaction with cytoskeletal components is required for normal migration and uropod formation. Flotillin microdomains thus constitute scaffolding platforms interacting with signaling components, cytoskeleton, and/or membrane proteins in various cellular processes.

References

Babuke T, Tikkanen R. Dissecting the molecular function of reggie/flotillin proteins. Eur J Cell Biol. 2007;86:525–32.
Babuke T, Ruonala M, Meister M, Amaddii M, Genzler C, Esposito A, Tikkanen R. Hetero-oligomerization of reggie-1/flotillin-2 and reggie-2/flotillin-1 is required for their endocytosis. Cell Signal. 2009;21:1287–97.

Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, Bickel PE, Pessin JE, Saltiel AR. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature. 2000;407:202–7.
Cremona ML, Matthies HJ, Pau K, Bowton E, Speed N, Lute BJ, Anderson M, Sen N, Robertson SD, Vaughan RA, Rothman JE, Galli A, Javitch JA, Yamamoto A. Flotillin-1 is essential for PKC-triggered endocytosis and membrane microdomain localization of DAT. Nat Neurosci. 2011;14:469–77.
Frick M, Bright NA, Riento K, Bray A, Merrified C, Nichols BJ. Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. Curr Biol. 2007;17:1151–6.
Ge L, Qi W, Wang LJ, Miao HH, Qu YX, Li BL, Song BL. Flotillins play an essential role in Niemann-Pick C1-like 1-mediated cholesterol uptake. Proc Natl Acad Sci USA. 2011;108:551–6.
Langhorst MF, Reuter A, Stuermer CA. Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. Cell Mol Life Sci. 2005;62:2228–40.
Liu J, Deyoung SM, Zhang M, Dold LH, Saltiel AR. The stomatin/prohibitin/flotillin/HflK/C domain of flotillin-1 contains distinct sequences that direct plasma membrane localization and protein interactions in 3T3-L1 adipocytes. J Biol Chem. 2005;280:16125–34.
Ludwig A, Otto GP, Riento K, Hams E, Fallon PG, Nichols BJ. Flotillin microdomains interact with the cortical cytoskeleton to control uropod formation and neutrophil recruitment. J Cell Biol. 2010;191:771–81.
Morrow IC, Parton RG. Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. Traffic. 2005;6:725–40.
Morrow IC, Rea S, Martin S, Prior IA, Prohaska R, Hancock JF, James DE, Parton RG. Flotillin-1/reggie-2 traffics to surface raft domains via a novel golgi-independent pathway. Identification of a novel membrane targeting domain and a role for palmitoylation. J Biol Chem. 2002;277:48834–41.
Otto GP, Nichols BJ. The roles of flotillin microdomains-endocytosis and beyond. J Cell Sci. 2011;124:3933–40.
Rajendran L, Masilamani M, Solomon S, Tikkanen R, Stuermer CA, Plattner H, Illges H. Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells. Proc Natl Acad Sci USA. 2003;100:8241–6.
Riento K, Frick M, Schafer I, Nichols BJ. Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase. J Cell Sci. 2009;122:912–18.
Rivera-Milla E, Stuermer CA, Málaga-Trillo E. Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. Cell Mol Life Sci. 2006;63:343–57.
Rossy J, Schlicht D, Engelhardt B, Niggli V. Flotillins interact with PSGL-1 in neutrophils and, upon stimulation, rapidly organize into membrane domains subsequently accumulating in the uropod. PLoS One. 2009;4:e5403.
Salzer U, Prohaska R. Flotillin 2. UCSD nature molecule pages. 2011. doi:10.1038/mp.a002226.01
Solis GP, Hoegg M, Munderloh C, Schrock Y, Malaga-Trillo E, Rivera-Milla E, Stuermer CA. Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. Biochem J. 2007;403:313–22.
Stuermer CA. The reggie/flotillin connection to growth. Trends Cell Biol. 2010;20:6–13.
Sugawara Y, Nishii H, Takahashi T, Yamauchi J, Mizuno N, Tago K, Itoh H. The lipid raft proteins flotillins/reggies interact with Galphaq and are involved in Gq-mediated p38 mitogen-activated protein kinase activation through tyrosine kinase. Cell Signal. 2007;19:1301–8.
Swanwick CC, Shapiro ME, Yi Z, Chang K, Wenthold RJ. NMDA receptors interact with flotillin-1 and −2, lipid raft-associated proteins. FEBS Lett. 2009;583:1226–30.

Flotillin-2 (*FLOT2*)

Rainer Prohaska and Ulrich Salzer
Max F. Perutz Laboratories (MFPL), Medical University of Vienna, Vienna, Austria

Synonyms

Epidermal surface antigen (ESA); ESA1; Membrane component, Chromosome 17, Surface marker 1 (M17S1); Reggie-1 (Reg-1)

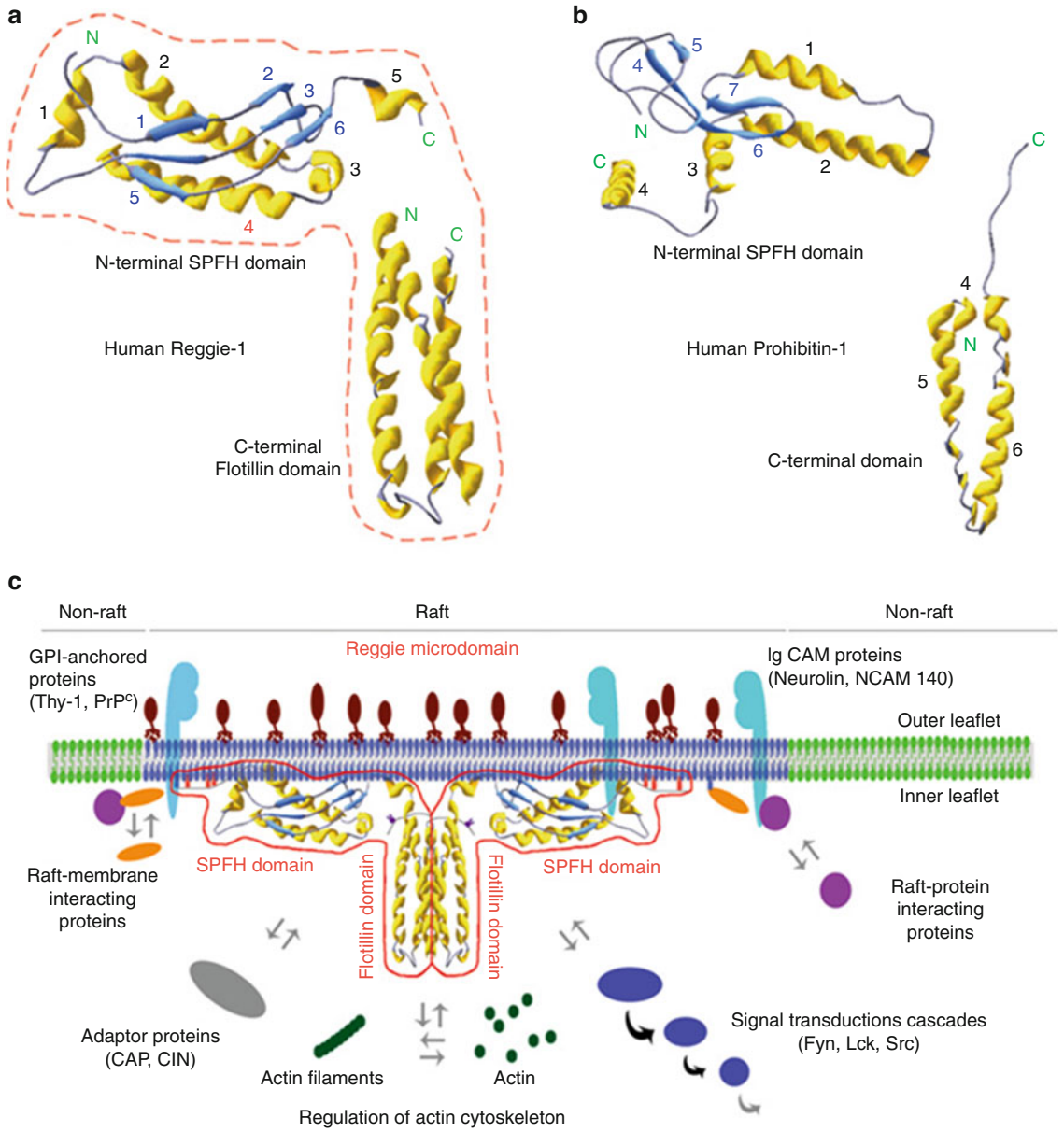
Historical Background

Flotillin-2 was originally cloned in 1994 by Schroeder et al. by immunoscreening of a human keratinocyte cDNA library using the monoclonal antibody ECS-1 against cultured keratinocytes. ECS-1 stained human epidermis and caused keratinocyte detachment in vitro, leading to the hypothesis that ECS-1 antigen may play a role in epidermal cell adhesion. Therefore, the immunoisolated cDNA and deduced protein was termed "epidermal surface antigen" (ESA), reviewed in Langhorst et al. (2005) and Morrow and Parton (2005). Western blot analysis identified a 35 kDa protein. In 1997, Bickel et al. analyzed caveolin-rich membrane fractions from mouse lung and isolated a 45 kDa protein. Microsequencing yielded novel and ESA-specific peptide sequences. Degenerate primers were used for PCR of mouse lung cDNA and the PCR product was then used as a probe for screening of a 3T3-L1 adipocyte cDNA library. The identified clone was characterized and its sequence showed similarity to the ESA sequence (47% identity). An anti-peptide antibody identified a 47 kDa protein on Western blots. Because this protein was detected in *flo*ating, Triton X-100-insoluble

membrane fractions together with caveolin-1 and ESA, it was termed "*flo*tillin-1" while the homologous ESA was renamed to "*flo*tillin-2." It turned out that the original, immunoisolated ESA was not the real ECS-1 antigen and not an epidermal surface antigen; therefore, this name was no longer appropriate. Also in 1997, Schulte et al. identified two proteins that were induced in *reg*enerating fish retinal ganglion cell axons after injury and thus were named "*reg*gie-1" and "*reg*gie-2." A striking 80% identity of fish reggie-1 and human flotillin-2 was recognized. In 1998, Lang et al. cloned the rat reggie proteins and found that rat reggie-1 is 99% identical to human flotillin-2, while rat reggie-2 is 98% identical to human flotillin-1. Reggie-1/flotillin-2 was identified at the plasma membrane of neurons and nonneuronal cells in non-caveolar micropatches co-clustering with reggie-2/flotillin-1 and GPI-anchored cell adhesion molecules. These micropatches localize along the axon and in lamellipodia and filopodia of growth cones. In 1999, Volonté et al. presented co-immunoprecipitation data that identified flotillin-1, flotillin-2, caveolin-1, and caveolin-2 in one complex. This led to the hypothesis that flotillins are resident components of caveolae; however, this hypothesis could not be verified by several groups, as reviewed in Langhorst et al. (2005). In 2001, Stuermer et al. identified clusters of reggies/flotillins with GPI-anchored proteins and Fyn kinase in non-caveolar plasma membrane microdomains of neurons and astrocytes. These microdomains had a diameter of less than 100 nm. In Jurkat cells, cross-linking of the GPI-anchored protein Thy-1 induced the colocalization of Thy-1, reggie/flotillin proteins, GM1, the T-cell receptor, and Fyn kinase, thus suggesting the formation of a raft-associated signal transduction center. Reggies/flotillins were also found in lysosomes, suggesting that the signaling complex becomes subject to degradation, reviewed in Langhorst et al. (2005). More details on the historical background can be found in Salzer and Prohaska (2011).

Protein Structure and Domain Organization

Mammalian flotillin-2 is a highly conserved, 48 kDa monotopic integral membrane protein of 428 amino acids that is divided into two large domains of similar size, an N-terminal "stomatin/prohibitin/flotillin/HflC/K" (SPFH) domain, also known as "prohibitin homology domain" (PHB) or "band 7 domain," and a C-terminal "flotillin domain" (Rivera-Milla et al. 2006). The N-terminal end of flotillin-2 contains a conserved hydrophobic region of about 30 amino acids that is associated with the cytoplasmic face of the bilayer but not spanning it. Both flotillins are not affected when proteinases are added to intact cells (Morrow and Parton 2005). It is not known if the hydrophobic region forms a "hairpin loop" similar to caveolin-1 or the related stomatin. Such a structure appears less likely in flotillin-2 due to several interspersed charged and hydrophilic residues. However, the N-terminus is truly membrane bound due to extensive modification by myristate at Gly-2 and palmitate at Cys-4, Cys-19, Cys-20, and probably Cys-38 (Neumann-Giesen et al. 2004; Rivera-Milla et al. 2006). The proximal SPFH/PHB/Band 7 domain is an ancient domain found in archaea and other prokaryotes; it comprises about 200 amino acids (Morrow and Parton 2005; Rivera-Milla et al. 2006). The solution structure of the mouse flotillin-2 SPFH domain was determined by NMR analysis (PDB ID: 1WIN; MMDB ID: 30602) and reveals a compact ellipsoid structure built from four β-strands on one side and four α-helices on the other. The crystal structure of the SPFH domain of an archaeal stomatin is surprisingly similar and both structures can be superposed very well (PDB ID: 3BK6; MMDB ID: 62565). The function of this domain is not clear. Because many SPFH-domain proteins are lipid raft associated, it is possible that this domain mediates raft association (Morrow and Parton 2005). Indeed, the membrane-facing surface of this domain contains hydrophobic patches that would favor interactions with membrane lipids. Moreover, the interaction with cortical actin of one SPFH-domain α-helix (see Fig. 1) has been predicted and identified (Rivera-Milla et al. 2006). The C-terminal "flotillin domain" is characterized by three contiguous blocks of coiled-coil heptad motifs that contain multiple Glu-Ala repeats as well as charged residues at the heptad positions 2, 3, and 5 (Rivera-Milla et al. 2006). This domain is predicted to form dimers, trimers, and oligomers. Using chemical cross-linking, it was determined that flotillins form homo- or hetero-tetramers as building blocks of larger oligomeric complexes (Solis et al. 2007). Moreover, it was shown that flotillin-2 stabilizes flotillin-1. Apparently, flotillin-2 homo-oligomers are relatively stable but flotillin-1 homo-oligomers are not. Downregulation of flotillin-2 causes the concomitant loss of flotillin-1 due to proteasomal degradation but not vice versa (Solis et al. 2007). A conserved PDZ3-binding consensus was



Flotillin-2 (*FLOT2*), Fig. 1 *Schematic model of flotillin*. Schematic model of flotillin structural and functional domains. (**a**) Predicted 3D models of human flotillin-2/reggie-1 SPFH- and flotillin-domains, based on 1win.pdb and Swiss-Model server (swissmodel.expasy.org//SWISS-MODEL.html). α-helices (in *yellow*) are numbered in *black*, with the exception of α-helix 4, which is predicted to interact with actin and is numbered in *red*. β-sheets are drawn and numbered in *blue*, with the exception of human β-sheet 4 (located between β-1 and β-5, not shown). The amino (N) and carboxyl (C) ends are indicated by *green letters* and are truncated. (**b**) For comparison, the ribbon diagram of the human prohibitin-1 SPFH and C-terminal domain is shown, based on 1lu7.pdb. Numbering of α-helices and β-sheets as in A (β-1 to β-3 are not shown). (**c**) Two-dimensional model for the assembly of a reggie/flotillin microdomain, showing the proposed basic interaction between two adjacent flotillin/reggie molecules within a larger hetero-oligomer. Lipid rafts (*blue*) have distinct lipid compositions that differ from non-raft membrane domains (*green*). A hetero-tetrameric cluster of flotillin-2/reggie-1 and flotillin-1/reggie-2, highlighted in *red*, is expected to interact with the inner leaflet of the plasma membrane via its SPFH domain and acylation (*jagged blue* and *red* features). The C-terminal flotillin domain coiled-coil structures are assumed to stabilize the tetrameric complex (Figure and legend taken from Rivera-Milla et al. (2006) by courtesy of Springer Netherlands)

recognized near the C-terminal end and could be involved in regulating oligomerization of both flotillins (Rivera-Milla et al. 2006). A schematic model of the flotillin/reggie structural and functional domains is shown in Fig. 1.

Interactions

Flotillin-2 forms homo-oligomers as well as hetero-oligomers with flotillin-1 by interacting with the C-terminal coiled-coil domains (Neumann-Giesen et al. 2004; Solis et al. 2007). Hetero-oligomerization is essential for flotillin-1 stability, because flotillin-2 depletion induces flotillin-1 degradation by proteasomes (Solis et al. 2007). Hetero-oligomerization is further required for flotillin endocytosis (Frick et al. 2007; Babuke et al. 2009), a process that is regulated by phosphorylation via EGF receptor/▶ Src (Neumann-Giesen et al. 2007) or Fyn kinase (Riento et al. 2009). ▶ Src phosphorylates Tyr-163 in flotillin-2 (Neumann-Giesen et al. 2007), Fyn phosphorylates Tyr-163 in flotillin-2 and Tyr-160 in flotillin-1 (Riento et al. 2009). Both flotillins were found to associate with Niemann-Pick C1-like 1 (NPC1L1) in a complex with clathrin heavy chain (CHC) and AP2 subunit μ2 (Ge et al. 2011). The localization of this complex is cholesterol dependent. Cholesterol depletion causes transport of flotillins and NPC1L1 to the plasma membrane, whereas cholesterol replenishment leads to endocytosis of both. Thus, flotillins play a role in NPC1L1-mediated cellular cholesterol uptake and regulation of lipid levels. Flotillin knockdown dramatically decreased NPC1L1 endocytosis and cholesterol uptake and reduced the association between the CHC-AP2 complex and NPC1L1. Because the knockdown of CHC had no effect on the flotillin-NPC1L1 association, it appears that flotillins are required for recruitment of the CHC-AP2 complex to NPC1L1 and thus act upstream to mediate the internalization of NPC1L1 and cholesterol (Ge et al. 2011). The NPC1L1-binding drug Ezetimibe was found to disrupt the flotillin–NPC1L1 complex. In this study, an important functional role of the flotillins was elucidated, namely, as mediators of cholesterol absorption via flotillin-NPC1L1-generated cholesterol-enriched membrane microdomains (Ge et al. 2011). Flotillins were also found to interact with the tetrameric *N*-methyl-D-aspartate receptor (NMDAR) by two-hybrid screening and co-immunoprecipitation with the subunits NR1, NR2A, and NR2B. The distal C-terminus of NR2B interacted with flotillin-1 and flotillin-2, whereas the C-terminus of NR2A bound only flotillin-1. Both flotillins were found to colocalize with NMDARs in hippocampal neurons (Swanwick et al. 2009). Moreover, both flotillins were shown to interact with the guanine-nucleotide-binding protein Gq subunit alpha (Gαq), independent of the Gαq-nucleotide-binding state (Sugawara et al. 2007). The flotillin N-terminal regions were essential for Gαq binding. Knockdown of flotillins, particularly flotillin-2, attenuated the UTP-induced activation of ▶ p38 mitogen-activated protein kinase (MAPK). Because the activation of MAPK depends on ▶ Src-family kinases and intact lipid rafts, these results suggest that flotillins mediate Gαq-induced MAPK activation through ▶ Src kinases in lipid rafts (Sugawara et al. 2007). In leukocytes, flotillin-2 interacts with P-selectin glycoprotein ligand-1 (PSGL-1), also known as SELPLG or CD162 (Rossy et al. 2009). When neutrophils are activated, flotillins rapidly form caps and later accumulate in the uropods of the polarized cells. While PSGL-1 accumulates in the uropods concomitantly, other uropod components such as ▶ CD43 and ERM proteins accumulate at a slower pace. Flotillins accumulate in uropods even in the absence of PSGL-1, indicating that PSGL-1 accumulation depends on flotillins (Rossy et al. 2009). This study was extended by using neutrophils from the flotillin-1 knockout mouse (Ludwig et al. 2010). They found that flotillin-2 was affected by the loss of flotillin-1, by showing weak, uniform staining of the plasma membrane and absence from detergent-resistant membranes. Importantly, the recruitment of flotillin-1-deficient neutrophils toward the chemoattractant fMLP was strongly reduced. Immunoisolation of tagged flotillin-2 followed by mass spectrometry of co-isolated proteins revealed the association of flotillin-2 with cytoskeletal proteins such as α- and β-spectrin and ▶ myosin IIa (Ludwig et al. 2010). The authors conclude that this association is important for neutrophil migration, uropod formation, and regulation of ▶ myosin IIa. More details on flotillin-2 interactions are described in Salzer and Prohaska (2011).

Cellular and Subcellular Localization

Flotillin-2 is expressed ubiquitously in tissues and diverse cell types. Gene expression analyses by

quantitative PCR of human and mouse (Mouse Genome Informatics MGI:103309) tissues have shown the expression in all tissues. In particular, flotillin-2 is highly expressed in hematopoietic cells (BioGPS GeneAtlas U133A, 201350_at), adipose tissue, spinal cord, and dorsal root ganglia (BioGPS GeneAtlas MOE430, 1417544_a_at). In cells, it is localized to the plasma membrane and internal vesicles such as Golgi vesicles, late endosomes and lysosomes (Neumann-Giesen et al. 2007; Langhorst et al. 2008), as well as centrosomes (Langhorst et al. 2005). At the plasma membrane, flotillin-2 is found in filopodia and lamellipodia, particularly when overexpressed (Neumann-Giesen et al. 2004; Langhorst et al. 2008). Flotillin hetero-oligomers in plasma membrane microdomains are endocytosed (Frick et al. 2007; Neumann-Giesen et al. 2007; Babuke et al. 2009; Riento et al. 2009) and apparently recycle back to the plasma membrane (Langhorst et al. 2008). Endocytosis takes place either in a clathrin- and caveolin-independent way (Riento et al. 2009) or in association with clathrin and NPC1L1 (Ge et al. 2011). Eventually the flotillin hetero-oligomeric complexes are transferred to the late endosomal compartment. In various cell types, flotillins are localized to recycling endosomes, late endosomes, multivesicular bodies, exosomes, lysosomes, phagosomes, and lipid droplets (Langhorst et al. 2005). In hematopoietic cells, flotillins are asymmetrically localized in stable, preassembled platforms of the plasma membrane, to which signaling components are recruited on activation (Rajendran et al. 2003). In chemoattracted neutrophils, flotillin microdomains accumulate rapidly in the uropod (Rajendran et al. 2009; Rossy et al. 2009) due to specific interaction with the cytoskeletal proteins α- and β-spectrin and ▶ myosin II (Ludwig et al. 2010). The role of flotillin microdomains in signaling, cytoskeletal regulation, and endocytosis, was reviewed and discussed recently (Otto and Nichols 2011).

Regulation of Expression and Activity

In fish and mammals, both flotillins are upregulated in retinal ganglion cells during axon regeneration after optic nerve section, reviewed in Langhorst et al. (2005). Flotillin-2 expression is essential for flotillin-1 stability (Solis et al. 2007) and flotillin-1 expression also correlates with flotillin-2 expression (Ludwig et al. 2010). Both flotillins co-assemble at the plasma membrane in roughly equal amounts and act together to generate microdomains (Frick et al. 2007), which are endocytosed after Fyn-dependent tyrosine phosphorylation (Riento et al. 2009). Deletion of flotillin-1 apparently results in destruction of flotillin microdomains (Ludwig et al. 2010). Flotillin-2 was found to be a direct transcriptional target of the ▶ p53 family members p73 and p63 (Sasaki et al. 2008) due to a specific response element in the human *FLOT2* gene. Expression of flotillin-2 was upregulated by p73 or p63 but not by ▶ p53. Moreover, transcription was activated in response to cisplatin but dependent on endogenous p73. siRNA, designed to target p73, silenced endogenous p73 and abolished the induction of flotillin-2 transcription following cisplatin treatment. A p73/p63 binding site located upstream of the *FLOT2* gene was identified and found to be responsive to the ▶ p53 family members. The flotillin-2 knockdown inhibited p63-mediated ▶ STAT3 activation. These data reveal a novel link between the ▶ p53 family and signal transduction via lipid rafts (Sasaki et al. 2008).

Variants and Mutants

There are three flotillin-2 isoforms described in the UniProtKB/Swiss-Prot database, accession number Q60634 (FLOT2_MOUSE). Isoform 1 (Q60634-1) has been chosen as the "canonical" sequence. Isoform 2 (Q60634-2) shows eight amino acid exchanges within the region of residues 50–68, the cause of which is unknown, and isoform 3 (Q60634-3) is missing the N-terminal 49 residues. The formation of isoform 3 may be caused by alternative splicing (skipping of exon 4, which is the first coding exon) and alternative translation initiation at Met-50. In *Drosophila*, flotillin-2 loss of function results in reduced spreading of the morphogens Wnt and Hedgehog (Hh), whereas its overexpression stimulates secretion of Wnt and Hh and expands their diffusion. The resulting changes in the morphogen gradients differently affect the short- and long-range targets. Apparently, flotillin-2 is an important component of the Wnt and Hh secretion pathway dedicated to formation of the mobile pool of these morphogens (Katanaev et al. 2008). In N2a neuroblastoma cells, the expression of a trans-negative flotillin-2 deletion mutant (R1EA), which interferes with flotillin oligomerization, inhibits insulin-like growth factor-induced

neurite outgrowth and impairs in vitro differentiation of primary rat hippocampal neurons (Langhorst et al. 2008). This mutant strongly perturbed Rac1 and Cdc42 activation caused by impaired recruitment of flotillin-associated CAP/ponsin to focal contacts.

Summary

Flotillin-2, also known as reggie-1, is a highly conserved, oligomeric, lipid-raft-associated, integral membrane protein of 48 kDa that is widely expressed in different tissues and cell types. It is associated with the cytosolic side of the plasma membrane and endosomes due to myristoylation, palmitoylation, and hydrophobic interaction. Flotillin-2 is mainly found in a hetero-oligomeric complex with its homologue flotillin-1, also known as reggie-2. Both flotillins and reggies are members of the SPFH (stomatin/prohibitin/flotillin/HflC/K) superfamily, more recently termed PHB (prohibitin homology domain) superfamily. Hetero-oligomerization is required for endocytosis of flotillin membrane microdomains either in a clathrin- and caveolin-independent way, such as Fyn-kinase-regulated endocytosis of GPI-anchored proteins, or in a clathrin-dependent way, such as cholesterol uptake mediated by Niemann-Pick C1-like 1 (NPC1L1). In neurons, flotillins/reggies control axon growth and growth cone elongation by coordinating the assembly of signaling complexes that regulate cortical cytoskeleton remodeling. In neutrophils, flotillin interaction with cytoskeletal components is required for normal migration and uropod formation. Flotillin microdomains thus constitute scaffolding platforms interacting with signaling components, cytoskeleton, and/or membrane proteins in various cellular processes.

References

Babuke T, Ruonala M, Meister M, Amaddii M, Genzler C, Esposito A, Tikkanen R. Hetero-oligomerization of reggie-1/flotillin-2 and reggie-2/flotillin-1 is required for their endocytosis. Cell Signal. 2009;21:1287–97.

Frick M, Bright NA, Riento K, Bray A, Merrified C, Nichols BJ. Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. Curr Biol. 2007;17:1151–6.

Ge L, Qi W, Wang LJ, Miao HH, Qu YX, Li BL, Song BL. Flotillins play an essential role in Niemann-Pick C1-like 1-mediated cholesterol uptake. Proc Natl Acad Sci USA. 2011;108:551–6.

Katanaev VL, Solis GP, Hausmann G, Buestorf S, Katanayeva N, Schrock Y, Stuermer CA, Basler K. Reggie-1/flotillin-2 promotes secretion of the long-range signalling forms of wingless and Hedgehog in *Drosophila*. EMBO J. 2008;27:509–21.

Langhorst MF, Reuter A, Stuermer CA. Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. Cell Mol Life Sci. 2005;62:2228–40.

Langhorst MF, Reuter A, Jaeger FA, Wippich FM, Luxenhofer G, Plattner H, Stuermer CA. Trafficking of the microdomain scaffolding protein reggie-1/flotillin-2. Eur J Cell Biol. 2008;87:211–26.

Ludwig A, Otto GP, Riento K, Hams E, Fallon PG, Nichols BJ. Flotillin microdomains interact with the cortical cytoskeleton to control uropod formation and neutrophil recruitment. J Cell Biol. 2010;191:771–81.

Morrow IC, Parton RG. Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. Traffic. 2005;6:725–40.

Neumann-Giesen C, Falkenbach B, Beicht P, Claasen S, Lüers G, Stuermer CA, Herzog V, Tikkanen R. Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. Biochem J. 2004;378:509–18.

Neumann-Giesen C, Fernow I, Amaddii M, Tikkanen R. Role of EGF-induced tyrosine phosphorylation of reggie-1/flotillin-2 in cell spreading and signaling to the actin cytoskeleton. J Cell Sci. 2007;120:395–406.

Otto GP, Nichols BJ. The roles of flotillin microdomains - endocytosis and beyond. J Cell Sci. 2011;124:3933–40.

Rajendran L, Masilamani M, Solomon S, Tikkanen R, Stuermer CA, Plattner H, Illges H. Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells. Proc Natl Acad Sci USA. 2003;100:8241–6.

Rajendran L, Beckmann J, Magenau A, Boneberg EM, Gaus K, Viola A, Giebel B, Illges H. Flotillins are involved in the polarization of primitive and mature hematopoietic cells. PLoS One. 2009;4:e8290.

Riento K, Frick M, Schafer I, Nichols BJ. Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase. J Cell Sci. 2009;122:912–18.

Rivera-Milla E, Stuermer CA, Málaga-Trillo E. Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. Cell Mol Life Sci. 2006;63:343–57.

Rossy J, Schlicht D, Engelhardt B, Niggli V. Flotillins interact with PSGL-1 in neutrophils and, upon stimulation, rapidly organize into membrane domains subsequently accumulating in the uropod. PLoS One. 2009;4:e5403.

Salzer U, Prohaska R. Flotillin 2. UCSD nature molecule pages. 2011. doi:10.1038/mp.a002226.01

Sasaki Y, Oshima Y, Koyama R, Maruyama R, Akashi H, Mita H, Toyota M, Shinomura Y, Imai K, Tokino T. Identification of flotillin-2, a major protein on lipid rafts, as a novel target of p53 family members. Mol Cancer Res. 2008;6:395–406.

Solis GP, Hoegg M, Munderloh C, Schrock Y, Malaga-Trillo E, Rivera-Milla E, Stuermer CA. Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. Biochem J. 2007;403:313–22.

Sugawara Y, Nishii H, Takahashi T, Yamauchi J, Mizuno N, Tago K, Itoh H. The lipid raft proteins flotillins/reggies

interact with Galphaq and are involved in Gq-mediated p38 mitogen-activated protein kinase activation through tyrosine kinase. Cell Signal. 2007;19:1301–8.
Swanwick CC, Shapiro ME, Yi Z, Chang K, Wenthold RJ. NMDA receptors interact with flotillin-1 and -2, lipid raft-associated proteins. FEBS Lett. 2009;583:1226–30.

FMLPR

▶ Formyl Peptide Receptor

FMLP-Related Receptor II (FMLP-R-II)

▶ FPR2/ALX

FMLPX

▶ FPR2/ALX

Fn14

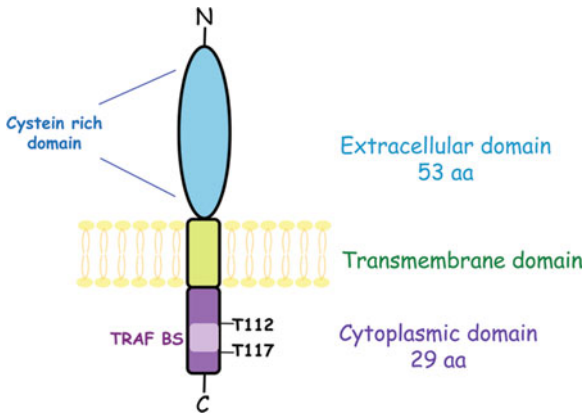
Ana B. Sanz[1], Maria C. Izquierdo[2], Maria D. Sanchez Niño[1], Alvaro C. Ucero[2] and Alberto Ortiz[3]
[1]IdI-Paz, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain
[2]IIS-Fundacion Jimenez Diaz and Universidad Autonoma de Madrid, Madrid, Spain
[3]Unidad de Dialisis, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain

Synonyms

CD266; TNFRSF12A; TWEAK receptor

Historical Background: Discovery and Structure

Numerous cellular processes, such as migration, proliferation, inflammation, and apoptosis are regulated by cytokines belonging to Tumor necrosis factor superfamily (TNFSF). TNFSF cytokines bind members of the TNF receptor superfamily (TNFRSF) (Bossen et al. 2006). TNFSF ligands are type II transmembrane proteins with a homology domain in the extracellular region that assemble as homotrimers. One trimeric ligand usually binds to three monomeric receptors, and this is a key step for signaling transduction. TNFRSF are type I transmembrane proteins characterized by the presence of several extracellular cystein-rich domains that mediate ligand binding. The TNFRSF protein Fn14 is the Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) receptor.



Fn14, Fig. 1 *Structural features of human Fn14*. Fn14 is a 129-aa type I transmembrane protein that is processed by signal peptidase into a mature 102-aa receptor. *TRAF BS* TRAF-binding sites, *T* Threonine phosphorylation sites

Fn14 was described in fibroblasts as Fibroblast Growth Factor–inducible-14, an immediate-early response gene to growth factors (Meighan-Mantha et al. 1999). Two years later, a cDNA clone encoding a TWEAK-binding cell surface molecule was named TWEAK receptor, and shown to have 100% sequence identity with Fn14 (Wiley and Winkles 2003). Phylogenetically Fn14 is much conserved and there is a 90% homology between the mouse and human protein (Wiley and Winkles 2003). The human Fn14 gene is localized at chromosomal position 16p13.3 and encodes a type I transmembrane protein of 129 aa, that is processed into a mature 102-aa protein. The molecular mass of mature Fn14 is 13,637 Da. Fn14 has structural features characteristic of TNFRSF members and is the smallest member of the family (Wiley and Winkles 2003) (Fig. 1). The extracellular domain (53-aa) that contains a cystein-rich domain, characteristic of TNFRSF members, is necessary

for TWEAK binding. The intracellular domain (29-aa) is the shortest of TNFRSF members, contains TNFR-associated factor (TRAF)-binding sites, but lacks a death domain (DD), thus differing from many TNFRSF members (Wiley and Winkles 2003). Fn14 does not contain asparagine or tyrosine residues, but it has two putative threonine phosphorylation sites in the cytoplasmic domain that may play a role in Fn14-TRAF association.

The only ligand for Fn14 is TWEAK (Wiley and Winkles 2003). Human TWEAK is a type II transmembrane glycoprotein of 249-aa. TWEAK contains an extracellular C-terminal domain with the receptor-binding site and an intracellular N-terminal domain with a potential protein kinase C phosphorylation site and several nuclear localization sequences (Chicheportiche et al. 1997). The extracellular domain is proteolytically processed, probably, at one or both of two furin consensus cleavage sites. As a result 156-aa soluble TWEAK (sTWEAK) is generated. Cells can express both full-length membrane-anchored TWEAK (mTWEAK) and sTWEAK.

The TWEAK/Fn14 system can regulates multiple functions with physiopathological relevance, such as, cell proliferation, cell death, cell migration, cell differentiation, tissue regeneration, neoangiogenesis, and inflammation (Winkles 2008). Moreover, TWEAK/Fn14 play a role in injury in different organs, including the central nervous system, liver, gut, the vasculature, skeletal muscle, heart, and kidney.

Fn14 Expression

Fn14 is expressed by many cell types, including epithelial, mesenchymal, and endothelial cells (Burkly et al. 2007). Fn14 was initially described as a fibroblast growth factor (FGF)-inducible immediate-early response gene in murine NIH 3T3 cells (Meighan-Mantha et al. 1999), and later it was observed that Fn14 expression is also quickly upregulated by additional cytokines, growth factors, serum, inflammatory cytokines, hormones, angiotensine II, or phorbol ester treatment in different cell types (Wiley and Winkles 2003; Sanz et al. 2009). Fn14 regulation is stimulus- and cell type-specific. Its own ligand, TWEAK, may upregulate Fn14 expression in glioma cells lines, suggesting the existence of a positive feedback loop that may amplify TWEAK/Fn14 signaling (Winkles 2008).

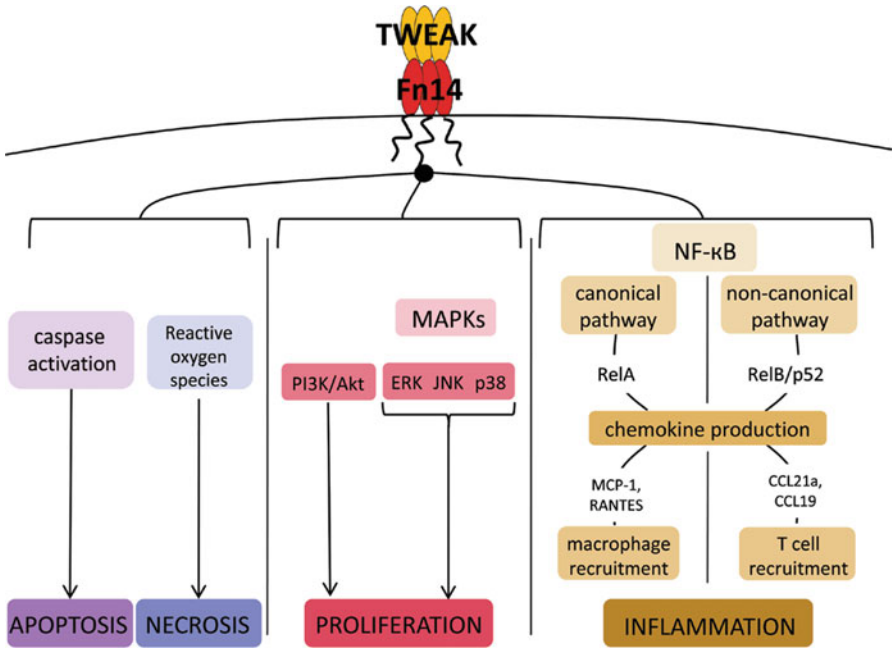
The human Fn14 promoter region contains several potential transcription factor-binding sites, including AP1 sites and an NFκB site, but the molecular mechanisms that regulate Fn14 expression are not well defined. In glioma cells Rac1 mediated basal Fn14 expression and TWEAK-induced Fn14 expression is mediated by Rac1/IKKβ/NFκB pathway (Winkles 2008; Burkly et al. 2007). The Rho/ROCK pathway is implicated in Fn14 upregulation in human aortic smooth muscle cells (hASMCs) and in cardiomyocytes (Ortiz et al. 2009; Chorianopoulos et al. 2010).

At the tissue level, Fn14 is expressed during mouse development and in adult tissue. In adults, murine Fn14 expression was high in heart and ovary, intermediate in kidney, lung, and skin, and low in brain, skeletal muscle, and testis (Meighan-Mantha et al. 1999). Despite this observation, Fn14 expression is relatively low in most resting tissues, but is considerably upregulated in injured tissues. There are numerous experimental models where high Fn14 expression has been observed, such as, chronic liver injury, denervation-induced skeletal muscle atrophy, myocardial infarction, colitis, autoimmune encephalomyelitis, the vasculature after balloon catheter injury, acute kidney injury, and others (Winkles 2008). Fn14 is also upregulated in human tissue injury and inflammation, including adipose tissue in severe obesity, periodontitis, ischemic stroke, atherosclerosis, and cancer (Winkles 2008). Finally, Fn14 is upregulated during tissue regeneration, as observed in experimental kidney or liver growth, following unilateral nephrectomy or partial liver resection, respectively (Sanz et al. 2009).

Fn14 Signaling

Most TNFRSF members contain a DD and signal through the DD, but Fn14 lacks this domain. However, Fn14 contains a TRAF-binding domain in the cytoplasmic tail that can associate with TRAFs 1, 2, 3, and 5 (Wiley and Winkles 2003). TRAF proteins can lead to the activation of cellular signaling pathways, such as MAPKs (ERK, p38, JNK), PI3K/Akt, and transcription factor NFκB (Winkles 2008) (Fig. 2).

Classical NFκB activation mediated by Fn14 required the Fn14 TRAF-binding domain and TRAF



Fn14, Fig. 2 *Fn14-induced intracellular signaling pathways*. Upon activation by the TWEAK cytokine Fn14 activates different signaling cascades. NF-κB activation has been observed in most of cultured cell types where it has been studied. Other pathways may be cell type-dependent

proteins 1, 2, 3, and 5 (Wiley and Winkles 2003). NFκB is a pleiotropic transcription factor that regulates the transcription of hundreds of genes that are involved in inflammation, immunity, apoptosis, cell proliferation, and differentiation. NFκB is composed of homo- and heterodimers of five members of the Rel family: p50 (generated from p105), p52 (generated from p100), RelA (p65), RelB, and c-Rel. The classical NFκB pathway results in nuclear migration of mainly a heterodimer formed by p50 and p65 (RelA) subunits, and the alternative NFκB pathway results in nuclear migration of a complex formed by p52 and RelB subunits (Sanz et al. 2010). Fn14 engagement, in contrast to TNF, activates both the classical and alternative NFκB pathways. Fn14-activated classical NFκB pathway occurs in numerous cell types and is usually an early, transient event that peaks at 1–6 h (Winkles 2008). However, alternative pathway activation does not occur in all cells. In fact, Fn14 activation of the alternative NFκB pathway was observed in MEFs and in renal tubular epithelial cells (Sanz et al. 2010). Alternative NFκB pathway activation by Fn14 is a late event that peaks at 24 h. In some cases, Fn14 stimulation by TWEAK leads to activation of different pathways in the same cellular line, but, the activated pathways may differ between the different cellular types.

Biological Activity

The cell response to Fn14 activation is stimulus- and cell type-specific. The cell response may include inflammation, proliferation, cell death, cell differentiation, and migration. The biological processes activated by Fn14 in each cell type likely depend of the intracellular signaling activated in these cells. Fn14-induced inflammation, cell death, and proliferation are reviewed.

Inflammation

Proinflammatory actions of Fn14 activation have been observed in numerous cell types, both in culture and in vivo. The first evidence of the inflammatory effect of TWEAK was induction of IL-8 secretion in tumor cell lines (Chicheportiche et al. 1997). Then, many studies showed that TWEAK, through Fn14 activation, induces chemokine, cytokine, and matrix metalloproteases production in various cell types, such as, endothelial cells, dermal and gingival fibroblasts, nervous system cells, mesangial and tubular renal cells, and others (Burkly et al. 2007; Ortiz et al. 2009).

The proinflammatory effect of Fn14 is mediated, mainly, by classical NFκB signaling. In this regard, NFκB blockade prevented Fn14-induced

chemokine production in human bronchial epithelial cells, macrophages, and renal tubular epithelial cells (Ortiz et al. 2009). Other intracellular pathways activated by Fn14 also promote inflammation. In this sense, PI3K mediates Fn14-induced ICAM and VCAM production in human gingival fibroblasts, alternative NFκB pathway activation mediates CCL21 and CCL19 production in renal tubular cells (Sanz et al. 2010), and MAPKs are involved in regulating chemokine production by mesangial cells and podocytes (Gao et al. 2009). Fn14 activation also promotes inflammatory in vivo. Intraperitoneal injection of recombinant TWEAK in mice induces cytokine and chemokine production in kidneys leading to inflammatory cells infiltration (Ortiz et al. 2009). Exogenous TWEAK administration in ApoE−/− mice increases chemokine expression as well as macrophage infiltration in atherosclerotic plaques and renal lesions (Munoz-Garcia et al. 2009). Intracerebral injection of TWEAK in wild-type mice induces matrix metalloproteinase-9 expression in the brain (Yepes 2007). The capacity of Fn14 activation to induce inflammation suggests a role in acute tissue inflammatory injury, as well as in autoimmune diseases.

Proliferation

Fn14 activation by TWEAK induces proliferation in different cell types, including endothelial, epithelial, tumor, and progenitor cells (Winkles 2008; Sanz et al. 2009; Tirnitz-Parker et al. 2010; Novoyatleva et al. 2010). Moreover, Fn14 activation can also induce proliferation in vivo. Thus, exogenous TWEAK injection induce proliferation in renal tubular cells (Sanz et al. 2009). TWEAK overexpression promotes proliferation in liver progenitor cells (Burkly et al. 2007; Tirnitz-Parker et al. 2010), and liver progenitor cell numbers are reduced in Fn14 knockout mice with chronic liver injury (Tirnitz-Parker et al. 2010).

Signaling for Fn14-induced proliferation may be cell type-specific. The principal mechanisms implicated include MAPKs, PI3K/Akt pathway and NFκB activation. In some cases, growth factors potentiate Fn14-induced proliferation. For example, renal epithelial cells Fn14 activation by TWEAK in presence of serum show higher rate of proliferation than cells cultured serum-free and a similar response is observed in human endothelial cells co-stimulated with TWEAK and FGF-2 (Winkles 2008; Sanz et al. 2009). Upregulation of Fn14 expression and positive regulation of intracellular pathways implicated in Fn14-induced proliferation, such as MAPKs signaling by growth factors may contribute to this observation. The ability of Fn14 activation to induce cell growth suggests a role in tissue repair after acute injury, as well as a role in pathological hyperplasia, such as cancer.

Cell Death

TWEAK was named for its capacity to induce apoptosis in interferonγ-treated HT29 adenocarcinoma cells (Chicheportiche et al. 1997) and later this effect of TWEAK was observed in different tumor cell lines. As is the case for other biological activities, TWEAK-induced cell death is mediated by Fn14 activation (Burkly et al. 2007). Fn14 activation promotes apoptosis in numerous cell types, including neurons, monocytes, tumor cell lines, resident renal cells, and others (Michaelson and Burkly 2009; Ortiz et al. 2009).

Fn14-induced cell death is relatively weak, characterized by a requiring long incubation periods, relatively high ligand concentrations and co-incubation with other sensitizing agents. As an example, in renal tubular cells Fn14 activation promotes proliferation, not death, in the absence of additional stimuli, but in presence of TNFα and interferonγ Fn14 activation promotes apoptosis (Ortiz et al. 2009; Sanz et al. 2009). Since, Fn14 does not contain a DD, the exact mechanism leading to Fn14-induced cell death is not well-known. Multiple context-dependent mechanisms have been characterized, including caspase-dependent apoptosis, caspase-independent death with features of both apoptosis and necrosis, and cathepsin B-dependent necrosis (Burkly et al. 2007).

The ability of Fn14 to induce cell death has pathophysiological relevance. When the TWEAK/Fn14 axis is targeted, cell death is prevented in different models of tissue injury, such as experimental acute kidney injury or cerebral ischemia (Yepes 2007; Sanz et al. 2009; Hotta et al. 2010).

The capacity of Fn14 to induce cell death in tumor cells could become in a strategy for cancer treatment.

Studies in Experimental Models and in Human Diseases: Fn14 Expression, Function, and TWEAK/Fn14 Targeting

The biological processes mediated by TWEAK/Fn14 described above suggest that this system may have a pathophysiological relevance in various disease conditions in which these processes play a key role. In this regard, functional experimental studies and descriptive human approaches have characterized the role of TWEAK/Fn14 in disease.

Acute Tissue Injury and Tissue Repair

Fn14 is extremely upregulated following acute tissue injury, both in animal experimental models, such as acute kidney injury (AKI), myocardial infarction, middle cerebral artery occlusion and colitis, and in human disease, including ischemic stroke and atherosclerosis.

Most of the pathologies studied present an initial inflammatory process and cell death. In this regard, functional approaches including TWEAK or Fn14 knockout mice and neutralizing anti-TWEAK or anti-Fn14 antibodies support a role for Fn14 in disease. For example, Fn14 or TWEAK blockade during acute kidney injury decrease renal inflammation and renal tubular cell death and improve renal function (Ortiz et al. 2009; Sanz et al. 2010; Hotta et al. 2010) and Fn14-Fc fusion protein or neutralizing anti-TWEAK inhibited features of atherosclerotic plaque progression (Schapira et al. 2009; Munoz-Garcia et al. 2009). Moreover, Fn14 knockout mice present reduced inflammatory responses after skeletal muscle injury (Burkly et al. 2007), and reduced cell death and inflammation after cerebral ischemia (Yepes 2007). All these studies indicate that TWEAK/Fn14 axis could be a therapeutic target in acute tissue injury.

Some of the diseases studied are also characterized by a regeneration phase. Fn14 activation induces proliferation in some progenitor cells and could play a role in tissue regeneration. In addition, inflammation can also contribute to tissue regeneration after an acute injury. First evidence of the contribution of Fn14 to tissue regeneration was suggested by its increased liver expression following partial hepatectomy, and confirmed by the oval cell hyperplasia observed in transgenic mice overexpressing TWEAK in hepatocytes and by the reduced oval cell proliferation in Fn14 knockout mice following partial hepatectomy (Burkly et al. 2007). TWEAK activates Fn14 to induce mitosis in liver progenitor cells in a model of chronic liver injury (Tirnitz-Parker et al. 2010). However, Fn14 role in muscle regeneration is unclear. Fn14 knockout mice exhibited reduced inflammatory response and delayed muscle fiber regeneration compared with wild-type mice in a model of cardiotoxin-induced skeletal muscle injury (Burkly et al. 2007). However, TWEAK knockout mice show enhanced muscle regeneration (Mittal et al. 2010). A potential explanation for the discrepancy is TWEAK-independent Fn14 activation and functions during muscle regeneration.

Autoimmune Disease

TWEAK attenuates the transition from innate to adaptive immunity and there is functional evidence for a role of TWEAK/Fn14 in autoimmune disease tissue injury (Burkly et al. 2007). However, there is little evidence for the participation of Fn14 in the genesis of autoimmunity and its role appears limited to involvement in tissue injury secondary to autoimmunity.

Autoimmune Neurological Disease: Multiple Sclerosis

Fn14 is expressed by astrocytes, and in these cells Fn14 activation results in inflammatory molecule release and proliferation, suggesting a Fn14 role in brain inflammatory diseases (Yepes 2007). Both, TWEAK and Fn14 are upregulated in multiple sclerosis brains compared with control brain samples (Serafini et al. 2008). Severity of experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis was enhanced in soluble TWEAK-overexpressing transgenic mice (Yepes 2007). In addition targeting the TWEAK/Fn14 system with different approaches ameliorated EAE (Yepes 2007). These results indicate that TWEAK/Fn14 may be a therapeutic target in CNS autoimmunity.

Systemic Lupus Erythematosus

Systemic lupus erythematosus is a multisystem autoimmune disease featuring autoantibody production, often with involvement of kidneys as lupus nephritis. Absence of Fn14 or TWEAK blockade decreased the severity of glomerulonephritis, glomerular IgG

deposition, kidney cytokine expression, and macrophage infiltration in a chronic murine model of lupus nephritis (Ortiz et al. 2009). The role of Fn14 expressed by resident kidney cells or by myeloid cells that infiltrate the kidney in lupus nephritis was approached using two groups of chimeric mice. One chimera expressed Fn14 only in kidney resident cells, while bone marrow-derived cells lacked Fn14. In the other chimera Fn14 expression was restricted to bone marrow-derived cells. Fn14 expressed only by kidney resident cells was enough to promote an early inflammatory response and glomerular injury, while Fn14 from bone marrow-derived cells plays a role at a later stage of the disease (Molano et al. 2009). This suggests that resident kidney cell Fn14 contribute to initiation of nephritis while infiltrating macrophage Fn14 is active only after localization of these cells to the kidney and that Fn14 is a therapeutic target in lupus nephritis. Urinary TWEAK (uTWEAK) correlated positively with lupus nephritis activity, indicating local inflammatory activity (Burkly et al. 2007). These data suggest that Fn14 may mediate kidney damage promoting local inflammation and could be a therapeutic target in this disease.

Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune disease characterized by chronic joint inflammation that leads to cartilage and bone destruction. Fn14 activation induces a proinflammatory process in cultured human dermal fibroblasts and synoviocytes (Burkly et al. 2007). Fn14 is expressed on other human joint cell types, such as chondrocytes, and osteoblast, that respond to TWEAK activation of Fn14 by expressing inflammatory mediators. Moreover, in an experimental model, TWEAK blockade reduced various arthritogenic mediators in serum and in joint and prevented arthritis (Burkly et al. 2007). In synovial cell isolated from rheumatoid arthritis patients Fn14 activation-induced proliferation and inflammatory cytokine production (Kamijo et al. 2008). Finally, serum levels of TWEAK were elevated and TWEAK and Fn14 expression is increased in synovial tissue of patients with rheumatoid arthritis (van Kuijk et al. 2010). Based on this evidence, clinical trials are underway for BIIB023 neutralizing anti-TWEAK antibodies in rheumatoid arthritis.

Cancer

There is evidence for a role of the TWEAK/Fn14 system in cancer. Thus, Fn14 is upregulated in numerous tumors (Michaelson and Burkly 2009). Fn14 may have pro-tumorigenic effects, promoting angiogenesis and cell survival, migration, proliferation, and in some tumor cells such as in glioma cells and in hepatocellular carcinoma cells. These data suggest that TWEAK/Fn14 targeting could be beneficial in cancer treatment. However, TWEAK was described as a cytokine that induced cell death in tumor cells. In this sense, Fn14 activation induces apoptosis in various tumor cell lines, such as adenocarcinoma cells, endometrial cancer cells, and human colon carcinoma cells (Michaelson and Burkly 2009; Dionne et al. 2010). Indeed, the lethal effect of Fn14 activation appears to be higher in tumor cells than in normal cells. Thus, Fn14 activation could be a therapeutic strategy for cancer treatment. An elegant approach to solve this conundrum was the development of a monoclonal anti-Fn14 antibody that both activates Fn14 and promotes antibody-mediated T cell cytotoxicity (Culp et al. 2010). This antibody can induce apoptosis in tumor cells sensitive to Fn14 activation-induced death and also in Fn14-expressing tumor cells resistant to Fn14 activation-induced death. This approach was successfully tested in cultured cells and in experimental animals in vivo (Culp et al. 2010). Indeed, phase I clinical trials in patients with solid tumors are underway.

Summary

Fn14 is the receptor for TWEAK, a cytokine of the TNF superfamily. Fn14 and TWEAK are broadly expressed and may regulate cell proliferation, cell death, cell differentiation, and inflammation. The pathways activated by TWEAK/Fn14 signaling include alternative and classic NFκB, MAPKs, and PI3K/Akt pathway. Fn14 lacks the DD domain, characteristic of the TNFR superfamily, but signals through TRAF-binding domains. In vivo, Fn14 expression in various healthy tissues is low, but is significantly upregulated after injury. Functional studies targeting TWEAK and or Fn14 implicate the system in promoting acute and chronic tissue injury, including in the course of autoimmune disease, as well as in cancer biology. Fn14 activation can also contribute to tissue

repair and any therapeutic intervention should consider this possibility. In this regard, while current preclinical data indicate that the TWEAK/Fn14 system may be a therapeutic target in various diseases, clinical trials are underway. A neutralizing anti-TWEAK antibody, BIIB023, is being tested in rheumatoid arthritis and PDL192 anti-Fn14 antibodies in subjects with advanced solid tumors. These results will provide the first data on the safety of targeting TWEAK/Fn14 in humans and will accelerate translational research in human disease.

References

Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, Ambrose C, Tschopp J, Schneider P. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. J Biol Chem. 2006;281:13964–71.

Burkly LC, Michaelson JS, Hahm K, Jakubowski A, Zheng TS. TWEAKing tissue remodeling by a multifunctional cytokine: role of TWEAK/Fn14 pathway in health and disease. Cytokine. 2007;40:1–16.

Chicheportiche Y, Bourdon PR, Xu H, Hsu YM, Scott H, Hession C, Garcia I, Browning JL. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. J Biol Chem. 1997;272:32401–10.

Chorianopoulos E, Heger T, Lutz M, Frank D, Bea F, Katus HA, Frey N. FGF-inducible 14-kDa protein (Fn14) is regulated via the RhoA/ROCK kinase pathway in cardiomyocytes and mediates nuclear factor-kappaB activation by TWEAK. Basic Res Cardiol. 2010;105:301–13.

Culp PA, Choi D, Zhang Y, Yin J, Seto P, Ybarra SE, Su M, Sho M, Steinle R, Wong MH, Evangelista F, Grove J, Cardenas M, James M, Hsi ED, Chao DT, Powers DB, Ramakrishnan V, Dubridge R. Antibodies to TWEAK receptor inhibit human tumor growth through dual mechanisms. Clin Cancer Res. 2010;16:497–508.

Dionne S, Levy E, Levesque D, Seidman EG. PPARgamma ligand 15-deoxy-delta 12,14-prostaglandin J2 sensitizes human colon carcinoma cells to TWEAK-induced apoptosis. Anticancer Res. 2010;30:157–66.

Gao HX, Campbell SR, Burkly LC, Jakubowski A, Jarchum I, Banas B, Saleem MA, Mathieson PW, Berman JW, Michaelson JS, Putterman C. TNF-like weak inducer of apoptosis (TWEAK) induces inflammatory and proliferative effects in human kidney cells. Cytokine. 2009;46:24–35.

Hotta K, Sho M, Yamato I, Shimada K, Harada H, Akahori T, Nakamura S, Konishi N, Yagita H, Nonomura K, Nakajima Y. Direct targeting of fibroblast growth factor-inducible 14 protein protects against renal ischemia reperfusion injury. Kidney Int. 2010;79(2):179–88.

Kamijo S, Nakajima A, Kamata K, Kurosawa H, Yagita H, Okumura K. Involvement of TWEAK/Fn14 interaction in the synovial inflammation of RA. Rheumatology (Oxford). 2008;47:442–50.

Meighan-Mantha RL, Hsu DK, Guo Y, Brown SA, Feng SL, Peifley KA, Alberts GF, Copeland NG, Gilbert DJ, Jenkins NA, Richards CM, Winkles JA. The mitogen-inducible Fn14 gene encodes a type I transmembrane protein that modulates fibroblast adhesion and migration. J Biol Chem. 1999;274:33166–76.

Michaelson JS, Burkly LC. Therapeutic targeting of TWEAK/Fnl4 in cancer: exploiting the intrinsic tumor cell killing capacity of the pathway. Results Probl Cell Differ. 2009;49:145–60.

Mittal A, Bhatnagar S, Kumar A, Lach-Trifilieff E, Wauters S, Li H, Makonchuk DY, Glass DJ. The TWEAK-Fn14 system is a critical regulator of denervation-induced skeletal muscle atrophy in mice. J Cell Biol. 2010;188:833–49.

Molano A, Lakhani P, Aran A, Burkly LC, Michaelson JS, Putterman C. TWEAK stimulation of kidney resident cells in the pathogenesis of graft versus host induced lupus nephritis. Immunol Lett. 2009;125:119–28.

Munoz-Garcia B, Moreno JA, Lopez-Franco O, Sanz AB, Martin-Ventura JL, Blanco J, Jakubowski A, Burkly LC, Ortiz A, Egido J, Blanco-Colio LM. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) enhances vascular and renal damage induced by hyperlipidemic diet in ApoE-knockout mice. Arterioscler Thromb Vasc Biol. 2009;29:2061–8.

Novoyatleva T, Diehl F, van Amerongen MJ, Patra C, Ferrazzi F, Bellazzi R, Engel FB, et al. TWEAK is a positive regulator of cardiomyocyte proliferation. Cardiovasc Res. 2010;85:681–90.

Ortiz A, Sanz AB, Munoz GB, Moreno JA, Sanchez Nino MD, Martin-Ventura JL, Egido J, Blanco-Colio LM. Considering TWEAK as a target for therapy in renal and vascular injury. Cytokine Growth Factor Rev. 2009;20:251–258.

Sanz AB, Sanchez-Nino MD, Izquierdo MC, Jakubowski A, Justo P, Blanco-Colio LM, Ruiz-Ortega M, Egido J, Ortiz A. Tweak induces proliferation in renal tubular epithelium: a role in uninephrectomy induced renal hyperplasia. J Cell Mol Med. 2009;13:3329–42.

Sanz AB, Sanchez-Nino MD, Izquierdo MC, Jakubowski A, Justo P, Blanco-Colio LM, Ruiz-Ortega M, Selgas R, Egido J, Ortiz A. TWEAK activates the non-canonical NFkappaB pathway in murine renal tubular cells: modulation of CCL21. PLoS One. 2010;5:e8955.

Schapira K, Burkly LC, Zheng TS, Wu P, Groeneweg M, Rousch M, Kockx MM, Daemen MJ, Heeneman S. Fn14-Fc fusion protein regulates atherosclerosis in ApoE−/− mice and inhibits macrophage lipid uptake in vitro. Arterioscler Thromb Vasc Biol. 2009;29:2021–7.

Serafini B, Magliozzi R, Rosicarelli B, Reynolds R, Zheng TS, Aloisi F. Expression of TWEAK and its receptor Fn14 in the multiple sclerosis brain: implications for inflammatory tissue injury. J Neuropathol Exp Neurol. 2008;67:1137–48.

Tirnitz-Parker JE, Viebahn CS, Jakubowski A, Klopcic BR, Olynyk JK, Yeoh GC, Knight B. Tumor necrosis factor-like weak inducer of apoptosis is a mitogen for liver progenitor cells. Hepatology. 2010;52:291–302.

van Kuijk AW, Wijbrandts CA, Vinkenoog M, Zheng TS, Reedquist KA, Tak PP. TWEAK and its receptor Fn14 in the synovium of patients with rheumatoid arthritis compared to psoriatic arthritis and its response to tumour necrosis factor blockade. Ann Rheum Dis. 2010;69:301–4.

Wiley SR, Winkles JA. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. Cytokine Growth Factor Rev. 2003;14:241–9.

Winkles JA. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. Nat Rev Drug Discov. 2008;7:411–25.
Yepes M. Tweak and FN14 in central nervous system health and disease. Front Biosci. 2007;12:2772–81.

FNDC1/Fibronectin Type III Domain Containing 1 (AGS8)

► Activators of G-Protein Signaling (AGS)

Follicle Stimulating Hormone Receptor (FSHR)

Masafumi Tetsuka[1], Motozumi Matsui[2] and Takashi Shimizu[3]
[1]Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan
[2]Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan
[3]Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

Synonyms

Follitropin receptor

Historical Background

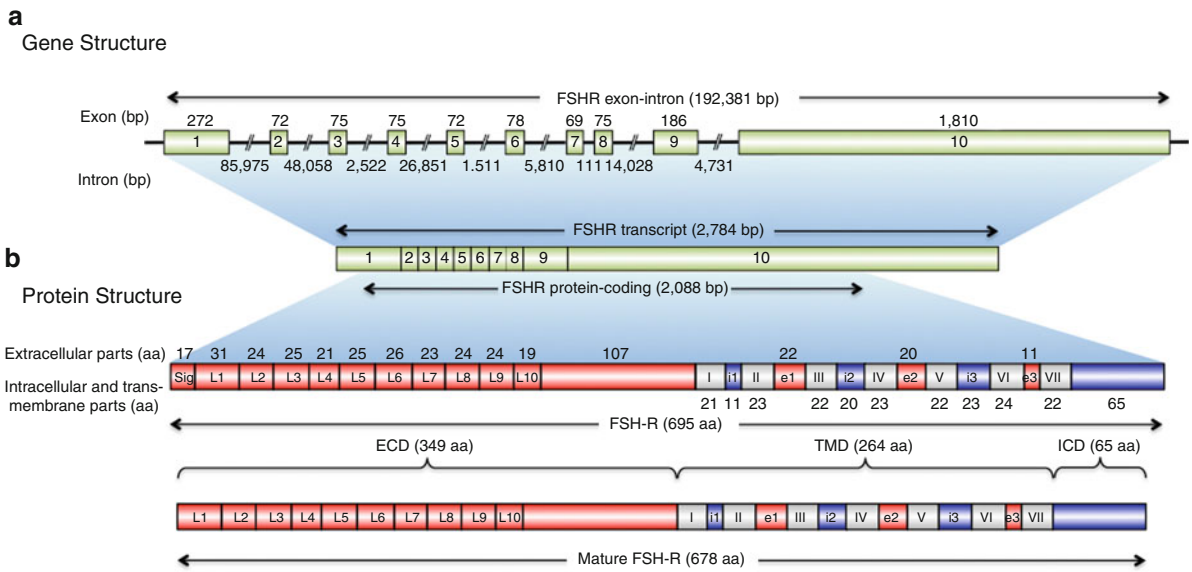
Although the importance of FSH in the reproduction has been well recognized for many years, FSH receptor (FSH-R) had not been identified until 1970s. High affinity, low capacity binding sites for FSH were demonstrated in 1972 using rat testicular tubules incubated with radiolabeled FSH (Simoni et al. 1997). In the following years, FSH-R was identified, isolated and purified in several species and cellular localization, ligand-receptor interaction and intracellular signaling molecules associated with the receptor were studied extensively (Simoni et al. 1997). FSH-R gene (*FSHR*) was cloned in 1990 by screening rat Sertoli cell cDNA library. Since then, *FSHR* has been cloned in several species ranging from human to reptile (Simoni et al. 1997). These findings led to identification of FSH-R mutations that either enhance (activating or gain-of-function mutation) or impair (inactivating or loss-of-function mutation) the function of the receptor (Lussiana et al. 2008). Advent in the molecular biology has made it possible to produce site-directed mutagenesis (Vassart et al. 2004) and *FSHR* knock out mouse, contributing to clarification of functional structure and physiological importance of FSH and FSH-R. The stereochemical structure and activating mechanism of FSH-R have not been fully clarified yet. Further understanding in the structure and the structure–function relationship of FSH-R will allow researchers to develop effective antagonists and agonists that can be used for better management of human and animal reproductive health.

Structures and Function of FSH-R

FSH-R, as for closely related glycoprotein hormone receptors, LH/hCG receptor and TSH receptor, is a member of the rhodopsin-like G protein-coupled receptor (GPCR) family. FSH-R is a glycoprotein that consists of a large N-terminal extracellular domain, a transmembrane domain, and an intracellular domain (Vassart et al. 2004) (Fig. 1b).

The human *FSHR* spans more than 192 kbp and constitutes of ten exons separated by nine introns (Fig. 1a). The exons 1–9 encode most of the extracellular domain whereas the exon ten encodes C-terminal part of the extracellular domain, transmembrane domain, and intracellular domain. Sequence homologies are high among glycoprotein hormone receptors with the homologies around 40% for the extracellular domain and 70% for the transmembrane domain (Vassart et al. 2004), indicating common evolutionary origin of these receptors (Dias et al. 2002). The mature human FSH-R constitutes of 678 aa (695 aa with N-terminal 17 aa signal peptide) with the calculated molecular mass around 78 kDa (UniProt 2010: Fig. 1b).

The extracellular domain of the receptor constitutes of 349 aa and is essential for FSH binding. This domain contains ten loosely related motifs called leucine-rich repeats (LRRs: Ensembl 2010). Together, they form a horseshoe-like structure that provides hydrophilic binding interface specific to FSH (Vassart et al. 2004;



Follicle Stimulating Hormone Receptor (FSHR), Fig. 1 Structural organization of the human FSH-R. (**a**) Gene structure. Human *FSHR* spans more than 192 kbp, constituting of ten exons and nine introns. *FSHR* transcript is 2,784 bp, of which 2,088 bp are translated. (**b**) Protein structure. FSH-R constitutes of 695 amino acids (*aa*), organized into the signal peptide (*Sig*), the extracellular domain (*ECD*), the transmembrane domain (*TMD*), and the intracellular domain (*ICD*). ECD contains ten leucine-rich repeats (LRR: L1-L10). TMD contains seven membrane spanning hydrophobic α helices (T1-7) connected by intra- (*i1-3*) and extracellular loops (*e1-3*). *Red* indicates extracellular potions of FSH-R whereas *blue* indicates intracellular potions of the receptor. Based on the online database (Ensembl 2010; UniProt 2010)

Fan and Hendrickson 2005: Fig. 2). The transmembrane domain constitutes of 264 aa and contains seven membrane spanning hydrophobic α helices connected by intra- and extracellular loops, the structure characteristic to the members of GPCR family (Figs. 1b and 2). The intracellular domain constitutes of 65 aa. Together with the intracellular loops of the transmembrane domain, this domain interacts with G proteins and other signaling molecules involved in signal transduction and receptor internalization (Ulloa-Aguirre et al. 2007: Fig. 2).

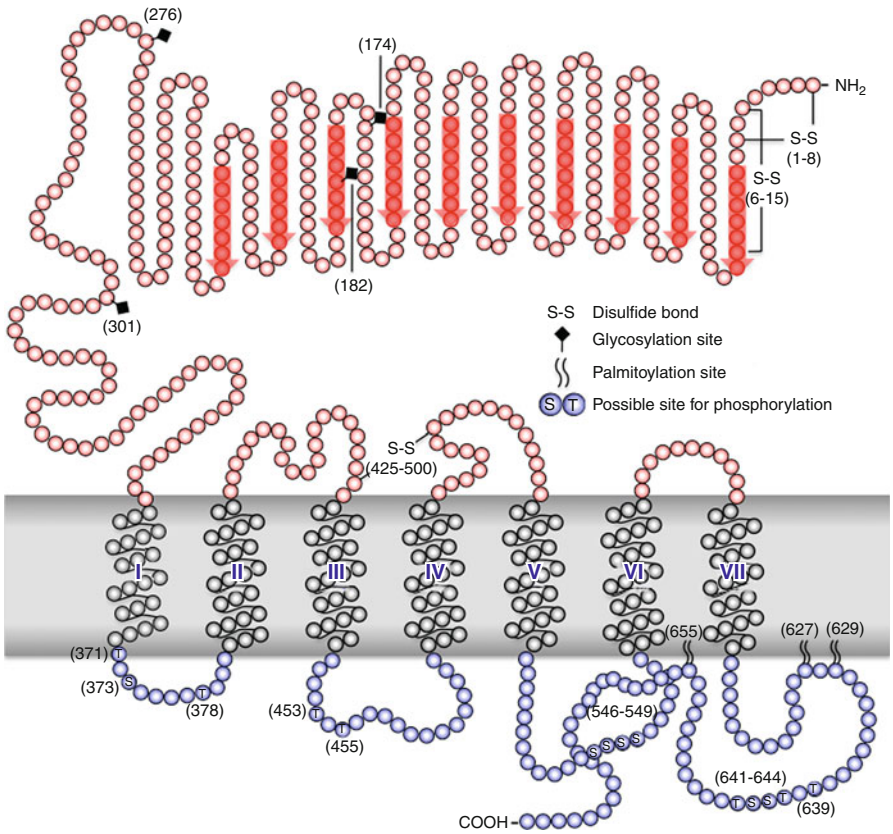
FSH-R is subjected to extensive post-translational modifications such as glycosylation, disulfide formation, palmitoylation and phosphorylation (Fig. 2). These modifications are essential for protein folding, trafficking, cell surface expression, activation, sequestration, and signal transduction of the receptor (Ulloa-Aguirre et al. 2007).

Accumulating evidences indicate that the FSH-R occurs as di/oligomeric units on the cellular membrane (Fan and Hendrickson 2005; Ulloa-Aguirre et al. 2007; Vassart 2010). The transmembrane α helices and the extracellular domain appear to be involved in di/oligomerization of the receptor (Vassart 2010). Although physiological significance of the receptor di/oligomerization is not well understood, it may enable positive cooperativity among receptor molecules (Ulloa-Aguirre et al. 2007).

FSH binding to the extracellular domain causes conformational change of the domain and triggers the FSH-R activation and subsequent signal transduction (Vassart et al. 2004). Although the exact mechanism that activates FSH-R has not been fully elucidated, interaction between the FSH-bound extracellular domain and the transmembrane domain appears to be necessary for full conformational change required for the activation (Vassart et al. 2004; Ulloa-Aguirre et al. 2007). This in turn causes conformational change of the transmembrane and intracellular domain, leading to the activation of associated G proteins and down-stream signaling pathways (see below).

Temporal and Special Expression of FSH-R

Temporal and special expression of FSH-R has been examined in several species including mouse, rat, cattle, and human by using various techniques



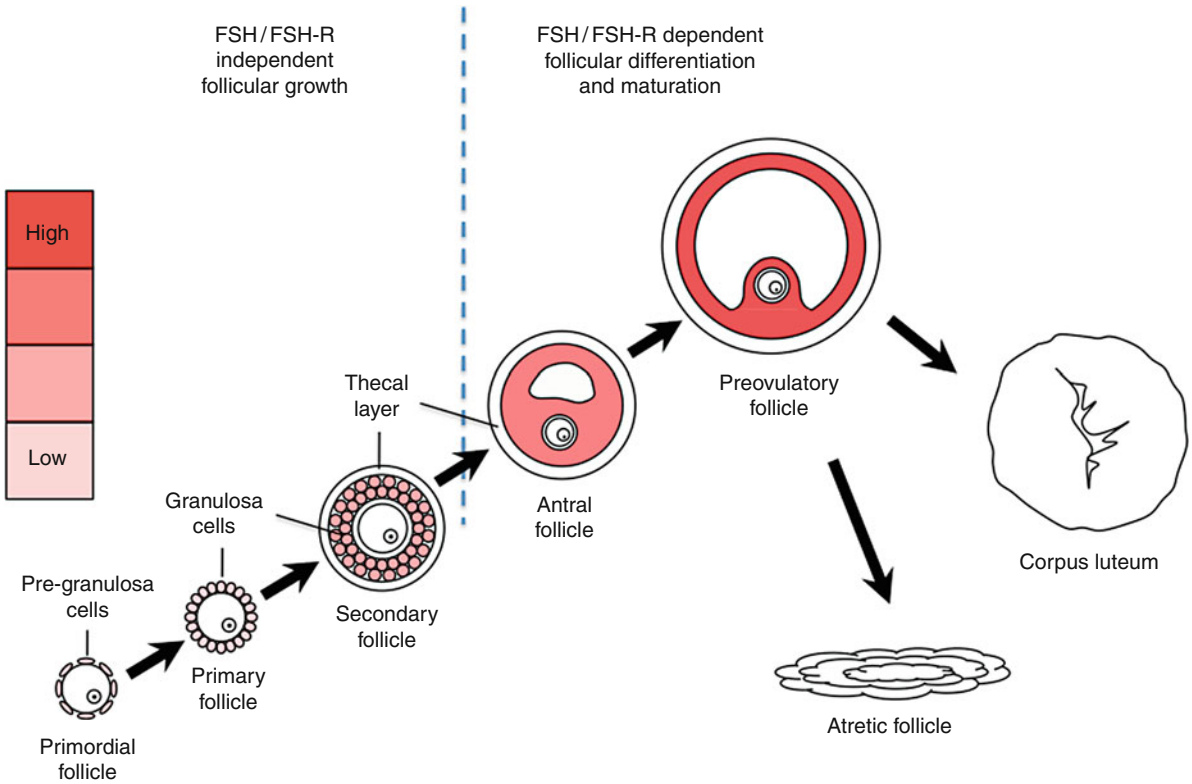
Follicle Stimulating Hormone Receptor (FSHR), Fig. 2 Schematic representation of the human FSH-R with post-translational modifications. ECD and extracellular loops are modified with three disulfied bonds and four glycosylations, whereas ICD and intracellular loops are modified three palmitoylations and several phosphorylations. Ten consecutive LRRs are indicated by *red arrows*. Numbers represent amino acid position in mature FSH-R (678 aa). Modified from Ulloa-Aguirre et al. (2007) using the data obtained from the online database (UniProt 2010)

such as ligand-binding study, in situ hybridization, immunohistochemistry, and quantitative RT-PCR. Irrespective of species examined, FSH-R is localized on the cellular membrane of the ovarian granulosa cells and the testicular Sertoli cells. The expression of FSH-R is developmentally regulated in both cell types (Simoni et al. 1997; O'Shaughnessy et al. 1996; Dias et al. 2002). In female mice and rats, FSH-R mRNA appears to be expressed in the fetal ovary and undifferentiated granulosa cells of primordial follicle. The presence of the functional FSH-R has been confirmed in small preantral follicles of various species and its expression increases as follicular development and maturation progress (O'Shaughnessy et al. 1996: Fig. 3). The expression of FSH-R sharply declines in preovulatory follicles after preovulatory LH-surge and in atretic follicles. Corpus luteum does not express FSH receptor (Simoni et al. 1997; O'Shaughnessy et al. 1996: Fig. 3).

In the rat, FSH-R mRNA is expressed in the fetal testis as early as fetal day 16.5 but it is not clear whether it plays any functional role (Simoni et al. 1997; O'Shaughnessy et al. 1996). In immature rats, number of FSH-R increases until day 7, and stays constant for 2 weeks (Simoni et al. 1997). The increase in FSH-R number coincides with the proliferation of Sertoli cells, indicating the functional role for FSH in this process. The expression of FSH-R decreases dramatically around day 40 when major initiation of spermatogenesis occurs (Simoni et al. 1997).

Spermatogenesis can be classified into several stages, defined by combination of germ cells at different stages of development in the seminiferous tubule. In the rat, where the spermatogenesis is organized into 14 stages, FSH-R is expressed stage dependently, being highest in stages XIII, XIV, and I, and lowest in VII and VIII (Simoni et al. 1997: Fig. 4). FSH responsiveness of the cells, in terms of cAMP production, follows similar pattern but a few stages behind the expression pattern, being highest in stages IV and V (Simoni et al. 1997: Fig. 4).

Ectopic expression of FSH-R has been reported in some tissues such as myometrium, prostate, bone, and ovarian surface epitherium (Simoni et al. 1997;



Follicle Stimulating Hormone Receptor (FSHR), Fig. 3 Expression of FSH-R during folliculogenesis. FSH-R is expressed in small growing follicles despite these follicles do not require FSH. FSH is absolutely necessary for antrum formation and subsequent follicular maturation. FSH-R expression increases as follicular maturation progresses whereas it decreases dramatically in corpora lutea and atretic follicles

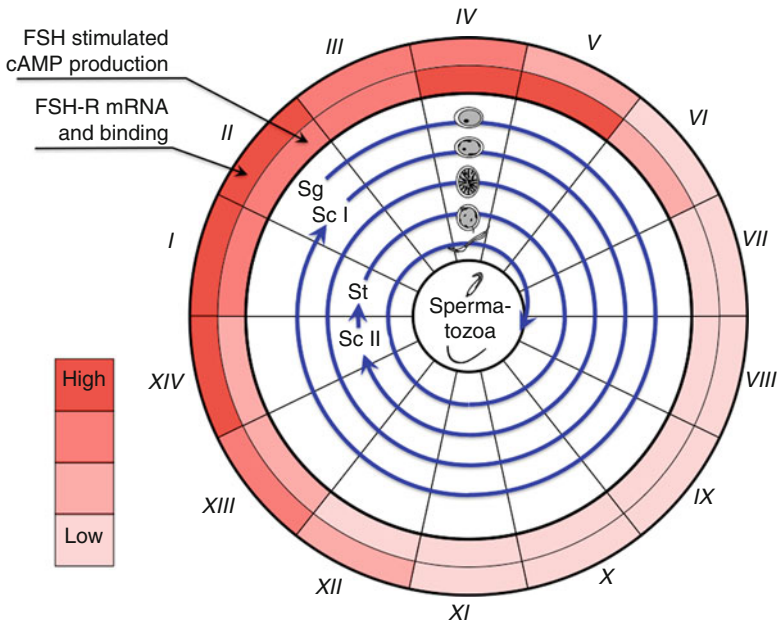
Dias et al. 2002; Ulloa-Aguirre et al. 2007; Bose 2008). Physiological significance of FSH-R in these tissues is not well understood but FSH-R may be involved in the development of osteoporosis (Ulloa-Aguirre et al. 2007) and ovarian cancer (Bose 2008).

Regulation of FSH-R Expression

The sensitivity of the target cells to FSH is determined by the number of functional receptor on the cells, which in turn is regulated by two mechanisms, the level of receptor expression and the rate of receptor turnover.

The expression of FSH-R is regulated by various endocrine, paracrine, and autocrine factors. FSH appears to be a prime hormone that regulates FSH-R expression. Effect of FSH on the receptor expression differs among species and between sexes. In male rats and mice, FSH down-regulates expression of FSH-R in Sertoli cells both *in vivo* and *in vitro* (Simoni et al. 1997). In the female, FSH effect appears to be biphasic; treatment of immature or hypophysectomized rats with eCG or FSH increased FSH-R mRNA and FSH binding, whereas subsequent treatment to induce ovulation with hCG or high dose of FSH down-regulated FSH-R mRNA and FSH binding (Simoni et al. 1997). In cultured rat granulosa cells, FSH prevented FSH-R down-regulation that otherwise occurs in the absence of FSH (Simoni et al. 1997). FSH was also shown to induce a dose-dependent decrease in FSH-R mRNA expression in cultured rat granulose cells (Minegishi 2004). These findings suggest that the expression of FSH-R is auto-regulated by FSH to maintain optimum levels of FSH stimulation.

Number of locally produced factors, either alone or together with FSH, modulate FSH-R expression in granulosa cells (Simoni et al. 1997; Minegishi 2004). Transforming growth factor (TGF)β increased while TGFα decreased basal expression of FSH-R mRNA



Follicle Stimulating Hormone Receptor (FSHR), Fig. 4 Stage specific FSH-R expression in the rat during spermatogenesis. In the rat, spermatogenesis is organized into 14 stages, defined by combination of germ cells at different stages of development in the seminiferous tubule. Germ cells therefore experience 4–5 cycles of the spermatogenic cycle to attain maturity. FSH-R is expressed stage dependently, being highest in stages *XIII*, *XIV*, and *I*, and lowest in *VII* and *VIII*. FSH responsiveness of the cells, in terms of cAMP production, follows similar pattern but a few stages behind the expression pattern, being highest in stages *IV* and *V*. Sg, spermatogonia; *Sc I*, spermatocytes in the first meiotic division; *Sc II*, spermatocytes in the second meiotic division; St, spermatids. Based on Simoni et al. (1997)

and protein in cultured rat granulosa cells. TGFα also dose dependently suppresses the effect of TGFβ. Epidermal growth factor (EGF) and basic ▶ fibroblast growth factor (bFGF) suppressed FSH stimulated FSH-R mRNA expression without affecting basal expression. Retinoic acid similarly suppressed FSH action without affecting basal expression of FSH-R (Minegishi 2004). GnRH likewise antagonized FSH action on the expression of FSH-R. Insulin-like growth factor I, on the other hand, synergistically increased FSH stimulated FSH-R mRNA expression without affecting the basal expression. Activin was shown to increase FSH-R mRNA level through increasing expression rate and mRNA half-life (Minegishi 2004).

The mechanism responsible for the transcriptional regulation of *FSHR* has been extensively studied in the mouse, rat, and human (Simoni et al. 1997; Hermann and Heckert 2007). Transient transfection experiments using sequential deletions of the 5'-flanking region revealed that only short promoter sequence immediately up-stream of the transcriptional start site is required to maintain transcriptional activity. When longer DNA constructs were used, this transcriptional activity was markedly suppressed, indicating the presence of repressor elements at the up-stream of the promoter region (Simoni et al. 1997; Hermann and Heckert 2007). The *FSHR* promoter region equips with a promoter element E-box (CACGTG) but does not equip usual TATA and CCAAT promoter elements. The E-box is bound by the basic helix-loop-helix transcription factors upstream stimulatory factor 1 (Usf1) and Usf2. Usf1 and 2 form homo- or heterodimer and initiate transcription in coordination with other transcription factors that bind proximal promoter elements (Hermann and Heckert 2007).

The expression of FSH-R is also affected by the alternate splicing (O'Shaughnessy et al. 1996). Many forms of *FSHR* splicing variants are reported in various species. The splicing variants are mostly formed by exon removal; in the human, short FSH-R isoform lacking 62 aa is formed by removal of exon 9. Physiological significance of these splicing variants is not clear but they may act as receptors with altered affinity for FSH or with altered response to FSH binding.

In fetal and neonatal mice, short splicing variants of *FSHR* have been reported both in male and female. In the bovine granulose cells undergoing luteinaization, the full length *FSHR* transcript disappears while the transcripts encoding the extracellular domain persist (O'Shaughnessy et al. 1996). These results indicate that the FSH action is also regulated at the post-transcriptional splicing step.

Receptor desensitization occurs through uncoupling of the receptor from the associated G protein followed by sequestration of the receptor from the membrane surface (Dias et al. 2002; Misrahi et al. 1996; Luttrell 2008). The intracellular domain and intracellular loops of the transmembrane domain play important roles in this process (Ulloa-Aguirre et al. 2007). Within seconds of ligand binding, the intracellular parts of the receptor are phosphorylated (see Fig. 2) that triggers an assembly of proteins responsible for receptor endocytosis. Once internalized, the receptor is taken into the endosome where it is sorted either for degradation or for resensitization and returns to the membrane surface (Misrahi et al. 1996; Luttrell 2008).

FSH-R Signaling Pathway

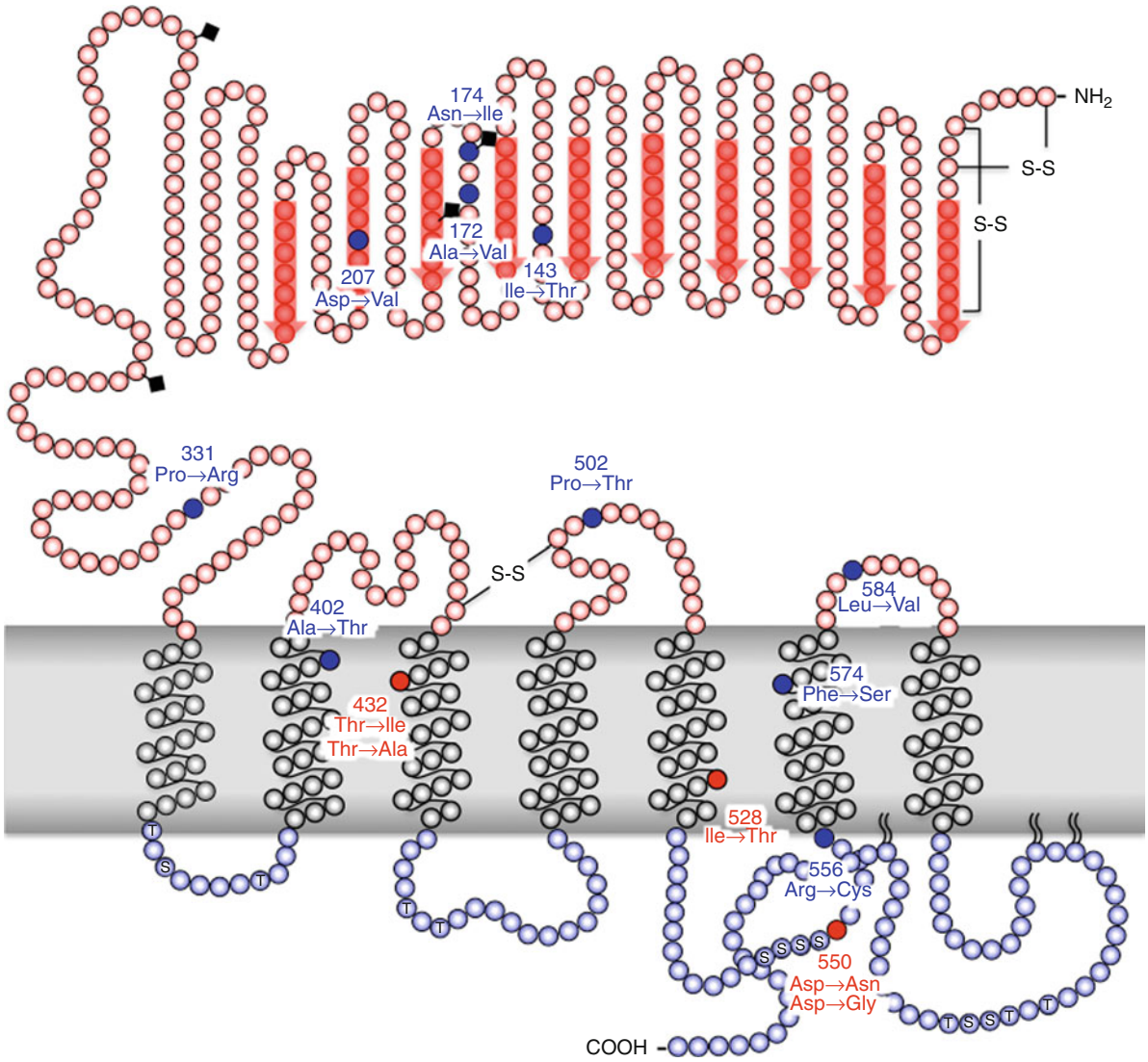
Upon activation, FSH-R transmits the activating signal into the target cells through series of events mediated by various protein kinases, second messengers and transcription factors. The most notable FSH-R signaling pathway is mediated by cAMP dependent protein kinase (PKA). Ligand binding induces a conformational change of the receptor, which activates heterotrimeric Gs proteins coupling with the intracellular domain of the receptor. The activated G proteins dissociate into ▶ G protein α and G protein β/γ heterodimer, and the former activates membrane-bound enzyme ▶ adenylyl cyclase, leading to synthesis of a second messenger cAMP. Cyclic AMP in turn activates PKA, which phosphorylates transcription factors such as cAMP responsive element (CRE) binding proteins (CREBs) and CRE modulators (CREMs), initiating transcription of FSH target genes (Simoni et al. 1997). Many lines of evidences indicate that the FSH-R led signal transduction is far more complicated with multiple signaling pathways interacting each other at various signaling steps. For example, PKA activates not only above mentioned transcription factors but also various ▶ mitogen-activated protein kinase (MAPK) cascades (Ulloa-Aguirre et al. 2007; Zhang et al. 2009). FSH-R also activates multiple kinases through PKA-independent signaling pathways. Some of these signaling pathways are also coupled with other receptor systems such as that of IGF-I and EGF, indicating FSH action is intricately modulated by the interacting factors at various signaling steps (Dias et al. 2002; Ulloa-Aguirre et al. 2007). Moreover, FSH has been shown to increase intracellular Ca2+ through cAMP-dependent and cAMP-independent signaling pathways in granulosa and Sertoli cells (Ulloa-Aguirre et al. 2007). These multiple signaling pathways allow FSH to exert complex, multiple facets effects on cellular functions of the ovarian granulosa and the testicular Sertoli cells.

Abnormality of FSH-R

FSH-R is indispensable for follicular development and maturation; using *FSHR* knockout female mice, it was demonstrated that the follicular development was blocked at the preantral stage and estradiol production was greatly reduced (Kumar 2005).

Unlike in the female, FSH-R is not absolutely necessary for spermatogenesis in the male. *FSHR* knockout caused smaller testis and accessory glands, decrease in testosterone production and reduction in qualitative and quantitative aspects of spermatogenesis, but they were nevertheless fertile (Kumar 2005; Sairam and Krishnamurthy 2001).

Mutagenesis may alter expression and function of FSH-R; mutagenesis in the promoter region may compromise promoter activity (Hermann and Heckert 2007), while that in the coding region may result in substitutions of amino acids that enhance (activating mutations) or impair (inactivating mutations) receptor functions. Amino acid substitutions may alter structural and/or physicochemical nature of the receptor that in turn alter binding affinity or specificity to hormones, or alter conformational change required for FSH-R activation. Reported activating mutations of human occur at the transmembrane domain (Fig. 5). Nevertheless, the mutations increase sensitivity to FSH or other tropic hormones such as hCG and TSH, allowing promiscuous activation of the receptor. Patients with these mutations suffer from spontaneous or iatrogenic ovarian hyperstimulation syndrome. The other type of activating



Follicle Stimulating Hormone Receptor (FSHR), Fig. 5 Schematic representation of amino acid substitutions in human FSH-R. Activating mutations are indicated with *red circles* whereas inactivating mutations with *blue circles*. Numbers represent amino acid position in mature FSH-R (678 aa). Modified from Ulloa-Aguirre et al. (2007) using the data obtained from Lussiana et al. (2008)

mutation causes ligand-independent constitutive activation of the receptor even in the absence of FSH. Inactivating mutations occur at any of the three domains (Fig. 5). These mutations may reduce the receptor response to FSH through altering FSH-R expression on the cell surface, reducing signal transduction efficiency, or impairing FSH binding. Patients carrying these mutations suffer from symptoms such as primary/secondary amenorrhea and precocious ovarian failure (Lussiana et al. 2008).

Summary

FSH-R is a transmembrane glycoprotein that directs FSH signal to the intracellular signaling pathways. FSH-R consists of three functional domains, extracellular, transmembrane, and intracellular domains. The extracellular parts of FSH-R, i.e., the extracellular domain and extracellular loops of the transmembrane domain, are responsible for high affinity specific binding to FSH, whereas the transmembrane and intracellular potions of the

receptor act as a switch to activate the intracellular signaling molecules, such as G proteins.

Ovarian granulosa cells and testicular Sertoli cells are primary cell types that express FSH-R where it plays pivotal roles in female and male reproduction. Expression of FSH-R is developmentally regulated in relation to the stages of folliculogenesis and spermatogenesis by various endocrine/paracrine/autocrine factors, such as FSH and growth factors. Upon activation, FSH-R transmits the signal through signal transduction pathways mediated by PKA and other signaling molecules, and stimulates cellular proliferation and differentiation. Mutations of *FSHR* may disrupt structural organization of the receptor, which result in partial or total loss of fertility. Structure–function relationship of FSH-R has been extensively studied in recent years by using molecular biological and biophysical techniques such as site-directed mutagenesis and crystallization, revealing complex ingenious molecular mechanism that regulates cellular response to FSH. Further understanding in the FSH-R will benefit to develop agonists/antagonists for better management of human and animal reproductive health.

References

Bose CK. Follicle stimulating hormone receptor in ovarian surface epithelium and epithelial ovarian cancer. Oncol. Res. 2008;17:231–8.

Dias JA, Cohen BD, Lindau-Shepard B, Nechamen CA, Peterson AJ, Schmidt A. Molecular, structural, and cellular biology of follitropin and follitropin receptor. Vitam. Horm. 2002;64:249–322.

Ensembl. Transcript: FSHR-001 (ENST00000406846). 2010. http://uswest.ensembl.org/Homo_sapiens/Transcript/Exons?db=core;g=ENSG00000170820;r=2:49189296–49381676;t=ENST00000406846.. Accessed 1 Dec 2010

Fan QR, Hendrickson WA. Structure of human follicle-stimulating hormone in complex with its receptor. Nature. 2005;433:269–77. doi:10.1038/nature03206.

Hermann BP, Heckert LL. Transcriptional regulation of the FSH receptor: new perspectives. Mol. Cell. Endocrinol. 2007;260–262:100–8. doi:10.1016/j.mce.2006.09.005.

Kumar TR. What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? Reproduction. 2005;130:293–302. doi:10.1530/rep. 1.00660.

Lussiana C, Guani B, Mari C, Restagno G, Massobrio M, Revelli A. Mutations and polymorphisms of the FSH receptor (FSHR) gene: clinical implications in female fecundity and molecular biology of FSHR protein and gene. Obstet. Gynecol. Surv. 2008;63:785–95. doi:10.1097/OGX.0b013e31818957eb.

Luttrell LM. Reviews in molecular biology and biotechnology: transmembrane signaling by G protein-coupled receptors. Mol. Biotechnol. 2008;39:239–64. doi:10.1007/s12033-008-9031-1.

Minegishi T. Regulation of gonadotropin receptor in ovary. In: Leung PCK, Adashi EY, editors. The ovary. San Diego: Elsevier Academic Press; 2004. p. 79–92.

Misrahi M, Beau I, Ghinea N, Vannier B, Loosfelt H, Meduri G, et al. The LH/CG and FSH receptors: different molecular forms and intracellular traffic. Mol. Cell. Endocrinol. 1996;125:161–7.

O'Shaughnessy PJ, Dudley K, Rajapaksha WR. Expression of follicle stimulating hormone-receptor mRNA during gonadal development. Mol. Cell. Endocrinol. 1996;125:169–75.

Sairam MR, Krishnamurthy H. The role of follicle-stimulating hormone in spermatogenesis: lessons from knockout animal models. Arch. Med. Res. 2001;32:601–8. doi: S0188-4409(01)00328-9 [pii].

Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr. Rev. 1997;18:739–73.

Ulloa-Aguirre A, Zarinan T, Pasapera AM, Casas-Gonzalez P, Dias JA. Multiple facets of follicle-stimulating hormone receptor function. Endocr. 2007;32:251–63. doi:10.1007/s12020-008-9041-6.

UniProtKB/Swiss-Prot. P23945 (FSHR_HUMAN). 2010. http://www.uniprot.org/uniprot/P23945.. Accessed 1 Dec 2010

Vassart G. An in vivo demonstration of functional G protein-coupled receptor dimers. Proc. Natl Acad. Sci. U.S.A. 2010;107:1819–20. doi:10.1073/pnas.0914432107.

Vassart G, Pardo L, Costagliola S. A molecular dissection of the glycoprotein hormone receptors. Trends Biochem. Sci. 2004;29:119–26. doi:10.1016/j.tibs.2004.01.006.

Zhang M, Ouyang H, Xia G. The signal pathway of gonadotrophins-induced mammalian oocyte meiotic resumption. Mol. Hum. Reprod. 2009;15:399–409. doi:10.1093/molehr/gap031.

Follitropin Receptor

► Follicle Stimulating Hormone Receptor (FSHR)

Formyl Peptide Receptor

Erica L. Southgate and Richard D. Ye
Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL, USA

Synonyms

FMLPR; FPR; NFPR

Historical Background

Formyl peptide receptor 1 (FPR1) was first discovered on human neutrophils through its ability to bind N-formylated peptides with high affinity (Schiffmann et al. 1975). Rabbit neutrophils exhibit similar binding properties. The 350-amino acid human FPR1 receptor was the first cloned leukocyte chemoattractant receptor (Boulay et al. 1990). Genes with homologous sequence (FPR2 and FPR3) were identified through low-stringency hybridization using the FPR1 cDNA. The FPR2 cDNA encodes a 351-residue protein and shares approximately 69% sequence identity with FPR1. FPR2 is a low-affinity receptor for the prototypic formyl peptide, N-formyl-Met-Leu-Phe (fMLF). It binds lipoxin A4 and therefore is termed FPR2/ALX (Ye et al. 2009). FPR3 encodes a 7TM receptor of 352 amino acids that shares 56% sequence identity with FPR1 but does not bind fMLF. Although these three human members of the formyl peptide receptor family are relatively similar in terms of sequence identity and receptor structure, they are quite divergent with respect to agonist selectivity and cellular and tissue distribution, indicating a variety of biological functions (Migeotte et al. 2006; Ye et al. 2009).

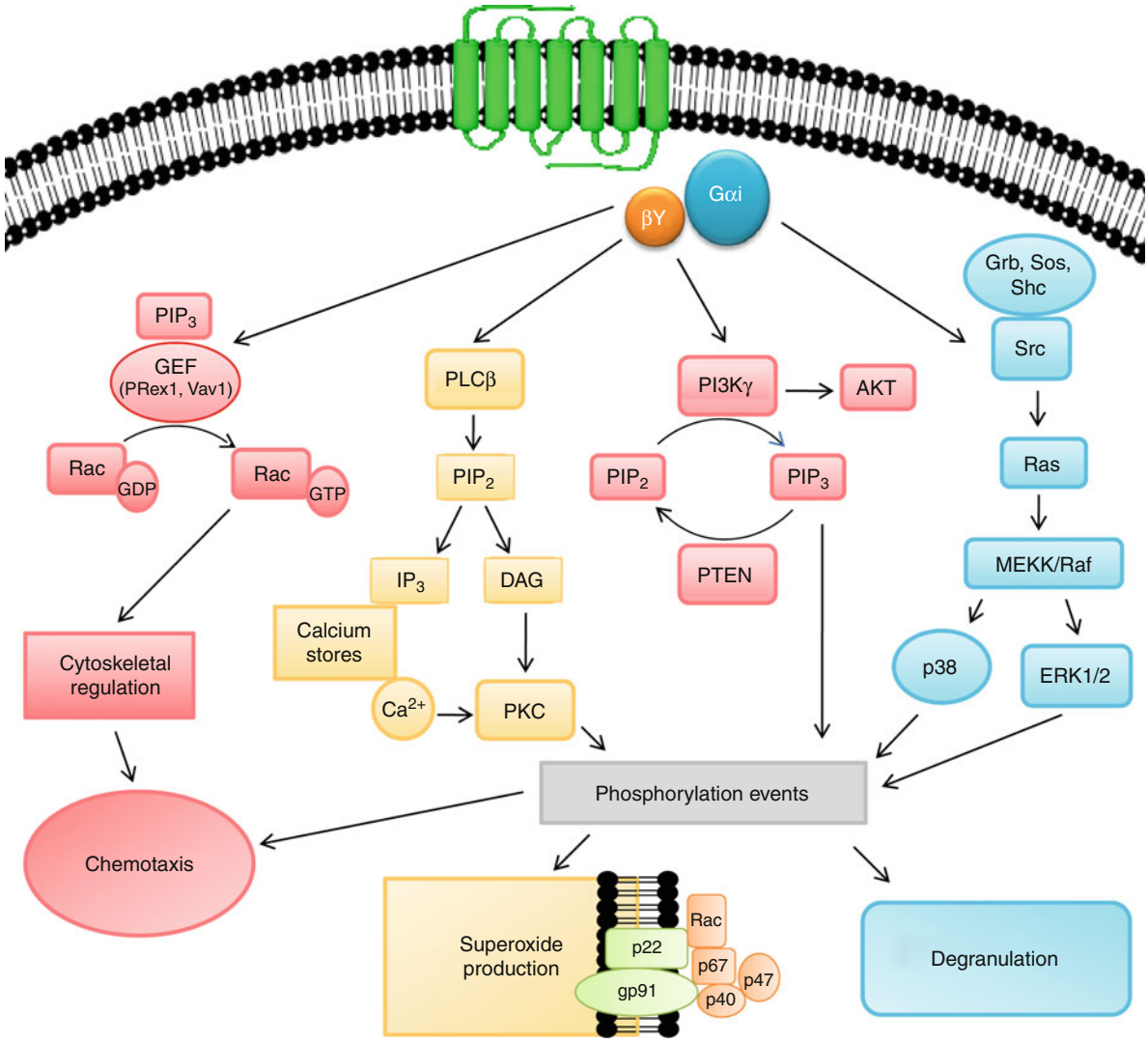
Activation of FPR1 was first described through ligation with N-formylmethionyl peptides (Schiffmann et al. 1975). The identification of the formyl peptide, fMLF, derived from *E. coli*, suggests that a major function of FPR1 is to detect bacterially derived formyl peptides (Marasco et al. 1984). Further discovery of numerous formyl peptides derived from various bacterial sources, such as *L. monocytogenes*, *S. aureus*, and even human mitochondria indicates that the agonists of this family are potent activators of FPR1 signaling (Rabiet et al. 2005). Although formyl peptides appear to be the dominant agonist for FPR1, this is not the case for other members of the FPR family such as FPR2. Even FPR1 has been shown to be activated by a variety of non-formylated microbe-derived peptides and synthetic peptides such as HIV-1 envelope proteins and WKYMVm, respectively (Le et al. 2002). Although a diverse group of agonists activate the FPR family as a whole, N-formyl peptides induce responses that are best characterized in neutrophils. This essay describes FPR1 for its signaling properties in neutrophils. A schematic representation of the major pathways downstream of an activated FPR1 is shown in Fig. 1.

Basic Signaling

Being the first characterized leukocyte chemoattractant receptor, FPR1 is extensively studied for its signaling mechanisms. FPR1 proves to be extremely important because of its ability to facilitate a number of downstream events in neutrophils leading to microbial killing. It has been known that different levels of FPR1 occupancy can lead to signaling pathways that vary between chemotaxis and cytotoxic functions such as degranulation and superoxide production (Korchak et al. 1984). However, the initial phase of receptor activation is similar for every aspect of FPR1 activation after fMLF binds to the receptor. After ligand binding, FPR1 undergoes a conformational change that enables the receptor to interact with the pertussis toxin (PTX) sensitive heterotrimeric G protein of the Gi family. Upon activation, there is an exchange of GDP for GTP in the Gαi subunit which leads to dissociation of the Gα and βγ subunit complex (Bokoch 1995). Following dissociation from the α subunit, the βγ subunit activates phospholipase C β2 ($PLC\beta_2$) and ▶ phosphoinositide 3-kinase γ (PI3Kγ). Activation of $PLC\beta_2$ hydrolyzes phophoinositiol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositiol trisphosphate (IP_3). Both hydrolyzation products are important second messengers, DAG activates protein kinase C (PKC), whereas IP3 binds the IP3 receptor on intracellular stores and mobilizes Ca^{2+}. The activation of PI3Kγ converts the membrane phosphoinositol-4,5-bisphosphate (PIP_2) into phosphoinositiol-3,4,5-trisphosphate (PIP_3). Activation of PKC, as well as numerous calcium-sensitive protein kinases, leads to further downstream signaling, which has been shown to account for a variety of neutrophil functions to aid in microbial killing (Bokoch 1995). For the remainder of this entry, the distinct signaling pathways for FPR1-activated processes such as chemotaxis, superoxide production, and degranulation will be identified separately to avoid confusion, although significant cross talk exists between these pathways.

FPR1-Mediated Phagocyte Functions

Chemotaxis. The directed migration, or chemotaxis, of neutrophils by N-formyl peptides was one of the initially identified functions mediated by FPR1 (Prossnitz and Ye 1997). Human neutrophils can detect a chemotactic gradient of N-formyl peptides which produces a bell-shaped curve in dose–response experiments indicating that past



Formyl Peptide Receptor, Fig. 1 A schematic representation of major signaling pathways downstream of FPR1 activation. Agonist binding to the receptor leads to dissociation of Gα and βγ subunits of the heterotrimeric Gi protein that couple to the receptor. The released βγ subunits activate downstream signaling pathways that involves GEFs for the Rho family small GTPase, PLCβ and PI3Kγ, which are critical to cellular functions including chemotaxis, superoxide production, and degranulation (see text for details). In addition, the α subunit is involved in the regulation of other pathways downstream of the activated receptor

optimal chemoattractant concentrations chemotaxis is reduced (Ye et al. 2009). Two mechanisms have been proposed to contribute to this phenomenon. First, saturation of these receptors with a high concentration of agonists eventually eliminates the chemoattractant gradient, thereby reducing chemotaxis. Secondly, FPR1 is desensitized at high concentrations of agonists, specifically fMLF, therefore reducing responsiveness to additional agonist stimulation (Prossnitz and Ye 1997). A number of events occur to aid in the chemotaxis activity such as polarization of the cell, integrin-mediated adhesion, and physical rearrangements of the cytoskeleton, requiring a variety of different signaling pathways. As previously mentioned, the initiation of chemotaxis through FPR1 activation begins with signaling from Gβγ. The βγ subunit activates PI3Kγ, which increases membrane concentration of PIP3 and helps distinguish leading edge of the chemotactic neutrophil from the

trailing edge, termed uropod. The exposure of neutrophils to a chemoattractant gradient creates an intracellular gradient of signaling molecules such as PIP3. The regulation of PIP3 at the leading edge of neutrophils during chemotaxis is also facilitated by the phosphatidylinositol-3 phosphatase, ▶ PTEN, converting PIP3 back to PIP2. PTEN is localized to the side and the back of a migrating cell and therefore the cell maintains a higher concentration of PIP3 at leading edge, allowing it to migrate. The localization of PTEN to these areas of the neutrophil appears to involve the p38 MAPK, although the direct mechanism of localization is unclear (Heit et al. 2008). The mechanisms that regulate PIP3 localization at the leading edge are important for polarization and forward migration of the cell have been shown to incorporate a number of downstream signaling molecules and an important feedback loop involving some of these molecules. One of these downstream signaling molecules is Akt (PKB). In a number of studies involving the Pleckstrin homology domain of Akt, there was evidence that PIP3 generation by PI3K was crucial for the translocation of Akt to the leading edge of the plasma membrane. Further studies involving knockouts of Akt isoforms, indicating that Akt2 translocation was vital for polarization and chemotaxis of neutrophils after chemoattractant stimulation. Generation of PIP3 has been indicated to be involved in the positive feedback loop involving the Rho GTPases Rac and Cdc42. The activation of Rac has been linked to actin filament formation and subsequent membrane ruffling, therefore inducing the protrusion of the pseudopod and aiding in chemotaxis toward chemoattractants. Additionally, concentration of PIP2 to the uropod from the PTEN conversion of PIP3 to PIP2 mediates the activation of Rho, which regulates stress fiber and focal adhesions and therefore aiding in the uropod movement. In addition to the regulation of Rho by PIP3, there have been other signaling molecules implicated in the complex action of chemotaxis. Gα12 and Gα13 have been shown to be involved in a divergent pathway from the canonical Gαi pathway activated by FPR1, and have been directly linked to activating Rho, a Rho-dependent kinase (p160-ROCK), and myosin II and aiding in the "backness" of the neutrophil (Xu et al. 2003). In addition to the described mechanisms of chemotaxis in neutrophils after FPR1 activation, directed cell movement has also been linked to alternative pathways for its regulation. One of the pathways involves tyrosine kinases such as Src. It is thought that the first dissociation of the βγ subunit which activates PI3K can also increase the activity of Src-like kinases. Further characterization of this pathway was shown to involve the activation of the Shc-Grb2-Sos-Ras pathway, which leads to enhancement of the MAPK pathway involving Raf-MEK-ERK. Although the mechanism of this pathway is biochemically sound, the functional outcome of the activation of this pathway has not been specifically implicated in chemotaxis or other functions of neutrophils (Rabiet et al. 2007).

Degranulation. Degranulation is an important mechanism of host defense against invading microorganisms. However, proper regulation of granule release in neutrophils is vital to evade tissue damage and excess inflammation. Generally four types of granules are distinguished in neutrophils, including primary or azurophilic granules which contain neutrophil myeloperoxidase (MPO) and many hydrolytic enzymes such as β-glucuronidase (Borregaard 1997). There are three peroxidase-negative granules which are classified as secondary (specific) granules, tertiary (gelatinase) granules, and secretory vesicles. The release of these different granules follows a strict kinetic hierarchy in which secretory vesicles containing adhesion molecules and receptors such as the FPR1 receptor are released promptly after activation. Gelatinase granule release follows whereas specific and primary granule releases occur at a much slower rate. Neutrophils can discharge the contents of the granules both into a phagosome containing engulfed particles of bacteria or to the external environment when activated by soluble stimuli such as fMLF (Borregaard 1997). fMLF-induced activation of degranulation downstream of FPR requires 10–50 times higher concentration of the agonist but the cells were committed to full degranulation abilities after only 10 s of receptor-agonist interaction (Korchak et al. 1984). The FPR1-mediated signaling pathway leading to degranulation involves the secondary messengers PLCβ and PI3K. PLCβ leads to the activation of diacylglycerol and release of Ca^{2+} from intracellular stores, both of which have been shown to modulate the activation of PKC. The role of PKC in granule release has been shown to be both Ca^{2+} dependent in the case of primary granules, whereas secretory and specific granule contents are released in a Ca^{2+}-independent manner. Pharmacological characterization of the signaling pathway leading to degranulation has implicated additional kinases, PI3K, p38 MAPK, and ▶ Src-family kinases such as Fgr and Hck (Ye et al. 2009). PI3K, which controls the phosphoinositide

population of PIP2 and PIP3, is involved in granule release by regulating the actin cytoskeleton. Tyrosine kinases such as the above-mentioned Src-family kinases Fgr and Hck are required for adhesion-dependent degranulation and are speculated to initiate actin cytoskeletal rearrangements leading to granule mobilization to the plasma membrane. In addition, the small GTPases Rac and Rho and their respective GDP dissociation inhibitors (GDIs) and guanine nucleotide exchange factors (GEFs) are directly involved in the signaling pathway that leads to granule release, due to their effect on the actin cytoskeleton (Bokoch 1995). Proper docking of the vesicles in neutrophils is one of the final steps of granule release after fMLF activation and requires a set of exocytosis machinery proteins. Activation of the cytoskeletal contractile components is followed by phosphorylation of the soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP) and receptor proteins (SNAREs). Many of the classic, neural-type SNARE proteins such as syntaxin 1, VAMP-1, and SNAP-25 are not detected in granulocytes but homologs such as syntaxin 4 and VAMP-2 have been found in granulocytes. Other homologs have been found in leukocytes, one being SNAP-23 which is the homolog of SNAP-25. At resting state, the Munc-18 family of proteins that serve as binding partners for targeting membrane SNAREs (t-SNAREs) assumes a closed conformation that prevents the SNARE complex from forming. Phosphorylation of these proteins by kinases such as PKC can induce dissociation of Munc18 and syntaxins and therefore facilitates vesicular fusion with the plasma membrane. Specific evidence in a mast cell line, RBL, and human neutrophils indicates that Munc 18–3, SNAP-23, and syntaxins 2, 4, and 6 are phosporylated after fMLF stimulation. Further evidence identified the cGMP-dependent kinase PKG-1 and PI3K are both involved in phosphorylating Munc18-3, SNAP-23, and syntaxins 2 and 4 and the subsequent granule release. Further studies will be necessary to link direct phosphorylation by kinases downstream of fMLF signaling pathway and regulation of the SNARE proteins involved in vesicular fusion for granule release.

Superoxide Generation. Superoxide production is initiated in neutrophils when the oxidase accepts an electron from reduced nicotinamide adenine dinucleotide phosphate (NADPH) and donates it to molecular oxygen, generating O_2^- in the phagosome or to the extracellular environment in cases of soluble stimuli such as fMLF. The O_2^- that is generated can be converted to cytotoxic products such as hydrogen peroxide by superoxide dismutase. The hydrogen peroxide can then be converted to the most potent bactericidal product, hypochlorous acid (HOCl), by the granular enzyme MPO. fMLF activation of superoxide generation in neutrophils requires a number of different proteins, and regulation of these proteins, as well as the coupling of responses to granulation, produces the appropriate amount of microbicidal response without resulting in injury to surrounding tissue. fMLF activation of FPR1 has been shown to require higher concentrations of the agonist than necessary for functions such as chemotaxis and degranulation. The mechanism underlying different concentration requirement of fMLF in activating these functions have not been fully appreciated; however, it is plausible that it helps to prevent tissue injury from migrating neutrophils. It could implicate that additional signaling pathways are required due to different receptor occupancy. Unlike degranulation that showed commitment to full granule release after finite receptor occupancy, O_2^- generation was shown to require continuous occupation of the receptor to initiate and maintain full superoxide response and generation (Korchak et al. 1984; Boxer et al. 1979).

Activation of the signaling pathway to stimulate O_2^- production downstream of FPR1 involves a number of additional proteins that are not necessary for chemotaxis and degranulation. The production of O_2^- is dependent on the translocation and activation of membrane and cytosolic components forming a fully active complex. In the resting state, the membrane components $gp91^{phox}$ and $p22^{phox}$ create the stable membrane complex cytochrome b_{558}. The cytosolic components of the NADPH oxidase, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac are physically separate from the membrane components and require phosphorylation and translocation to the membrane to assemble the functioning complex. $P47^{phox}$ contains an N-terminal phox homology (PX) domain that targets $p47^{phox}$ to the membrane to interact with the membrane component $p22^{phox}$ and PIP2. Phosophorylation of $p47^{phox}$ is required before translocation to change the conformation and expose the PX domain for the membrane. Evidence has been shown for a number of kinases to phosphorylate $p47^{phox}$. Several PKC isoforms, including α, β, δ, and ζ have been implicated in $p47^{phox}$ phosphorylation (Ye et al. 2009). With

regard to fMLF-specific activation of $p47^{phox}$ and subsequent superoxide production, the PKCδ isoform has been implicated and shown to directly phosphorylate the autoinhibitory region of $p47^{phox}$ (Babior et al. 2002). Additional kinases, including AKT and the MAPKs, ERK and p38 have been shown to phosphorylate $p47^{phox}$ such as the SH3 domain and the C-terminal proline-rich domain. The $p67^{phox}$ protein is a larger protein that interacts with almost all proteins involved in the NADPH complex. Its N-terminal tetratricopeptide repeat domains (TPR) interact with activated Rac. Phosphorylation of $p67^{phox}$ in its activation domain enhances binding of the protein to the $gp91^{phox}$ membrane component of the oxidase. Additional interaction with both $p47^{phox}$ and $p40^{phox}$ proteins allows for full activation and regulation of the oxidase system. $P40^{phox}$ has been indicated as a regulatory protein in the NADPH oxidase complex, specifically through its association with 67^{phox} in the resting state. Both basal phosphorylation and subsequent phosphorylation of $p40^{phox}$ by a PKC isoform after stimulation with fMLF have been suggested for its involvement in both regulation and activation. Rac is a cytosolic component of the NADPH oxidase complex essential for activation (Abo et al. 1991). Rac2 binds directly to $p67^{phox}$ at the membrane but does not bind $p40^{phox}$ or $p47^{phox}$. The activation of Rac involves guanine nucleotide exchange factors that exchange the inactive GDP for GTP. A Rac GEF, ▶ P-Rex1, is activated by PIP3 and Gβγ downstream of FPR1 stimulation, and therefore generates active GTP bound Rac. Additional GEFs, such as Vav1, have also been shown to play a role in fMLF-induced activation of NADPH oxidase (Welch et al. 2002).

Regulation of FPR1

Activation of FPR1 is tightly regulated through mechanisms including phosphorylation, desensitization, and internalization, which are necessary for the regulation of the levels of G protein activation and subsequent expansion of the signal through secondary messengers. The first step in FPR1 desensitization is the rapid phosphorylation of the C-terminal tail which is both time- and agonist-concentration dependent. Phosphorylation of FPR1 is PKC independent unlike other GPCR chemoattractant receptors such as the C5a receptor. The C-terminal tail of FPR1 contains a total of 11 serine and threonine residues, which are arranged in two domains characteristic of G protein receptor kinase (GRK) phosphorylation sites. GRK2 has been shown as a primary kinase in phosphorylating FPR1, whereas other GRKSs have minimal (GRK3) or no effect (GRK5,6) on the receptor (Prossnitz 1997). The phosphorylation of the clusters of serine and threonine residues modulates the affinity of the receptor to both β-arrestins and agonists. The affinity of FPR1 for β-arrestins is controlled by the phosphorylation level on the C-terminal tail of the receptor, allowing β-arrestin to form a high affinity complex and sterically inhibit Gαi protein coupling. Desensitization of FPR1 also uncouples it from G proteins independently of β-arrestin, indicating that phosphorylation of the receptor alone is sufficient in uncoupling the receptor from G protein–mediated signaling cascade. In addition to uncoupling receptors from their respective G proteins, β-arrestins have also been shown to colocalize with FPR1 during internalization of the receptor through an endocytic pathway. There are a number of reviews that address desensitization and internalization of GPCRs with details of the involvement of GRKs and β-arrestins (Ye et al. 2009; Prossnitz and Ye 1997; Rabiet et al. 2007; Migeotte et al. 2006).

Diversity of Formyl Peptide Receptor Family Ligand Binding and Downstream Effects

Although this review focuses on the downstream signaling and functional response of FPR1 activation after fMLF stimulation, it is important to note that different members of the family, such as FPR2, can mediate response through an entirely separate system or pathway. One notable difference is in the primary response, calcium mobilization, after receptor activation. The cyclic adenosine 5′-diphosphate ribose (cADPR) is a known regulator of calcium signaling. It is synthesized by CD38 ADP ribosyl cyclase and regulates intracellular calcium release from the ryanodine receptor–gated stores (Migeotte et al. 2006). Human FPR2 has been shown to be dependent on the presence of cyclic ADP-ribose for intracellular calcium release, whereas FPR1 calcium release is independent of this specific calcium metabolite. There is some evidence that FPR2 can couple to the Gq family of G proteins, which could lead to differential downstream effects for the calcium signaling. In addition to calcium mobilization, there is evidence for different endocytic machinery involved in FPR1 and FPR2 signaling. The classical GPCR internalization pathway involving β-arrestin, dynamin, and clathrin, is absent in FPR1 internalization. However, FPR2 undergoes

clathrin-mediated and dynamin-dependent endocytosis and has also been shown to require at least one β-arrestin for internalization. Another interesting phenomenon is the diversity in ligand binding between FPR1 and FPR2. These two receptors share 68% sequence identity, yet they bind the same agonists, fMLF, with drastically different affinity (Rabiet et al. 2005). It is surprising that FPR2 can bind a wider range and more structural diverse group of agonists than FPR1, including the eicosanoid lipoxin A4. These agonists can induce similar signaling pathways as described above; however, many can also trigger divergent signaling pathways with different functional outcomes (Ye et al. 2009).

Summary

FPR1 is a "classic" chemoattractant receptor characterized with its ability to mediate strong neutrophil activation of a full set of bactericidal functions, and functionally couple to the Gαi family of G proteins. FPR1 is one of the first cloned and well-characterized leukocyte chemoattractant receptors, and is often used as a model for studies of G protein–mediated signaling pathways in leukocytes. Although a tremendous amount of work has been completed on the formyl peptide receptor family, many questions still remain. These include the diverse ligand-binding properties of the FPR family receptors, the ability to mediate both pro-inflammatory and anti-inflammatory activities, and the physiological functions of these receptors when bound to bacterial formyl peptides compared to endogenous ligands. Studies have begun in recent years for the characterization of mouse FPR family receptors, which may result in genetic models for a better understanding of the signaling properties of this family of G protein–coupled chemoattractant receptors.

References

Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. Nature. 1991;353(6345):668–70.

Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. Arch Biochem Biophys. 2002;397(2):342–4.

Bokoch GM. Chemoattractant signaling and leukocyte activation. Blood. 1995;86(5):1649–60.

Borregaard N. Development of neutrophil granule diversity. Ann NY Acad Sci. 1997;832:62–8.

Boulay F, Tardif M, Brouchon L, Vignais P. Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. Biochem Biophys Res Commun. 1990;168:1103–9.

Boxer LA, Yoder M, Bonsib S, Schmidt M, Ho P, Jersild R, et al. Effects of a chemotactic factor, N-formylmethionyl peptide, on adherence, superoxide anion generation, phagocytosis, and microtubule assembly of human polymorphonuclear leukocytes. J Lab Clin Med. 1979;93(3):506–14.

Heit B, Robbins SM, Downey CM, Guan Z, Colarusso P, Miller BJ, et al. PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils. Nat Immunol. 2008;9(7):743–52.

Korchak HM, Wilkenfeld C, Rich AM, Radin AR, Vienne K, Rutherford LE. Stimulus response coupling in the human neutrophil. Differential requirements for receptor occupancy in neutrophil responses to a chemoattractant. J Biol Chem. 1984;259(12):7439–45.

Le Y, Murphy PM, Wang JM. Formyl-peptide receptors revisited. Trends Immunol. 2002;23(11):541–8.

Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, et al. Purification and identification of formyl-methionyl-leucyl- phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. J Biol Chem. 1984;259:5430–9.

Migeotte I, Communi D, Parmentier M. Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. Cytokine Growth Factor Rev. 2006;17(6):501–19.

Prossnitz ER. Desensitization of N-formylpeptide receptor-mediated activation is dependent upon receptor phosphorylation. J Biol Chem. 1997;272(24):15213–9.

Prossnitz ER, Ye RD. The N-formyl peptide receptor: a model for the study of chemoattractant receptor structure and function. Pharmacol Ther. 1997;74(1):73–102.

Rabiet MJ, Huet E, Boulay F. Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. Eur J Immunol. 2005;35(8):2486–95.

Rabiet MJ, Huet E, Boulay F. The N-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. Biochimie. 2007;89(9):1089–106.

Schiffmann E, Corcoran BA, Wahl SM. N-formylmethionyl peptides as chemoattractants for leucocytes. Proc Natl Acad Sci USA. 1975;72:1059–62.

Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, et al. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell. 2002;108(6):809–21.

Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, et al. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell. 2003;114(2):201–14.

Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, et al. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol Rev. 2009;61(2):119–61.

FoxO1

Dae Hyun Kim, Steven Ringquist and H. Henry Dong
Division of Immunogenetics, Department of Pediatrics, Rangos Research Center, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Synonyms

FKHR (forkhead in rhabdomyosarcoma)

Historical Background

FoxO1 (NCBI Accession:NM002015) belongs to the forkhead box O family that is characterized by a highly conserved DNA binding motif, known as forkhead box or winged helix domain (Accili and Arden 2004). FoxO1 is initially described by Galili et al. (1993) as the product of the t(2;13)(q35;q14) translocation associated with alveolar rhabdomyosarcoma, a cancer of connective tissue that usually develops in children. FoxO1 is also known as forkhead in rhabdomyosarcoma (FKHR). Subsequent investigation characterizes FoxO1 as a key nuclear factor that mediates the inhibitory effect of insulin or insulin-like growth factor 1 (IGF-1) on the expression of genes, whose functions are instrumental for cell growth, differentiation, and metabolism in mammals (Accili and Arden 2004). FoxO1 orthologues dFoxO in Drosophila melanogaster and DAF16 in *C. elegans* contribute to the regulation of longevity (Hwangbo et al. 2004; Lee et al. 2003).

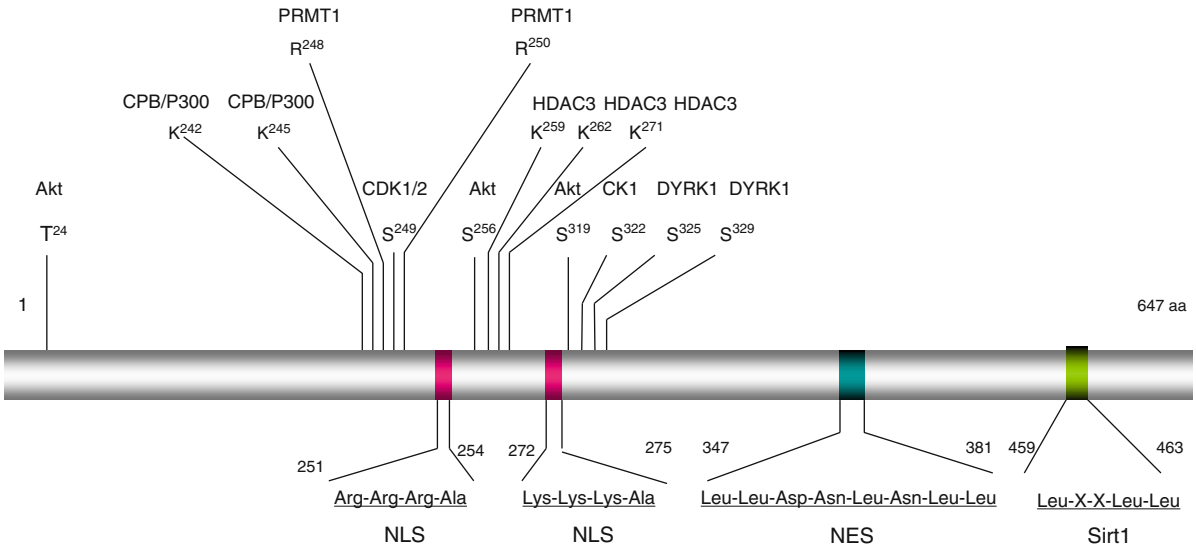
FoxO1 and Insulin Action

FoxO1 is a target of protein kinase B (PKB, also known as Akt) and serum/glucocorticoid-induced kinase (SGK), whose activities are tightly controlled by insulin or IGF-1. Insulin or IGF-1 exerts its inhibitory effect on gene expression via a highly conserved sequence (TG/ATTTT/G), termed insulin response element (IRE) in target promoters (Accili and Arden 2004). In the absence of insulin, FoxO1 resides in the nucleus and binds as a trans-activator to the IRE DNA motif, enhancing promoter activity. In response to insulin, FoxO1 is phosphorylated at three highly conserved phosphorylation sites (Thr24, Ser256, and Ser319) via the PI3K-dependent pathway, resulting in its nuclear exclusion and inhibition of target gene expression (Accili and Arden 2004) (Fig. 1). Three additional phosphorylation sites (S322, S325, and S329) are identified and their phosphorylation by CK1 (casein kinase 1) and DYRK1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) seems to accelerate FoxO1 nuclear exclusion (Accili and Arden 2004). The three mammalian homologues of FoxO1, namely, FoxO3, FoxO4, and FoxO6, play redundant and complementary functions in cell survival, differentiation, and metabolism. Except for FoxO6, all members of the FoxO family undergo insulin-dependent phosphorylation and nuclear exclusion. Failure in phosphorylation of FoxO1 results in its permanent nuclear localization and constitutive transactivation of gene expression (Accili and Arden 2004).

Insulin (or IGF-1) inhibition of FoxO1 activity can occur without necessarily altering FoxO1 subcellular redistribution, but this inhibition depends on the ability of FoxO1 to undergo insulin-dependent phosphorylation (Accili and Arden 2004). Phosphorylation distorts FoxO1 DNA binding domain, preventing its cognate binding to target promoters. Consistent with this notion is the presence of both Thr24 and Ser256 phosphorylation sites in its DNA binding domain (Accili and Arden 2004). However, phosphorylation of *C. elegans* ortholog DAF-16 does not affect its DNA binding activity (Accili and Arden 2004). Instead, DAF-16 phosphorylation promotes its association with the multifunctional factor 14-3-3, perturbing DAF-16 binding to target DNA. Likewise, FoxO1 is capable of interacting with 14-3-3 via two consensus sites for 14-3-3 binding (Thr24 and Ser253) (Accili and Arden 2004). A prevailing notion is that 14-3-3 in complex with FoxO1 masks its DNA binding motif and disables its binding to target promoters. In the cytoplasm, 14-3-3 serves as an anchor to sequester FoxO1, precluding its reentry into the nucleus (Accili and Arden 2004).

FoxO1 and Glucose Metabolism

FoxO1 plays a pivotal role in mediating insulin action on gluconeogenesis, a metabolic pathway that



FoxO1, Fig. 1 Schematic depiction of FoxO1 protein. FoxO1 comprises two structural domains, the amino DNA binding and carboxyl trans-activation domains. Localized in the DNA binding domain are two highly conserved basic nuclear localization signals (*NLS*). The first NLS (^{251}Arg–Arg–Arg–Ala^{254}) has been shown to mediate FoxO1 nuclear import, but the function of the second NLS (^{272}Lys–Lys–Lys–Ala^{275}) remains to be determined. The predicted leucine-rich nuclear export signal (*NES*) is located within 374–381 amino acid residues. This region is thought to be associated with nuclear export factors Ran-GTP and Crm-1, promoting FoxO1 nuclear export. A conserved Sirt1 binding site (^{459}Leu-X–X-Leu–Leu^{463}) is located in the carboxyl domain. FoxO1 has six well-characterized phosphorylation sites, three of which (T24, S256, and S319) are phosphorylated in the PI3K-dependent pathway by Akt/PKB and SGK, two of which (S322 and S325) are phosphorylated by casein kinase 1 (CK1) and one site (S329) is phosphorylated by the dual-specificity tyrosine-phosphorylated and regulated kinase (*DYRK*). The acetylation and deacetylation sites are marked along their respective enzymes (CPB/P300 and HDAC3). In addition, FoxO1 contains two sites (Arg248 and Arg250) that are methylated by PRMT1

takes place mainly in liver for the generation of glucose from non-carbohydrate substrates. Gluconeogenesis is controlled by phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). PEPCK catalyzes the conversion of oxaloacetate into phosphoenolpyruvate, the rate-limiting step of gluconeogenesis. G6Pase carries out the final step of gluconeogenesis by converting glucose-6-phosphate to glucose. FoxO1 stimulates PEPCK and G6Pase expression via selective binding to the IRE motif in the PEPCK and G6Pase promoters, promoting gluconeogenesis. This effect is enhanced by glucagon and inhibited by insulin (Accili and Arden 2004). FoxO1 gain-of-function is associated with augmented hepatic gluconeogenesis, contributing to fasting hyperglycemia in mice (Matsumoto et al. 2007; Qu et al. 2006). FoxO1 loss-of-function is associated with diminished hepatic gluconeogenesis, resulting in reduced fasting blood glucose levels in mice (Altomonte et al. 2003).

FoxO1-dependent regulation of gluconeogenesis provides an acute mechanism for the liver to adjust the rate of glucose production. In response to postprandial insulin release, hepatic gluconeogenic activity is suppressed to limit glucose production and prevent excessive postprandial blood glucose excursion. In response to fasting, hepatic gluconeogenesis is stimulated, resulting in increased glucose output from liver (Accili and Arden 2004). Such a reciprocal mechanism of hepatic insulin action is crucial for rapid adaptation of liver to metabolic shift between fed and fasting states to maintain blood glucose levels within the physiological range. Consistent with its action in gluconeogenesis, FoxO1 activity in liver is enhanced in response to fasting and is inhibited in fed states (Qu et al. 2006).

FoxO1 and Lipid Metabolism

The role of FoxO1 in lipid metabolism culminates in its ability to mediate insulin action on hepatic expression of microsomal triglyceride transfer protein (MTP)

and apolipoprotein C-III (ApoC-III), two rate-limiting steps in triglyceride metabolism. MTP (MW, 88 kDa) is a molecular chaperone that is responsible for transporting lipid droplets to nascent apolipoprotein B (apoB) for very low-density lipoprotein (VLDL) production in liver (Kamagate et al. 2008). ApoC-III functions as an inhibitor of lipoprotein lipase (LPL) and hepatic lipase (HL), key enzymes in the hydrolysis of triglyceride in VLDL and chylomicrons (Altomonte et al. 2004). MTP deficiency is associated with abnormal production of triglyceride-rich particles, resulting in abetalipoproteinemia or Bassen-Kornzweig syndrome, a rare autosomal recessive disorder in humans (Berriot-Varoqueaux et al. 2000). Elevated ApoC-III levels are associated with impaired clearance of TG-rich particles, leading to the accumulation of TG-rich lipoprotein remnants in plasma (Altomonte et al. 2004). FoxO1 is shown to mediate insulin-dependent regulation of both MTP and ApoC-III production in liver (Altomonte et al. 2004). This effect coincides with the ability of FoxO1 to bind to the IRE motif within both MTP and ApoC-III promoters (Altomonte et al. 2004).

These findings provide important mechanistic insights into the pathophysiology of hypertriglyceridemia, the most common lipid disorder that is characterized by increased production of very low-density lipoprotein (VLDL) and/or decreased clearance of TG-rich particles (VLDL and chylomicrons). Unbridled FoxO1 activity, resulting from insulin resistance, promotes hepatic MTP and ApoC-III overproduction, contributing to increased secretion of VLDL from the liver and decreased clearance of triglyceride-rich particles in plasma. These data have spurred the idea of targeting FoxO1 for improving triglyceride metabolism and ameliorating hypertriglyceridemia in insulin resistant subjects with diabetic dyslipidemia.

FoxO1 and Nonalcoholic Fatty Liver Disease

Qu et al. (2006) show that elevated FoxO1 production in liver is associated with increased intrahepatic fat infiltration. Similar findings are reported by Valenti et al. (2008), who detect an enhanced FoxO1 activity along with increased fat deposition in the liver of patients with nonalcoholic fatty liver disease (NAFLD). To dissect the underlying mechanism, Zhang et al. (2006) show that hepatic lipogenic genes including SREBP-1c, FAS, and ACC are upregulated in transgenic mice expressing a constitutively active FoxO1 allele. These findings are reproduced by two independent studies showing that adenovirus-mediated FoxO1 production results in augmented lipogenesis with concomitant fat accumulation in the liver (Qu et al. 2006; Matsumoto et al. 2006). It appears that unchecked FoxO1 activity contributes to the upregulation of lipogenic gene expression, accounting in part for the development of NAFLD under insulin resistant conditions.

To understand the underlying mechanism, two independent studies show that FoxO1 gain-of-function, resulting from adenovirus-mediated FoxO1 production or transgenic overexpression of its constitutively active allele, is associated with impaired insulin action in liver, accompanied by inappropriately increased hepatic glucose production (Accili and Arden 2004). FoxO1 loss-of-function, caused by hepatic expression of its dominant-negative allele or antisense oligonucleotide-mediated FoxO1 knockdown in liver, is associated with improved insulin action and blood glucose profiles in insulin resistant obese mice (Altomonte et al. 2003). FoxO1 haploinsufficiency protects mice from developing high fat diet-induced insulin resistance and rescues the diabetic phenotype in insulin receptor substrate 2 (IRS2)-deficient diabetic mice (Accili and Arden 2004). Liver-specific FoxO1 knockout results in near normalization of metabolic disorders in insulin resistant mice with both IRS1 and IRS2 depletion in liver (Dong et al. 2008). These results support the notion that FoxO1 is a counter-regulator of insulin signaling in liver, suggesting that aberrant FoxO1 production is deleterious to hepatic insulin signaling, contributing to abnormal metabolism in diabetes.

Insulin Regulation of FoxO1 Activity

FoxO1 is subject to insulin inhibition. Insulin inhibits FoxO1 activity by stimulating its phosphorylation and promoting its trafficking from the nucleus to cytoplasm. Loss of insulin inhibition, due to insulin deficiency or insulin resistance, results in unbridled FoxO1 activity, contributing to the dual pathogenesis of fasting hyperglycemia and hyperlipidemia in diabetes (Matsumoto et al. 2006). Insulin-dependent regulation

of FoxO1 activity provides an acute mechanism for suppressing hepatic glucose and triglyceride production after meals. This mechanism is critical for maintaining blood glucose and lipid levels within the physiological range. Apart from its acute regulation by insulin, FoxO1 is regulated at multiple levels. At the transcriptional level, FoxO1 mRNA expression is induced in response to fasting, correlating with its enhanced activity in augmenting hepatic gluconeogenesis (Qu et al. 2006).

FoxO1 Feedback Regulation

FoxO1 activity is subject to feedback regulation. FoxO1 stimulates the expression of its upstream effector gene encoding insulin receptor (IR), which in turn activates insulin signaling and inhibits FoxO1 activity. To understand the underlying physiology, Kamagate et al. (2010) show that hepatic FoxO1 activity is subject to feedback regulation in an IR- and IRS2-dependent manner. Unchecked FoxO1 activity, resulting from molecular defects in the FoxO1 feedback loop, is deleterious to hepatic metabolism, culminating in unrestrained glycogen breakdown and excessive ER stress in liver. ER is the principal organelle for biosynthesis of proteins and steroids, and VLDL assembly. Perturbation of ER homeostasis such as accumulation of misfolded proteins or altered glycosylation triggers adaptive ER stress (Hotamisligil 2010). Unresolved ER stress results in apoptosis and causes insulin resistance (Hotamisligil 2010). FoxO1 feedback loop is thought to serve as a safeguarding mechanism for keeping FoxO1 activity in check to avert hepatic glycogen depletion and ER stress.

Non-insulin-dependent Regulation of FoxO1 Activity

Aside from its regulation by insulin (or IGF-1), FoxO1 activity is regulated by other signaling molecules, such as cyclin-dependent kinases (CDKs). CDK1 catalyzes FoxO1 phosphorylation at Ser249. This effect disrupts FoxO1 association with 14-3-3 proteins, promoting FoxO1 nuclear retention and augmenting FoxO1-dependent transcription, leading to cellular apoptosis (Yuan et al. 2008). In contrast, CDK2-mediated phosphorylation of FoxO1 at Ser249 promotes its cytoplasmic localization, resulting in inhibition of FoxO1 transcriptional activity (Huang et al. 2006). This effect favors cell survival in response to DNA damage. Further studies are warranted for clarifying the mechanism underlying the opposite effects of CDK1 and CDK2 on FoxO1 activity.

FoxO1 also undergoes phosphorylation by extracellular signal regulated kinase (ERK) and p38, two members of the Mitogen-activated protein kinase (MAPK) family in endothelial cells (Asada et al. 2007). MAPK-mediated phosphorylation of FoxO1 enhances its transcriptional activity in stimulating vascular endothelial growth factor receptor 2 (VEGFR2) in modulating angiogenesis (Asada et al. 2007).

FoxO1 Acetylation and Deacetylation

FoxO1 activity is modulated by acetylation via cAMP response element-binding (CREB)-binding protein (CBP) and/or p300 factor. FoxO1 is acetylated by the intrinsic acetyltransferase activity of p300, resulting in its enhanced trans-activation activity (Perrot and Rechler 2005). In contrast, FoxO1 acetylation at the basic residues (Lys-242, Lys-245, and Lys-262) attenuates FoxO1 activity by inhibiting its ability to bind target DNA (Matsuzaki et al. 2005). Acetylation of FoxO1 at these three residues by CBP appears to enhance its phosphorylation at Ser-253 by Akt/PKB, resulting in inhibition of FoxO1 activity (Matsuzaki et al. 2005). These data reflect diverse effects of acetylation on FoxO1 transcriptional activity.

FoxO proteins are substrates of SIRT1, the mammalian ortholog of the yeast Sir2 deacetylase (Motta et al. 2004). Deacetylation of FoxO proteins modulates their trans-activation activities, which has been viewed as an important mechanism for cells to defend against oxidative stress. FoxO1 contains a consensus site (459-LXXLL-463) within its carboxyl trans-activation domain that is responsible for SIRT1 binding. Mutations in the LXXLL motif (L462A and L463A) disrupt FoxO1 binding to SIRT1 and abolish SIRT1-mediated deacetylation (Nakae et al. 2006).

FoxO1 is also subject to deacetylation by the Class IIa histone deacetylases (HDACs) (Mihaylova et al. 2011). In response to glucagon, HDACs are dephosphorylated and translocated to the nucleus, where HDACs recruits HDAC3 for deacetylating FoxO1 in hepatocytes. This effect enhances FoxO1 activity in promoting hepatic gluconeogenesis during fasting.

FoxO1 Ubiquitination and Degradation

FoxO1, when phosphorylated in response to insulin or IGF-1, is targeted for ubiquitination and proteolytic degradation (Matsuzaki et al. 2003). Efficient ubiquitination depends upon both phosphorylation and cytoplasmic retention. Likewise, FoxO3 undergoes proteosome-mediated degradation in response to Akt signaling. To gain insight into the molecular basis of ubiquitin-mediated FoxO1 degradation, studies show that MDM2 serves as an E3 ligase to promote FoxO1 and FoxO4 ubiquitination (Fu et al. 2009). COP1 also possess E3 ligase activity to stimulate cytosolic FoxO1 ubiquitination and degradation (Kato et al. 2008). FoxO1 ubiquitination serves as a distinct posttranslational mechanism by which insulin (IGF-1) inhibits FoxO1 activity in cells.

FoxO1 Dephosphorylation and Reverse Translocation

Not all phosphorylated FoxO1 proteins are destined for proteosome-mediated degradation in the cytoplasm. There is evidence that cytosolic FoxO1 proteins undergo Sirt1-dependent deacetylation, resulting in FoxO1 reverse translocation to the nucleus in cultured hepatocytes in response to oxidative stress (Accili and Arden 2004). Sirt1 activation promotes FoxO1 nuclear retention and enhances its activity in resveratrol-treated cells. Yan et al. (2008) show that FoxO1 is a substrate of protein phosphatase 2A (PP2A), which dephosphorylates FoxO1 and promotes FoxO1 nuclear localization in cultured lymphoid FL5.12 cells. Inhibition of PP2A protects FoxO1 from dephosphorylation and perturbs FoxO1 nuclear localization (Yan et al. 2008). FoxO1 dephosphorylation and reverse translocation may serve as a fine-tuning mechanism for recycling cytosolic FoxO1 and promoting its reentry to the nucleus for function.

FoxO1 Methylation

FoxO1 methylation constitutes a new paradigm for posttranslational modification of FoxO1 activity. Yamagate et al. (2008) show that FoxO1 is methylated by the protein arginine methyltransferase 1 (PRMT1), a ubiquitous enzyme that catalyzes methylation of terminal nitrogens of guanidinium side chains at arginine residues of target proteins. As PRMT1 methylates FoxO1 at Arg248 and Arg250 within a consensus motif for Akt phosphorylation, the resulting methylation interferes with Akt-mediated phosphorylation of FoxO1 at Ser253. As a result, FoxO1 is confined in the nucleus, accounting for enhanced transcriptional activity. Arginine methylation seems to serve as an antagonistic mechanism for counterbalancing Akt/PKB-mediated phosphorylation of target proteins.

FoxO1 O-Linked Glycosylation

O-linked glycosylation is an enzymatic process by which sugar moieties are added to serine and threonine residues by O-N-acetylglucosamine (O-GlcNAc) transferase. As this action takes place on serines or threonines, residues that are targeted for phosphorylation by serine/threonine kinases, O-linked glycosylation consequently interferes with phosphorylation, resulting in alterations in stability, folding or subcellular distribution of target proteins. There is evidence that FoxO1 undergoes O-linked glycosylation in cultured hepatocytes at high glucose levels. O-linked glycosylation of FoxO1 enhances its transcriptional activity in cultured cells (Housley et al. 2008). These data help explain why FoxO1 activity is enhanced in the liver under hyperglycemic conditions, presaging that inhibiting O-linked glycosylation would reduce FoxO1 activity and alleviate hyperglycemia in diabetes.

Summary

Characterization of FoxO1 signaling has provided important insights into the mechanism by which insulin (or IGF-1) regulates the expression of target genes in cell growth, differentiation, metabolism, oxidative stress, and aging. Ubiquitously expressed, FoxO1 plays important roles in different organs. Due to space limitation, this article focuses on reviewing research progress made in the liver toward delineating the PI3K-Akt-FoxO1 cascade in glucose and lipid metabolism. Insulin signaling through FoxO1 is instrumental for adjusting the rate of hepatic glucose and triglyceride production in response to nutritional cues. FoxO1 is subject to insulin inhibition in liver. Unchecked FoxO1 activity, resulting from insulin

resistance, promotes unrestrained hepatic glucose and triglyceride production. This effect contributes to the dual pathogenesis of hyperglycemia and hypertriglyceridemia. The available preclinical studies support the notion of inhibiting FoxO1 for improving glucose and lipid metabolism in diabetes.

Cross-References

► FoxO1

References

Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell. 2004;117:421–6.

Altomonte J, et al. Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. Am J Physiol. 2003;285:E718–28.

Altomonte J, et al. Foxo1 mediates insulin action on ApoC-III and triglyceride metabolism. J Clin Invest. 2004;114:1493–503.

Asada S, et al. Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate FoxO1. Cell Signal. 2007;19:519–27.

Berriot-Varoqueaux N, Aggerbeck LP, Samson-Bouma M, Wetterau JR. The role of the microsomal triglygeride transfer protein in abetalipoproteinemia. Annu Rev Nutr. 2000;20:663–97.

Dong XC, et al. Inactivation of hepatic FoxO1 by insulin signaling is required for adaptive nutrient homeostasis and endocrine growth regulation. Cell Metab. 2008;8:65–76.

Fu W, et al. MDM2 acts downstream of p53 as an E3 ligase to promote FOXO ubiquitination and degradation. J Biol Chem. 2009;284:13987–4000.

Galili N, et al. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet. 1993;5:230–5.

Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell. 2010;140:900–17.

Housley MP, et al. O-GlcNAc regulates FoxO activation in response to glucose. J Biol Chem. 2008;283:16283–92.

Huang H, Regan KM, Lou Z, Chen J, Tindall DJ. CDK2-dependent phosphorylation of FoxO1 as an apoptotic response to DNA damage. Science. 2006;314:294–7.

Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature. 2004;429:562–6.

Kamagate A, et al. FoxO1 mediates insulin-dependent regulation of hepatic VLDL production in mice. J Clin Invest. 2008;118:2347–64.

Kamagate A, et al. FoxO1 links hepatic insulin action to endoplasmic reticulum stress. Endocrinology. 2010;151:3521–35.

Kato S, Ding J, Pisck E, Jhala US, Du K. COP1 functions as a FoxO1 ubiquitin E3 ligase to regulate FoxO1-mediated gene expression. J Biol Chem. 2008;283:35464–73.

Lee SS, Kennedy S, Tolonen AC, Ruvkun G. DAF-16 target genes that control *C. elegans* life-span and metabolism. Science. 2003;300:644–7.

Matsumoto M, Han S, Kitamura T, Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest. 2006;116:2464–72.

Matsumoto M, Pocai A, Rossetti L, Depinho RA, Accili D. Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor FoxO1 in liver. Cell Metab. 2007;6:208–16.

Matsuzaki H, Daitoku H, Hatta M, Tanaka K, Fukamizu A. Insulin-induced phosphorylation of FKHR (FoxO1) targets to proteasomal degradation. Proc Natl Acad Sci USA. 2003;100:11285–90.

Matsuzaki H, et al. Acetylation of FoxO1 alters its DNA-binding ability and sensitivity to phosphorylation. Proc Natl Acad Sci USA. 2005;102:11278–83.

Mihaylova MM, et al. Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. Cell. 2011;145:607–21.

Motta MC, et al. Mammalian SIRT1 represses forkhead transcription factors. Cell. 2004;116:551–63.

Nakae J, et al. The LXXLL motif of murine forkhead transcription factor FoxO1 mediates Sirt1-dependent transcriptional activity. J Clin Invest. 2006;116:2473–83.

Perrot V, Rechler MM. The coactivator p300 directly acetylates the forkhead transcription factor FoxO1 and stimulates FoxO1-induced transcription. Mol Endocrinol. 2005;19(9):2283–98.

Qu S, et al. Aberrant Forkhead box O1 function is associated with impaired hepatic metabolism. Endocrinology. 2006;147:5641–52.

Valenti L, et al. Increased expression and activity of the transcription factor FoxO1 in nonalcoholic steatohepatitis. Diabetes. 2008;57:1355–62.

Yamagata K, et al. Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. Mol Cell. 2008;32:221–31.

Yan L, et al. PP2A regulates the pro-apoptotic activity of FoxO1. J Biol Chem. 2008;283:7411–20.

Yuan Z, et al. Activation of FoxO1 by Cdk1 in cycling cells and postmitotic neurons. Science. 2008;319:1665–8.

Zhang W, et al. FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. J Biol Chem. 2006;281:10105–17.

FPR

► Formyl Peptide Receptor

FPR2

▶ FPR2/ALX

FPR2/ALX

Karen Nolan and Catherine Godson
UCD Diabetes Research Centre, UCD Conway Institute, School of Medicine and Medical Sciences, University College Dublin, Dublin, Ireland

Synonyms

ALX; ALXR; FMLP-related receptor II (FMLP-R-II); FMLPX; FPR2; FPR2A; FPRH1; FPRH2; FPRL1; HM63; LXA4R; Receptor related to FPR (RFP)

Historical Background

The FPR2/ALX receptor is a member of the formyl peptide receptor (FPR) superfamily which in humans also includes formyl peptide receptor 1 (FPR1) and formyl peptide receptor 3 (FPR3). Recent interest in this specific receptor reflects its intriguing anti-inflammatory and proresolving bioactions in contrast to other FPR family members (Serhan 2007; Maderna et al. 2010). FPR2/ALX was identified as a specific receptor for lipoxin A_4 (LXA_4) in 1992 (Fiore et al. 1992). Using radiolabeled [11,12-^{3}H]LXA_4 and human polymorphonuclear leukocytes (PMNs) in which LXA_4-mediated action had previously been identified, specific stereoselective binding with a Kd of approximately 0.5 nM was demonstrated. Modulation of [11,12-^{3}H]LXA_4 binding by guanosine analogs indicated that this binding site was a G-protein-coupled receptor (GPCR). Screening of orphan receptors in differentiated HL-60 cells, which displayed specific LXA_4 binding, was carried out in parallel and it identified a GPCR with high affinity for and selective binding by [11,12-^{3}H](6R)-LXA_4. Leukotriene B_4 (LTB_4), lipoxin B_4 (LXB_4), (6S)-LXA_4, or 11-trans-LXA_4 did not compete with LXA_4 for binding of this orphan receptor when expressed in CHO cells, indicating specificity for LXA_4. LXA_4 stimulated GTPase activity and promoted the release of esterified arachidonate in this model system. A pertussis toxin sensitive response further supported the hypothesis that the cDNA encoded a GPCR. The receptor cDNA sequence was found to be ~70% homologous to FPR1 and was thus named formyl peptide receptor-like1 (FPRL1) (for more detail refer to Chiang et al. 2006; Ye et al. 2009). It has also been referred to by a number of other names in the literature (see synonyms). In accordance with the International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature 2009 (Ye et al. 2009), this receptor is referred as FPR2/ALX in this entry. FPR2/ALX was subsequently identified and cloned in several cell types including monocytes, T cells, synovial fibroblasts, and intestinal epithelial and renal mesangial cells. The gene encoding FPR2/ALX is located at 19q13.3 and is a single copy gene with an intronless open reading frame (for more detail refer to Chiang et al. 2006; Ye et al. 2009). FPR2/ALX is now known to bind endogenous and exogenous proteins and lipid ligands eliciting distinct proinflammatory or anti-inflammatory responses indicating that FPR2/ALX can thus serve as a stereoselective multirecognition receptor in immune responses (Maderna et al. 2010).

FPR2/ALX Expression, Structure, and Agonists

FPR2/ALX is expressed in cells of diverse lineage: myeloid, epithelial, and mesenchymal. Upregulation of FPR2/ALX by proinflammatory cytokines (e.g., tumor necrosis factor-α [▶ TNF-α]) suggests the regulation of the receptor under inflammatory conditions. Additionally, upregulation of FPR2/ALX expression by anti-inflammatory glucocorticoid-stimulated signaling in human PMNs and monocytes as well as murine dermal tissues is thought to contribute to their potent anti-inflammatory effects (Perretti and D'Acquisto 2009).

FPR2/ALX is a 351 amino acid protein, the overall homology between human, mouse, and rat FPR2/ALX is 74% at nucleotide and 65% at the amino acid sequence level. One hundred percent homology across

these species is found in the second intracellular loop, 97% homology in the sixth transmembrane domain (TMD), followed by the second, third, and seventh TMD as well as the first extracellular loop (87–89%). ChemR23 is a GPCR which like FPR2/ALX binds both peptide and lipid ligands and is implicated in anti-inflammatory signal transduction. ChemR23 shares 36.4% overall homology with FPR2/ALX amino acid sequence. It has been suggested that highly conserved domains within the second intracellular loop and the seventh TMD which share 75% and 69.5% identity with FPR2/ALX, respectively, may contribute to their anti-inflammatory and proresolving properties. Such conservation between species and among similar receptors suggests these regions of the receptor play an essential role in ligand recognition and functional G-protein coupling. Studies using chimeric receptors show that the seventh TMD and its adjacent regions are essential for LXA_4 recognition. Ser-236, Ser-237, and Tyr-302 are essential for LXA_4-stimulated FPR2/ALX signaling and a role for conserved glycosylation sites present on Asn-4 and Asn-179 are important for peptide binding (for more detail refer to Chiang et al. 2006). The ability of FPR2/ALX to interact with ligands of diverse structural and chemical nature (i.e., lipid, peptide, protein) is thought to be related to certain properties within its binding pocket, which is large enough to accommodate ligands like the protein Annexin A1 (AnxA1) (~40 kDa), yet flexible enough to contact the smaller peptide ligands like the synthetic proinflammatory peptide WKYMVm. Hydrophobic interactions and possibly multiple ligand-binding sites may also facilitate interaction with both peptide and lipid agonists (for more detail refer to Ye et al. 2009). FPR2/ALX agonists elicit either proinflammatory or anti-inflammatory responses depending on the ligand and cell type. The bacterial peptide *N*-formyl-methionine-leucine-phenylalanine (fMLF) is one of the smallest and most potent formyl peptides and one of the first identified chemotactic peptides. Although FPR2/ALX shows low affinity for fMLF (Kd = 430 nM) compared to FPR1 (Kd = 1 nM), it does show high affinity for mitochondrial derived formyl peptides as well as several *Listeria monocytogenes*–derived formyl peptides (for more detail refer to Ye et al. 2009). It is hypothesized that during inflammation, impaired mitochondrial function results in *N*-formyl peptide secretion which attracts inflammatory cells to the site of tissue damage (Godson et al. 2000). In vitro studies show that a number of mitochondrial *N*-formylated peptides are agonists at FPR2/ALX. Some of these naturally produced peptides interact with FPR2/ALX in the nanomolar to subnanomolar range. FPR2/ALX also shows affinity for nonformylated peptides such as major histocompatibility complex (MHC)–binding peptide, a potent necrotactic peptide derived from mitochondrial NADH dehydrogenase subunit 1, which competes with $[^3H]LXA_4$ for FPR2/ALX binding and stimulates PMN chemotaxis (Chiang et al. 2000).

A number of FPR2/ALX ligands have antimicrobial properties and are stored in human neutorphil granules. LL-37 is expressed by leukocytes and epithelial cells and secreted into wounds and onto the airway surface where it attracts monocytes, neutrophils, and T lymphocytes through FPR2/ALX activation (for more detail refer to Ye et al. 2009). LL-37-stimulated release of proinflammatory LTB_4 from PMNs is inhibited by LXA_4 (Wan et al. 2011). Temporin A, a frog-derived antimicrobial peptide also acts through FPR2/ALX, stimulating monocyte, macrophage, and neutrophil migration. Nonformylated peptides from *Helicobacter pylori* attract monocytes and basophils to the gastric mucosa and those from human immunodeficiency virus-1 (HIV-1) activate phagocytic leukocytes through their agonistic activity at FPR2/ALX (for more detail refer to Ye et al. 2009). Furthermore, FPR2/ALX has been shown to recognize phenol-soluble modulin produced by strains of highly pathogenic *Staphylococcus aureus* and to initiate a proinflammatory neutrophil response (Rautenberg et al. 2011).

Host-derived peptides signaling through FPR2/ALX have been associated with inflammatory and amyloidogenic diseases. Serum amyloid A (SAA) is an acute-phase protein best known for its role in the pathogenesis of inflammatory arthritis. SAA serum concentrations increase dramatically in response to infection, trauma, and other physiological stress and it acts through FPR2/ALX to promote chemotaxis of monocytes, neutrophils, mast cells, and T lymphocytes to the wounded area. SAA is also known to induce matrix metalloproteinase expression in fibroblast-like synoviocytes and monocytes and this activity is mimicked by the proinflammatory peptide and FPR2/ALX agonist WKYMVm (for more detail refer to Ye et al. 2009). $A\beta_{42}$ the 42 amino acid peptide cleavage

product of the β-amyloid peptide, is implicated in the pathology of Alzheimer's disease. FPR2/ALX mediates $A\beta_{42}$-stimulated activation and accumulation of monocytic phagocytes as well as facilitating $A\beta_{42}$ uptake, thus contributing to fibrillar formation (Cui et al. 2002). Humanin is an endogenous neuroprotective peptide which displays increased potency at FPR2/ALX when *N*-formylated. Humanin may competitively inhibit $A\beta_{42}$ at FPR2/ALX. Other proinflammatory FPR2/ALX ligands include truncated chemotactic peptides (e.g., CKβ8-1), a urokinase-type plasminogen activator receptor fragment and prion protein (Ye et al. 2009).

FPR2/ALX ligands which display anti-inflammatory properties are of particular interest. AnxA1 is a glucocorticoid-inducible protein and FPR2/ALX agonist which is expressed by a wide range of immune cells and tissues. Following cellular activation, AnxA1 is mobilized to the cell surface and secreted, where it colocalizes with FPR2/ALX. AnxA1 and AnxA1 peptide derivatives have been shown to inhibit neutrophil adhesion and transmigration through endothelial barriers both in vitro and in vivo. Additionally, increased secretion of the anti-inflammatory cytokine, interleukin-10 (IL-10), in response to AnxA1 exposure has been described (Perretti and D'Acquisto 2009). Endogenous AnxA1 and peptide derivatives are released from apoptotic cells and act on macrophages to promote efferocytosis of apoptotic leukocytes (Scannell et al. 2007). Interestingly, it has been shown that LXA_4 and Antiflammin-2, the FPR2/ALX peptide agonist, which corresponds to the region 246–254 of AnxA1, also provoke FPR2/ALX-dependent mobilization of AnxA1 to the plasma membrane of PMNs (Brancaleone et al. 2011).

LXA_4 was the first identified endogenous ligand described for FPR2/ALX. Unlike the diverse array of peptide ligands for FPR2/ALX discussed so far, lipoxins (LXs) are arachidonate-derived lipid mediators. LXs are generated in vivo within an inflammatory milieu where they possess anti-inflammatory activity as well as the ability to promote the resolution of inflammation. LXA_4 displays multilevel control of processes relevant in acute inflammation via specific and selective actions on multiple cell types through specific receptors (Serhan 2007). Well-established effects of LXA_4 include limiting of leukocyte infiltration, inhibition of neutrophil and eosinophil activation, stimulation of efferocytosis of apoptotic leukocytes by macrophages as well as stimulation of genes which promote inflammatory resolution (reviewed in Maderna and Godson 2009). In vitro studies have shown that LXA_4 inhibits production of proinflammatory cytokines in synovial fibroblasts and intestinal and bronchial epithelial cells (for more detail refer to Ye et al. 2009). Furthermore, LXA_4 stimulates the expression of anti-inflammatory IL-10 in endothelial cells (Baker et al. 2009). More recently, LXA_4 has been shown to have antifibrotic activity in experimental models of lung and kidney fibrosis as will be discussed later (Rodgers et al. 2005; Wu et al. 2006; Martins et al. 2009; Borgeson et al. 2011).

Aspirin-triggered lipoxins (ATLs) such as 15-epi-LXA_4 are generated by the activity of aspirin-acetylated cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO). 15-epi-LXA_4 displays similar activity to native LXA_4 and acts with higher affinity for and potency at FPR2/ALX. Given the rapid metabolic degradation of native LXA_4, several generations of analogs have been synthesized. These include a new class of chemically stable LX analogs featuring replacement of the tetraene unit of native LXA_4 with a substituted benzo-fused ring. These benzo-LXs have also been shown to have potent anti-inflammatory properties that are likely to be mediated through FPR2/ALX (O'Sullivan et al. 2007; Sun et al. 2009).

Recent investigations using mice deficient in the murine homologue of FPR2/ALX have provided important insights into this receptor. In experimental models the animals showed exaggerated inflammatory responses to arthrogenic stimuli (Dufton et al. 2010). Bone marrow–derived macrophages from these mice were unable to phagocytose apoptotic PMNs in response to stimulation with LXA_4 or Ac2-26 (Maderna et al. 2010). These data highlight the role of FPR2/ALX as an anti-inflammatory and proresolution receptor in the context of LXA_4 or AnxA1 stimulation. It is now appreciated that the murine FPR2/ALX homologues targeted in these models may also have deleted Fpr3.

Resolvin D1 (RvD1), like LXA_4, is generated during the resolution phase of inflammation and also displays potent and stereoselective anti-inflammatory actions. Using [^{3}H-RvD1], FPR2/ALX was identified as one of two specific RvD1 recognition sites. RvD1 increased macrophage phagocytosis of apoptotic PMNs and its ability to do so was enhanced by overexpression of FPR2/ALX

and GPR32, the orphan receptor identified to bind RvD1 (Krishnamoorthy et al. 2010).

The potential of computational platform design to identify novel, potent FPR2/ALX agonists with anti-inflammatory and cardioprotective effects has been demonstrated using inflamed air pouch and myocardial ischemia-reperfusion-injury (IRI) in rodent models (Hecht et al. 2009).

Antagonists of the formyl peptide receptor family include t-Boc which was developed by replacing the *N*-formyl group of fMLF with a tertiary butyloxy-carbonyl. Similar antagonists include Boc1 and Boc2 which act at high micromolar concentrations to partially inhibit FPR2/ALX. Selective antagonists for FPR2/ALX like WRWWWW have been identified through screening hexapeptide libraries for the ability to inhibit binding of a synthetic peptide agonist, WKYMVm to FPR2/ALX (for more detail refer to Ye et al. 2009) and as described above FPR2/ALX knockout mice have been developed for in vivo studies (Dufton et al. 2010).

FPR2/ALX Signal Transduction

FPR2/ALX is phosphorylated in response to ligand activation, but as of yet little is known about the kinase(s) involved in the process. Lipid rafts are known to play an important role in receptor coupling to G-proteins and FPR2/ALX signaling is sensitive to cholesterol depletion in PMNs. Although the current understanding of the downstream signaling following FPR2/ALX activation remains incomplete, it does appear that the nature of signaling elicited is dependent on the ligand concentration and cell type in which FPR2/ALX is activated (for more detail refer to Chiang et al. 2006; Ye et al. 2009).

Proinflammatory responses mediated by FPR2/ALX include the activation of nuclear factor-κB (► NF-κB) by SAA in human neutrophils resulting in interleukin-8 (IL-8) secretion (He et al. 2003). SAA induces neutrophil Ca^{2+} mobilization and activation of ERK1/2 and p38. SAA also promotes the survival of neutrophils by delaying apoptosis. The underlying mechanism is thought to involve SAA activation of ERK1/2 and Akt which in turn leads to downstream phosphorylation events which regulate apoptosis (El Kebir et al. 2008).

The signaling pathways involved in the anti-inflammatory and proresolving effects of FPR2/ALX agonists are of great interest. LXA_4 and ATLs suppress the expression of neutrophil and endothelial adhesion molecules and thus attenuate adhesion and the production of proinflammatory cytokines through modulation of MAPK signaling, superoxide generation, and ► NF-κB activity (Filep et al. 2005). LXA_4 stimulates the non-phlogistic uptake of apoptotic PMNs by monocyte-derived macrophages (Godson et al. 2000; Mitchell et al. 2002) by a process coupled to Rho- and Rac-dependent cytoskeletal rearrangement (Maderna et al. 2002). In human enterocytes, ATL analogs downregulate *Salmonella typhimurium*–induced gene expression (Gewirtz et al. 1998). A subset of these genes are known to be regulated by the transcription factor ► NF-κB through a FPR2-/ALX-dependent mechanism (for more detail refer to Chiang et al. 2006). LXA_4 and ATLs attenuate nuclear accumulation of ► NF-κB and AP-1 transcription factors in human leukocytes thus inhibiting LPS-induced IL-8 secretion. Mesangial cell proliferation is a feature of glomerular inflammation and LXA_4 inhibits LTD_4-stimulated mesangial proliferation (McMahon et al. 2000). The underlying mechanisms involve LXA_4's inhibition of LTD_4-stimulated platelet-derived growth factor (PDGF) receptor transactivation (McMahon et al. 2002). LXA_4 and Ac2-26 attenuated PDGFRβ phosphorylation but did not alter the receptor tyrosine kinase activity, suggesting the involvement of protein tyrosine phosphatases. Using cells stably transfected with FRP2/ALX, transient transfection with PDGFRβ and stimulation with PDGF-BB resulted in a decrease in PDGFRβ phosphorylation compared to control cells. These data confirmed that the reduced phosphorylation was FPR2/ALX dependent. LXA_4 was shown to promote SHP-2-mediated dephosphorylation of PDGFRβ. Site-directed mutagenesis of the cytoplasmic domain of PDGFRβ indicated the importance of the binding site of the p85 subunit of ► PI3K in LXA_4-mediated transinactivation of the receptor (Mitchell et al. 2007). LXA_4 has also been shown to inhibit epidermal growth factor (EGF) receptor and vascular endothelial growth factor (VEGF) receptor 2 activation (Baker et al. 2009). These studies highlighted the complex cross talk between GPCRs and receptor tyrosine kinases in an inflammatory milieu and suggested a protective role for LXA_4 in renal inflammation and

fibrosis in which activation of the PDGFRβ is known to play a pathological role (Mitchell et al. 2007). Additionally LXA_4 inhibits TNF-α induced mesangial cell proliferation (Wu et al. 2005).

The best characterized mechanism of FPR2/ALX internalization is described following stimulation with a synthetic proinflammatory peptide agonist WKYMVm and results in lipid raft–dependent, clathrin-mediated FPR2/ALX endocytosis. However it has recently been shown that when stimulated with anti-inflammatory ligands, FPR2/ALX colocalizes with caveolin-1 and flotillin-1 but not clathrin and that internalization of FPR2/ALX is required for LXA_4 and Ac2-26 stimulated macrophage phagocytic activity and is dependent on PKC (for more detail refer to Ye et al. 2009; Maderna et al. 2010).

FPR2/ALX: Anti-inflammatory, Proresolution, and Therapeutic Potential

As outlined, FPR2/ALX agonists have been shown to modulate specific actions of cells involved in immune-inflammatory responses and thus FPR2/ALX has been proposed as a target for therapeutic intervention. Several experimental models of disease further support this idea.

Transgenic mice with myeloid-selective expression of human FPR2/ALX (generated by DNA injection of 3.8 kb transgene consisting of full-length human FPR2/ALX cDNA driven by a fragment of human CD11b promoter) were challenged via dermal ear skin with proinflammatory LTB_4 and prostaglandin E_2 (PGE_2). The FPR2/ALX transgenic mice showed a significant reduction in neutrophil infiltration compared to nontransgenic littermate controls. In a model of zymosan-induced peritonitis, transgenic mice exhibited a reduced level of neutrophil infiltration and increased sensitivity to i.v. administration of 15-epi-LXA_4 (Devchand et al. 2003).

The potential of LXA_4 as a therapeutic mediator has been demonstrated in numerous animal models including a variety of renal injury models, inflammatory skin conditions, inflammatory intestinal disorders, lung injury, and asthma. Of particular interest in the context of Alzheimer's disease, LXA_4 has been shown to attenuate $A\beta_{42}$-induced expression of proinflammatory cytokines, interleukin-1β (IL-1β) and ▶ TNFα, to inhibit the degradation of IκBα and the translocation of ▶ NF-κB p65 subunit into the nucleus in the cortex and hippocampus of mice (Wu et al. 2011). An orally stable LX analog ZK-192 has been shown to have antiinflammatory activity in vivo (Guilford et al. 2004). The chronic perpetuation of an inflammatory response can result in fibrosis and eventual organ failure. The antifibrotic actions of LXA_4 and a synthetic-LX analog have been demonstrated in a murine model of early renal fibrosis. LXs attenuated collagen deposition and renal apoptosis and shifted the inflammatory cytokine milieu toward resolution by inhibiting ▶ TNF-α and IFN-γ expression, while stimulating proresolving IL-10. LXs attenuated unilateral ureteric obstruction–induced activation of ▶ MAP kinases, Akt, and Smads in injured kidneys. Further analysis suggests that renal fibroblast responses to TGF-β_1 may be among the targets of LXA_4 in this context (Borgeson et al. 2011). Furthermore, the antifibrotic activity of an ATL analog has been demonstrated in bleomycin-induced lung injury (Martins et al. 2009).

In murine models of IRI, FPR2/ALX ligands have been shown to have protective effects. Hundred microgram of Ac2-26, 30 min prior to renal artery clamping was shown to be effective at preventing loss of glomerular filtration rate and decreases in urinary osmolarity as well as protecting against the development of acute tubular necrosis, preventing neutrophil extravasation, and attenuating macrophage infiltration as well as affording structural protection against IRI. In vitro ischemia-reperfusion studies using isolated proximal tubules from control mice with no inflammatory cell influence showed that Ac2-26 protected against IRI. It has been proposed that this indicates that the protective effects are directly related to the intracellular actions of this peptide in tubular epithelial cells and suggest an important role for AnxA1 in renal epithelial cell defense against IRI (Facio et al. 2011). Analogous to findings with Ac2-26, a synthetic analog of 15-epi-LXA_4, 15-epi-16-(para-fluorophenoxy)-LXA_4-methyl ester was shown to be protective against murine renal IRI (Leonard et al. 2002).

Compound 43, a nitrosylated pyrazolone derivative, was identified as a FPR2/ALX agonist in a high-throughput compound library screen. It was found to inhibit PMN migration and to have good solubility characteristics and bioavailability following i.v.

administration to rats. In a murine model of prostaglandin E_2 and LTB_4 induced ear inflammation, oral administration of Compound 43 (50 mg/kg) 1 h before induction of inflammation afforded protection from edema which was comparable to established therapy (i.e., dexamethasone,1 mg/kg i.v.) (Burli et al. 2006).

Together, these in vivo studies using a number of molecules known to be FPR2/ALX agonists make a convincing argument for targeting this receptor for therapeutic purposes in a diverse range of inflammatory conditions. The development of stable and potent FPR2/ALX agonists makes it an even more attractive target. Recent developments have shown that human PMN-derived microparticles contain precursors for proresolving lipid mediator biosynthesis and display anti-inflammatory properties via FPR2/ALX. Using microparticle scaffolds, nanoparticles containing aspirin-triggered RvD1 or a stable analog of LXA_4, *o*-[9,12]-benzo-ω6-epi-LXA_4 have been developed. These enriched nanoparticles dramatically reduce PMN influx in murine zymosan–induced peritonitis, accelerate keratinocyte wound healing, and protect against inflammation in the temporomandibular joint (Norling et al. 2011).

Summary

Since its identification in 1992, FPR2/ALX has been shown to be a receptor with the ability to interact with a diverse array of ligands and to transduce signals which elicit completely contrasting physiological responses, which are either proinflammatory or anti-inflammatory in nature. Precise mechanisms through which differential responses are mediated remain incompletely understood. Yet this promiscuous receptor continues to attract research interest as a possible therapeutic target. Future studies will reveal the true potential of FPR2/ALX in anti-inflammatory and proresolving circuits where its modulation may help restore tissue homeostasis and prevent fibrosis, the common, progressive pathologic feature of numerous inflammatory conditions.

Acknowledgments The authors thank Dr. Aisling Kennedy and Dr. Eoin Brennan for their helpful comments. Work in the authors' lab is funded by Science Foundation Ireland, The Health Research Board, and The Government of Ireland Programme for Research in Third-Level Institutions. Karen Nolan is an IRCSET postgraduate Scholar.

References

Baker N, O'Meara SJ, Scannell M, Maderna P, Godson C. Lipoxin A4: anti-inflammatory and anti-angiogenic impact on endothelial cells. J Immunol. 2009;182:3819–26.

Borgeson E, Docherty NG, Murphy M, Rodgers K, Ryan A, O'Sullivan TP, Guiry PJ, Goldschmeding R, Higgins DF, Godson C. Lipoxin A4 and benzo-lipoxin A4 attenuate experimental renal fibrosis. FASEB J. 2011;25:2967–79.

Brancaleone V, Dalli J, Bena S, Flower RJ, Cirino G, Perretti M. Evidence for an anti-inflammatory loop centered on polymorphonuclear leukocyte formyl peptide receptor 2/lipoxin A4 receptor and operative in the inflamed microvasculature. J Immunol. 2011;186:4905–14.

Burli RW, Xu H, Zou X, Muller K, Golden J, Frohn M, Adlam M, Plant MH, Wong M, McElvain M, Regal K, Viswanadhan VN, Tagari P, Hungate R. Potent hFPRL1 (ALXR) agonists as potential anti-inflammatory agents. Bioorg Med Chem Lett. 2006;16:3713–8.

Chiang N, Fierro IM, Gronert K, Serhan CN. Activation of lipoxin A(4) receptors by aspirin-triggered lipoxins and select peptides evokes ligand-specific responses in inflammation. J Exp Med. 2000;191:1197–208.

Chiang N, Serhan CN, Dahlen SE, Drazen JM, Hay DW, Rovati GE, Shimizu T, Yokomizo T, Brink C. The lipoxin receptor ALX: potent ligand-specific and stereoselective actions in vivo. Pharmacol Rev. 2006;58:463–87.

Cui Y, Le Y, Yazawa H, Gong W, Wang JM. Potential role of the formyl peptide receptor-like 1 (FPRL1) in inflammatory aspects of Alzheimer's disease. J Leukoc Biol. 2002;72:628–35.

Devchand PR, Arita M, Hong S, Bannenberg G, Moussignac RL, Gronert K, Serhan CN. Human ALX receptor regulates neutrophil recruitment in transgenic mice: roles in inflammation and host defense. FASEB J. 2003;17:652–9.

Dufton N, Hannon R, Brancaleone V, Dalli J, Patel HB, Gray M, D'Acquisto F, Buckingham JC, Perretti M, Flower RJ. Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. J Immunol. 2010;184:2611–9.

El Kebir D, Jozsef L, Filep JG. Opposing regulation of neutrophil apoptosis through the formyl peptide receptor-like 1/lipoxin A4 receptor: implications for resolution of inflammation. J Leukoc Biol. 2008;84:600–6.

Facio Jr FN, Sena AA, Araujo LP, Mendes GE, Castro I, Luz MA, Yu L, Oliani SM, Burdmann EA. Annexin 1 mimetic peptide protects against renal ischemia/reperfusion injury in rats. J Mol Med. 2011;89:51–63.

Filep JG, Khreiss T, Jozsef L. Lipoxins and aspirin-triggered lipoxins in neutrophil adhesion and signal transduction. Prostaglandins Leukot Essent Fatty Acids. 2005;73:257–62.

Fiore S, Ryeom SW, Weller PF, Serhan CN. Lipoxin recognition sites. Specific binding of labeled lipoxin A4 with human neutrophils. J Biol Chem. 1992;267:16168–76.

Gewirtz AT, McCormick B, Neish AS, Petasis NA, Gronert K, Serhan CN, Madara JL. Pathogen-induced chemokine secretion from model intestinal epithelium is inhibited by lipoxin A4 analogs. J Clin Invest. 1998;101(9):1860–9.

Godson C, Mitchell S, Harvey K, Petasis NA, Hogg N, Brady HR. Cutting edge: lipoxins rapidly stimulate

nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. J Immunol. 2000; 164:1663–7.

Guilford WJ, Bauman JG, Skuballa W, Bauer S, Wei GP, Davey D, Schaefer C, Mallari C, Terkelsen J, Tseng JL, Shen J, Subramanyam B, Schottelius AJ, Parkinson JF. Novel 3-oxa lipoxin A4 analogues with enhanced chemical and metabolic stability have anti-inflammatory activity in vivo. J Med Chem. 2004;47(8):2157–65.

He R, Sang H, Ye RD. Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA4R. Blood. 2003;101(4):1572–81.

Hecht I, Rong J, Sampaio AL, Hermesh C, Rutledge C, Shemesh R, Toporik A, Beiman M, Dassa L, Niv H, Cojocaru G, Zauberman A, Rotman G, Perretti M, Vinten-Johansen J, Cohen Y. A novel peptide agonist of formyl-peptide receptor-like 1 (ALX) displays anti-inflammatory and cardioprotective effects. J Pharmacol Exp Ther. 2009;328:426–34.

Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee CH, Yang R, Petasis NA, Serhan CN. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. Proc Natl Acad Sci USA. 2010;107:1660–5.

Leonard MO, Hannan K, Burne MJ, Lappin DW, Doran P, Coleman P, Stenson C, Taylor CT, Daniels F, Godson C, Petasis NA, Rabb H, Brady HR. 15-Epi-16-(para-fluorophenoxy)-lipoxin A(4)-methyl ester, a synthetic analogue of 15-epi-lipoxin A(4), is protective in experimental ischemic acute renal failure. J Am Soc Nephrol. 2002; 13:1657–62.

Maderna P, Cottell DC, Berlasconi G, Petasis NA, Brady HR, Godson C. Lipoxins induce actin reorganization in monocytes and macrophages but not in neutrophils: differential involvement of rho GTPases. Am J Pathol. 2002;160(6): 2275–83.

Maderna P, Godson C. Lipoxins: resolutionary road. Br J Pharmacol. 2009;158:947–59.

Maderna P, Cottell DC, Toivonen T, Dufton N, Dalli J, Perretti M, Godson C. FPR2/ALX receptor expression and internalization are critical for lipoxin A4 and annexin-derived peptide-stimulated phagocytosis. FASEB J. 2010;24:4240–9.

Martins V, Valenca SS, Farias-Filho FA, Molinaro R, Simoes RL, Ferreira TP, Silva PM, Hogaboam CM, Kunkel SL, Fierro IM, Canetti C, Benjamim CF. ATLa, an aspirin-triggered lipoxin A4 synthetic analog, prevents the inflammatory and fibrotic effects of bleomycin-induced pulmonary fibrosis. J Immunol. 2009;182:5374–81.

McMahon B, Stenson C, McPhillips F, Fanning A, Brady HR, Godson C. Lipoxin A4 antagonizes the mitogenic effects of leukotriene D4 in human renal mesangial cells. Differential activation of MAP kinases through distinct receptors. J Biol Chem. 2000;275(36):27566–75.

McMahon B, Mitchell D, Shattock R, Martin F, Brady HR, Godson C. Lipoxin, leukotriene, and PDGF receptors cross-talk to regulate mesangial cell proliferation. FASEB J. 2002;16(13):1817–9.

Mitchell S, Thomas G, Harvey K, Cottell D, Reville K, Berlasconi G, Petasis NA, Erwig L, Rees AJ, Savill J, Brady HR, Godson C. Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. J Am Soc Nephrol. 2002;13 (10):2497–507.

Mitchell D, O'Meara SJ, Gaffney A, Crean JK, Kinsella BT, Godson C. The Lipoxin A4 receptor is coupled to SHP-2 activation: implications for regulation of receptor tyrosine kinases. J Biol Chem. 2007;282:15606–18.

Norling LV, Spite M, Yang R, Flower RJ, Perretti M, Serhan CN. Cutting edge: humanized nano-proresolving medicines mimic inflammation-resolution and enhance wound healing. J Immunol. 2011;186:5543–7.

O'Sullivan TP, Vallin KS, Shah ST, Fakhry J, Maderna P, Scannell M, Sampaio AL, Perretti M, Godson C, Guiry PJ. Aromatic lipoxin A4 and lipoxin B4 analogues display potent biological activities. J Med Chem. 2007;50:5894–902.

Perretti M, D'Acquisto F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nat Rev Immunol. 2009;9:62–70.

Rautenberg M, Joo HS, Otto M, Peschel A. Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence. FASEB J. 2011;25:1254–63.

Rodgers K, McMahon B, Mitchell D, Sadlier D, Godson C. Lipoxin A4 modifies platelet-derived growth factor-induced pro-fibrotic gene expression in human renal mesangial cells. Am J Pathol. 2005;167(3):683–94.

Scannell M, Flanagan MB, deStefani A, Wynne KJ, Cagney G, Godson C, Maderna P. Annexin-1 and peptide derivatives are released by apoptotic cells and stimulate phagocytosis of apoptotic neutrophils by macrophages. J Immunol. 2007;178:4595–605.

Serhan CN. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu Rev Immunol. 2007;25:101–37.

Sun YP, Tjonahen E, Keledjian R, Zhu M, Yang R, Recchiuti A, Pillai PS, Petasis NA, Serhan CN. Anti-inflammatory and pro-resolving properties of benzo-lipoxin A(4) analogs. Prostaglandins Leukot Essent Fatty Acids. 2009;81: 357–66.

Wan M, Godson C, Guiry PJ, Agerberth B, Haeggstrom JZ. Leukotriene B4/antimicrobial peptide LL-37 proinflammatory circuits are mediated by BLT1 and FPR2/ALX and are counterregulated by lipoxin A4 and resolvin E1. FASEB J. 2011;25:1697–705.

Wu SH, Lu C, Dong L, Zhou GP, He ZG, Chen ZQ. Lipoxin A4 inhibits TNF-alpha-induced production of interleukins and proliferation of rat mesangial cells. Kidney Int. 2005;68(1): 35–46.

Wu SH, Wu XH, Lu C, Dong L, Chen ZQ. Lipoxin A4 inhibits proliferation of human lung fibroblasts induced by connective tissue growth factor. Am J Respir Cell Mol Biol. 2006;34 (1):65–72.

Wu J, Wang A, Min Z, Xiong Y, Yan Q, Zhang J, Xu J, Zhang S. Lipoxin A4 inhibits the production of proinflammatory cytokines induced by beta-amyloid in vitro and in vivo. Biochem Biophys Res Commun. 2011;408:382–7.

Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN, Murphy PM. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol Rev. 2009;61:119–61.

FPR2A

▶ FPR2/ALX

FPRH1

▶ FPR2/ALX

FPRH2

▶ FPR2/ALX

FPRL1

▶ FPR2/ALX

Frabin

Hiroyuki Nakanishi[1] and Yoshimi Takai[2]
[1]Department of Molecular Pharmacology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan
[2]Division of Molecular and Cellular Biology, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, Kobe, Japan

Synonyms

FGD4

Historical Background

Dynamic reorganization of the actin cytoskeleton is essential for many cellular activities, such as cell shape changes, cell migration, cell adhesion, and cytokinesis. The Rho family small GTP-binding proteins (G proteins), including Cdc42, Rac, and Rho, regulate these actin cytoskeleton-dependent cellular activities (Takai et al. 2001; Hall 2005). In fibroblasts such as NIH 3T3 and Swiss 3T3 cells, Cdc42 regulates the formation of filopodia; Rac regulates the formation of lamellipodia and ruffles; and Rho regulates the formation of stress fibers and focal adhesions. Cdc42 and Rac activate the Arp2/3 complex through their respective target proteins, Wiskott-Aldrich syndrome protein (WASP)/neural (N-) WASP and WASP-family verprolin-homologous protein (WAVE) (Takenawa and Suetsugu 2007). The Arp2/3 complex interacts with the sides of preexisting actin filaments (F-actin) to promote actin polymerization and generate a branched F-actin network. Rho promotes actin polymerization through two distinct targets, p160 and mDia (Hall 2005). In addition to these actin cytoskeleton-dependent activities, the Rho family small G proteins regulate other cellular activities, such as the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) cascade, an NADPH oxidase enzyme complex and the transcription factor ▶ NF-κB (Hall 2005).

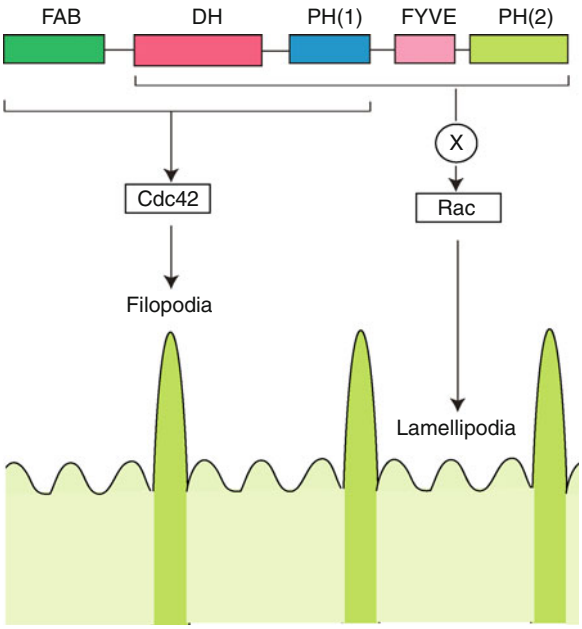
The Rho family members cycle between the GDP-bound inactive and GTP-bound active forms (Takai et al. 2001; Hall 2005). This cycling is tightly controlled by three types of regulators: guanine nucleotide exchange factors (GEFs) that stimulate conversion from the GDP-bound form to the GTP-bound form; GDP dissociation inhibitors that inhibit this reaction; and GTPase activating proteins that stimulate conversion from the GTP-bound form to the GDP-bound form. Most GEFs for the Rho family members share two conserved domains: a Dbl homology (DH) domain of about 250 amino acids, for which the Dbl oncogene product is the prototype, and a pleckstrin homology (PH) domain of about 100 amino acids. Many GEFs for the Rho family members were originally identified as oncogenes (Takai et al. 2001; Hall 2005), but FGD1, which encodes a GEF specific to Cdc42, was discovered by positional cloning to be the gene responsible for faciogenital dysplasia (FGDY) (Pasteris et al. 1994). FGD1 homologues were subsequently identified: FGD2, FGD3, FGD5, and FGD6 were identified by genetic searches; in contrast, frabin/FGD4 was identified by protein purification as an F-actin-binding protein (Obaishi et al. 1998; Nakanishi and Takai 2008).

Molecular Structure, Tissue Distribution, and Splicing Variants

Frabin/FGD4 possesses a domain organization similar to those of FGD1, FGD2, FGD3, FGD5, and FGD6, and these molecules constitute a family of GEFs; however, each FGD has a unique N-terminal region (Nakanishi and Takai 2008). Frabin has an F-actin-binding (FAB) domain at the N-terminal region (Fig. 1). Following the FAB domain, a DH domain, a first PH domain adjacent to the DH domain, a FYVE domain, and a second PH domain are located in order from the N-terminus to the C-terminus. The FAB domain binds along the sides of F-actin (Obaishi et al. 1998). Full-length frabin causes F-actin to associate into bundles, while the FAB domain alone does not show the bundling activity in a cell-free assay system. It is likely that frabin forms an oligomer with multiple FAB domains, and thereby shows F-actin-bundling activity.

DH domains encode a principal GEF catalytic unit and are required to stimulate GDP release from the Rho family small G proteins (Takai et al. 2001; Hall 2005). PH domains not only serve to target cellular membranes by binding to phosphoinositides, but also participate in the binding of small G proteins to facilitate guanine nucleotide exchange reactions (Rossman et al. 2002; Lemmon 2004). The amino acid sequence of the DH domain and the first PH domain of frabin shows significant homology to those of FGD1, FGD2, FGD3, FGD5, and FGD6 (Nakanishi and Takai 2008). A fragment of frabin containing the DH domain and the first PH domain stimulates the guanine nucleotide exchange reaction of Cdc42 in a cell-free assay system (Umikawa et al. 1999). This GEF activity is specific to Cdc42. The frabin fragment does not show the GEF activity toward Rho or Rac.

FYVE domains have been found in many proteins involved in membrane trafficking and phosphoinositide metabolism, and have been shown to specifically interact with one of phosphoinositides, phosphatidylinositol 3-phosphate (PI3P) (Kutateladze 2006). PI3P is found on the limiting membrane domain of early endosomes. However, frabin as well as FGD1 possesses an atypical FYVE domain, which recognizes not only PI3P but also another phosphoinositide, phosphatidylinositol 5-phosphate (PI5P) (Sankaran et al. 2001). PI5P is proposed to be present on late endosomes and at the plasma membrane (Lecompte et al. 2008). It is, therefore, likely that the two PH and FYVE domains of frabin serve to target to specific membrane domains or structures by binding to phosphoinositides.



Frabin, Fig. 1 Domain organization and roles of the domains. Frabin consists of an FAB domain, a DH domain, a first PH [PH(1)] domain, an FYVE domain, and a second PH [PH(2)] domain from the N-terminus and to the C-terminus. The N-terminal region of frabin containing the FAB domain, the DH domain, and the first PH domain induces the formation of filopodia through the direct activation of Cdc42. The C-terminal region of frabin containing the DH domain, the first PH domain, the FYVE domain and the second PH domain induces the formation of lamellipodia through the indirect activation of Rac

Rat frabin is expressed in all the tissues, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (Umikawa et al. 1999). In cultured rat hippocampal neurons, frabin is highly concentrated at filopodia in growth cones. Mouse frabin has two smaller splicing variants (Ikeda et al. 2001a). The original biggest, middle, and smallest variants are named frabin-α, -β and -γ, respectively. Frabin-β lacks the second PH domain, whereas frabin-γ lacks the FYVE domain and the second PH domain. These three splicing variants are expressed in all the tissues, but their expression levels differ among tissues. In this entry, unless otherwise indicated, frabin represents the α form. Human frabin has other splicing variants, one of which is deprived of the FAB domain (Delague et al. 2007). The splicing variants of frabin induce partly

different morphological changes, suggesting that the variants may have different physiological functions (Ikeda et al. 2001a).

Role of Domains in Cellular Activities

In fibroblasts, exogenous expression of full-length frabin induces the formation of filopodia and the activation of JNK through the activation of Cdc42 (Obaishi et al. 1998; Umikawa et al. 1999; Ono et al. 2000). The DH domain and the first PH domain are sufficient for the activation of Cdc42 in a cell-free assay system, but these two domains alone do not induce the formation of filopodia or the activation of JNK in fibroblasts (Obaishi et al. 1998; Umikawa et al. 1999). The FAB domain is additionally required for the formation of filopodia, suggesting that the association of frabin with the actin cytoskeleton is necessary for the formation of filopodia (Fig. 1). The FAB domain is recruited to the constitutively active mutant (CA) of Cdc42-formed filopodia, but not to Rho CA-formed stress fibers (Kim et al. 2002). Furthermore, co-expression of this domain inhibits the formation of filopodia induced by full-length frabin. It is likely that the FAB domain competes with full-length frabin for the association with a specific actin structure (s), and thereby inhibits the formation of filopodia.

Expression of frabin induces the formation of not only filopodia, but also lamellipodia, in fibroblasts (Ono et al. 2000) (Fig. 1). This morphological change is inhibited by a dominant-negative mutant (DN) of Rac, indicating that the formation of lamellipodia is mediated by the activation of Rac. The FYVE domain and the second PH domain, in addition to the DH domain and the first PH domain, are necessary for the formation of lamellipodia and the activation of JNK, suggesting that the association of frabin with membranes is required for these activities (Umikawa et al. 1999; Ono et al. 2000) (Fig. 1). Expression of the fragment containing the mutated DH, first PH, FYVE, and second PH domains inhibits the formation of membrane ruffles induced by full-length frabin; however, expression of shorter fragments, such as the FYVE domain alone, does not result in this inhibitory action. The mutated DH domain is constructed to lack the GEF activity. It is, therefore, likely that this fragment, containing the DH, first PH, FYVE, and second PH domains, competes with full-length frabin for the association with a specific membrane structure(s). The highly ordered structure of this fragment may be required for its interaction with the specific membrane structure(s), because shorter fragments, such as the FYVE domain alone, do not show a DN effect.

In epithelial cells, such as MDCK cells, frabin induces the formation of microspikes at the basal area of the lateral membrane through the activation of both Cdc42 and Rac, although Cdc42 CA alone, Rac CA alone, or both do not induce the formation of microspikes (Yasuda et al. 2000). However, microspikes are formed when Cdc42 CA is co-expressed with a fragment of frabin minimally including the FAB domain and a mutated DH domain, which lacks the GEF activity (Ikeda et al. 2001b). These data suggest that the region containing the FAB and DH domains directly reorganizes the actin cytoskeleton in a Cdc42-independent manner, and that both the Cdc42-GEF and F-actin-modulating activities of frabin are required for the generation of microspikes in MDCK cells.

Possible Mode of Action in Morphological Changes

A model for the mode of action of frabin in the formation of filopodia and lamellipodia is proposed as follows: initially, frabin is targeted to a preexisting specific actin structure through the FAB domain. Once recruited, frabin reorganizes the actin cytoskeleton through the action of its N-terminal region, including the FAB domain and the DH domain, in a Cdc42-independent manner. In addition, frabin activates Cdc42 through the DH domain and the first PH domain in the vicinity of the actin structure(s), resulting in the WASP/N-WASP-induced generation of branched F-actin. The F-actin-bundling activity of frabin may contribute to the formation of bundled F-actin in filopodia. Furthermore, Cdc42 stimulates actin polymerization via mDia2 (Hall 2005). The cooperation of Cdc42-independent and Cdc42-dependent actin reorganization finally induces the formation of filopodia. This newly formed actin structure further recruits frabin in a positive feedback cycle to lengthen the filopodia. Frabin is also recruited to a specific membrane structure(s) through the region including the DH domain, the first PH domain, the FYVE domain, and the second PH domain (Kim et al. 2002). Frabin activates Rac in the vicinity of

membrane structure(s), resulting in the WAVE-induced generation of branched F-actin. WAVE furthermore induces outward membrane protrusion at the plasma membrane (Takenawa and Suetsugu 2007). The synergistic reorganization of the actin cytoskeleton and the plasma membrane finally induces the formation of lamellipodia.

Involvement in Human Diseases

Many pathogen microbes, including viruses, bacteria, and parasites, utilize the host-cell actin cytoskeleton for multiple actions, such as attachment, entry into cells, and movement within and between cells (Gruenheid and Finlay 2003). *Cryptosporidium parvum*, an intracellular parasite, is one of the most commonly reported enteric pathogens worldwide. Frabin was shown to mediate the cellular invasion of this parasite (Chen et al. 2004). *C. parvum* recruits ▶ phosphoinositide 3-kinase to the host cell–parasite interface, an event that then results in the recruitment of frabin, leading to the activation of Cdc42 and subsequent host-cell actin reorganization during cellular invasion.

Mutations in human frabin have been identified to be responsible for the Charcot–Marie–Tooth (CMT) disorder Type 4H (Delague et al. 2007; Stendel et al. 2007). CMT disorders are clinically and genetically heterogeneous hereditary motor and sensory neuropathies characterized by muscle weakness and wasting, foot and hand deformities, and electrophysiological changes. The CMT4H subtype is an autosomal recessive demyelinating form of CMT disorder. Patients show early disease onset, but slowly progressive sensorimotor neuropathy. Nerve biopsy specimens display a severe loss of myelinated fibers, thinly myelinated axons, and outfolding of myelin sheaths. These data indicate that frabin plays an important role in the proper myelination of the peripheral nervous system.

Summary

Frabin/FGD4, together with at least FGD1, FGD2, FGD3, FGD5, and FGD6, is a member of a family of Cdc42-specific GEFs. Frabin consist of FAB, DH, first PH, FYVE, and second PH domains, in order, from the N-terminus to the C-terminus. Frabin associates with specific actin and membrane structures and activates Cdc42 and Rac in the vicinity of these structures, resulting in the reorganization of the actin cytoskeleton coupled with membrane dynamics. Thus, it has been shown how frabin induces cell shape changes. However, important questions still remain to be solved: (1) how external stimuli and intracellular signals induce the activation of frabin; (2) the physiological significance of a variety of splicing variants; (3) how frabin induces the activation of Rac; and (4) how frabin regulates myelination. Solving these questions will lead to a better understanding of the modes of action and activation of frabin.

References

Chen XM, Splinter PL, Tietz PS, Hunag BQ, Billadeau DD, LaRusso NF. Phosphatidylinositol 3-kinase and frabin mediate *Cryptosporidium parvum* cellular invasion via activation of Cdc42. J Biol Chem. 2004;279:31671–8.

Delague V, Jacquier A, Hamadouche T, Poitelon Y, Baudot C, Boccaccio I, Chouery E, Chaouch M, Kassouri N, Jabbour R, Grid D, Mégarbané A, Haase G, Lévy N. Mutations in FGD4 encoding the Rho GDP/GTP exchange factor FRABIN cause autosomal recessive Charcot-Marie-Tooth type 4H. Am J Hum Genet. 2007;81:1–16.

Gruenheid S, Finlay BB. Microbial pathogenesis and cytoskeletal function. Nature. 2003;422:775–81.

Hall A. Rho GTPases and the control of cell behavior. Biochem Soc Trans. 2005;33:891–5.

Ikeda W, Nakanishi H, Takekuni K, Itoh S, Takai Y. Identification of splicing variants of frabin with partly different functions and tissue distribution. Biochem Biophys Res Commun. 2001a;286:1066–72.

Ikeda W, Nakanishi H, Tanaka Y, Tachibana K, Takai Y. Cooperation of Cdc42 small G protein-activating and actin filament-binding activities of frabin in microspike formation. Oncogene. 2001b;20:3457–63.

Kim Y, Ikeda W, Nakanishi H, Tanaka Y, Takekuni K, Itoh S, Monden M, Takai Y. Association of frabin with specific actin and membrane structures. Genes Cells. 2002;7:413–20.

Kutateladze TG. Phosphatidylinositol 3-phosphate recognition and membrane docking by the FYVE domain. Biochim Biophys Acta. 2006;1761:868–77.

Lecompte O, Poch O, Laporte J. PtdIns5P regulation through evolution: role in membrane trafficking? Trends Biochem. Sci.. 2008;33:453–60.

Lemmon MA. Pleckstrin homology domains: not just for phosphoinositides. Biochem Soc Trans. 2004;32:707–11.

Nakanishi H, Takai Y. Frabin and other related Cdc42-specific guanine nucleotide exchange factors couple the actin cytoskeleton with the plasma membrane. J Cell Mol Med. 2008;12:1169–76.

Obaishi H, Nakanishi H, Mandai K, Satoh K, Satoh A, Takahashi K, Miyahara M, Nishioka H, Takaishi K, Takai Y. Frabin, a novel FGD1-related actin filament-binding protein capable of changing cell shape and activating c-Jun N-terminal kinase. J Biol Chem. 1998;273:18697–700.

Ono Y, Nakanishi H, Nishimura M, Kakizaki M, Takahashi K, Miyahara M, Satoh-Horikawa K, Mandai K, Takai Y. Two actions of frabin: direct activation of Cdc42 and indirect activation of Rac. Oncogene. 2000;19:3050–8.
Pasteris NG, Cadle A, Logie LJ, Porteous ME, Schwartz CE, Stevenson RE, Glover TW, Wilroy RS, Gorski JL. Isolation and characterization of the faciogenital dysplasia (Aarskog-Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor. Cell. 1994;79:669–78.
Rossman KL, Worthylake DK, Snyder JT, Siderovski DP, Campbell SL, Sondek J. A crystallographic view of interactions between Dbs and Cdc42: PH domain-assisted guanine nucleotide exchange. EMBO J. 2002;21:1315–26.
Sankaran VG, Klein DE, Sachdeva MM, Lemmon MA. High-affinity binding of a FYVE domain to phosphatidylinositol 3-phosphate requires intact phospholipid but not FYVE domain oligomerization. Biochemistry. 2001;40:8581–7.
Stendel C, Roos A, Deconinck T, Pereira J, Castagner F, Niemann A, Kirschner J, Korinthenberg R, Ketelsen UP, Battaloglu E, Parman Y, Nicholson G, Ouvrier R, Seeger J, De Jonghe P, Weis J, Krüttgen A, Rudnik-Schöneborn S, Bergmann C, Suter U, Zerres K, Timmerman V, Relvas JB, Senderek J. Peripheral nerve demyelination caused by a mutant Rho GTPase guanine nucleotide exchange factor, frabin/FGD4. Am J Hum Genet. 2007;81:158–64.
Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev. 2001;81:153–208.
Takenawa T, Suetsugu S. The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat Rev Mol Cell Biol. 2007;8:37–48.
Umikawa M, Obaishi H, Nakanishi H, Satoh-Horikawa K, Takahashi K, Hotta I, Matsuura Y, Takai Y. Association of frabin with the actin cytoskeleton is essential for microspike formation through activation of Cdc42 small G protein. J Biol Chem. 1999;274:25197–200.
Yasuda T, Ohtsuka T, Inoue E, Yokoyama S, Sakisaka T, Kodama A, Takaishi K, Takai Y. Importance of spatial activation of Cdc42 and Rac small G proteins by frabin for microspike formation in MDCK cells. Genes Cells. 2000;5:583–91.

Fractalkine

► CX3CL1

Fragile Histidine Triad Gene

► FHIT

Frizzled-1

► FZD (Frizzled)

Frizzled-10

► FZD (Frizzled)

Frizzled-2

► FZD (Frizzled)

Frizzled-3

► FZD (Frizzled)

Frizzled-4

► FZD (Frizzled)

Frizzled-5

► FZD (Frizzled)

Frizzled-6

► FZD (Frizzled)

Frizzled-7

► FZD (Frizzled)

Frizzled-8

► FZD (Frizzled)

Frizzled-9

► FZD (Frizzled)

FRS2

Noriko Gotoh
Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan

Synonyms

FRS2 family members: FRS2α; FRS2β; FRS3; suc 1-associated neurotrophic factor target (SNT-1); suc 1-associated neurotrophic factor target (SNT-2)

Historical Background

FRS2 stands for fibroblast growth factor (FGF) receptor substrate 2. The FGF receptor substrate 1 is phospholipase (PLC) γ, which was identified as the first substrate of FGF receptor tyrosine kinases (Gotoh et al. 2004). Historically, SNT was identified as a protein that is tyrosine phosphorylated in response to nerve growth factor (NGF) or ▶ FGF in PC12 cells and can bind to $p13^{suc1}$, a yeast cyclin–dependent kinase binding protein, immobilized on agarose (Rabin et al. 1993). It was reported that there is a good correlation between the tyrosine phosphorylation of SNT and differentiation of PC12 cells. Then FRS2 was purified and cloned as a novel protein that is tyrosine phosphorylated in NIH3T3 cells in response to FGF (Kouhara et al. 1997). It turned out to be the same protein as SNT. Another protein that has homology with FRS2 was found in one of the expression sequence tag (EST) fragments. After the full-length cDNA of this protein was cloned, it was named as FRS2β and the original FRS2 was renamed as FRS2α {Gotoh, 2004 #1}. Other groups also identified this protein and named it SNT-2 or FRS3 (Xu et al. 1998; McDougall et al. 2001).

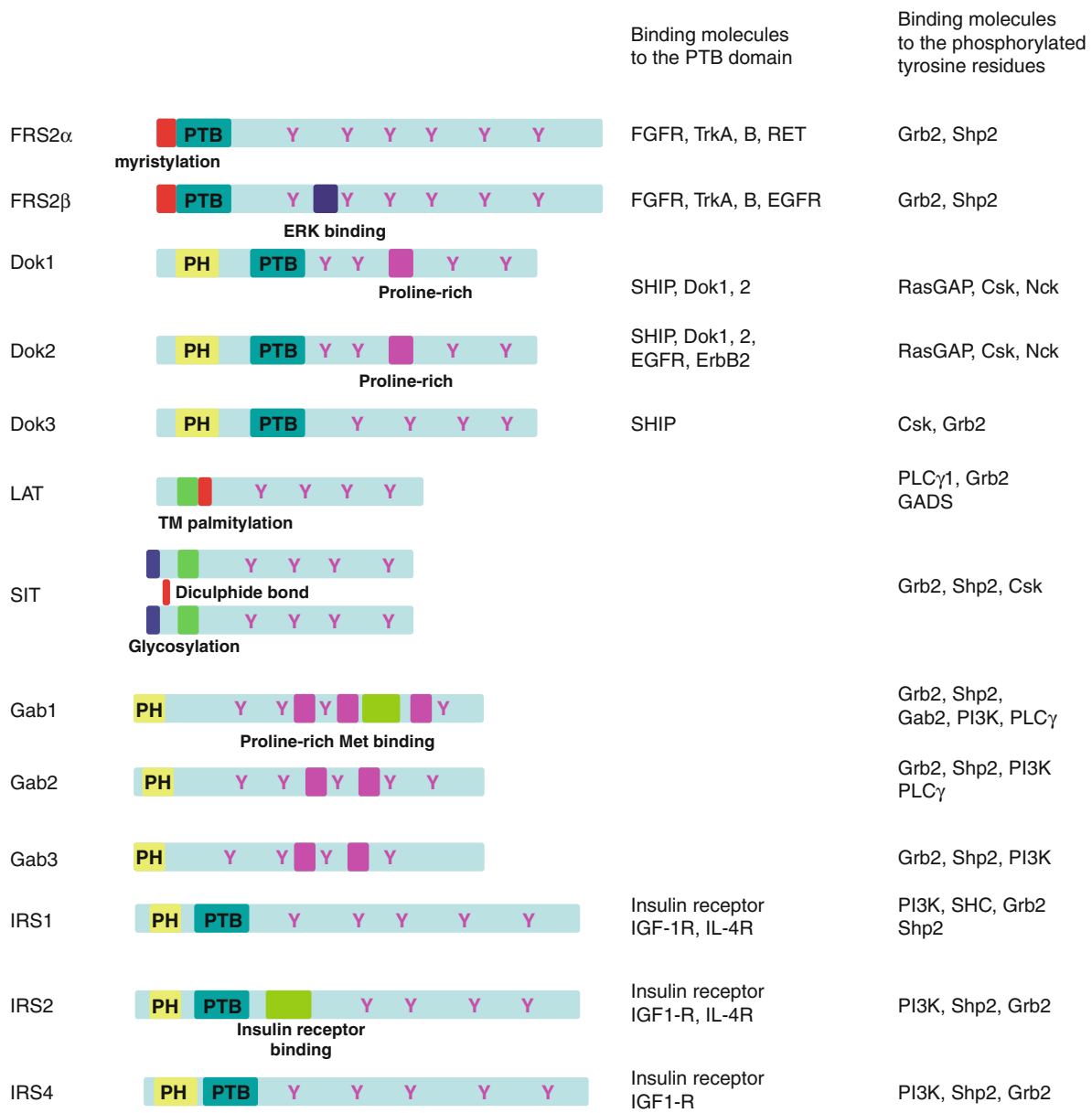
FRS2 Family Proteins Belong to a Group of Membrane-Linked Docking/Adaptor Proteins (MLDP)

Signal transduction pathways through receptor tyrosine kinases (RTKs) play pivotal roles in a number of aspects of physiological and pathological biology including cancer (Lemmon and Schlessinger 2010). Ample amounts of evidence indicate that proteins lacking catalytic activity, so-called scaffolding adaptor proteins, relay many key events of signal transduction from upstream components such as receptors to downstream elements (Pawson 2007). The scaffolding adaptor proteins that are upstream of RTK signal transduction are classified into two groups. One is comprised of docking proteins that have multiple tyrosine phosphorylation sites to dock downstream signaling proteins. They also often have other domains to bind other molecules. This group includes FRS2-, a new SH2-containing sequence (Shc), Grb2-associated binder (Gab)-, insulin receptor substrate (IRS)-, downstream of kinase (Dok)-family proteins-, and transmembrane adaptor proteins (TRAP). Several proteins in this group are subdivided into a group of membrane-linked docking protein (MLDP) (Fig. 1) (Gotoh and Tsuchida 2008). MLDP is localized in the lipid component of plasma membrane, since the protein contains membrane-anchor domain of a stretch of hydrophobic amino acid residues, pleckstrin homology (PH) domain, or transmembrane domain at or close to N-terminus. The other is comprised of adaptor proteins in a narrow sense. They have only SH3 or/and SH2 domains to bind signaling proteins. This group includes Grb2, Crk, and Nck.

Some of the scaffolding adaptor proteins have phosphotyrosine binding (PTB) domain or Src homology (SH)2 domain to bind specific residues containing a tyrosine residue that becomes phosphorylated by activated RTKs or other tyrosine kinases. All the scaffolding adaptor proteins act in the specification and/or amplification of the signal transduction pathway. Since these scaffolding proteins were first discovered almost two decades ago, signal transduction pathways have emerged as a very complex network. FRS2 proteins are typical scaffolding adaptor proteins.

Domain Structure of FRS2 Proteins

FRS2α and FRS2β are similar in structure. Both proteins contain amino acid residues MGSCCS, a consensus myristylation sequence (MGXXXS/T), at the N-terminus for binding to lipids in the membrane structure, including plasma membrane, constitutively (Fig. 2). Each has a PTB domain and multiple tyrosine phosphorylation sites at the C-terminus. FRS2α has six tyrosine phosphorylation sites and FRS2β has five. The PTB domains are highly homologous and 72% of

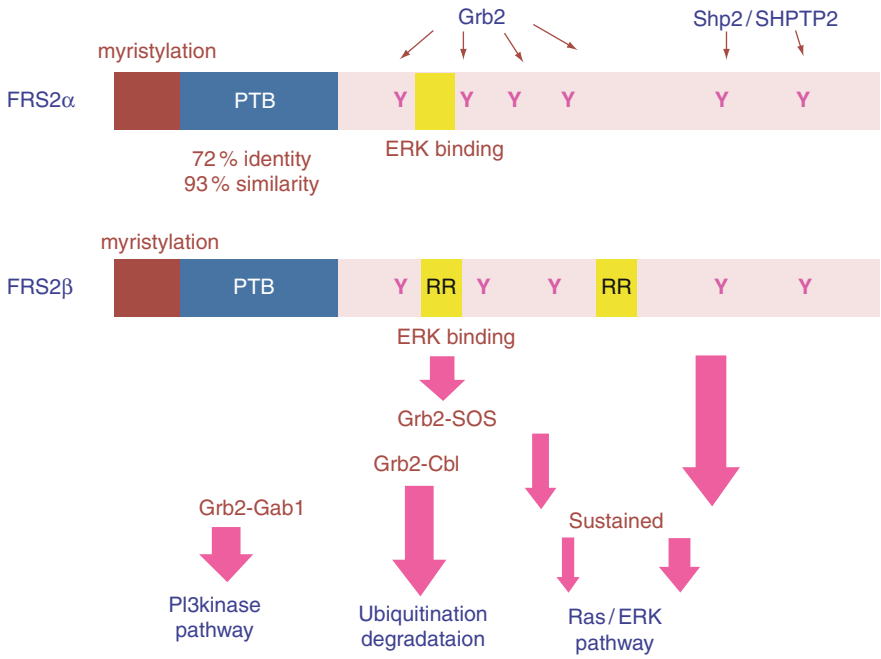


FRS2, Fig. 1 Model of signaling through membrane-linked docking protein (MLDP). Schematic structure of several members of MLDPs. The phosphotyrosine binding domain (PTB) binds to phosphrylated tyrosine residues on receptors or signaling proteins. The pleckstrin homology (PH) domain binds to phospholipids such as phosphatidyl inositol (PI)-3 phosphate. The proline-rich domains are important for binding to SH3 containing proteins such as Src family tyrosine kinases or Grb2. Y designates potential tyrosine (Y) phosphorylation sites. The ERK binding domain in FRS2β, Met binding domain in Gab1, or insulin receptor binding domain in IRS2 contains a unique sequence for binding to ERK, Met or insuling receptor

amino acids are identical. The residues surrounding each tyrosine phosphorylation site are similar but more than ten amino acids from the sites there is no similarity. Both proteins have binding sites for ERK; arginine residues (R) are critical for ERK binding to FRS2β.

Molecular Functions of FRS2α

FRS2α proteins act as docking proteins downstream in certain types of RTKs, including FGF receptors (FGFR)s, neurotrophin receptors (TrkA, TrkB, TrkC), Eph (EphA4), RET, and ALK (Gotoh 2008). In particular, emerging evidence indicates that FRS2α



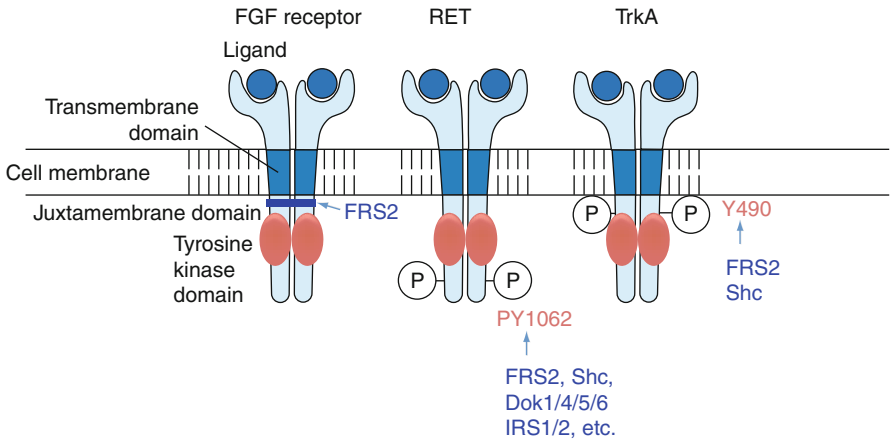
FRS2, Fig. 2 Schematic structures of FRS2 family proteins. The PTB domains of FRS2α and FRS2β have 72% identity and 93% similarity in amino acids. Tyrosine-phosphorylated Grb2 binding sites activate Ras/ERK, ubiquination/degradation, and PI-3 kinase pathways. Tyrosine-phosphorylated Shp2 binding sites activate Ras/ERK pathway stronger than the Grb2 binding site. Arginine residues (R) in the ERK binding domain of FRS2β are essetinal for binding to ERK

acts as a central mediator for intracellular signaling in response to FGF, as described in detail below. FRS2α proteins bind to these RTKs via the PTB domain and become phosphorylated on tyrosine residues upon activation of these RTKs. In contrast, FRS2α proteins are not good substrates for other RTKs, insulin receptors, platelet-derived growth factor receptors, epidermal growth factor receptors (EGFR), and so on. Selectivity, in that FRS2α proteins choose only some tyrosine kinases for phosphorylation, is characteristic of this protein family.

The PTB domain is able to bind to specific peptides with or without tyrosine-phosphorylated residues. FRS2 proteins bind to non-phosphorylated peptides at the juxtamembrane domain of the FGFR via their PTB domains independently on ligand binding and receptor activation (Fig. 3). In contrast, binding between the PTB domain of FRS2α, TrkA and TrkB, or RET is mediated by tyrosine-phosphorylated peptides that have NPXY motif (X is any amino acid reside and Y is phosphorylated) in the receptors and is dependent on activation of the RTKs.

Molecular functions of FRS2α are well studied in FGF signaling (Fig. 2) (Lemmon and Schlessinger 2010; Gotoh 2008; Eswarakumar et al. 2005). When FGFR is activated, FRS2α becomes tyrosine phosphorylated and creates two specific binding sites for Shp2, the SH2-containing tyrosine phosphatase, and four binding sites for the SH2 domain of Grb2. Several proteins are constitutively bound via two SH3 domains of Grb2: SOS, Cbl and Gab1. SOS is a guanine nucleotide exchange factor for Ras, and FRS2α-mediated recruitment of Grb2-SOS induces activation of the Ras/extracellular signal-regulated kinase (ERK) pathway. Cbl functions as an E3 ubiquitin ligase and the ternary complex FRS2α-Grb2-Cbl results in ubiquitination and degradation of FRS2α and its receptors. This is one of the negative feedback mechanisms of FGF signaling. The ternary complex FRS2α-Grb2-▶ Gab1 enables tyrosine phosphorylation of Gab1 followed by recruitment of phosphatidyl inositol (PI)-3 kinase and activation of a cell survival pathway. SOS binds to the N-terminal SH3 domain of Grb2, Gab1 binds to the C-terminal SH3 domain of Grb2, and Cbl binds to both N- and C-terminal SH3 domains of Grb2. Thus, FRS2α assembles both positive and negative signaling proteins to mediate a balanced signal transduction through Grb2. The FRS2α-Shp2 complex induces tyrosine phosphorylation of Shp2 followed by strong activation of ERK in response to FGF. Tyrosine-phosphorylated Shp2 activates its own tyrosine phosphatase, resulting in strong activation of the Ras/ERK pathway in numerous cell contexts. The Grb2-binding sites of FRS2α activate ERK at more moderate levels.

Signaling pathways activated through Trks and RET downstream of FRS2α upon activation of these



FRS2, Fig. 3 Binding sites of FRS2 proteins in RTKs. FRS2 binds to unphosphorylated peptides in the juxamembrane domain of FGF receptor. It binds to tyrosine-phosphorylated peptides in RET and TrkA and the binding sites are shared with those of other PTB domain-containing signaling proteins

RTKs appear to be similar to FGF signaling. Tyrosine phosphorylation of FRS2α is also induced by the activation of EphA4. FGFRs and EphA4 form complex proteins by direct interactions between the juxtamembrane domain of FGFRs and the N-terminal portion of the tyrosine kinase domain of EphA4. Activation of EphA4 leads to the activation of FGFRs and tyrosine phosphorylation of FRS2α.

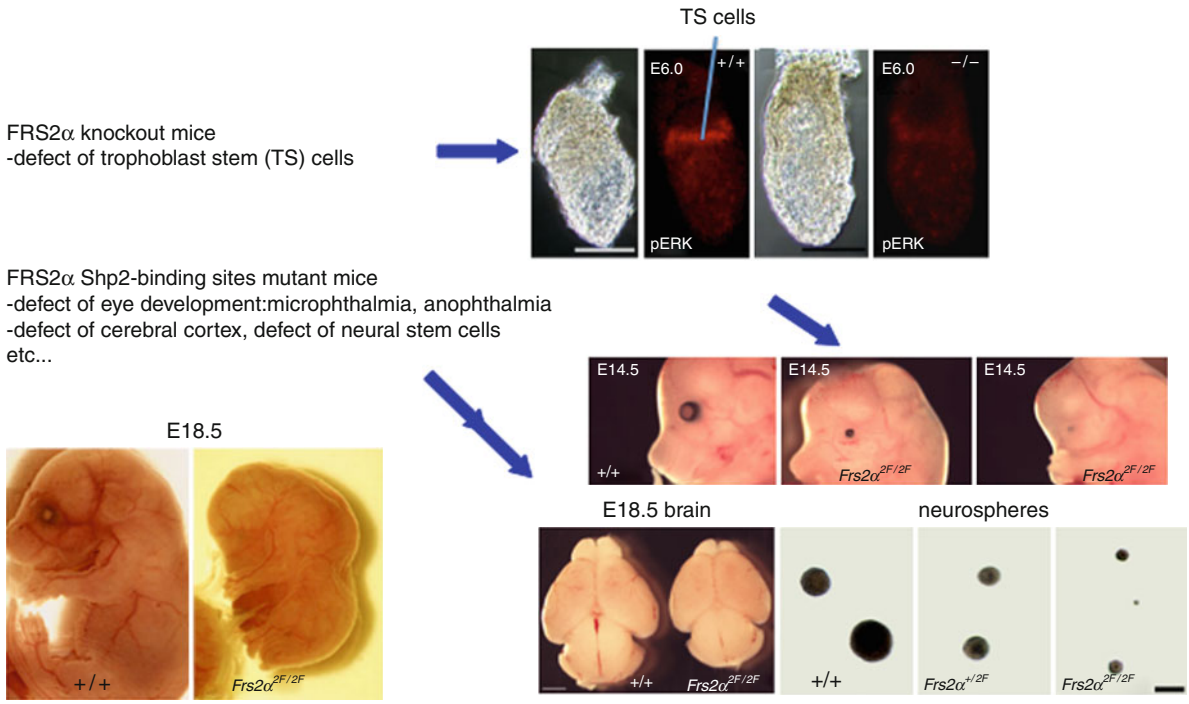
FRS2α is phosphorylated by ERK at multiple threonine residues in response to a variety of ligands, FGF, insulin, EGF, and PDGF. These include extracellular stimuli that do not induce tyrosine phosphorylation of FRS2α. There are eight canonical ERK phosphorylation sites (PXTP motif) in FRS2α. In addition, activated ERK binds to threonine phosphorylated FRS2α (Fig. 2). Thus there is an ERK-mediated negative feedback mechanism for the control of signaling pathways that are dependent on FRS2α.

Physiological Roles of FRS2α

The expression of *Frs2α* can be detected at embryonic day (E) 5.5 in mouse embryos and is expressed ubiquitously during development. FRS2α play critical roles for a variety of FGF-induced developmental processes and maintenance of tissue type–specific stem cells that are dependent on FGF. Several *Frs2α* mutant mice were generated: *Frs2α*-knockout mice and two knock-in mice that express a mutant form of FRS2α whose tyrosine residues of Grb2 ($Frs2\alpha^{4F}$ mutant)- or Shp2 ($Frs2\alpha^{2F}$ mutant)-binding sites are replaced with phenylalanine (Fig. 4) (Gotoh 2008). Embryos of the *Frs2α*-knockout mouse have defects in the FGF4-dependent maintenance of trophoblast stem cells and show developmental retardation, resulting in embryonic lethality by E8 (Gotoh et al. 2005; Murohashi et al. 2010). $Frs2\alpha^{2F/2F}$ mice display multiple developmental defects and show perinatal death, though $Frs2\alpha^{4F/4F}$ mice can survive as adults and show no gross morphological defects except an eyelid developmental defects. Embryos of $Frs2\alpha^{2F/2F}$ mice lack carotid body, have defective eye development, and show anophthalmia (no eyes) or microphthalmia (small eyes). Neural progenitor cells of $Frs2\alpha^{2F/2F}$ mice show reduced proliferation in response to FGF stimulation. FRS2α is also essential for self-renewing activity of neural stem/progenitor cells (NSPCs) in response to FGF (Sato et al. 2010). Specific ablations of *Frs2α* in the prostate epithelium inhibit prostatic branching morphogenesis and growth (Zhang et al. 2008).

Molecular Functions of FRS2β

FRS2β binds to a few RTKs including FGFR, Trk, and ALK through the PTB domain and becomes phosphorylated on tyrosine residue, similar to FRS2α. Like FRS2α, Grb2 and Shp2 are recruited to phosphorylated tyrosine residues on FRS2β upon activation of RTKs, leading to the activation of ERK (Fig. 2). FRS2β also binds to EGFR family members, including EGFR and ErbB2, constitutively with its PTB domain regardless of the absence or presence of ligands (Fig. 5).



FRS2, Fig. 4 FRS2α is a central mediator in FGF signaling in vivo. *Frs2α* null mouse embryos showed early embryonic lethality due to a failure of maintenance of trophoblast stem (TS) cells. TS cells are dependent on FGF4 and localized in extraembryonic ectoderm (ExE). The wild type ExE is positive in pERK staining but it is weak in the mutant. The *Frs2α* $^{2F/2F}$ mice have a variety of developmental defects and some of them are reasonably explained by failure of FGF signaling

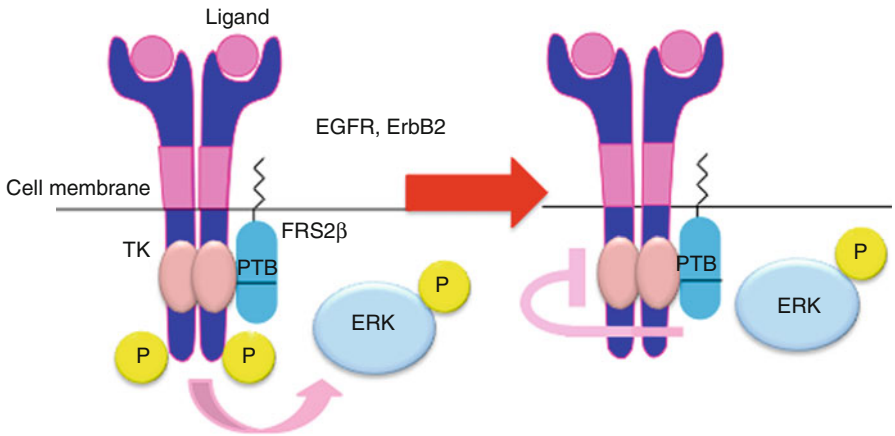
However, FRS2β is not phosphorylated by EGFR RTK in this case. Instead, FRS2β inhibits the activity of EGFR. Expression of FRS2β inhibits EGF-induced autophosphorylation of EGFR, and also decreases activation of its downstream signaling proteins. The binding between FRS2β and phosphorylated ERK is important for inhibition of EGFR signaling. This forms a negative feedback loop after the activation of ERK downstream of the activated EGFR, or to maintain the EGFR in an inactive state after the activation of ERK by other stimuli. Therefore, it appears that FRS2β is a unique scaffolding adaptor that serves as both a negative and a positive regulator for receptor species–dependent RTK signaling (Iejima et al. 2010).

Physiological Roles of FRS2β

In contrast to the rather ubiquitous expression pattern of *Frs2α*, *Frs2β* is expressed only in a limited number of tissues, including central nervous systems (CNS) and other epithelial tissues, such as lung epithelial cells and the gastrointestinal tract (Gotoh et al. 2004; Minegishi et al. 2009). Though both *Frs2α* and *Frs2β* are expressed in CNS, the expression pattern of these genes tends to be mutually exclusive. For example, though *Frs2α* is strongly expressed in the ventricular zone of mesencephalon, expression of *Frs2β* is weak there. The distinct expression patterns of *Frs2α* and *Frs2β* suggest the possibility that they have different physiological roles. In contrast to the well-known functions of FRS2α, the functions of FRS2β have long been unclear.

Relevance of FRS2 Proteins to Cancer

It is known that FGF ligands and their recepors are overexpressed in a variety of cancers, including of the breast, stomach, prostate, pancreas, bladder, and colon (Sato and Gotoh 2009). Activating mutations in FGFR3 are observed frequently in bladder and cervical carcinoma and in some cases of multiple myeloma, while activating mutations in FGFR2 are found in gastric carcinoma. Further, several types of fusion proteins involving FGFR1 caused by chromosomal translocation are found in hematologic malignancies. Given that aberrant FGF signaling is important in tumorigenesis based on all these reports, it is reasonable to predict that FRS2 proteins are also involved in tumorigenesis.



FRS2, Fig. 5 FRS2β constitutively binds to EGFR family members. Activated EGFR family members activate the ERK pathway. The activated ERK binds to FRS2β and inhibits the EGFR signalling

Somatic rearrangements of the TrkA gene are detected in a fraction of papillary thyroid carcinomas and produce chimeric proteins. All of them contain a portion of TrkA, including tyrosine kinase domain. The resultant thyroid Trk oncoproteins activate FRS2α and FRS2β. Gene rearrangements leading to fusion of the kinase domain of RET with heterologous proteins containing dimerization motifs result in constitutively activated RET proteins. Such fusion proteins are expressed in some cases of papillary thyroid carcinoma. Germ line point mutation in RET results in inherited multiple endocrine neoplasm types 2A and 2B and familial medullary thyroid carcinoma. FRS2α is tyrosine phosphorylated by ligand-stimulated and constitutively activated oncogenic forms of RET, leading to activation of ERK. The NPM-ALK oncoprotein activates FRS2α and FRS2β and transforms NIH3T3 cells.

Expression of FRS2β suppresses EGF-induced cell transformation and proliferation. High expression levels of *FRS2β* significantly correlate with good prognosis of non-small lung cancer patients (Iejima et al. 2010). These findings suggest that FRS2β is suppressive for tumorigenesis in which aberrant EGF signaling is involved.

Summary

There are two members – FRS2α and FRS2β – in the FRS2 family of docking/scaffolding adaptor proteins. These proteins function downstream of certain kinds of RTKs that are important for tumorigenesis, including the FGFR, Trk, RET, and ALK. Activation of these RTKs allows FRS2 proteins to become phosphorylated of tyrosine residues and then bind to Grb2 and Shp2, a SH2 domain-containing adaptor and a tyrosine phosphatase, respectively. Subsequently, Shp2 activates a Ras/ERK pathway and Grb2 activates a Ras/ERK, PI-3 kinase, and ubiquitination/degradation pathways by binding to SOS, Gab1, and Cbl via the SH3 domains of Grb2. FRS2α acts as a central mediator in FGF signaling. FRS2α mutant mice exhibit a variety of defects in developmental processes and in maintenance of tissue type–specific stem cells, such as trophoblast stem cells and neural stem cells; many of them are due to defects in FGF signaling. Although FRS2β binds to EGFR family RTKs, including EGFR and ErbB2, it does not become tyrosine phosphorylated. Instead, it inhibits EGF signaling, resulting in inhibition of EGF-induced cell proliferation and cell transformation. FRS2β may have a tumor suppressive role for non-small cell lung cancer.

References

Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 2005;16(2):139–49.

Gotoh N. Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins. Cancer Sci. 2008; 99(7):1319–25.

Gotoh N, Tsuchida N. Membrane-lined docking protein. Encyclopedia of Cancer. 2nd ed. Heiderlberg/Germany: Springer; 2008. p. 1819–23.

Gotoh N, Laks S, Nakashima M, Lax I, Schlessinger J. FRS2 family docking proteins with overlapping roles in activation of MAP kinase have distinct spatial-temporal patterns of expression of their transcripts. FEBS Lett. 2004;564(1–2):14–8.

Gotoh N, Manova K, Tanaka S, Murohashi M, Hadari Y, Lee A, et al. The docking protein FRS2{alpha} is an essential component of multiple fibroblast growth factor responses

during early mouse development. Mol Cell Biol. 2005;25(10):4105–16.
Iejima D, Minegishi Y, Takenaka K, Siswanto A, Watanabe M, Huang L, et al. FRS2beta, a potential prognostic gene for non-small cell lung cancer, encodes a feedback inhibitor of EGF receptor family members by ERK binding. Oncogene. 2010;29(21):3087–99.
Kouhara H, Hadari YR, Spivak-Kroizman T, Schilling J, Bar-Sagi D, Lax I, et al. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell. 1997;89(5):693–702.
Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141(7):1117–34.
McDougall K, Kubu C, Verdi JM, Meakin SO. Developmental expression patterns of the signaling adapters FRS-2 and FRS-3 during early embryogenesis. Mech Dev. 2001; 103(1–2):145–8.
Minegishi Y, Iwanari H, Mochizuki Y, Horii T, Hoshino T, Kodama T, et al. Prominent expression of FRS2beta protein in neural cells and its association with intracellular vesicles. FEBS Lett. 2009;583(4):807–14.
Murohashi M, Nakamura T, Tanaka S, Ichise T, Yoshida N, Yamamoto T, et al. An FGF4-FRS2alpha-Cdx2 axis in trophoblast stem cells induces Bmp4 to regulate proper growth of early mouse embryos. Stem Cells. 2010;28(1):113–21.
Pawson T. Dynamic control of signaling by modular adaptor proteins. Curr Opin Cell Biol. 2007;19(2):112–6.
Rabin SJ, Cleghon V, Kaplan DR. SNT, a differentiation-specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. Mol Cell Biol. 1993;13(4):2203–13.
Sato T, Gotoh N. The FRS2 family of docking/scaffolding adaptor proteins as therapeutic targets of cancer treatment. Expert Opin Ther Targets. 2009;13(6):689–700.
Sato T, Shimazaki T, Naka H, Fukami S, Satoh Y, Okano H, et al. FRS2alpha regulates Erk levels to control a self-renewal target Hes1 and proliferation of FGF-responsive neural stem/progenitor cells. Stem Cells. 2010;28(9):1661–73.
Xu H, Lee KW, Goldfarb M. Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. J Biol Chem. 1998; 273(29):17987–90.
Zhang Y, Zhang J, Lin Y, Lan Y, Lin C, Xuan JW, et al. Role of epithelial cell fibroblast growth factor receptor substrate 2alpha in prostate development, regeneration and tumorigenesis. Development. 2008;135(4):775–84.

FRS2 Family Members: FRS2α

▶ FRS2

FRS2β

▶ FRS2

FRS3

▶ FRS2

Fus3 (*Saccaromyces cerevisiae*)

▶ ERK1/ERK2

Fyn-Binding Protein (FYB)

▶ ADAP

FZD

▶ FZD (Frizzled)

FZD (Frizzled)

Masaru Katoh
Division of Integrative Omics and Bioinformatics, National Cancer Center, Tokyo, Japan

Synonyms

Dfz1; Dfz2; Frizzled-1; Frizzled-2; Frizzled-3; Frizzled-4; Frizzled-5; Frizzled-6; Frizzled-7; Frizzled-8; Frizzled-9; Frizzled-10; FZD; FZD1; FZD2; FZD3; FZD4; FZD5; FZD6; FZD7; FZD8; FZD9; FZD10; Hfz5; Hfz6

Historical Background

Drosophila frizzled (Dfz1) was originally identified as a causative gene for a mutant with disoriented cuticular hairs, and then was cloned and characterized as a seven-transmembrane-type protein similar to G protein-coupled receptors (GPCRs) (Vinson and Adler 1987; Lagerström and Schiöth 2008).

The phenotype of *Dfz1* mutant was aberrant polarization of epithelial cells within the epithelial plane, or dysregulation of planar cell polarity (PCP). *Drosophila* mutants of *dishevelled*, *Van Gogh (strabismus)*, *prickle*, *diego*, and *starry night (flamingo)* show phenotypes similar to *Dfz1* mutant. Dfz1, Dishevelled, Van Gogh (strabismus), prickle, diego, and starry night are characterized as core PCP components (Katoh 2005; Wang 2009; Wu and Mlodzik 2009).

Drosophila frizzled-2 (Dfz2) was cloned and characterized as a seven-transmembrane-type receptor for secreted glycoprotein Wingless, which is involved in the patterning of embryonic segments and their adult derivatives (Bhanot et al. 1996; Zhang and Carthew 1998). Genetic interaction experiments revealed that Dfz2, Wingless, Dishevelled, and Armadillo belong to the WNT group of segment polarity components.

FZD1 (Frizzled-1), FZD2 (Frizzled-2), FZD3 (Frizzled-3), FZD4 (Frizzled-4), FZD5 (Frizzled-5 or Hfz5), FZD6 (Frizzled-6 or Hfz6), FZD7 (Frizzled-7), FZD8 (Frizzled-8), FZD9 (Frizzled-9), and FZD10 (Frizzled-10) constitute the human Frizzled family of atypical GPCRs functioning as WNT receptors (Zhao et al. 1995; Wang et al. 1996; Koike et al. 1999; Katoh 2007).

Structural Features of Frizzled Receptors

Frizzled family members share the conserved domain architecture consisting of N-terminal extracellular cysteine-rich domain (Frizzled-like CRD), seven transmembrane domains, and cytoplasmic K-T-X-X-X-W motif. Frizzled-like CRD is a WNT-binding domain, which is conserved among Frizzled family members (Wang et al. 1996), secreted-type WNT antagonist SFRPs (Elston and Clifton-Bligh 2010), ROR family of WNT co-receptors (Green et al. 2008), and single transmembrane-type Frizzled-related protein (MFRP) (Katoh 2001). The K-T-X-X-X-W motif in the C-terminal cytoplasmic region is a binding motif for the PDZ domain of Dishevelled family of scaffold proteins (Wong et al. 2003).

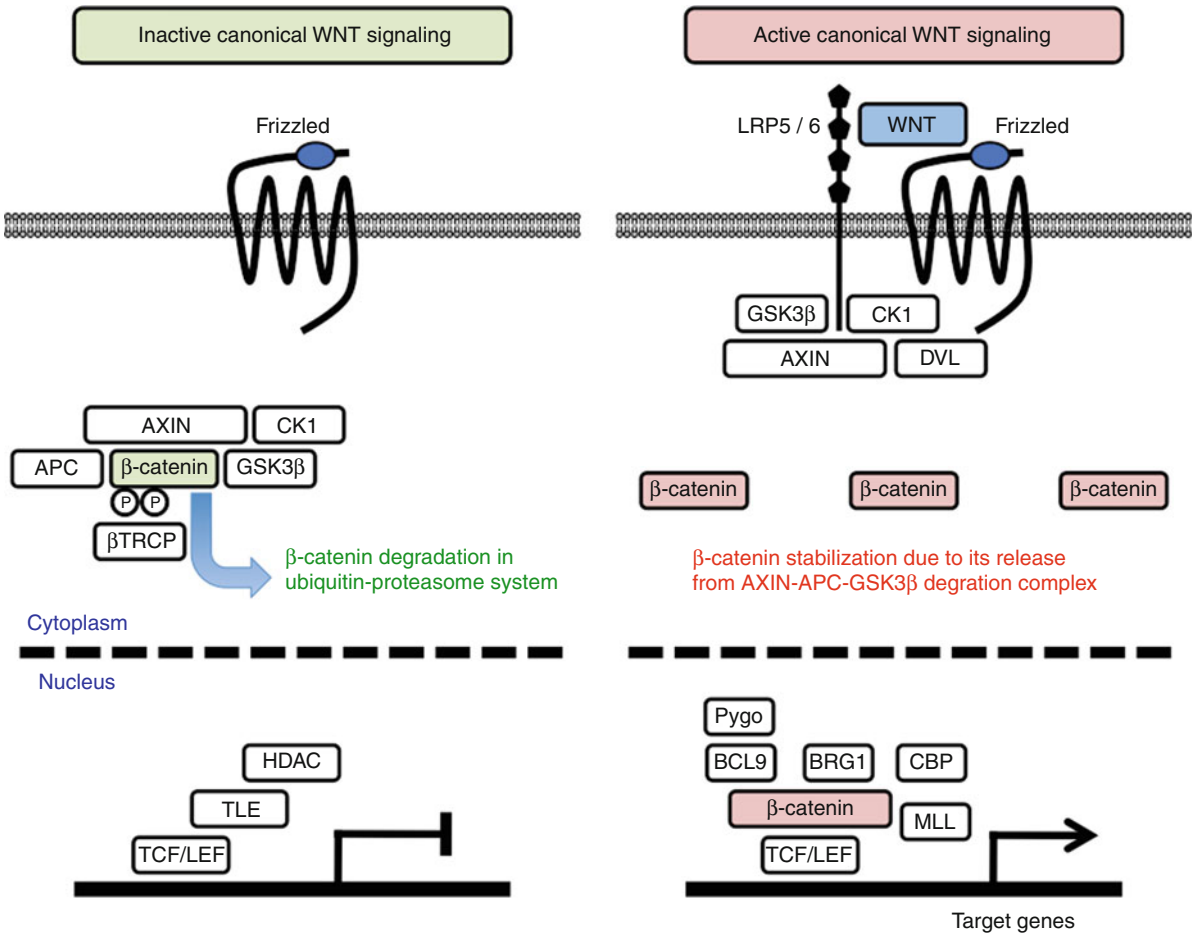
Context-Dependent WNT Signaling Through Frizzled Receptors

Frizzled receptors transduce WNT signals via cytoplasmic Dishevelled family members to β-catenin-dependent TCF/LEF transactivation cascade (Barker and Clevers 2006) and β-catenin-independent RhoA-ROCK, RhoB-Rab4, Rac-JNK, and aPKC signaling cascades (Chien et al. 2009). Frizzled receptors also transduce WNT signals via phospholipase C (PLC) to β-catenin-independent MAP3K7-NLK, Calcineurin-NFAT, and DAG-PKC signaling cascades (Chien et al. 2009). Canonical WNT signaling cascade is defined as the β-catenin-dependent TCF/LEF branch, whereas noncanonical WNT signaling cascades as a variety of β-catenin-independent branches. WNT signals are transduced to canonical and noncanonical signaling cascades in a context-dependent manner based on the combination of WNT ligands, Frizzled receptors, other co-receptors, and WNT antagonists (Swain et al. 2005; Mikels and Nusse 2006; Katoh and Katoh 2007).

Canonical WNT Signaling Cascade

In the absence of canonical WNT signals, β-catenin forms a ternary complex with AXIN and APC to undergo CK1-mediated priming phosphorylation at Ser 45 and GSK3β-mediated subsequent phosphorylation at Thr 41, Ser 37, and Ser 33 (Fig. 1). Phosphorylated β-catenin is recognized by F-box proteins βTRCP1 or βTRCP2 to be poly-ubiquitinated by an E3 ubiquitin ligase complex for its degradation in the proteasome system (Frescas and Pagano 2008).

In the presence of canonical WNT signals, Frizzled-Dishevelled complex is indirectly associated with LRP5/6 co-receptor via extracellular WNT ligand (Fig. 1). Dishevelled recruits AXIN based on DIX-DIX hetero-dimerization, and then AXIN directly binds to LRP5/6, which results in the assembly of canonical WNT signalosome consisting of WNT ligand, Frizzled-Dishevelled complex, and LRP5/6-AXIN complex (Fig. 1). Due to its release from the AXIN-APC degradation complex, β-catenin is stabilized for nuclear accumulation and subsequent association with TCF/LEF family of HMG-box transcription factors. Because nuclear β-catenin is also associated with BCL9-PYGO co-activator complex, SWI/SNF-related chromatin regulator BRG1, histone acetyl transferase CBP/p300, TRRAP, and histone methyltransferase MLL, stabilized β-catenin induces transcriptional activation of TCF/LEF target genes



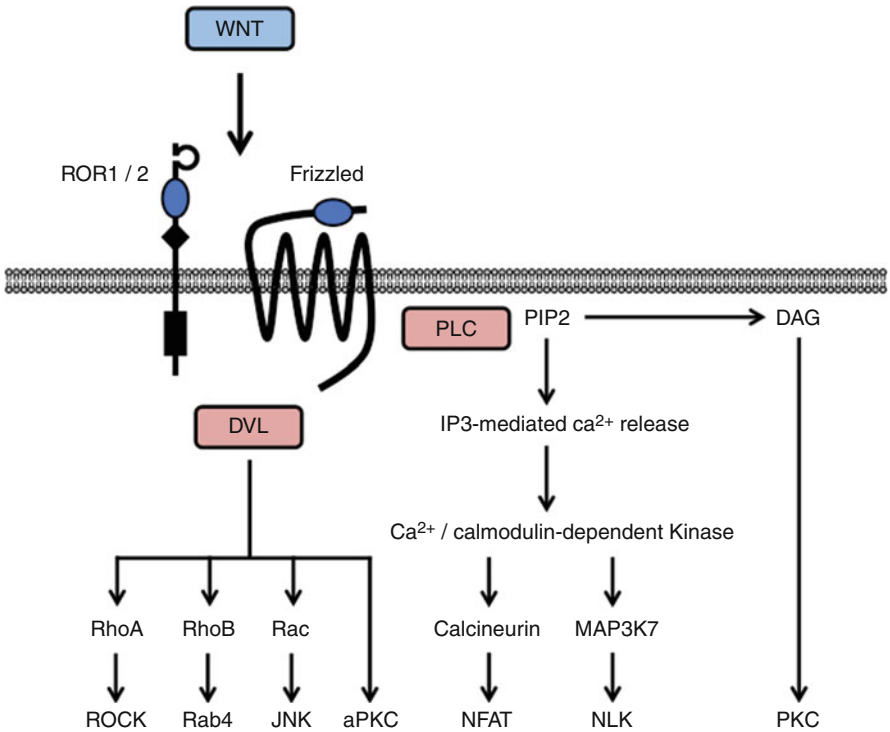
FZD (Frizzled), Fig. 1 Canonical WNT signaling cascade. Canonical WNT signals are transduced through Frizzled to the TCF/LEF transactivation cascade in a β-catenin-dependent manner. (*Left*) In the absence of canonical WNT signals, β-catenin is phosphorylated by CK1 and GSK3β in the AXIN-APC degradation complex. Phosphorylated β-catenin is poly-ubiquitinated by an E3 ubiquitin ligase complex for its degradation in the proteasome system. (*Right*) In the presence of canonical WNT signals, assembly of canonical WNT signalosome consisting of WNT ligand, Frizzled-Dishevelled complex, and LRP5/6-AXIN complex leads to the release of β-catenin from the AXIN-APC degradation complex. Stabilized β-catenin is translocated to the nucleus for the subsequent association with TCF/LEF transcription factors. Because nuclear β-catenin is also associated with multiple co-activators, stabilized β-catenin induces transcriptional activation of TCF/LEF target genes such as *FZD7, LEF1, DKK1, AXIN2, βTRCP1, MYC*, and *CCND1*

(Fig. 1). *FZD7, LEF1, DKK1, AXIN2, βTRCP1, MYC*, and *CCND1* are representative target genes of the canonical WNT signaling cascade (Barker and Clevers 2006; Katoh and Katoh 2007). FZD7 and LEF1 are involved in positive-feedback regulation, whereas DKK1, AXIN2, βTRCP1 in negative-feedback regulation. Because MYC and CCND1 induce cell-cycle progression, canonical WNT signals promote self-renewal of stem cells and proliferation of progenitor or transit-amplifying cells in physiological and pathological situations.

Noncanonical WNT Signaling Cascades

Noncanonical WNT signals are transduced to a variety of signaling cascades, including RhoA-ROCK, RhoB-Rab4, Rac-JNK, aPKC, MAP3K7-NLK, Calcineurin-NFAT, and DAG-PKC branches (Fig. 2).

Noncanonical WNT signals are transduced to RhoA-ROCK, RhoB-Rab4, Rac-JNK, and aPKC signaling cascades in a Dishevelled-dependent and β-catenin-independent manner. Dishevelled-dependent RhoA-ROCK and Rac-JNK signaling branches are involved



FZD (Frizzled),
Fig. 2 Noncanonical WNT signaling cascades. Noncanonical WNT signals are transduced through Frizzled receptors to a variety of downstream cascades in a β-catenin-independent manner. Dishevelled mediates noncanonical WNT signaling to RhoA-ROCK, RhoB-Rab4, Rac-JNK, and aPKC branches, whereas phospholipase C (PLC) mediates noncanonical WNT signaling to MAP3K7-NLK, Calcineurin-NFAT, and DAG-PKC branches

in PCP or polarized cell movements. DAAM1 and DAAM2 are Formin family proteins regulating the actin-filament dynamics, such as nucleation, elongation, and barbed-end capping. DAAM proteins are tethered in the cytoplasm based on intramolecular interaction in the absence of noncanonical WNT signals, while DAAM proteins are recruited to the membranous Frizzled-Dishevelled complex based on intermolecular interaction between DAAM and Dishevelled in the presence of noncanonical WNT signals. Because DAAM in the Frizzled-Dishevelled complex preferentially interacts with GTP-bound RhoA, noncanonical WNT signals activate the RhoA-ROCK signaling cascade via Dishevelled and DAAM to regulate actin remodeling during fetal morphogenesis and tumor invasion (Katoh 2005; Wang 2009; Chien et al. 2009).

Noncanonical WNT signals activate PLC for the catalysis of phosphatidylinositol diphosphate (PIP2) to inositol triphosphate (IP3) and DAG, because Frizzled receptors are GPCR superfamily members (Lagerström and Schiöth 2008; Chien et al. 2009). IP3 triggers Ca^{2+} release from endoplasmic reticulum for the activation of MAP3K7-NLK and Calcineurin-NFAT signaling cascades, while DAG induces PKC activation. MAP3K7 is a mitogen-activated protein kinase kinase kinase (MAP3K), which phosphorylates NLK, IKK, MAP2K3 (MEK3), and MAP2K6 (MEK6). Noncanonical WNT signals induces PLC-mediated Ca^{2+} release for the MAP3K7 activation, which leads to phosphorylation and subsequent activation of NLK. NLK then phosphorylates TCF/LEF to inhibit the transcriptional activation of the canonical WNT signaling cascade. Therefore, noncanonical WNT signaling to the MAP3K7-NLK branch switches off the canonical WNT signaling cascade.

Pathophysiology of Frizzled Family Members

FZD3 and *FZD6* genes are human orthologs of *Dfz1*, which is involved in PCP (Katoh 2005). Double knockout mice for *Fzd3* and *Fzd6* show PCP phenotypes characterized by neural tube closure defect (NTD) and disoriented sensory hair cells of inner ear (Wang 2009), whereas single nucleotide polymorphisms (SNPs) of human *FZD3* or *FZD6* were not associated with NTD in a case-control study, including 338 cases of NTD infants and 338 non-malformed infants (Wen et al. 2010).

FZD4 gene at human chromosome 11q14.2 is mutated in autosomal-dominant familial exudative vitreoretinopathy (FEVR) and sporadic exudative vitreoretinopathy featured by variable clinical manifestations, such as retinal exudates, retinal neovascularization, retinal detachment, vitreous hemorrhage, and total blindness (Kirikoshi et al. 1999; Kondo et al. 2003).

FZD7 is expressed in blastocyst, undifferentiated embryonic stem (ES) cells, ES-derived endodermal progenitors, ES-derived neural progenitors, fetal cochlea, adult gastrointestinal tract, and regenerating liver. *FZD7* expression on mesenchymal stem cells is downregulated during osteogenesis. *FZD7* is upregulated in gastric cancer, esophageal cancer, colorectal cancer, and liver cancer. *FZD7* is one of the target genes of the canonical WNT signaling cascade as mentioned above. FZD7 transduces WNT3, WNT6, or WNT8B signals to the canonical WNT signaling cascade, and also WNT11 or WNT5A signals to the noncanonical WNT signaling cascades. FZD7 orchestrates cellular proliferation, epithelialization, migration, and tissue movement in a context-dependent manner during embryogenesis, adult-tissue homeostasis, and carcinogenesis (Sagara et al. 1998; Vincan and Barker 2008).

FZD9 gene at human chromosome 7q11.23 was initially cloned and characterized as one of genes within the region commonly deleted in patients with Williams syndrome, which is a neurodevelopmental disorder with dysmorphic facial features, cardiovascular disease, mild mental retardation, and a unique cognitive profile; however, because *FZD9* gene is not always deleted in patients with Williams syndrome, it is believed that *FZD9* is not responsible for the major features of the Williams syndrome (Botta et al. 1999).

FZD10 gene at human chromosome 12q24.33 was cloned and characterized as the last member of the Frizzled family (Koike et al. 1999). *FZD10* is upregulated in several types of human cancer, such as cervical cancer, glioblastoma, colorectal cancer, and synovial sarcoma (Koike et al. 1999; Nagayama et al. 2005). Anti-FZD10 polyclonal antibody effectively mediates antibody-dependent cell-mediated cytotoxicity (ADCC) against synovial sarcoma cells in nude mice xenograft model (Nagayama et al. 2005), and phase I study of anti-FZD10 monoclonal antibody conjugated with radioisotope started for its clinical application.

Summary

Frizzled family members are atypical GPCRs functioning as WNT receptors. Canonical WNT signals are transduced through Frizzled to the TCF/LEF transactivation cascade in a β-catenin-dependent manner to upregulate target genes, such as *FZD7, LEF1, DKK1, AXIN2, βTRCP1, MYC*, and *CCND1*. Noncanonical WNT signals are transduced through Frizzled to a variety of downstream cascades in a β-catenin-independent manner. Dishevelled-mediated noncanonical WNT signaling to RhoA-ROCK and Rac-JNK signaling cascades are involved in PCP and cellular migration during embryonic morphogenesis and tumor invasion, whereas PLC-mediated noncanonical WNT signaling to MAP3K7-NLK signaling cascade is involved in the inhibition of the canonical WNT signaling cascade. Because WNT-Frizzled signaling cascades play key roles in a variety of cellular processes during embryogenesis, adult-tissue homeostasis, and carcinogenesis, Frizzled receptors are utilized as stem-cell markers in the field of regenerative medicine, and also as therapeutic targets in the field of clinical oncology.

Cross-References

- Beta-Catenin
- Myc
- ROCK Kinases

References

Barker N, Clevers H. Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov. 2006;5:997–1014.

Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, et al. A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. Nature. 1996;382:225–30.

Botta A, Novelli G, Mari A, Novelli A, Sabani M, Korenberg J, et al. Detection of an atypical 7q11.23 deletion in Williams syndrome patients which does not include the *STX1A* and *FZD9* genes. J Med Genet. 1999;36:478–80.

Chien AJ, Conrad WH, Moon RT. A Wnt survival guide: from flies to human disease. J Invest Dermatol. 2009;129:1614–27.

Elston MS, Clifton-Bligh RJ. Identification of Wnt family inhibitors: a pituitary tumor directed whole genome approach. Mol Cell Endocrinol. 2010;326(1–2):48–54.

Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and β-TrCP: tipping the scales of cancer. Nat Rev Cancer. 2008;8:438–49.

Green JL, Kuntz SG, Sternberg PW. Ror receptor tyrosine kinases: orphans no more. Trends Cell Biol. 2008;18:536–44.
Katoh M. Molecular cloning and characterization of MFRP, a novel gene encoding a membrane-type Frizzled-related protein. Biochem Biophys Res Commun. 2001;282:116–23.
Katoh M. WNT/PCP signaling pathway and human cancer. Oncol Rep. 2005;14:1583–8.
Katoh M. Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. Stem Cell Rev. 2007;3:30–8.
Katoh M, Katoh M. WNT signaling pathway and stem cell signaling network. Clin Cancer Res. 2007;13:4042–5.
Kirikoshi H, Sagara N, Koike J, Sekihara H, Hirai M, Katoh M. Molecular cloning and characterization of human *Frizzled-4* on chromosome 11q14-q21. Biochem Biophys Res Commun. 1999;264:955–61.
Koike J, Takagi A, Miwa T, Hirai M, Terada M, Katoh M. Molecular cloning of *Frizzled-10*, a novel member of the *Frizzled* gene family. Biochem Biophys Res Commun. 1999;262:39–43.
Kondo H, Hayashi H, Oshima K, Tahira T, Hayashi K. *Frizzled-4* gene *(FZD4)* mutations in patients with familial exudative vitreoretinopathy with variable expressivity. Br J Ophthalmol. 2003;87:1291–5.
Lagerström MC, Schiöth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov. 2008;7:339–57.
Mikels AJ, Nusse R. Purified Wnt5a protein activates or inhibits β-catenin-TCF signaling depending receptor context. PLoS Biol. 2006;4:e115.
Nagayama S, Fukukawa C, Katagiri T, Okamoto T, Aoyama T, Oyaizu N, et al. Therapeutic potential of antibodies against FZD10, a cell-surface protein, for synovial sarcomas. Oncogene. 2005;24:6201–12.
Sagara N, Toda G, Hirai M, Terada M, Katoh M. Molecular cloning, differential expression, and chromosomal localization of human *Frizzled-1*, *Frizzled-2*, and *Frizzled-7*. Biochem Biophys Res Commun. 1998;252:117–22.
Swain RK, Katoh M, Medina A, Steinbeisser H. *Xenopus* frizzled-4 S, a splicing variant of Xfz4, is a context-dependent activator and inhibitor of Wnt/β-catenin signaling. Cell Commun Signal. 2005;3:12.
Vincan E, Barker N. The upstream components of the Wnt signalling pathway in the dynamic EMT and MET associated with colorectal cancer progression. Clin Exp Metastasis. 2008;25:657–63.
Vinson CR, Adler PN. Directional non-cell autonomy and the transmission of polarity information by the *frizzled* gene of *Drosophila*. Nature. 1987;329:549–51.
Wang Y. Wnt/Planar cell polarity signaling: a new paradigm for cancer therapy. Mol Cancer Ther. 2009;8:2103–9.
Wang Y, Macke JP, Abella BS, Andreasson K, Worley P, Gilbert DJ, et al. A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene *frizzled*. J Biol Chem. 1996;271:4468–76.
Wen S, Zhu H, Lu W, Mitchell LE, Shaw GM, Lammer EJ, et al. Planar cell polarity pathway genes and risk for spina bifida. Am J Med Genet. 2010;152A:299–304.
Wong HC, Bourdelas A, Krauss A, Lee HJ, Shao Y, Wu D, et al. Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. Mol Cell. 2003;12:1251–60.
Wu J, Mlodzik M. A quest for the mechanism regulating global planar cell polarity of tissues. Trends Cell Biol. 2009;19:295–305.
Zhang J, Carthew RW. Interactions between Wingless and Dfz2 during *Drosophila* wing development. Development. 1998;125:3075–85.
Zhao Z, Lee CC, Baldini A, Caskey CT. A human homolog of the *Drosophila* polarity gene *frizzled* has been identified and mapped to 17q21.1. Genomics. 1995;27:370–3.

FZD1

► FZD (Frizzled)

FZD10

► FZD (Frizzled)

FZD2

► FZD (Frizzled)

FZD3

► FZD (Frizzled)

FZD4

► FZD (Frizzled)

FZD5

► FZD (Frizzled)

FZD6

► FZD (Frizzled)

FZD7

► FZD (Frizzled)

FZD8

► FZD (Frizzled)

FZD9

► FZD (Frizzled)

G

G Alpha (o)

▶ G Protein α i/o/z

G Alpha z

▶ G Protein α i/o/z

G Beta

▶ G Protein Beta/Gamma

G Gamma

▶ G Protein Beta/Gamma

G Protein Alpha 12

Thomas E. Meigs[1] and Alex Lyakhovich[2]
[1]Dept. of Biology, University of North Carolina Asheville, Asheville, NC, USA
[2]Duke-National University of Singapore Graduate Medical School, Singapore

Synonyms

Concertina (*Drosophila melanogaster* homolog); Gα12; G12α; gep; Gna12; gpa-12 (*Caenorhabditis elegans* homolog)

Historical Background

Gα12, along with the closely related Gα13, is a member of the G12 subfamily of heterotrimeric guanine nucleotide-binding protein α-subunits (G proteins). These proteins are activated by GDP-GTP exchange, which is triggered by external stimulation of heptahelical G protein coupled receptors (GPCRs) and causes the Gα to dissociate from the βγ dimer of the heterotrimeric complex and serve as an active signaling moiety until inactivated by its intrinsic GTPase activity. Proteins of the G12 subfamily are posttranslationally modified by palmitoylation, facilitating their localization at the inner face of the plasma membrane. Gα12 was discovered in a PCR-based screen of a mouse cDNA library, utilizing oligonucleotides derived from conserved sequences within known Gα subunits of the ▶ Gs and Gi subfamilies (Strathmann and Simon 1991). Independent of these studies, a genetic screen for female sterile mutations in the fruit fly yielded a mutant form of the gene *concertina*, which encoded a protein harboring ~54% amino acid identity with the murine Gα12 (Parks and Wieschaus 1991). Furthermore, the *concertina* gene product was found to be necessary for proper gastrulation during embryogenesis. Northern blotting and immunoblot analyses revealed the presence of Gα12 in essentially all mammalian tissues (Radhika and Dhanasekaran 2001). The potential role of Gα12 in cell growth and tumorigenesis was discovered when Chan et al. (1993) generated a combined cDNA library from Ewing's sarcoma and synovial tumor-derived cell lines and performed a screen for cDNAs that induced aberrant growth in cultured fibroblasts. The cDNA with highest transforming potency in these

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

assays was revealed to encode human Gα12, and subsequent studies of mutationally activated (GTPase-deficient) Gα12 revealed its acute oncogenic potential in several cell types (Radhika and Dhanasekaran 2001).

The first purification of Gα12 was performed by Kozasa and Gilman (1995), who utilized baculovirus vectors to overexpress Gα12 along with mammalian heterotrimeric β and γ subunits in cultured *Spodoptera frugiperda* cells. Hexa-histidine tagging of the recombinant γ subunit enabled capture of the heterotrimer on nickel resin, and activation of Gα12 by AlF_4^- triggered its elution from the immobilized G protein βγ dimer. Although this approach did not yield levels of Gα12 necessary for crystallographic studies, it allowed the first biochemical characterization of this 43-kDa protein and revealed striking differences from the well-studied ▶ Gs and Gi subfamilies in its slow rate of nucleotide exchange, exceptionally slow rate of GTP hydrolysis, and lack of ADP-ribosylation by cholera toxin or pertussis toxin (Kozasa and Gilman 1995). Follow-up studies showed Gα12 to have the unusual property among Gα subunits as a substrate for phosphorylation by protein kinase C. In similar fashion to Gαz of the Gi subfamily, phosphorylation of Gα12 inhibited its binding to the βγ dimer, suggesting a role of this covalent modification in prolonging the activated, monomeric state of Gα12. Several years later, a chimeric Gα12 harboring the N-terminal 28 amino acids of the Gi subfamily member $Gαi_1$ was purified in sufficient quantity to allow crystallographic analysis of Gα12 in its activated conformation (Kreutz et al. 2006).

The downstream mechanisms of Gα12-mediated signaling remained unknown until the small G protein Rho was found to mediate Gα12-driven cytoskeletal changes, specifically the formation of stress fibers and assembly of focal adhesion complexes (Buhl et al. 1995). These findings led to a wide range of downstream processes that required activation of Rho by Gα12 or Gα13, and helped lead investigators to the first direct downstream binding partners of the Gα12 subfamily, a subset of Rho-specific guanine nucleotide exchange factors, or RhoGEFs (Kozasa et al. 1998). In addition to linking Gα12 to Rho, these effector proteins were shown to act as GTPase activating proteins (GAP) for the G12 subfamily, a finding of particular significance given the slow GTP hydrolysis rate of purified Gα12. However, it has become increasingly evident that Gα12 stimulates pathways in addition to a RhoGEF-Rho axis, as nearly 20 non-receptor proteins have been reported as interactors with Gα12 specifically or with both G12 subfamily members (Kelly et al. 2007).

Role of Gα12 in Cell Growth, Apoptosis, and Oncogenic Transformation

Human Gα12 was discovered as a potential transforming oncogene in a soft tissue sarcoma-derived cDNA library, and comparison to several cDNAs isolated from non-cancerous mammary epithelial cells verified this Gα12 clone as the wild-type form. Significantly, this revealed Gα12 as the only heterotrimeric Gα protein among the four subfamilies – ▶ Gs, Gi, Gq, and G12 – to harbor transforming ability as an overexpressed but not mutationally activated protein. Gα12-induced transformation is serum-dependent; however, introduction of activating point mutations, analogous to those discovered in Gαs isolated from pituitary and thyroid tumors, allow Gα12 to drive cellular transformation in the absence of serum (Radhika and Dhanasekaran 2001). The mechanisms of Gα12-induced growth activation and cellular transformation are not fully understood; however, several signaling pathways have been implicated in these responses, particularly pathways harboring mitogen-activated protein kinases (MAPK) and small G proteins of the Rho and Ras families. One of the first downstream signaling proteins reported to be activated by Gα12 signaling was the c-Jun-N-terminal kinase (JNK), which plays a role in stress responses and apoptotic signaling but also has been linked to cell proliferation and tumorigenesis. Subsequent studies revealed several transcriptional responses downstream of Gα12; constitutively active Gα12 stimulated AP-1-mediated gene expression via a mechanism sensitive to dominant-negative Ras, and also activated several primary response genes through stimulation of serum response factor (SRF). Gα12 was also shown to mediate a signaling pathway in which extracellular thrombin stimulation triggered AP-1 activation (Sah et al. 2000). Also, activated Gα12 was found to stimulate G_1-to-S phase progression in a manner dependent on Ras, Rho and Rac. Furthermore, acylation of Gα12 plays an important role in its growth signaling; mutation of a native palmitoylation target Cys in Gα12 to Ser abolished the transforming ability of the protein in cultured NIH3T3 fibroblasts. Introduction of a residue allowing myristoylation of Gα12 near its N-terminus rescued transforming ability in this Cys-to-Ser mutant,

suggesting that membrane tethering of Gα12 is essential to its function in cell growth and transformation (Kelly et al. 2007).

It is apparent that Rho activation is a critical aspect of the mechanism through which Gα12 stimulates cell proliferative pathways. Gα12 stimulation of JNK and SRF are blocked by Rho inhibition, and Gα12-mediated transformation of cultured fibroblasts requires Rho signaling to SRF (Radhika and Dhanasekaran 2001). Gα12-mediated responses disrupted by expression of a dominant-negative RhoGEF include stimulation of ▶ phospholipase D activity and angiotensin-triggered activation of JNK and p38 MAPK. However, mutationally activated Rho lacks the potency of activated Gα12 in triggering cellular transformation, supporting a model in which Gα12 engages additional, Rho-independent pathways in order to activate growth and tumorigenesis (Suzuki et al. 2009). For example, Gα12 interaction with the ▶ cadherin cytoplasmic domain triggers dissociation of the transcriptional co-activator ▶ β-catenin from ▶ cadherin, and in specific cell types up-regulates the expression of several ▶ β-catenin target genes. Gα12 binding to axin, a protein required for regulated degradation of ▶ β-catenin, provides an additional point at which Gα12 potentially regulates ▶ β-catenin-mediated pathways (Kelly et al. 2007). In addition, a dominant-negative Rac inhibited Gα12-induced transformation in cultured cells, and Gα12 activation of JNK was blocked by dominant-negative forms of Rac, Ras, and MAP kinase kinase kinase 1 (MEKK1), although these effects were specific to certain cell types. Furthermore, Gα12-mediated stimulation of ERK5, a MAPK harboring an extended C-terminal region, was unaffected by Rho inhibition. Finally, the proline-rich tyrosine kinase PYK2 has been implicated in Gα12-mediated mitogenic signaling; a kinase-deficient PYK2 mutant blocked stimulation of SRF by constitutively active Gα12 (Radhika and Dhanasekaran 2001; Kelly et al. 2007). Several effects of Gα12 on growth signaling appear cell type specific; for example, constitutively active Gα12 weakly stimulated extracellular signal-regulated kinase (ERK) in NIH3T3 fibroblasts, but in a separate study utilizing COS-7 fibroblasts, Gα12 inhibited ERK while simultaneously stimulating JNK. Also, Gα12 inhibited p38 MAPK signaling and stimulated JNK in NIH3T3 cells, whereas in neonatal rat cardiomyocytes, Gα12 stimulated p38 MAPK along with JNK (Radhika and Dhanasekaran 2001).

Evidence is mounting that Gα12-mediated pathways regulate cell growth by interfacing with pathways driven by receptor tyrosine kinases, or by stimulating autocrine loops that promote cell proliferation. Specific inhibitors of the platelet-derived growth factor (PDGF) receptor and the protein tyrosine kinase JAK3 blunted the ability of constitutively active Gα12 to stimulate cell proliferation in the absence of serum. Dominant-negative variants of the PDGF receptor and ▶ STAT3 hindered Gα12 in triggering focus formation in NIH3T3 cells (Kelly et al. 2007). Also, Gα12 stimulated expression of cyclooxygenase-2 (COX-2), and specific inhibitors of COX-2 blocked Gα12-mediated activation of DNA synthesis. Taken in the context of a previous finding that Gα12 stimulates ▶ phospholipase A_2 to catalyze arachidonic acid release from cell membranes, an event predicted to up-regulate prostaglandin biosynthesis, these results suggest multiple points at which Gα12 may regulate prostaglandin signaling in cell growth and transformation (Radhika and Dhanasekaran 2001). In addition to its complex roles in growth signaling, Gα12 can activate programmed cell death pathways through JNK. Activated Gα12 was shown to induce apoptosis in COS-7 fibroblasts through two separate pathways that converge on JNK activation, one via MEKK1 and the other via apoptosis signal-regulating kinase. More recently, Gα12 was found to stimulate apoptosis through a pathway dependent on PP2A, leading to degradation of the anti-apoptotic protein ▶ Bcl-2 (Juneja and Casey 2009). Other studies revealed the polycystic kidney disease–related protein polycystin-1 as a negative regulator of the apoptotic signaling generated by expression of constitutively active Gα12, or by thrombin stimulation, in human embryonic kidney cells (Yu et al. 2011). Stimulation of JNK by Gα12 was recently implicated in yet another cellular response: the transcription factor ▶ Nrf2, a protein activated by oxidative stress, was stimulated through a Gα13-dependent pathway but negatively balanced by Gα12. Additional findings showed ▶ Nrf2 to be up-regulated in the absence of Gα12, due to reduced JNK-dependent proteolysis of ▶ Nrf2 (Cho et al. 2007).

Role of Gα12 in Cell Polarity, Adhesion, Migration, and Invasion

Two early lines of investigation implicated Gα12 and its homologs as regulators of cell shape change. In fruit flies homozygous for a mutation in the

concertina gene, apical constriction of cells was severely disrupted during the ventral furrow formation stage that initiates gastrulation (Parks and Wieschaus 1991). Subsequently, expression of a constitutively active mammalian Gα12 in cultured cells was found to trigger formation of actin stress fibers, as well as assembly of focal adhesion complexes (Buhl et al. 1995). These discoveries led to Gα12 being implicated in mechanisms regulating cell polarity, which is an essential aspect of proper cytoskeletal rearrangement and directed migration. Gα12 was found to regulate actin-mediated protrusions, as well as reorientation of the microtubule-organizing center toward the leading edge of the cell (Juneja and Casey 2009). Activated Gα12 also triggered neurite retraction and cell rounding in cultured PC12 and 1321N1 cell lines (Sah et al. 2000), and signaling through the ▶ sphingosine-1-phosphate receptor was shown to stimulate stress fiber formation in a Gα12-dependent manner (Riobo and Manning 2005). Also, expression of constitutively active Gα12 in human embryonic kidney cells stimulated phosphorylation of the focal adhesion proteins paxillin, focal adhesion kinase, and p130 Crk-associated substrate (▶ p130 CAS), although studies in Madin-Darby canine kidney epithelial cells revealed Gα12 to inhibit phosphorylation of paxillin and focal adhesion kinase (Radhika and Dhanasekaran 2001; Kong et al. 2009).

Many cytoskeletal effects of Gα12 are Rho-dependent. The first reports of Gα12 triggering the generation of stress fibers and focal adhesions revealed these events as sensitive to *Clostridium botulinum* C3 exoenzyme, a specific inhibitor of Rho (Buhl et al. 1995). Guanine nucleotide exchange on Rho, activated by a Gα12-stimulated RhoGEF, results in downstream activation of the Rho target proteins Rho kinase (ROCK) and ▶ myosin II. These Rho effectors mediate contraction of the trailing edge of the cell, a critical step in migration. Gα12 stimulation of this pathway was shown to mediate polarity of neutrophils and contraction at the back and sides of the cell during migration (Xu et al. 2003). In cultured neonatal cardiomyocytes, either Rho inhibition or expression of a peptide mimicking the C-terminal 55 amino acids of Gα12 blocked α-adrenergic receptor-induced hypertrophic responses, including cytoskeletal rearrangements (Kelly et al. 2007). Also, stimulation of cell migration by lysophosphatidic acid (LPA) was hindered by inhibitors of Rho or ROCK. The downstream effects of Gα12 signaling on the cytoskeleton are complex; for instance, thrombin was found to stimulate endothelial cell permeability through a Gα12-Rho axis, with ROCK mediating cytoskeletal rearrangements and protein kinase C-related kinase mediating the remodeling of focal adhesions (Gavard and Gutkind 2008). Although Rho signaling is crucial in Gα12-mediated cytoskeletal changes and migration, it is apparent that other downstream targets of Gα12 are involved in these events. Constitutively active Gα12 was demonstrated to overcome epithelial cadherin (▶ E-cadherin)-mediated blockage of breast cancer cell migration in wound-filling assays, and this effect of Gα12 was insensitive to a specific inhibitor of ROCK (Kelly et al. 2007). Glycogen synthase kinase-3 (GSK3) is involved in neurite retraction induced by LPA, and Gα12 and the closely related Gα13 were found to up-regulate GSK3 activity. Interestingly, the effect of Gα12, but not Gα13, was refractory to C3 exoenzyme, suggesting a Rho-independent mechanism specific to Gα12. Constitutively active Gα12 also stimulated invasiveness of several breast cancer and prostate cancer cell lines through an artificial extracellular matrix (Kelly et al. 2006, 2007).

Gα12 has been revealed as a regulator of cell adhesion. Following the discovery of Gα12 interaction with several members of the ▶ cadherin superfamily of cell-cell adhesion glycoproteins, this binding event was found to disrupt ▶ cadherin association with ▶ β-catenin, allowing the latter protein to accumulate in the cytoplasm and nucleus of colon carcinoma cells and drive transcriptional activation of key cell proliferative genes (Kelly et al. 2007). Gα12-▶ cadherin interaction was also shown to negatively regulate the cell-cell adhesive function of E-cadherin, as well as overcome ▶ cadherin-induced inhibition of breast cancer cell migration. Gα12 may regulate cell adhesion through other downstream proteins. Its interaction with p120 catenin, a protein implicated in stabilizing ▶ cadherin at the plasma membrane, increases the affinity of p120 catenin for E-cadherin; furthermore, Gα12 modulates the ability of p120 catenin to bind and negatively regulate Rho. In addition, Gα12 may regulate cell adhesion through interaction with an additional ▶ cadherin-associated protein, αSNAP, that has been shown to stabilize ▶ cadherin at the cell surface and regulate endothelial barrier function (Kelly et al. 2007). In addition to its role in regulating cell-cell adhesion mechanisms, Gα12 has been

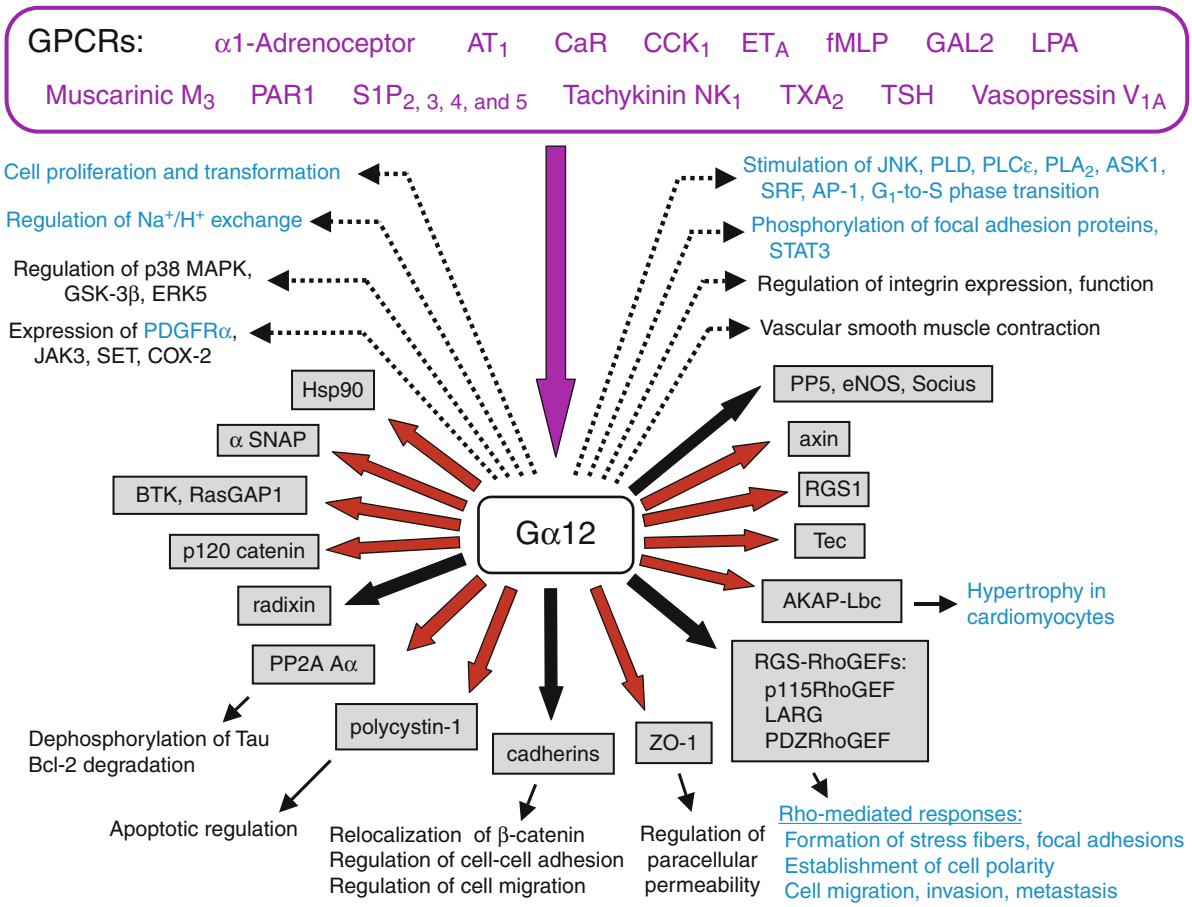
implicated as a modulator of cell-substrate interaction. Attachment of Madin-Darby canine kidney cells to laminin-5 was negatively regulated by Gα12-mediated transcriptional inhibition of the α6 integrin gene, which encodes a protein that adheres specifically to the laminin-5 substrate. Gα12 was also reported to negatively regulate epithelial cell attachment and migration on collagen-I, via inhibition of α2β1 integrin through an "inside-out" signaling pathway that requires Gα12, Rho, and ▶ Src, but apparently does not involve transcriptional regulation of integrins (Kong et al. 2009).

Receptors and Effectors Coupled to Gα12

A diverse group of cell surface, heptahelical G protein coupled receptors (GPCRs) have been reported as activators of Gα12. For several of these GPCRs, it is not clear whether coupling to Gα12, Gα13, or both proteins occurs (see below), but a number of GPCRs have been demonstrated to bind and/or activate Gα12 directly (Fig. 1). Most of these receptors have also been found to stimulate Gα13. Receptors were implicated as Gα12-coupled by experiments employing either a dominant-negative Gα12 mutant, a blocking peptide matching the Gα12 C-terminus, [^{32}P]GTP-azidoanilide photolabeling or [^{35}S]GTPγS binding to detect guanine nucleotide exchange on Gα12, or microinjection of antibodies to bind and inactivate Gα12 (Riobo and Manning 2005). In addition, the Ca^{++}-sensing receptor was shown to co-immunoprecipitate with Gα12 in lysates of breast cancer cells (Huang et al. 2009). These putative Gα12-coupled receptors bind a wide range of extracellular molecules, including peptides, phospholipid-derived ligands, and other chemical messages (Fig. 1). Also found to couple to Gα12 is the protease-activated receptor PAR1, which is activated by cleavage within its extracellular N-terminal region catalyzed by thrombin (Riobo and Manning 2005). For several GPCRs not shown in Fig. 1, signaling is blocked by expression of a dominant-negative RhoGEF, which binds the activated forms of Gα12 and Gα13 and, therefore, does not distinguish between these G12 subfamily members. These GPCRs include the chemokine receptor CXCR4, the orphan receptor GPR56, and the muscarinic M1, lysophosphatidylcholine G2A, and Smoothened receptors (Riobo and Manning 2005; Dorsam and Gutkind 2007; Juneja and Casey 2009). Domain-exchange mutants within the G12 subfamily may shed light on specific receptor-Gα couplings. A recent study utilizing Gα12 and Gα13 chimeras with interchanged N-terminal regions suggested thrombin-induced signaling as preferentially Gα12-mediated, whereas LPA-generated signaling appeared to utilize Gα13 rather than Gα12 (Suzuki et al. 2009).

Several Gα12-coupled receptors have independently been linked to cell proliferation and cancerous progression. PAR1 is overexpressed in advanced breast and prostate cancers, and appears to contribute to metastatic invasion. Also, the LPA receptor appears involved in an autocrine signaling mechanism that promotes the growth of ovarian cancer cells, whereas endothelin receptors have been implicated in metastatic progression in prostate tumors. The ▶ sphingosine-1-phosphate receptor, which couples to Gα12, has been shown to induce proliferation and migration in vascular smooth muscle cells (Juneja and Casey 2009). Also, loss of physiological regulation of the ▶ Sonic hedgehog-stimulated receptor Smoothened has been linked to basal cell carcinomas. The receptor CXCR4, which binds exclusively to stromal cell–derived factor 1, is particularly interesting due to its aberrantly high expression in several metastatic cancers (Dorsam and Gutkind 2007); however, direct coupling of this receptor to Gα12 remains to be demonstrated. Because essentially all of these receptors couple to heterotrimeric G proteins outside the G12 subfamily, it remains to be determined whether the interface between Gα12 and specific receptors will provide a viable target for cancer drug development.

After the discovery of Gα12 (see Historical Background), nearly a decade elapsed before the first direct effector of Gα12 was identified: the Rho-stimulatory guanine nucleotide exchange factor p115RhoGEF. This protein bound to Gα12 and Gα13 and accelerated GTP hydrolysis on both proteins (Kozasa et al. 1998). During the next several years, yeast two-hybrid screening and other approaches yielded a structurally and functionally diverse collection of Gα12-interacting proteins. Some of these are targets for both Gα12 and Gα13, while others appear selective for Gα12 within the G12 subfamily. As illustrated in Fig. 1, the former group includes leukemia-associated RhoGEF (LARG), p115RhoGEF and PDZ-RhoGEF, several ▶ cadherins, the actin-binding protein radixin of the ezrin/radixin/moesin family, protein phosphatase-5, endothelial nitric oxide synthase, and Socius, whereas the Gα12-specific interactors include heat shock protein-90, the scaffolding subunit of the protein



G Protein Alpha 12, Fig. 1 Receptors, effectors, and signaling responses mediated by Gα12. G protein coupled receptors (GPCRs) demonstrated to couple to Gα12 are enclosed by a rounded rectangle at top. Effector proteins that directly interact with Gα12 are indicated by *shaded boxes*. *Thick black arrows* indicate binding to both Gα12 and Gα13, and *thick red arrows* indicate binding that appears selective for Gα12. *Non-boxed text* and *dashed lines/arrows* indicate downstream cellular responses demonstrated to be mediated by Gα12, and *solid, thin lines/arrows* indicate responses for which the effector protein immediately upstream of the *arrow* has been implicated. *Lighter* text indicates downstream responses shown to be dependent on Rho activation. Abbreviations for GPCRs are as follows: *AT* angiotensin, *CCK* cholecystokinin, *ET* endothelin, *fMLP* formyl peptide, *GAL* galanin, *LPA* lysophosphatidic acid, *PAR* protease-activated receptor, *S1P* sphingosine-1-phosphate, *TXA_2* thromboxane A_2, *TSH* thyroid-stimulating hormone. Abbreviations for effector proteins and downstream responses are as follows: *AKAP* A-kinase anchoring protein, *ASK* apoptosis signal-regulating kinase, *BTK* Bruton's tyrosine kinase, *COX* cyclooxygenase, *eNOS* endothelial nitric oxide synthase, *ERK* extracellular signal-regulated kinase, *GEF* guanine nucleotide exchange factor, *GSK* glycogen synthase kinase, *Hsp* heat shock protein, *JAK* Janus kinase, *JNK* c-Jun N-terminal kinase, *LARG* leukemia-associated RhoGEF, *PDGFR* platelet-derived growth factor receptor, *PDZ* PSD-95/Dlg/ZO-1, *PL* phospholipase, *PP* protein phosphatase, *RGS* regulator of G protein signaling, *SET* leukemia associated protein SET, *SNAP* soluble NSF-associated protein, *SRF* serum response factor, *STAT* signal transducer and activator of transcription, *ZO* zonula occludens

phosphatase-2A (PP2A) holoenzyme, non-receptor tyrosine kinases (Tec, Bmx, Bruton's tyrosine kinase), potential regulators of ▶ cadherin stabilization (p120 catenin and αSNAP) the tight junctional protein zonula occludens-1, proteins harboring a regulator of G protein signaling domain (axin and RGS1), the polycystic kidney disease–related protein polycystin-1, and the Rho-stimulatory A-kinase-anchoring protein AKAP-Lbc (Kelly et al. 2007; Yu et al. 2011). Efforts are ongoing to connect specific Gα12-target interactions to known Gα12-mediated signaling responses. The pathways in which RhoGEF

interaction drives Rho-mediated signaling are the best characterized, although the specific roles of Gα12 and Gα13 in these pathways are not resolved. Likewise, the effects of Gα12 and Gα13 on cadherin-mediated cell biology remain to be determined. Regulation of cell polarity and migration by Gα12 has been shown to involve LARG, which associates with pericentrin and localizes to the microtubule-organizing center (Juneja and Casey 2009). Gα12 interaction with zonula occludens-1 has been linked to regulation of paracellular permeability, and AKAP-Lbc appears to mediate Gα12 signaling leading to hypertrophy in cardiomyocytes (Appert-Collin et al. 2007). Gα12 interaction with the scaffolding subunit of PP2A revealed a potential role of Gα12 as a regulatory subunit in the PP2A holoenzyme, and implicated the Gα12-PP2A signaling axis in regulation of Tau phosphorylation in neural cells (Juneja and Casey 2009). Furthermore, the discovery of Bruton's tyrosine kinase, a protein involved in B-lymphocyte maturation, as a Gα12 target suggests a role for Gα12 in signaling pathways that govern this process (Worzfeld et al. 2008).

It remains to be determined whether subgroups of these Gα12-binding partners participate in common signaling pathways, or compete for interaction with Gα12. Also, for some Gα12 targets it is not clear whether the protein serves as a direct effector of Gα12 activation (i.e., interacts exclusively with GTP-bound Gα12 to propagate a signal). At least one protein, the scaffolding subunit of PP2A, has been reported to bind Gα12 in both its active and inactive (GDP-bound) conformation (Kelly et al. 2007). Also, the region of polycystin-1 mapped as interacting with Gα12 harbors a motif found in a number of GPCRs (Yu et al. 2011). It is possible that some Gα12-interacting proteins do not serve as classical downstream effectors, but instead play other roles such as facilitating Gα12 binding to effectors, specifying Gα12 coupling to receptors, regulating the intracellular location of Gα12, participating in non-receptor activation of guanine nucleotide exchange, or other, unknown functions.

Role of Gα12 at the Organismal Level: Development and Morphogenesis

Although many investigations of Gα12 have focused on its function at the single-cell level (e.g., signaling mechanisms, structural aspects of interaction with effector proteins, transcriptional responses), a number of studies have examined its role in larger-scale physiological events such as morphogenesis, organ system development, and embryogenesis. The first such studies revealed the role of the *Drosophila* Gα12 homolog Concertina in formation of the posterior midgut and ventral furrows during gastrulation (Parks and Wieschaus 1991). In studies of the roundworm *Caenorhabditis elegans*, RNA interference targeting the Gα12 homolog Gpa-12 caused an egg-laying defect, was embryonic lethal in most of these offspring, and caused locomotion and sensory defects in the few survivors. RNA interference of CeRhoGEF, a homolog of the G12-coupled mammalian RhoGEFs, phenocopied the Gpa-12-impaired roundworms to a lesser degree, suggesting that the Gα12-RhoGEF-Rho axis is an ancient signaling pathway. However, the greater severity of Gpa-12 knockdown than Ce-RhoGEF knockdown, along with Gpa-12 expression in several cell types lacking expression of Ce-RhoGEF, suggest the presence of other, Rho-independent Gpa-12-driven effector pathways in *C. elegans* (Suzuki et al. 2009). Initial studies of Gα12 knockout mice were less than encouraging; these mice appeared normal while mice engineered to lack the G12 subfamily member Gα13 died at embryonic day 9.5 with severe defects in angiogenesis. However, it has become apparent that Gα12 function is not completely eclipsed by Gα13 during embryogenesis. In mice haploinsufficient for Gα13, at least one allele of Gα12 was required to avoid embryonic lethality, and mice lacking both Gα12 and Gα13 died at least 1 day earlier in embryogenesis than Gα13 ($^{-/-}$) mice (Worzfeld et al. 2008). In more recent studies, approaches such as conditional gene inactivation and expression of dominant-negative proteins have revealed novel roles for Gα12; for example, knockout of Gα12 and Gα13 in neurons and glial cells resulted in over-migration of cortical neurons in certain brain subregions, suggesting Gα12 as a negative regulator of this migratory stage. A similar outcome was observed in marginal zone B-lymphocytes lacking Gα12 and Gα13, and in neutrophils harboring dominant-negative versions of these G12 subfamily proteins; these cells showed an increase in random migration that disrupted the ability of B-lymphocytes to settle properly in the marginal zone of the spleen. Also, Gα12 was shown to mediate migration of neural progenitor cells in response to stimulation of the orphan receptor GPR56

(Juneja and Casey 2009). In addition, Gα12 has been implicated in vascular smooth muscle contraction; studies in mice harboring conditional Gα12/Gα13 knockout in smooth muscle cells revealed Gα12 activation as necessary for the increased vascular tone that underlies salt-induced hypertension, but not basal vascular tone (Worzfeld et al. 2008). Finally, in studies examining the role of Gα12 in cancer progression, a dominant-negative, G12-specific RhoGEF hindered metastatic invasion of breast cancer cells implanted in the mammary fat pad of athymic mice. In contrast to the extensive results in cultured fibroblasts (see Role of Gα12 in Cell Growth, Apoptosis, and Oncogenic Transformation), Gα12 did not stimulate proliferation of these implanted cells (Kelly et al. 2006). These results suggest that the differential effects of Gα12 on proliferation versus migration/invasion are cell type specific, and that aberrant Gα12 signaling in some tumors may be most important during the progression of aberrantly dividing cells to a metastatic, invasive state.

Gα12 is essential for embryonic development in zebrafish, as suppression of Gα12 function was shown to disrupt convergence and extension during gastrulation. This effect of Gα12 requires signaling through a RhoGEF-Rho pathway; however, Gα12 also appears to act through other downstream effectors. Epiboly is a stage of embryogenesis in which the blastoderm spreads over the yolk cell, and involves the coordinated shape changes and movements of several cell populations. Interaction between Gα12 and the cytoplasmic domain of E-cadherin has been demonstrated as a critical step during epiboly in zebrafish, and gastrulae with disrupted Gα12 signaling phenocopy E-cadherin mutants, displaying aberrant movement and dissociation of several cell types. Gα12 signaling during epiboly appears to occur via two distinct mechanisms: a Rho-independent down-regulation of E-cadherin adhesive function and a Rho-dependent reorganization of the actin cytoskeleton (Lin et al. 2009).

Summary

This entry describes the molecules, cellular responses, and organismal processes regulated by Gα12, but it must be noted that many of these signaling functions of Gα12 overlap with functions of its G12 subfamily counterpart, Gα13. However, many properties of Gα12, particularly in terms of downstream target proteins, appear to be exclusive to Gα12 or show differential regulation by Gα12 and Gα13. For example, one of the earliest signaling responses reported for the G12 subfamily was stimulation of Na^+/H^+ ion exchange via the integral plasma membrane NHE proteins. This occurred through a protein kinase C-dependent pathway for Gα12, whereas activation of Na^+/H^+ exchange by Gα13 did not require protein kinase C. Furthermore, the ubiquitous Na^+/H^+ exchanger NHE1 was stimulated by Gα13 but, surprisingly, was inhibited by Gα12 (Lin et al. 1996). In *Drosophila* and *C. elegans*, the G12 subfamily is comprised of a single protein that harbors roughly equal similarity to the two mammalian members; for example, Concertina shares approximately 54% identity with Gα12 and Gα13. Therefore, many questions in the field of Gα12 signaling concern the respective signaling properties and biological roles of the two mammalian G12 α-subunits, as well as the evolutionary advantages afforded by the gene duplication and divergence that yielded these similar yet distinct signaling proteins.

The G12 subfamily has several interesting distinctions among heterotrimeric G proteins. First, it regulates an unusually wide variety of cellular processes that have potential to become aberrant in cancers (e.g., cell migration, growth, polarity, cell-cell adhesion, and cell-substrate adhesion). Second, the α-subunits of the G12 subfamily appear to be unique among G protein α-subunits in their ability to transform cells as an overexpressed rather than constitutively GTP-bound form. Therefore, it is possible that aberrantly high production or impaired turnover of Gα12 is sufficient to exert oncogenic effects in cells. Third, Gα12 interacts with a more diverse group of target proteins than any other Gα subunit. These discoveries have revealed intriguing possibilities for cellular events in which Gα12 plays a role; however, this diversity of downstream targets has created a maze of potential signaling mechanisms and possible regulatory relationships between different Gα12 effector proteins. Furthermore, cross-talk with other signaling pathways must be considered; for example, the G protein Gαq has been shown to stimulate Rho via activation of a recently discovered guanine nucleotide exchange factor, p63RhoGEF (Suzuki et al. 2009). Toward the goal of a comprehensive understanding of Gα12 signaling, it will be important to elucidate the structural features of Gα12 that mediate specific

target protein interactions. Mutational analyses of Gα12 have revealed amino acid determinants of its binding to several proteins, including RhoGEFs, polycystin-1, and the scaffolding subunit of PP2A (Kelly et al. 2007; Yu et al. 2011). A crystallographic analysis of Gα12 in complex with a downstream target protein has yet to be reported. However, it is promising that crystal structures have been published of recombinant Gα13 bound to the RGS domain of RhoGEFs (Suzuki et al. 2009). Similar structural studies of Gα12 may reveal subtle differences in the mechanisms through which Gα12 and Gα13 engage their respective effector proteins.

Although evidence is compelling that Gα12 can induce oncogenic transformation of cells, as well as initiate progression of existing cancer cells toward metastasis, it is important to note that human tumor samples harboring activating mutations in Gα12 have yet to be reported. Studies in breast and prostate cancer cell lines suggest that overexpression of Gα12 may suffice as a transforming agent in the absence of a GTPase-impaired mutant of Gα12 (Kelly et al. 2006). Also, recent results in a small cell lung carcinoma line showed RNA interference of Gα12 to block cell proliferation in culture and in a mouse xenograft model (Grzelinski et al. 2010). Clearly, a broad analysis of tumor samples from breast, prostate, lung, and other tissues will be valuable in assessing the significance of Gα12 in the pathology of specific human cancer types. Also, because of the mounting evidence that aberrantly high levels of Gα12 may drive cancerous growth and/or invasion, studies that examine factors regulating Gα12 gene expression, transcript stability (e.g., microRNAs), and protein turnover may be increasingly vital to understanding the role of Gα12 in cancer progression. Furthermore, continued efforts to identify the key Gα12 target proteins and pathways that mediate its growth, migratory, and invasive effects should aid the development of reagents for manipulating Gα12-mediated signaling as a treatment for cancers and other diseases.

References

Appert-Collin A, Cotecchia S, Nenniger-Tosato M, Pedrazzini T, Diviani D. The A-kinase anchoring protein (AKAP)-Lbc-signaling complex mediates alpha1 adrenergic receptor-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA. 2007;104:10140–5.

Buhl AM, Johnson NL, Dhanasekaran N, Johnson GL. Gα12 and Gα13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly. J Biol Chem. 1995;270:24631–4.

Chan AM, Fleming TP, McGovern ES, Chedid M, Miki T, Aaronson SA. Expression cDNA cloning of a transforming gene encoding the wild-type Gα12 gene product. Mol Cell Biol. 1993;13:762–8.

Cho MK, Kim WD, Ki SH, Hwang JI, Choi S, Lee CH, Kim SG. Role of Gα12 and Gα13 as novel switches for the activity of Nrf2, a key antioxidative transcription factor. Mol Cell Biol. 2007;27:6195–208.

Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. Nat Rev Cancer. 2007;7:79–94.

Gavard J, Gutkind JS. Protein kinase C-related kinase and ROCK are required for thrombin-induced endothelial cell permeability downstream from $G\alpha_{12/13}$ and $G\alpha_{11/q}$. J Biol Chem. 2008;283:29888–96.

Grzelinski M, Pinkenburg O, Buch T, Gold M, Stohr S, Kalwa H, Gudermann T, Aigner A. Critical role of Gα12 and Gα13 for human small cell lung cancer cell proliferation in vitro and tumor growth in vivo. Clin Cancer Res. 2010;16:1402–15.

Huang C, Hydo LM, Liu S, Miller RT. Activation of choline kinase by extracellular Ca^{2+} is Ca^{2+}-sensing receptor, Gα12 and Rho-dependent in breast cancer cells. Cell Signal. 2009;21:1894–900.

Juneja J, Casey PJ. Role of G12 proteins in oncogenesis and metastasis. Br J Pharmacol. 2009;158:32–40.

Kelly P, Moeller BJ, Juneja J, Booden MA, Der CJ, Daaka Y, Dewhirst MW, Fields TA, Casey PJ. The G12 family of heterotrimeric G proteins promotes breast cancer invasion and metastasis. Proc Natl Acad Sci USA. 2006;103:8173–8.

Kelly P, Casey PJ, Meigs TE. Biologic functions of the G12 subfamily of heterotrimeric G proteins: growth, migration, and metastasis. Biochemistry. 2007;46:6677–87.

Kong T, Xu D, Yu W, Takakura A, Boucher I, Tran M, Kreidberg JA, Shah J, Zhou J, Denker BM. Gα12 inhibits α2β1 integrin-mediated Madin-Darby canine kidney cell attachment and migration on collagen-I and blocks tubulogenesis. Mol Biol Cell. 2009;20:4596–610.

Kozasa T, Gilman AG. Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits: characterization of α12 and inhibition of adenylyl cyclase by αz. J Biol Chem. 1995;270:1734–41.

Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G, Sternweis PC. p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. Science. 1998;280:2109–11.

Kreutz B, Yau DM, Nance MR, Tanabe S, Tesmer JJG, Kozasa T. A new approach to producing functional Gα subunits yields the activated and deactivated structures of Gα12/13 proteins. Biochemistry. 2006;45:167–74.

Lin X, Voyno-Yasenetskaya TA, Hooley R, Lin CY, Orlowski J, Barber DL. Gα12 differentially regulates Na^+-H^+ exchanger isoforms. J Biol Chem. 1996;271:22604–10.

Lin F, Chen S, Sepich DS, Panizzi JR, Clendenon SG, Marrs JA, Hamm HE, Solnica-Krezel L. $G\alpha_{12/13}$ regulate epiboly by inhibiting E-cadherin activity and modulating the actin cytoskeleton. J Cell Biol. 2009;184:909–21.

Parks S, Wieschaus E. The Drosophila gastrulation gene *concertina* encodes a Gα-like protein. Cell. 1991;64:447–58.

Radhika V, Dhanasekaran N. Transforming G proteins. Oncogene. 2001;20:1607–14.

Riobo NA, Manning DR. Receptors coupled to heterotrimeric G proteins of the G12 family. Trends Pharmacol Sci. 2005; 26:146–54.
Sah VP, Seasholtz TM, Sagi SA, Brown JH. The role of Rho in G protein-coupled receptor signal transduction. Annu Rev Pharmacol Toxicol. 2000;40:459–89.
Strathmann MP, Simon MI. Gα12 and Gα13 subunits define a fourth class of G protein α subunits. Proc Natl Acad Sci USA. 1991;88:5582–6.
Suzuki N, Hajicek N, Kozasa T. Regulation and physiological functions of G12/13-mediated signaling pathways. Neurosignals. 2009;17:55–70.
Worzfeld T, Wettschureck N, Offermanns S. G12/G13-mediated signaling in mammalian physiology and disease. Trends Pharmacol Sci. 2008;29:582–9.
Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, Takuwa Y, Sugimoto N, Mitchison T, Bourne HR. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell. 2003;114:201–14.
Yu W, Ritchie BJ, Su X, Zhou J, Meigs TE, Denker BM. Identification of polycystin-1 and Gα12 binding regions necessary for regulation of apoptosis. Cell Signal. 2011;23:213–21.

G Protein Alpha i

▶ G Protein α i/o/z

G Protein Alpha o

▶ G Protein α i/o/z

G Protein Alpha Transducin

Hoon Shim and Ching-Kang Chen
Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA, USA

Synonyms

Gαt; GNAT1; GNAT2; Gt; T

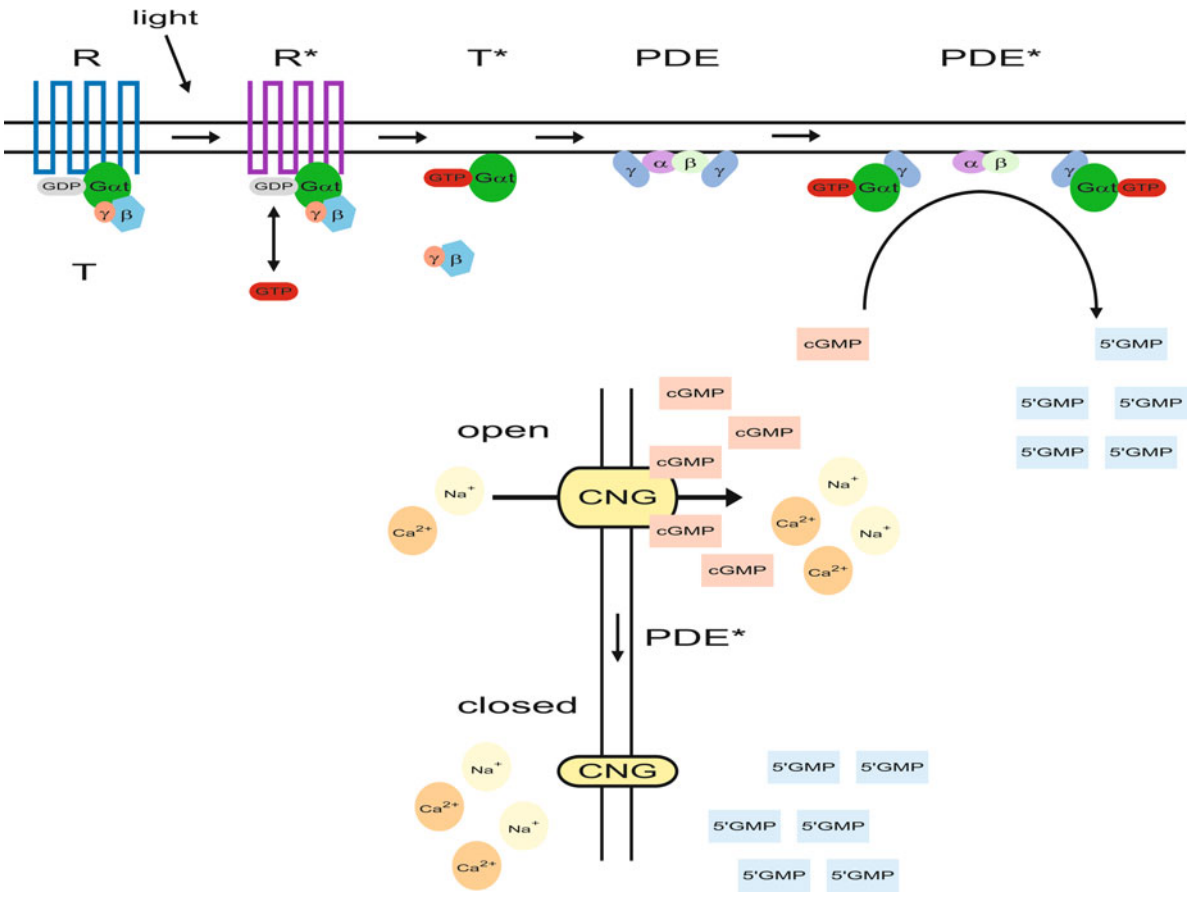
Historical Background

G protein alpha transducin was purified from bovine retinal extracts more than three decades ago. The discovery of transducin was the culmination of a series of findings that started in the mid-1970s (see Bourne 2006 for a recent review), beginning with the identification of the effector molecule cGMP phosphodiesterase (Bitensky et al. 1975). Shortly afterward, the presence of light-induced GTPase activity in rod outer segment extracts was described (Wheeler and Bitensky 1977). In 1978, activation of cGMP phosphodiesterase was shown to require activated rhodopsin and the presence of GTP (Yee and Liebman 1978). In 1979, a soluble guanine nucleotide binding protein with light-activated GTP–GDP exchange was purified (Godchaux and Zimmerman 1979). However, only two components, the α and β subunits, of the heterotrimeric G protein transducin were described. In 1981, the full heterotrimeric G protein was purified in Lubert Stryer's group, and they named it "transducin." Using purified transducin and rhodopsin, Fung et al. were able to demonstrate light-dependent binding and hydrolysis of GTP (Fung et al. 1981). These pioneered discoveries were later augmented by human and mouse genetics along with suction-electrode recordings to elucidate the role of transducin in phototransduction. However, many questions remain unresolved.

Role of Transducin in Phototransduction

In the absence of light, photoreceptors maintain a high concentration of intracellular cGMP. The dark cGMP level is sufficient to keep up to 10% of cyclic nucleotide-gated (CNG) channels on the plasma membrane open to allow an inward "dark current" of sodium and calcium ions to depolarize outer segment membranes to a resting voltage of approximately −35 mV. At this depolarized state, photoreceptors tonically release glutamate at their synaptic terminals. When light is introduced, a cascade of events involving transducin and collectively called phototransduction is activated, which leads to photoreceptor hyperpolarization and cessation of the tonic glutamate release.

The rod phototransduction cascade involves the G-protein-coupled receptor (GPCR) rhodopsin (R), the heterotrimeric G protein transducin (Tαβγ), and the effector cGMP phosphodiesterase ($PDE\alpha\beta\gamma_2$). In the dark, R is in an inactive conformation comprised of an apoprotein opsin and a covalently attached chromophore 11-*cis*-retinal. Light isomerizes the 11-*cis*-retinal to all-*trans*-retinal, which induces conformational changes of rhodopsin leading to an



G Protein Alpha Transducin, Fig. 1 Rod phototransduction cascade. Refer to the text for a description of the rod phototransduction cascade. An asterisk (*) denotes the activated state of a molecule

activated form called Metarhodopsin II (R^*). As shown in Fig. 1, rhodopsin (R) is coupled to the heterotrimeric G protein transducin (T); after light is introduced, rhodopsin is activated (R^*) to catalyze the exchange of GDP on Gαt for GTP. The GTP bound Gαt is the activated form (T^*) of G protein transducin. The activated transducin α subunit (T^*) dissociates from its Gβγ subunits and interacts with the PDEγ subunit, preventing it from inhibiting the catalytically active PDEαβ subunits. With PDEγ inhibition removed, PDEαβ (PDE^*) hydrolyzes cGMP into 5′GMP, which leads to a decline in intracellular cGMP concentration. This results in the closure of cyclic nucleotide-gated (CNG) cation channels and a decreased Na^+ and Ca^{2+} influx, which leads to membrane hyperpolarization. Cone phototransduction is similar to rod phototransduction; however, cones have a distinct set of signaling molecules with presumably similar functions (see Table 1).

Transducin Activation

The exchange of GTP for GDP on the α subunit of transducin, catalyzed by light-activated rhodopsin, constitutes the first amplification step in the phototransduction cascade. Transducin has a fairly fast guanine nucleotide exchange rate at approximately 1,000/s (Vuong et al. 1984). During its effective lifetime, R^* may activate multiple transducin molecules. The estimated rate of transducin activation by a single R^* has been reported in the range of 10–3,000/s (Fu and Yau 2007). In mouse rods, the activation rate of transducin by R* is reported to be ~240/s (Fu and Yau 2007). Rods have a large pool of transducin molecules and this characteristic has partly been attributed to the ability of rods to sense a single photon of light. The activation rate of rod transducin is approximately tenfold higher than cone transducin by cone pigment, which also contributes to rod's higher sensitivity to

G Protein Alpha Transducin, Table 1 Distinct phototransduction molecules in mouse rods and cones

Phototransduction molecule	In rods	In cones
GPCR[a]	Rhodopsin (*Rho*)	Green-sensitive opsin (*Opn1mw*), blue-sensitive opsin (*Opn1sw*)
G protein alpha	$G\alpha t_1$ (*Gnat1*)	$G\alpha t_2$ (*Gnat2*)
G protein beta/gamma	$G\beta_1\gamma_1$ (*Gnb1*, *Gngt1*)	$G\beta_3\gamma_c$ (*Gnb3*, *Gngt2*)
Effector	PDE6A (*Pde6a*), PDE6B (*Pde6b*)	PDE6C (*Pde6c*)

Gene names are in parentheses
[a]Mouse cones do not contain red-sensitive opsin (*Opn1lw*), which is present in primate cones

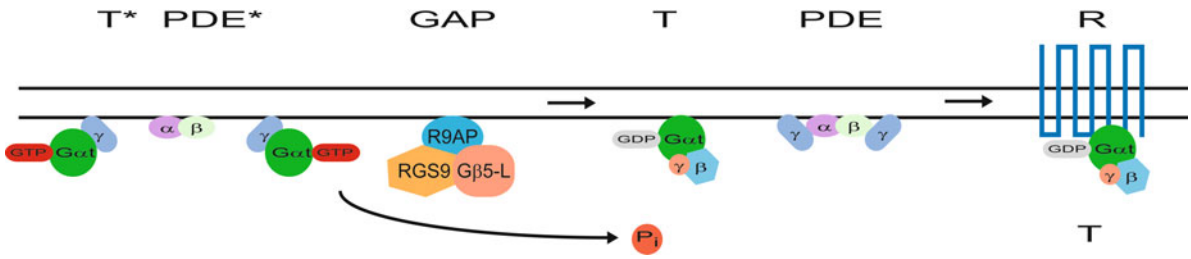
light (Luo et al. 2008). The effector cGMP phosphodiesterase (PDE) is a tetrameric membrane-associated protein consisting of two active and highly similar catalytic subunits αβ and two identical inhibitory γ subunits. The interaction of T^* with PDEγ removes the inhibitory PDEγ from the two catalytic PDEαβ subunits. Since there are two PDEγ subunits in the tetrameric PDEαβγ_2, two activated T^* molecules are required to achieve full PDE activation.

Transducin Deactivation

Deactivation of T^* requires its intrinsic GTPase activity to hydrolyze the bound GTP to GDP. When GTP is hydrolyzed by T^*, conformational changes in T^* lead to the release of PDEγ, which reassociates with and inhibits PDEαβ catalytic subunits. The rate at which GTP is hydrolyzed by T* determines the rate at which PDE^* is deactivated. As seen in Fig. 2, Regulator of G protein signaling 9 (RGS9-1) with the signature 120 amino acid RGS domain forms the so-called transducin GTPase accelerating protein (GAP) complex along with Gβ5-L and ▶ RGS9 anchoring protein (R9AP). The GAP complex binds T^* when it is bound to PDEγ to accelerate GTP hydrolysis (Keresztes et al. 2004; Krispel et al. 2003). Therefore, T^* will not be prematurely deactivated before it associates with PDEγ. The members of the transducin (GAP) complex, RGS9-1, Gβ5-L, and R9AP, are obligate protein partners. Knockout mice lacking any of these proteins, all result in a common phenotype: a delay in the recovery phase of the phototransduction cascade (Chen et al. 2000; Keresztes et al. 2004; Krispel et al. 2003). In photoreceptors, the rate-limiting step of rod recovery is determined by the level of the transducin GAP complex (Krispel et al. 2006). The GAP level is then determined by the R9AP expression level. In mice with different degrees of R9AP overexpression, which have corresponding overexpression of the entire transducin GAP complex, rod recovery can be accelerated to approach an asymptote with a time constant of ~70 ms. This suggests that when GAP concentration is significantly elevated, rod recovery is rate-limited not by transducin deactivation but by a different reaction(s) (Krispel et al. 2006).

Light-Dependent Redistribution of Transducin

In darkness, transducin is localized to the outer segment (OS) of rod photoreceptors. After prolonged light exposure, transducin can redistribute throughout the photoreceptor (Slepak and Hurley 2008). Interestingly, the Gβγ subunits also redistribute, but follow a different time course. Upon return to darkness, both Gαt and Gβγ return to the rod OS. Two lipid modifications are present in heterotrimeric rod transducin: Gαt is N-acylated and Gγ is S-prenylated on the C-terminus with a farnesyl group. A mutant form of Gαt (Q200L) that lacks the intrinsic GTPase activity was developed. The Q200L Gαt is constitutively bound to GTP and maintained in the active state (T*), and thus cannot reassociate with Gβγ. The Q200L Gαt is unable to stay localized to the rod OS in darkness, suggesting that reassociation with Gβγ was necessary for localization to the rod OS (Artemyev 2008; Slepak and Hurley 2008). Interestingly, cone transducin does not redistribute in response to light. One reason for this difference is the fact that Gβγ in cones has a stronger affinity for cone transducin even in the activated form. When a transgenic mouse was developed replacing rod transducin with cone transducin, the transgenic cone transducin in rods redistributed in a light-dependent manner (Chen et al. 2010). This suggests that it is not the proteins, but rather the cells that are responsible for the lack of transducin redistribution in cones. However, a recent paper by Lobanova et al. demonstrates that intense



G Protein Alpha Transducin, Fig. 2 Recovery phase of the rod phototransduction cascade. The intrinsic GTPase activity of Gαt is accelerated by the transducin GAP complex composed of RGS9-1, Gβ5-L, and R9AP. Hydrolysis of GTP to GDP on Gαt releases PDEγ to reassociate with PDEαβ. Gαt-GDP returns to heterotrimeric form by binding to its Gβγ and then recouples to rhodopsin (R). An asterisk (*) denotes the activated state of the molecule

light exposure for a prolonged period of time can cause cone transducin to redistribute in cones. This entry further shows that increasing cone sensitivity by ectopically expressing rhodopsin also moves cone transducin out of the outer segment in a light-dependent manner (Lobanova et al. 2010).

Role of Transducin in Photoreceptor Sensitivity and Response Decay

Rods are more sensitive to light than cones. Since rods and cones have distinct set of transducin molecules (see Table 1), the distinct proteins found in rods versus cones may have inherent properties that contribute to differences in their responses to light. To study whether transducin has any contribution, a transgenic mouse was generated with full-length mouse cone transducin (*Gnat2*) expressed in rods (Chen et al. 2010). This transgenic mouse was mated onto the *Gnat1*−/− mouse background to produce the GNAT2C mouse line. In GNAT2C rods, where cone transducin replaces rod transducin, GNAT2 expression level is similar to that of GNAT1 in normal mouse rods. Using suction-electrode recordings, GNAT2C rods were found to be three times less sensitive than wild-type and required brighter light to produce responses of the same amplitude (Chen et al. 2010). GNAT2C rods also displayed accelerated response decay compared to wild-type. When GNAT2C mice were mated to a mouse line overexpressing transducin GAP, the response recovery was accelerated even further beyond what was achieved from the transducin substitution (Chen et al. 2010). In the *RGS9*−/− background, GNAT2C rods could still turn off twofold faster than wild-type rods (Chen et al. 2010). These results suggested that the species of Gαt may indeed play an important role in setting the response kinetics and sensitivity of rods and cones.

GNAT1-Independent Phototransduction in Rods

Researchers frequently utilize the *Gnat1*−/− mouse as a model for loss of rod phototransduction. In 2010, Allen et al. mated *Gnat1*−/−; *Cnga3*−/−, *Opn4*−/− together to generate a triple knockout (TKO) mouse line. The *Cnga3*−/− mouse has a loss of cone vision, while the *Opn4*−/− mouse lacks melanopsin, a visual pigment expressed in intrinsically light-sensitive retinal ganglion cells. The TKO mouse theoretically should not have any light response. However, when they were used as "negative" controls in electroretinography (ERG) experiments, they were found to have a reproducible flash ERG response at high stimulus intensities >0.5 log cd/m^2 (Allen et al. 2010). Spectral analysis suggests that the light responses of TKO mice are mediated by rhodopsin. Since GNAT2 is known to couple to rhodopsin (Chen et al. 2010), *Gnat1*−/− was mated to a spontaneous mutant *Gnat2*^{cpfl3} mice, which have progressive loss of cone vision. The results from *Gnat1*−/−, *Gnat2*^{cpfl3} mice showed that the ERG response was reduced, but not eliminated. Consistent with their findings: the original characterization of the *Gnat1*−/− mouse by Calvert et al. showed that one (out of 213 examined) *Gnat1*−/− rod was found to have robust light responses similarly mediated by rhodopsin (Calvert et al. 2000). Allen et al. speculated that some ERG responses in TKO mice are mediated by GNAT2 (Allen et al. 2010). In an era in which genetics and technology are rampantly expanding the edge of scientific knowledge, the presence of robust

light responses in TKO mice exemplifies the opportunities that exist in the vision research field, where scientific dogma can be frequently challenged to reveal underappreciated intricacy and beauty of the visual system that are yet to be fully comprehended.

Summary

The critical role of $G\alpha t_1$ in the phototransduction cascade has been elucidated in rods, while the role of $G\alpha t_2$ in cone phototransduction is less understood. Custom-made transgenic animals, such as the GNAT2C mouse, should be useful to further dissect the difference of rod and cone phototransduction.

References

Allen AE, Cameron MA, Brown TM, Vugler AA, Lucas RJ. Visual responses in mice lacking critical components of all known retinal phototransduction cascades. PLoS One. 2010;5:e15063.

Artemyev NO. Light-dependent compartmentalization of transducin in rod photoreceptors. Mol Neurobiol. 2008; 37:44–51.

Bitensky MW, Miki N, Keirns JJ, Keirns M, Baraban JM, Freeman J, Wheeler MA, Lacy J, Marcus FR. Activation of photoreceptor disk membrane phosphodiesterase by light and ATP. Adv Cyclic Nucleotide Res. 1975;5:213–40.

Bourne HR. G-proteins and GPCRs: from the beginning. Ernst Schering Found Symp Proc. 2006;2:1–21.

Calvert PD, Krasnoperova NV, Lyubarsky AL, Isayama T, Nicoló M, Kosaras B, Wong G, Gannon KS, Margolskee RF, Sidman RL, Pugh Jr EN, Makino CL, Lem J. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha-subunit. Proc Natl Acad Sci USA. 2000;97:13913–8.

Chen CK, Burns ME, He W, Wensel TG, Baylor DA, Simon MI. Slowed recovery of rod photoresponse in mice lacking the GTPase accelerating protein RGS9-1. Nature. 2000; 403:557–60.

Chen CK, Woodruff ML, Chen FS, Shim H, Cilluffo MC, Fain GL. Replacing the rod with the cone transducin subunit decreases sensitivity and accelerates response decay. J Physiol. 2010;588:3231–41.

Fu Y, Yau KW. Phototransduction in mouse rods and cones. Pflugers Arch. 2007;454:805–19.

Fung BK, Hurley JB, Stryer L. Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc Natl Acad Sci USA. 1981;78:152–6.

Godchaux 3rd W, Zimmerman WF. Membrane-dependent guanine nucleotide binding and GTPase activities of soluble protein from bovine rod cell outer segments. J Biol Chem. 1979; 254:7874–84.

Keresztes G, Martemyanov KA, Krispel CM, Mutai H, Yoo PJ, Maison SF, Burns ME, Arshavsky VY, Heller S. Absence of the RGS9.Gbeta5 GTPase-activating complex in photoreceptors of the R9AP knockout mouse. J Biol Chem. 2004;279:1581–4.

Krispel CM, Chen CK, Simon MI, Burns ME. Prolonged photoresponses and defective adaptation in rods of Gbeta5−/− mice. J Neurosci. 2003;23:6965–71.

Krispel CM, Chen D, Melling N, Chen YJ, Martemyanov KA, Quillinan N, Arshavsky VY, Wensel TG, Chen CK, Burns ME. RGS expression rate-limits recovery of rod photoresponses. Neuron. 2006;51:409–16.

Lobanova ES, Herrmann R, Finkelstein S, Reidel B, Skiba NP, Deng WT, Jo R, Weiss ER, Hauswirth WW, Arshavsky VY. Mechanistic basis for the failure of cone transducin to translocate: why cones are never blinded by light. J Neurosci. 2010;30:6815–24.

Luo DG, Xue T, Yau KW. How vision begins: an odyssey. Proc Natl Acad Sci USA. 2008;105:9855–62.

Slepak VZ, Hurley JB. Mechanism of light-induced translocation of arrestin and transducin in photoreceptors: interaction-restricted diffusion. IUBMB Life. 2008;60:2–9.

Vuong TM, Chabre M, Stryer L. Millisecond activation of transducin in the cyclic nucleotide cascade of vision. Nature. 1984;311:659–61.

Wheeler GL, Bitensky MW. A light-activated GTPase in vertebrate photoreceptors: regulation of light-activated cyclic GMP phosphodiesterase. Proc Natl Acad Sci USA. 1977;74:4238–42.

Yee R, Liebman PA. Light-activated phosphodiesterase of the rod outer segment. Kinetics and parameters of activation and deactivation. J Biol Chem. 1978;253:8902–9.

G Protein Alpha z

► G Protein α i/o/z

G Protein Beta

► G Protein Beta/Gamma

G Protein Beta/Gamma

Denis J. Dupré[1] and Terence E. Hébert[2]
[1]Department of Pharmacology, Dalhousie University, Nova Scotia, Canada
[2]Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada

Synonyms

G beta; G gamma; G protein beta; G protein gamma; Gb; Gnb; Gng; Guanine nucleotide binding protein;

Guanine nucleotide binding protein, beta; Guanine nucleotide binding protein, beta polypeptide; Guanine nucleotide binding protein (G protein), gamma; Guanine nucleotide binding protein, gamma

Isoforms

Five different isoforms of Gbeta have been identified, numbered 1 to 5. Ggamma isoforms are numbered from 1 to 5 and 7 to 13.

G protein beta5 can also be known as Flailer, Flail, Flr, Hug.

G protein gamma2 is also known as G protein gamma6.

Historical Background

G protein β and γ subunits were first discovered as components of G proteins over 30 years ago. Since then, their role has evolved significantly from simple inhibitors of ► G protein α subunits to independent signaling regulators modulating a number of different cellular effectors. Although several isoforms of Gα were cloned in the early 1980s, it was not until 1986 that the transducin Gβ subunit was cloned (Fong et al. 1986). The following year, a second Gβ subunit was identified and the count rapidly increased to five different genes. Gβ1-4 shared 82–92% identity, while Gβ5 shared only 51–53% identity with the other four isoforms. As was the case for Gα and Gβ, the first Gγ subunit to be cloned was also from transducin (Hurley et al. 1984). The Gγ subunits are more structurally diverse than the Gβ subunits; 12 nonallelic mammalian Gγ genes encode proteins of between 68 and 75 amino acids that share between 27% and 76% sequence homology. If divided into subfamilies, the sequence similarities among family members are much greater. For example, Gγ1, Gγ11, and Gγ13 share 62–73% homology. Gγ subunits undergo several posttranslational modifications including isoprenylation of an invariant cysteine residue in a conserved CAAX motif at the carboxyl end of the protein which have been demonstrated to be important for membrane localization of Gβγ. Most Gγ subunits have a leucine at the carboxyl terminus which directs addition of a geranylgeranyl group, while some (Gγ1, Gγ8, Gγ11, and Gγ13) have a serine which permits addition of a farnesyl group. Gγ subunits form tight (essentially non-dissociable) complexes with Gβ, and confer structural diversity and functional diversity to the Gβγ signaling complex. Although Gβγ is made up of two polypeptides, Gβ and Gγ, it essentially exists as a single protein as they do not dissociate in the absence of denaturing agents. The high resolution structure of the Gβγ subunit was first elucidated in the context of the G protein heterotrimer (Lambright et al. 1996). Subsequently, the structure of the Gβγ dimer alone was solved (Sondek et al. 1996). The Gβ subunit is folded into four stranded β-sheets forming each of the seven blades of the prototypical circular β-propeller. The circular structure is held closed by a molecular "Velcro snap" in the seventh blade of the propeller. The first 57–70 amino acids N-terminal to the β-propeller form an α-helical domain, which is tightly associated with the Gγ subunit in a coiled-coil interaction.

Five different Gβ subunits and 12 different Gβγ subunits genes have been identified in the human and mouse genomes. The different subunits can pair to form unique $G\beta_x\gamma_x$ combinations, which contribute to the functional diversity of Gβγ signaling. Interpretation of phenotypes resulting from knockout of individual Gβγ subunits has failed to provide a clear picture of the role of each subunit, as Gβγ subunits participate in multiple, integrated functional interactions with receptors, Gα subunits, and effectors. Surprisingly, knockout of the Gβ1 subunit, despite its homology with Gβ2-4, was embryonic lethal (Okae and Iwakura). This suggests that the different Gβ subunits are developmentally regulated. This has been demonstrated directly for Gβ3 subunits in cardiomyocytes (Rybin and Steinberg 2008). Nevertheless, there is evidence suggesting that specific Gβγ subtypes interact with particular G protein-coupled receptors (GPCR). Genetic deletion of specific Gγ subunits in mice results in specific phenotypes. For example, deletion of Gγ7 resulted in distinct behavioral changes associated with loss of cAMP production in the striatum, while deletion of Gγ3 induced changes in metabolism resulting in resistance to a high fat diet (Schwindinger et al. 2003).

If all Gβ subunits interact and randomly form dimers with all Gγ subunits, there would be 60 possible combinations in total. Most can form pairs in vitro but some exceptions have been reported. For example, Gβ1 can combine with all known Gγ subunits, while

Gβ2 was shown to be more selective, associating with Gγ2, but not Gγ1. The region of Gγ defining the specificity of interaction with Gβ subunits has been localized to a 14-amino acid segment located toward the middle of the molecule (Spring and Neer 1994). Gβ5 is clearly an outlier with respect to sequence and its capacity to interact with Gγ. While Gβ5 can interact with some Gγ subunits, it appears to be weakly bound and the complex can be separated under non-denaturing conditions. Biochemical studies demonstrated that RGS7 formed stable complexes with Gβ5, but not other Gβ subunits (Cabrera et al. 1998) and was co-purified as a tight complex, while no Gγ subunit was found to co-purify or form stable complexes with Gβ5 (Witherow et al. 2000). Another study examining Gβ5 complex formation with different potential partners showed that Gβ5 slightly prefers Gγ2 relative to RGS7, suggesting that in native tissues, Gγ2 could potentially assemble with Gβ5 unless there is a tight regulation of this interaction by molecular chaperones (Yost et al. 2007).

While functional Gα subunits can be synthesized in almost any expression system, Gβγ synthesis seems more tightly regulated. This is not simply owing to differential posttranslational modification as both Gα and Gγ subunits are modified by the addition of lipid moieties that facilitate their association with lipid bilayers. For example, Gβγ can be synthesized in vitro in rabbit reticulocyte lysates. However, either cotranslationally or by subsequent attempts at assembly in vitro, formation of functional Gβγ dimers is inefficient; only 30–50% of the synthesized Gβ and Gγ subunits can form functional Gβγ dimers. Interestingly, Gβ subunits can be synthesized separately from Gγ subunits in rabbit reticulocytes and wheat germ extract, but these will not interact efficiently with Gγ subunits. By contrast, Gγ subunits can be synthesized in rabbit reticulocyte lysates, wheat germ extracts, and bacteria, and will efficiently associate with Gβ subunits. This specificity suggests that molecular chaperones are necessary for the proper folding of Gβ and subsequent assembly into a Gβγ dimer. Recent studies have indicated that there are preferential associations of such chaperone candidates for different Gβγ subunits in living cells. Members of the phosducin family were originally proposed to act as inhibitors of G protein signaling via sequestration of the Gβγ subunits from Gα and effector molecules. Phosducin-like proteins (PhLP 1–3) have been shown to serve as co-chaperones with the cytosolic chaperonin complex (CCT) to assist in folding a variety of nascent proteins (Martin-Benito et al. 2004; Lukov et al. 2006). CCT is an essential chaperone required for protein folding in the cytosol of eukaryotic cells. Among the known substrates of CCT are Gα and multiple proteins with β-propeller WD40 structures similar to Gβ. PhLP acts as a co-chaperone by binding above the CCT cavity and occluding the cavity to stabilize folding processes until native protein formation occurs. PhLP1 may act as a co-chaperone for the folding of the Gβ subunit until Gβγ reaches its native stable state. This idea is consistent with observations that when PhLP1 is deleted in *Dictyostelium*, Gβ does not colocalize with Gγ at the plasma membrane but is expressed in the cytosol, as if the Gγ interaction was inhibited. To facilitate Gβγ dimer formation, PhLP1 must be phosphorylated on serine residues by ▶ casein kinase 2 (CK2). A mutant of PhLP1 that cannot be phosphorylated (S18–20A) inhibits both Gβ release from CCT and subsequent Gβγ assembly. The mechanism for Gβ release from CCT may involve steric repulsion, thereby triggering release of a PhLP1-Gβ complex intermediate. Here, Gβγ subunits are not yet in their native form because the intermediate complex of PhLP1-Gβ does not contain Gγ subunits (Dupre et al. 2009; Lukov et al. 2006; Willardson and Howlett 2007).

Interestingly, Gγ was not found to interact with CCT either directly or in a complex with Gβ. A separate chaperone has also been identified for Gγ subunits. Dopamine receptor interacting protein 78 (DRiP78) is an ER membrane–bound HSP40 co-chaperone that regulates receptor transport to the plasma membrane of GPCRs such as β_2-adrenergic (β_2AR), dopamine D1, M2 muscarinic cholinergic, and angiotensin II AT1 receptors (Bermak et al. 2001; Leclerc et al. 2002; Dupre et al. 2007). Gγ subunits and DRiP78 initially colocalize in the ER, presumably facing the cytosolic compartment where they can interact with Gβ. It was suggested that DRiP78 maintains the stability of nascent Gγ in the absence of its heterotrimeric partners. Furthermore, DRiP78 interacts directly with PhLP1, suggesting that PhLP-Gβ complex might interact with DRiP78-Gγ complex, thus participating in the assembly of the native Gβγ dimer. DRiP78 acts as a co-chaperone for Gβγ assembly, protecting Gγ from degradation until both subunits can be assembled into their native form (Dupre et al. 2009).

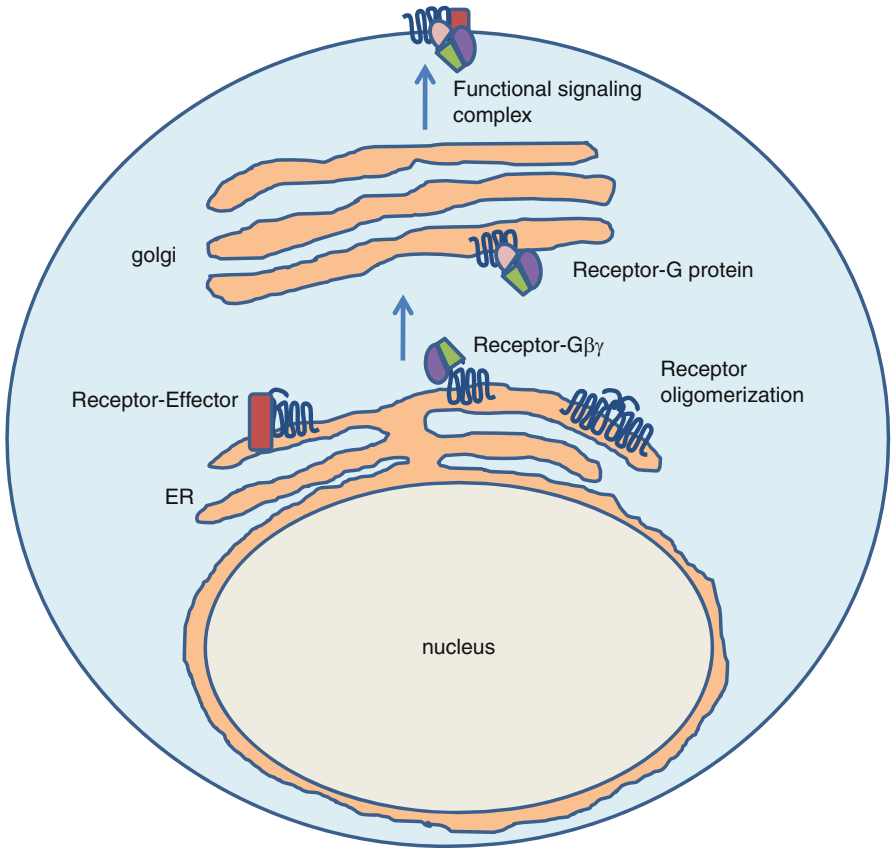
Role in G Protein Coupled Receptor Assembly, Organization, and Signaling

Receptors, G proteins, effectors, as well as several scaffolding/chaperone proteins are observed as multimeric complexes at the plasma membrane. Some studies have suggested that several of these signaling partners can form complexes before receptor activation by agonist, that is, they are pre-associated complexes. A rather confusing picture regarding the trafficking of individual components of GPCR signaling complexes has appeared in recent years. While it is clear that receptor oligomers themselves are assembled in the endoplasmic reticulum, the site of the assembly of the rest of the core signaling components was still unclear. Constitutive trafficking of some GPCR-regulated effectors, such as adenylyl cyclase isoforms or various ion channels, demonstrates that components of these signaling pathways can make their way to the membrane independently of the receptor or G protein. However, there is now significant evidence that like GPCR oligomers, these complexes are also formed early in their maturation steps. A number of studies have also demonstrated that receptors can directly interact with Gβγ subunits as well as Gα subunits (Wu et al. 1998; Wu et al. 2000; Mahon et al. 2006) and that many of these proteins interact initially in the endoplasmic reticulum (ER), including receptor dimers, receptor and Gβγ subunits, and effectors such as Kir3 channels and ▶ adenylyl cyclase with nascent Gβγ (Dupre et al. 2006; Rebois et al. 2006). If these complexes are preformed during protein biosynthesis and maturation, they would need to be trafficked inside the cell as a complex and not necessarily as individual proteins (Fig. 1). It is clear that both GPCRs and their effector molecules interact with G protein subunits before targeting to the plasma membrane. It has been suggested that Gβγ subunits might play an organizing role for assembly of GPCR-based signaling complexes as they interact with all of the relevant components, receptor, Gα, and effectors before any of them reach their destination. A potential mechanism may rely on early interactions with G protein subunits which may regulate assembly of receptor signaling complexes in the ER as when Gβγ function is inhibited by using a membrane-localized GRK2ct construct, Kir3.1/Gβγ complex formation in the ER can be blocked. Although still in its infancy, it seems that current research points toward an important role of Gβγ in signaling complex organizations.

G Protein βγ Regulation of Effectors

Early reconstitution studies with receptors and purified G proteins indicate that Gβγ is required for GPCR-induced nucleotide exchange. While some studies suggested Gβγ was necessary for the targeting of Gα subunits to the receptor, others have suggested that Gβγ might not only promote coupling, but also organize the structure of the Gα subunit so it is a substrate for GPCRs. Gβγ subunits can also regulate a wide range of effectors. Although various Gβγ binding motifs within effectors have been identified, no single consensus sequence or structure has been identified. However, energetic hot spots have been identified on the surface of Gβγ which allow several types of chemical interactions (ionic, hydrophobic), with multiple structural and chemical motifs at a single binding site (DeLano 2002; Fairbrother et al. 1998; Ma et al. 2001; Scott et al. 2001). Two general mechanisms for Gβγ-dependent regulation of effectors were proposed, according to the localization of the effector. In the case of cytosolic proteins like PLCβ2 or GRK2, whose substrates are membrane-bound, a potential mechanism for activation is via membrane-bound Gβγ recruitment to the effector. Membrane-embedded targets like Kir3 channels and adenylyl cyclase would be regulated via conformational changes. Some examples of different Gβγ effectors are given in Table 1, and a brief overview of the mechanisms used to regulate some effectors is presented here.

Gβγ is known to regulate several ion channels, such as Kir3 (inward rectifier G protein-gated K^+ channel) and voltage-gated calcium channels. The model of activation of membrane-bound channels such as Kir3 suggests that Gβγ binding to an intracellular cytosolic domain of the channel strengthens interactions between PIP_2 and the channel, which alters the structure at the mouth of the conductance pore to increase channel activity. PLCβ activation by Gβγ relies on the alteration of its enzymatic activity either through conformational alteration of the active isoforms or modulation of the orientation of PLC with respect to membrane surface (Romoser et al. 1996; Runnels et al. 1996). Evidence for the alteration of enzymatic activity comes from studies showing that fragments of PLCβ2 could compete for the PLCβ2 activation by Gβγ in transfected cells. Two overlapping fragments from the catalytic Y domain of PLC blocked activation by Gβγ or a Gαi coupled C5A receptor, but not Gαq



G Protein Beta/Gamma, Fig. 1 Schematic representation of a cell and assembly sites of G protein subunits, receptors, and effectors

G Protein Beta/Gamma, Table 1 Some Gβγ targets (Clapham and Neer 1997)

Effector	Regulation	Direct
N type, P/Q type Ca^{2+} channels	+	+
Inwardly rectifying K^+ channel GIRK/GIRK2, GIRK1/GIRK4	+	+
Phospholipase A_2	+	
PLCβ1, PLCβ2, PLCβ3	+	+
Adenylyl cyclase type II, type IV, type VII (activation)	+	+
Adenylyl cyclase type I, type III, type V, type VI (inhibition)	+	+
G protein coupled receptor kinases (GRK2 and GRK3)	+	+
Phosphoinositide 3 kinase γ	+	+
Raf-1	+	
SNAP-25	+	+
Bruton tyrosine kinase	+	
Dynamin	+	
RGS3, RGS4	+	
P-Rex1 Rac GEF	+	+

coupled α_1-adrenergic receptor (Kuang et al. 1996). A triple substitution (E574, L575, K576) in the PLCβ2 catalytic domain disrupted direct binding of Gβγ to PLCβ2, strongly suggesting that the region involved in the activation of PLCβ2 is also mediating the interaction (Bonacci et al. 2005; Sankaran et al. 1998). Supporting the notion that a pleckstrin homology (PH) domain is involved is the observation that the isolated PH domain from PLCβ2 interacts with Gβγ on membrane surfaces and splicing of the PH domain to PLCδ confers the ability for PLCδ to be activated by Gβγ (Wang et al. 2000). Further biochemical and structural analysis will be required to determine the exact mechanism involved in the process.

Gβγ will not only regulate classic GPCR signaling effectors, but also seems to be involved in vesicular traffic. Heterotrimeric G proteins are associated with intracellular membrane compartments. For example, it was shown that purified Gβγ subunits inhibited the export of a marker protein from the endoplasmic

reticulum (Schwaninger et al. 1992). Although the exact mechanisms are still unclear, both Gαs and Gβγ interact with the small GTPase ARF (Colombo et al. 1995), important for vesicular trafficking. Also, the association of ARF and β-COP with Golgi membranes is sensitive to a number of reagents that modulate heterotrimeric G protein function (Donaldson et al. 1991; Ktistakis et al. 1992). Interestingly, ARF might be an exception in the small GTPases family, as Rab, Ras, Rho, and their relatives did not display binding capacity to Gβγ subunits. The Ras exchange factor ▶ $CDC25^{Mm}$ or $p140^{Ras\text{-}GRF}$ could become constitutively activated following co-expression with Gβ1γ2 or Gβ1γ5, classifying it as a Gβγ-sensitive pathway.

Receptor-Independent Signaling by G Protein βγ

The classical view holds that GPCR signaling was mediated solely via activation of G proteins and their downstream effectors. However, an emerging area of research is non-receptor and nucleotide exchange-independent mechanisms for G protein activation (Luttrell 2005). Some of the mechanisms used to activate Gβγ involve binding to Gα, leading to the release of free Gβγ. Other proteins have been found to directly activate Gβγ via a direct interaction; however, the mechanisms remain poorly understood. Below is a sampling of these events.

Several activators of G protein signaling (AGS) have been identified to date. Group I AGS proteins are guanine nucleotide exchange factors that promote receptor-independent G protein activation by facilitating GDP dissociation from, and thus GTP binding to, Gα subunits. Group II AGS proteins (also called GPR or GoLoco proteins), in contrast, *inhibit* GDP dissociation, but may promote Gβγ signaling by altering the association between Gα and Gβγ. Group III AGS proteins differ from the others in that they do not appear to bind appreciably to Gα subunits but rather they produce their effects by binding directly to Gβγ. This interaction could promote dissociation of the heterotrimer subunits or simply compete for interaction with Gα. AGS2, a light chain component of the dynein motor in the cytoplasm may also be a direct Gβγ effector important for the modulation of neurite outgrowth and other processes required dynamic modulation of the cytoskeleton (Sachdev et al. 2007). Another study indicated that AGS9, a Group III AGS protein, modulated signaling events via interactions with an intact G protein heterotrimer, and may in fact form a signaling complex with the G protein heterotrimer and one of the classic Gβγ effectors, PLCβ (Yuan et al. 2007). However the exact function of the Group III AGS proteins remains unclear. Gβ1 can be phosphorylated on histidine 266 by histidine kinase and this high energy phosphate can be transferred to Gα-GDP, yielding Gα-GTP, by nucleoside diphosphate kinase B (NDPK B) (Wieland 2007). This may represent a mechanism for heterotrimeric G protein activation which does require a GPCR per se. In rat cardiomyocytes, Gβ1 H266L, a mutant which cannot be phosphorylated by histidine kinase showed reduced cAMP stimulation and reduced levels of cardiac contractility and decreased phosphorylation of phospholamban on serine 16 following receptor stimulation by agonist (Hippe et al. 2007).

G Protein βγ as a Target for Therapeutic Development

The diversity of physiological Gβγ functions in the cell suggests that their specific manipulation might be of significant therapeutic interest. Although the blockade of Gβγ in cells is a highly promising target, selective manipulation would be required. Indeed, the type of compound that would be preferred would be a small molecule capable of binding to Gβγ and affecting particular downstream signaling events, without affecting other vital functions of the heterotrimeric G protein (Gαβγ) and GPCR activation. Interestingly, some molecules have been found to act in such way. For example, the carboxy terminus of GRK2 (GRK2ct) (Koch et al. 1995) was used in cardiac cells to demonstrate the therapeutic potential of targeting Gβγ in cardiac function and failure. During heart failure, a loss of β-adrenergic receptor (βAR)-dependent expression is observed, where it has been shown that receptor desensitization following GRK2 phosphorylation of the receptor is occurs. GRK2 is controlled by Gβγ which, following GPCR activation, recruits GRK2 to the receptor leading to receptor phosphorylation. GRK2ct blocks this recruitment and permits continuation of receptor function, as demonstrated in transgenic cardiac overexpression

of GRK2ct in mice where cardiac performances were improved following β-adrenergic receptor stimulation. Another study demonstrated in cardiomyocytes isolated from human biopsies that expression of GRK2ct could significantly improve contractile function (Williams et al. 2004).

Peptides with the capacity to bind directly to Gβγ as observed for the SIRK/SIGK peptides. Although these peptides were shown to block Gβγ-dependent PLCβ2 and PI3Kγ activation in vitro, while they had no effect on the inhibition of ▶ adenylate cyclase, demonstrating selectivity for inhibition of some Gβγ targets. Cell-permeable versions of the peptides predicted to inhibit G protein signaling demonstrated rapid, efficient, and potent activation of the ERK/MAPK pathway, while a Gβ mutant (W332A), unable to bind the peptide showed significantly inhibited SIRK-dependent ERK activation (Malik et al. 2005).

These molecules could represent potentially specific Gβγ targets in inflammation, as well as drug addiction. Although one major therapeutic target, chemokines and their receptors physiology is extremely complex, due to their natural tendency for redundancy and the multitude of chemokines and receptors that can be expressed in a single cell. These considerations make it difficult to convincingly identify the factors responsible for inflammatory diseases such as arthritis. Gβγ regulates some downstream effectors such as PI3Kγ, which was shown to be involved in the regulation of neutrophil migration in response to chemoattractants and inhibition of inflammation. In this system, inhibition of PI3Kγ would bypass the inhibited interactions between Gβγ and targets that are critical for chemoattractant-dependent directed migration or reactive oxygen species production by neutrophils or other monocytes (Lehmann et al. 2008). For example, M119 blocked membrane translocation of ▶ P-Rex, a PIP_3- and Gβγ-regulated Rac2 exchange factor, in neutrophils. This action could result from direct blockade of P-Rex binding to Gβγ in addition to blocking PIP_3 production by ▶ PI 3-kinase (Lehmann et al. 2008; Zhao et al. 2007). In vivo validation of small molecules targeting Gβγ has been shown in studies of opioid-dependent nociception (Mathews et al. 2008). M119, when injected intracerebroventricularly in mice, could cause tenfold and sevenfold increases in the potencies of morphine and the μ-opioid receptor peptide DAMGO, respectively, while having little or no effect on the κ- or δ-opioid receptor dependent analgesic pathways. M119 also inhibited μ-opioid receptor dependent activation of PLC, and systemic administration of M119 resulted in a fourfold shift increase in potency of systemically administered morphine. A recent study showed that inhibition of Gβγ signaling with small molecules based on these peptides (M119) was protective in heart failure models (Casey et al.). These results suggest that small organic compounds that specifically regulate Gβγ signaling could have important therapeutic applications in several diseases.

Summary

Gβγ are important modulators of cellular function, highly implicated in G protein signaling, and possibly the scaffolding of receptors with their signaling partners. Despite years of investigation, the selectivity of assembly of the different subunits and the specific function of all these pairs is still poorly understood. It is likely that in the near future, new properties, mechanisms of action and functions will emerge which might provide us with a better understanding of the role of this key component in signal transduction. Given the large potential for therapeutic strategies targeting Gβγ, understanding how these proteins work in physiological systems will likely provide answers as to how we can manipulate them to develop novel therapeutic approaches to several diseases.

References

Bermak JC, Li M, et al. Regulation of transport of the dopamine D1 receptor by a new membrane-associated ER protein. Nat Cell Biol. 2001;3(5):492–8.

Bonacci TM, Ghosh M, et al. Regulatory interactions between the amino terminus of G-protein betagamma subunits and the catalytic domain of phospholipase Cbeta2. J Biol Chem. 2005;280(11):10174–81.

Cabrera JL, de Freitas F, et al. Identification of the Gbeta5-RGS7 protein complex in the retina. Biochem Biophys Res Commun. 1998;249(3):898–902.

Casey LM, Pistner AR, et al. Small molecule disruption of G beta gamma signaling inhibits the progression of heart failure. Circ Res. 107(4):532–9.

Clapham DE, Neer EJ. G protein beta gamma subunits. Annu Rev Pharmacol Toxicol. 1997;37:167–203.

Colombo MI, Inglese J, et al. Heterotrimeric G proteins interact with the small GTPase ARF. Possibilities for the regulation of vesicular traffic. J Biol Chem. 1995;270(41):24564–71.

DeLano WL. Unraveling hot spots in binding interfaces: progress and challenges. Curr Opin Struct Biol. 2002;12(1): 14–20.

Dessal AL, Prades R, et al. Rational design of a selective covalent modifier of G protein βγ subunits. Mol Pharmacol. 2011;79(1):24–33.

Donaldson JG, Kahn RA, et al. Binding of ARF and beta-COP to Golgi membranes: possible regulation by a trimeric G protein. Science. 1991;254(5035):1197–9.

Dupre DJ, Robitaille M, et al. Seven transmembrane receptor core signaling complexes are assembled prior to plasma membrane trafficking. J Biol Chem. 2006;281(45):34561–73.

Dupre DJ, Robitaille M, et al. Dopamine receptor-interacting protein 78 acts as a molecular chaperone for Ggamma subunits before assembly with Gbeta. J Biol Chem. 2007; 282(18):13703–15.

Dupre DJ, Robitaille M, et al. The role of Gbetagamma subunits in the organization, assembly, and function of GPCR signaling complexes. Annu Rev Pharmacol Toxicol. 2009;49: 31–56.

Fairbrother WJ, Christinger HW, et al. Novel peptides selected to bind vascular endothelial growth factor target the receptor-binding site. Biochemistry. 1998;37(51):17754–64.

Fong HK, Hurley JB, et al. Repetitive segmental structure of the transducin beta subunit: homology with the CDC4 gene and identification of related mRNAs. Proc Natl Acad Sci USA. 1986;83(7):2162–6.

Hippe HJ, Luedde M, et al. Regulation of cardiac cAMP synthesis and contractility by nucleoside diphosphate kinase B/G protein beta gamma dimer complexes. Circ Res. 2007; 100(8):1191–9.

Hurley JB, Fong HK, et al. Isolation and characterization of a cDNA clone for the gamma subunit of bovine retinal transducin. Proc Natl Acad Sci USA. 1984;81(22):6948–52.

Illenberger D, Walliser C, et al. Specificity and structural requirements of phospholipase C-beta stimulation by Rho GTPases versus G protein beta gamma dimers. J Biol Chem. 2003;278(5):3006–14.

Jezyk MR, Snyder JT, et al. Crystal structure of Rac1 bound to its effector phospholipase C-beta2. Nat Struct Mol Biol. 2006;13(12):1135–40.

Koch WJ, Rockman HA, et al. Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. Science. 1995;268(5215):1350–3.

Ktistakis NT, Linder ME, et al. Action of brefeldin A blocked by activation of a pertussis-toxin-sensitive G protein. Nature. 1992;356(6367):344–6.

Kuang Y, Wu Y, et al. Identification of a phospholipase C beta2 region that interacts with Gbeta-gamma. Proc Natl Acad Sci USA. 1996;93(7):2964–8.

Lambright DG, Sondek J, et al. The 2.0 A crystal structure of a heterotrimeric G protein. Nature. 1996;379(6563):311–9.

Leclerc PC, Auger-Messier M, et al. A polyaromatic caveolin-binding-like motif in the cytoplasmic tail of the type 1 receptor for angiotensin II plays an important role in receptor trafficking and signaling. Endocrinology. 2002;143(12): 4702–10.

Lehmann DM, Seneviratne AM, et al. Small molecule disruption of G protein beta gamma subunit signaling inhibits neutrophil chemotaxis and inflammation. Mol Pharmacol. 2008;73(2):410–8.

Lukov GL, Baker CM, et al. Mechanism of assembly of G protein betagamma subunits by protein kinase CK2-phosphorylated phosducin-like protein and the cytosolic chaperonin complex. J Biol Chem. 2006;281(31):22261–74.

Luttrell LM. Composition and function of g protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. J Mol Neurosci. 2005;26(2–3):253–64.

Ma B, Wolfson HJ, et al. Protein functional epitopes: hot spots, dynamics and combinatorial libraries. Curr Opin Struct Biol. 2001;11(3):364–9.

Mahon MJ, Bonacci TM, et al. A docking site for G protein betagamma subunits on the parathyroid hormone 1 receptor supports signaling through multiple pathways. Mol Endocrinol. 2006;20(1):136–46.

Malik S, Ghosh M, et al. Ric-8 enhances G protein betagamma-dependent signaling in response to betagamma-binding peptides in intact cells. Mol Pharmacol. 2005;68(1):129–36.

Martin-Benito J, Bertrand S, et al. Structure of the complex between the cytosolic chaperonin CCT and phosducin-like protein. Proc Natl Acad Sci USA. 2004;101(50): 17410–5.

Mathews JL, Smrcka AV, et al. A novel Gbetagamma-subunit inhibitor selectively modulates mu-opioid-dependent antinociception and attenuates acute morphine-induced antinociceptive tolerance and dependence. J Neurosci. 2008;28(47):12183–9.

Okae H, Iwakura Y. Neural tube defects and impaired neural progenitor cell proliferation in Gbeta1-deficient mice. Dev Dyn. 239(4):1089–101.

Rebois RV, Robitaille M, et al. Heterotrimeric G proteins form stable complexes with adenylyl cyclase and Kir3.1 channels in living cells. J Cell Sci. 2006;119(Pt 13):2807–18.

Romoser V, Ball R, et al. Phospholipase C beta2 association with phospholipid interfaces assessed by fluorescence resonance energy transfer. G protein betagamma subunit-mediated translocation is not required for enzyme activation. J Biol Chem. 1996;271(41):25071–8.

Runnels LW, Jenco J, et al. Membrane binding of phospholipases C-beta 1 and C-beta 2 is independent of phosphatidylinositol 4,5-bisphosphate and the alpha and beta gamma subunits of G proteins. Biochemistry. 1996;35(51):16824–32.

Rybin VO, Steinberg SF. G protein betagamma dimer expression in cardiomyocytes: developmental acquisition of Gbeta3. Biochem Biophys Res Commun. 2008;368(2):408–13.

Sachdev P, Menon S, et al. G protein beta gamma subunit interaction with the dynein light-chain component Tctex-1 regulates neurite outgrowth. EMBO J. 2007;26(11): 2621–32.

Sankaran B, Osterhout J, et al. Identification of a structural element in phospholipase C beta2 that interacts with G protein betagamma subunits. J Biol Chem. 1998;273(12):7148–54.

Schwaninger R, Plutner H, et al. Multiple GTP-binding proteins regulate vesicular transport from the ER to Golgi membranes. J Cell Biol. 1992;119(5):1077–96.

Schwindinger WF, Betz KS, et al. Loss of G protein gamma 7 alters behavior and reduces striatal alpha(olf) level and cAMP production. J Biol Chem. 2003;278(8):6575–9.

Scott JK, Huang SF, et al. Evidence that a protein-protein interaction 'hot spot' on heterotrimeric G protein betagamma subunits is used for recognition of a subclass of effectors. EMBO J. 2001;20(4):767–76.

Sondek J, Bohm A, et al. Crystal structure of a G-protein beta gamma dimer at 2.1A resolution. Nature. 1996;379(6563):369–74.
Spring DJ, Neer EJ. A 14-amino acid region of the G protein gamma subunit is sufficient to confer selectivity of gamma binding to the beta subunit. J Biol Chem. 1994;269(36):22882–6.
Stow JL, Heimann K. Vesicle budding on Golgi membranes: regulation by G proteins and myosin motors. Biochim Biophys Acta. 1998;1404(1–2):161–71.
Wang T, Dowal L, et al. The pleckstrin homology domain of phospholipase C-beta(2) links the binding of gbetagamma to activation of the catalytic core. J Biol Chem. 2000;275(11):7466–9.
Wieland T. Interaction of nucleoside diphosphate kinase B with heterotrimeric G protein betagamma dimers: consequences on G protein activation and stability. Naunyn Schmiedebergs Arch Pharmacol. 2007;374(5–6):373–83.
Willardson BM, Howlett AC. Function of phosducin-like proteins in G protein signaling and chaperone-assisted protein folding. Cell Signal. 2007;19(12):2417–27.
Williams ML, Hata JA, et al. Targeted beta-adrenergic receptor kinase (betaARK1) inhibition by gene transfer in failing human hearts. Circulation. 2004;109(13):1590–3.
Witherow DS, Wang Q, et al. Complexes of the G protein subunit gbeta 5 with the regulators of G protein signaling RGS7 and RGS9. Characterization in native tissues and in transfected cells. J Biol Chem. 2000;275(32):24872–80.
Wu G, Benovic JL, et al. Receptor docking sites for G-protein betagamma subunits. Implications for signal regulation. J Biol Chem. 1998;273(13):7197–200.
Wu G, Bogatkevich GS, et al. Identification of Gbetagamma binding sites in the third intracellular loop of the M(3)-muscarinic receptor and their role in receptor regulation. J Biol Chem. 2000;275(12):9026–34.
Yost EA, Mervine SM, et al. Live cell analysis of G protein beta5 complex formation, function, and targeting. Mol Pharmacol. 2007;72(4):812–25.
Yuan C, Sato M, et al. Signaling by a non-dissociated complex of G protein betagamma and alpha subunits stimulated by a receptor-independent activator of G protein signaling, AGS8. J Biol Chem. 2007;282(27):19938–47.
Zhao T, Nalbant P, et al. Signaling requirements for translocation of P-Rex1, a key Rac2 exchange factor involved in chemoattractant-stimulated human neutrophil function. J Leukoc Biol. 2007;81(4):1127–36.

G Protein Gamma

▶ G Protein Beta/Gamma

G Protein α i

▶ G Protein α i/o/z

G Protein α i/o/z

Joe B. Blumer[1,2] and Gregory G. Tall[3]
[1]Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA
[2]Department of Neurosciences, Medical University of South Carolina, Charleston, SC, USA
[3]Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY, USA

Synonyms

Adenylyl cyclase inhibitory Gi alpha subunit; G alpha (o); G alpha z; G protein alpha i; G protein alpha o; G protein alpha z; G protein α i; G protein α o; G protein α z; Gi protein alpha subunit; GNAI; GNAO; GNAO1; GNAZ; Go alpha subunit; Guanine nucleotide binding protein, alpha inhibiting; Guanine nucleotide binding protein, alpha o; Guanine nucleotide binding protein, alpha z subunit; Guanine nucleotide binding regulatory protein, alpha i; Gz alpha subunit

Historical Background: Discovery of G Protein α i as the Inhibitor of Hormone-Stimulated Adenylyl Cyclase Activity

During the discovery purification that identified G protein (alpha) s (Gαs) as the ▶ adenylyl cyclase activator, Gilman and colleagues noted a 41 kDa contaminating protein (now known as G protein alpha i (Gαi)) that persisted into the final stages of the Gsαβγ heterotrimer purification. Hormonal activation *and* inhibition of ▶ adenylyl cyclase were known at the time to be GTP-dependent. The 41 kDa protein was found to be an ADP-ribosylation substrate of *Bordetella pertussis* toxin (PTX). Importantly, PTX was known to block hormonal inhibition of ▶ adenylyl cyclase activity. Activity-based purification of the inhibitor (G protein i) from rabbit liver correlated with PTX-catalyzed ADP-ribosylation of the 41 kDa (Gαi) substrate. The purified G protein i heterotrimer consisted of the 41 kDa GTP binding α subunit, the 35 kDa Gβ subunit, and a later recognized 8–10 kDa Gγ

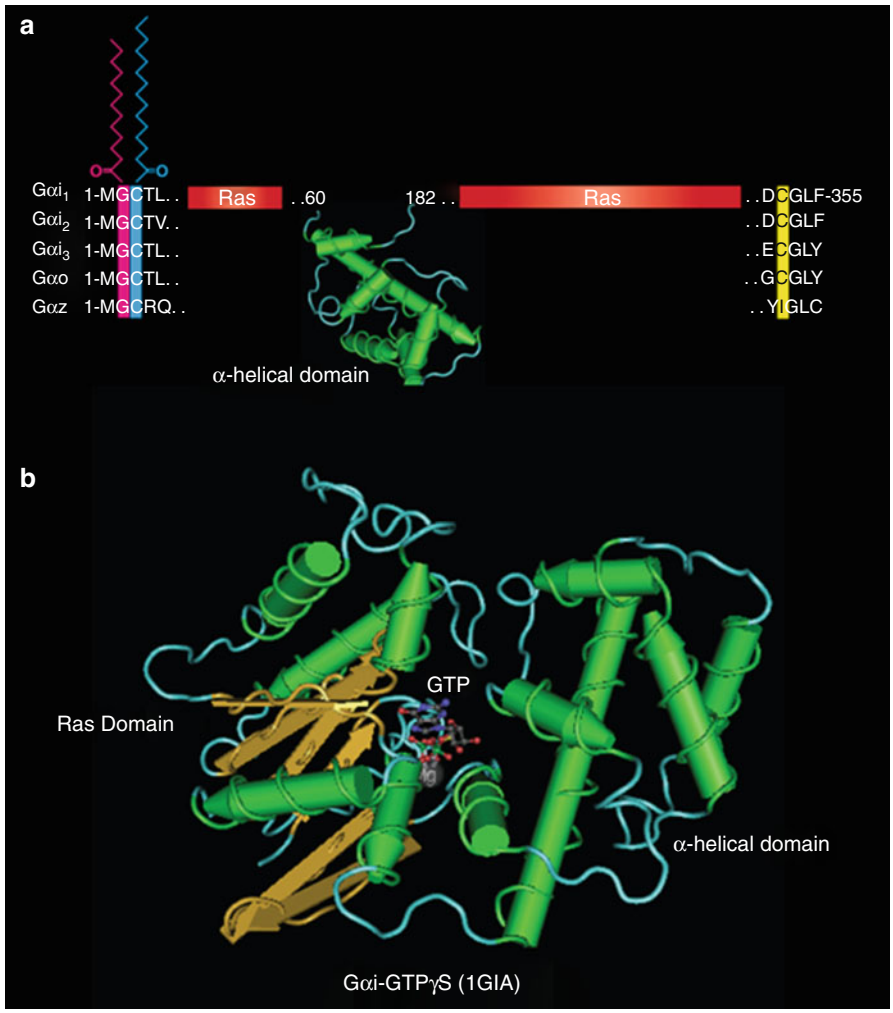
subunit. When reconstituted into cellular membranes pretreated with PTX, purified G protein i heterotrimer restored the ability of hormone and GTP to inhibit ▶ adenylyl cyclase activity (for review and a comprehensive historical account of primary references, see Gilman (1995)). Subsequent molecular cloning endeavors collectively identified three closely related G protein αi genes that encode the $G\alpha i_1$ (NP_002060), $G\alpha i_2$ (NP_002061), and $G\alpha i_3$ (or in some current databases, GαK) (NP_006487) proteins (Didsbury and Snyderman 1987; Jones and Reed 1987; Suki et al. 1987). Gαo was discovered by purification of an "other" G protein substrate of PTX from bovine brain and is estimated to constitute a substantial percentage (~1%) of total brain membrane protein (Neer et al. 1984; Sternweis and Robishaw 1984). Two alternatively spliced Gαo gene products encode two nearly identical proteins, $G\alpha o_A$ (or $G\alpha o_1$) (NP_066268) and $G\alpha o_B$ (or $G\alpha o_2$) (NP_620073). Later, peptide antisera raised against translated Gαz cDNA sequences were used to purify Gαz from bovine brain (Casey et al. 1990). Unlike all other Gαi-class G proteins, Gαz (NP_002064) does not contain the specific cysteine residue located in the fourth position from the carboxyl terminus that is the site of *Pertussis* toxin-catalyzed ADP-ribosylation Fig. 1. G protein α T (Transducin), and G protein α Gust (Gustducin) are tissue-specific G protein αi-class family members in the visual transduction and tastant sensing pathways, respectively, and are the subjects of a separate essay. The Gαi G protein subfamily comprises the largest number of individual members and constitutes the bulk of expressed G proteins in most tissue types.

Gi Biosynthesis

Gi heterotrimers follow a similar biosynthetic route as most G proteins, but aspects of Gαi-class subunit biosynthesis include unique processing. Gα subunits are translated on free cytosolic ribosomes and the initiator methionine is cleaved. The resultant amino terminal Gαi/o/z glycine residue becomes myristoylated. *N*-myristoyl transferase catalyzes the covalent attachment of a 14-carbon myristate chain to the glycine amino group during protein translation (Mumby et al. 1990). The nascent, myristoylated Gαi chain is folded by action of the cytosolic chaperone complex (CCT), as documented for Gαi family member G protein α Transducin (Farr et al. 1997). A new role for Gα non-receptor ▶ Ric-8 guanine nucleotide exchange factors (GEFs) showed that Ric-8A may aid Gαi folding with the CCT, or function after folding is mostly complete to promote the initial membrane association of nascent Gαi/o subunits. In *Ric-8A*$^{-/-}$ cells, Gαi (and also Gαq and Gα12/13) subunits exhibit membrane targeting defects and were subjected to rapid turnover (Gabay et al. 2011). Gαi binds to Gβγ on the endoplasmic reticulum membrane to form the nascent Gi heterotrimer, which is requisite for trafficking of the intact heterotrimer to the plasma membrane. Posttranslational Gαi/o/z palmitoylation at cysteine 3 is also required for G protein plasma membrane targeting (Fig. 1a). ER or Golgi-enriched DHHC palmitoyl transferases are likely responsible. The actual G protein heterotrimer trafficking mechanism has not been elucidated, but may involve a diffusive or membrane sampling type of mechanism until plasma membrane residence is achieved. Once G protein heterotrimers reach the inner leaflet of the plasma membrane, they are considered mature and sufficient to transduce signals from G protein coupled receptors (GPCRs). Plasma membrane residence is not static and Gαi/o and Gβγ subunits undergo agonist-dependent and -independent translocation to (and from) other cellular residences, including the Golgi during a process that may intersect a dynamic palmitoylation and depalmitoylation cycle (for comprehensive reviews of G protein trafficking mechanisms and accounts of primary references therein, see Chisari et al. (2007), Marrari et al. (2007), Saini et al. (2009)).

Gαi Structure and G Protein Catalytic Mechanism

Gαi/o/z subunits share primary structural features common to all heterotrimeric G protein α subunits. Each contain a core Ras small GTP-binding protein homology domain consisting of Gαi amino terminal amino acids ~1–60, interrupted by a region of ~120 amino acids, followed by the carboxyl-terminal ~175 amino acids that complete the Ras homology domain. The intervening region has high α-helical content and is commonly referred to as the Gα subunit α-helical domain. It is not found in small GTP binding proteins (Fig. 1a). Guanine nucleotide and its Mg^{+2} cofactor bind the Ras domain and are sandwiched between the



G Protein α i/o/z, Fig. 1 (**a**) Members of the Gα(i) family comprise a conserved Ras small GTP binding domain containing a ~120 amino acid insertion termed the α-helical domain. The initiator methionine is cleaved from all Gαi species leaving an amino-terminal glycine 2 residue that becomes irreversibly *N*-myristoylated (14 carbon chain) (shown in *magenta*). The sulfhydryl group of Cysteine 3 is reversibly palmitoylated (16 carbon chain) (shown in *light blue*). The fourth residue from the carboxyl-terminus is the site of *Pertussis* toxin-catalyzed ADP-ribsolyation of all Gαi species (cysteine) except Gαz (isoleucine) (highlighted in *yellow*). (**b**) Rendering of the structure of $G\alpha i_1$ bound to Mg^{+2} and GTPγS. GTPγS is bound in the guanine nucleotide-binding pocket located between the Ras and α-helical domains. Pivoting and/or rotation about the two loop/hinge regions that connect the two domains is thought to be the mechanism by which guanine nucleotide exchange occurs. The $G\alpha i_1$-GTPγS structure was obtained from RCSB PDB (www.pdb.org) of PDB ID: 1GIA (Coleman et al. 1994) and modified using the Cn3D v.4.3 macromolecular structure viewer software available from the NCBI

Ras and α-helical domains. Like other Gα subunits, the Gαi carboxyl terminal residues constitute one region responsible for the specificity of G protein-receptor coupling.

G protein catalytic mechanisms have been elucidated biochemically and structurally, predominantly using $G\alpha i_1$, as well as Gαs and Gαq as the prominent model G proteins (for review and primary references therein, see Elliott (2008), Gilman (1987), Sprang (1997)). The G protein catalytic cycle consists of three primary steps, GDP release (intrinsic $G\alpha i_1$ rate: ~0.02–0.05 min^{-1}), subsequent GTP binding to the open, nucleotide-free Gαi subunit (predicted to be very fast), and subsequent GTP hydrolysis ($G\alpha i_1$ rate ~3 min^{-1}). As is evident, GDP release is rate limiting to the steps of Gαi activation and single turnover and

steady-state hydrolysis of GTP. GPCRs act as guanine nucleotide exchange factors (GEFs) for Gi heterotrimers and accelerate the GDP release rate such that it may no longer be the slowest step in the G protein catalytic cycle. In many activated-GPCR signaling contexts, the intrinsic Gαi GTP hydrolysis rate becomes limiting, underscoring the relevancy of the action of RGS GTPase activating proteins. RGS proteins bind to Gαi (and Gαq and Gα12/13-class) subunits and accelerate the GTP hydrolysis rate to keep pace with the rate of GPCR-stimulated GDP release (and apparent GTP binding) (Berman and Gilman 1998). The mechanism of *Pertussis* toxin inhibition of Gi is to ADP-ribosylate Gαi subunits. This renders Gi heterotrimers as non-substrates for GPCR-mediated activation.

The X-ray crystal structures of various forms of Gαi helped reveal the important features of G protein function and catalysis, and demonstrated the changes in the G protein Ras domain switch regions that occur during the transition from the GDP-bound to GTP-bound state. The conformational differences between these two states enable the G protein to interact with different sets of protein binding partners. G protein structures and mechanisms of action are reviewed in exquisite detail by Sprang (1997). Many structures of G proteins were produced using Gαi subunits as the model G protein and prominent examples include the following structures with PDB ID numbers: RCSB PDB (www.pdb.org): $G\alpha i_1$-GTPγS (1GIA) (Fig. 1b) (Coleman et al. 1994), $G\alpha i_1$-GDP-AlF_4^- (1GFI) (Coleman et al. 1994), $G\alpha i_1$-GDP (1GDD) (Mixon et al. 1995), $G\alpha i_1$-GDP:Gβγ (1GP2) (Wall et al. 1995), Gαi/αT chimera:Gβγ (1GOT) (Lambright et al. 1996), and $G\alpha i_1$-GDP-AlF_4^-:RGS4 (1AGR) (Tesmer et al. 1997).

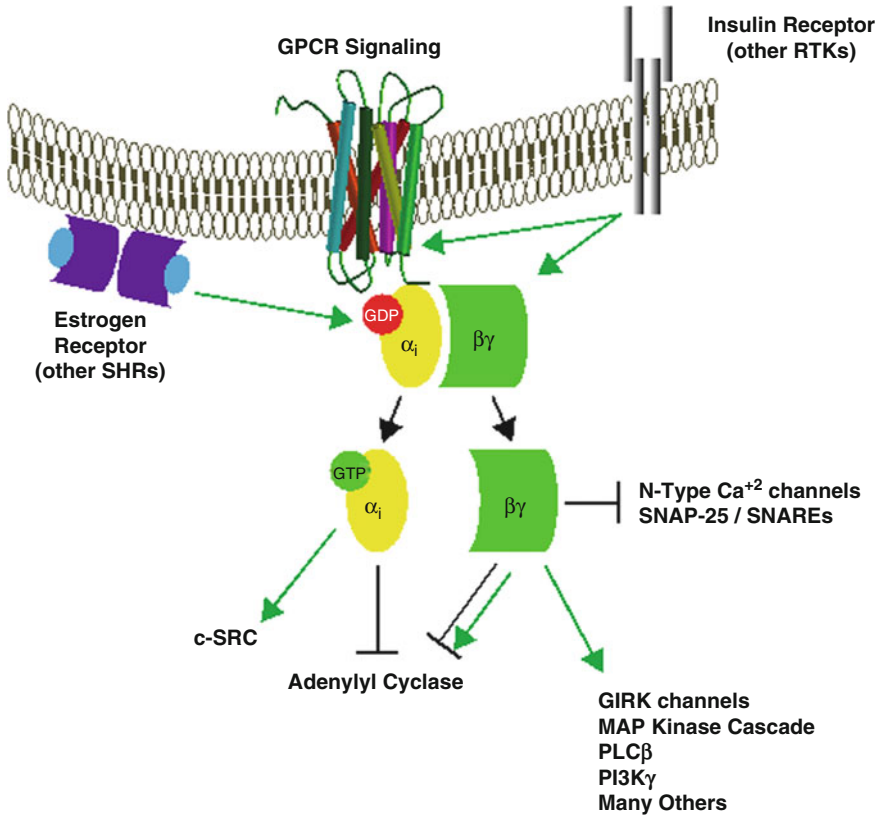
GPCR-Regulated Gi Signaling

GPCR regulation of Gi heterotrimer activation and downstream signaling pathways can broadly be grouped into two classes: effector enzyme targets regulated directly by Gαi/o/z-GTP and those regulated by Gβγ subunits released from Gi-class heterotrimers (Fig. 2).

Gαi-GTP targets – Agonist-stimulated GPCRs coupled to Gi-class heterotrimers produce Gαi-GTP and Gβγ. Gαi/z-GTP interacts directly with ▶ adenylyl cyclase (AC) isoforms I, V, and VI to inhibit catalytic production of the soluble second messenger cAMP. In comparison to Gαi, Gαo has reduced ability to inhibit adenylyl cyclase I. Gαs-GTP is the G protein stimulator of all membrane-bound ▶ adenylyl cyclase isoforms. The Gαi-GTP interaction site of AC is distinct from the Gαs-GTP binding site. Combined mutagenesis, biochemical, and kinetic modeling studies show that the opposed allosteric action of these G proteins may occur in simultaneous fashion (Chen-Goodspeed et al. 2005; Dessauer et al. 1998; Taussig et al. 1994). Gβγ liberated from Gi heterotrimers also influences AC activities in an AC isoform-dependent manner (activation or inhibition depending on isoform). The activities of Gβγ and the small molecule AC activator, forskolin are synergistic to the action of Gα-GTP (for comprehensive reviews and accounts of primary references therein, see Sadana and Dessauer (2009), Sunahara et al. (1996), Taussig and Gilman (1995)).

Free Gαi subunits bisect tyrosine kinase signaling pathways in ways that are unique from Gβγ (Gi) regulation. In vitro ▶ c-SRC tyrosine kinase activity was activated directly by purified Gαi-GTPγS (and Gαs-GTPγS), and ▶ c-SRC-dependent phosphorylation of cellular substrates appeared to be enhanced by co-expression of (activated) GTPase-deficient Gαi or Gαs (Ma et al. 2000). Reciprocally, ▶ c-SRC can phosphorylate Gαi and Gαs on tyrosine residues to alter adrenergic receptor coupling to the phosphorylated G proteins (Hausdorff et al. 1992). There are multiple modes of pathway integration and crosstalk between Gi and SRC or other tyrosine kinases (Natarajan and Berk 2006). Gαi and Gβγ subunits liberated from Gi heterotrimers have both redundant and opposed contextual effects toward tyrosine kinase signaling outputs.

Gi-dependent Gβγ targets – The majority of cellular responses to GPCR-induced Gi heterotrimer activation are arguably manifested by Gβγ. One explanation of why Gβγ dimers released from Gαi-GTP elicit signaling responses that other Gα/GPCR species do not is that Gi-class heterotrimers constitute the majority of expressed G proteins in many tissues. The "dose" of Gβγ produced from Gi heterotrimers may be above a particular response threshold for a given effector enzyme. This threshold may not be attainable by release of Gβγ from lower expressed G protein heterotrimer subtypes (Gq, ▶ Gs, G12/13). Regulated subcellular localization and



G Protein α i/o/z, Fig. 2 *Major Gαi signaling pathways and mechanisms of Gαi regulation*. G protein coupled receptor (GPCR) activation of Gi heterotrimers results in the exchange of GDP for GTP bound to the Gαi subunit. Gαi-GTP and Gβγ are dissociated or altered in conformation such that they functionally interact with downstream effector enzymes. Gαi-GTP inhibits the enzymatic activity of its major target, adenylyl cyclase and appears to functionally activate c-SRC kinase activity through direct binding. Gβγ subunits interact with a variety of effector targets. Major Gβγ effector targets are shown, but the list is not exclusive in this figure. Crosstalk from other receptor signaling systems regulates the activity of Gi. Estrogen receptors and other steroid-hormone-receptors (SHRs) regulate Gαi and Gβγ signaling pathways using a mechanism that might not include Gαi guanine nucleotide exchange (Kumar et al. 2007). The insulin receptor and other receptor tyrosine kinases (RTKs) exhibit extensive cross-talk with GPCR systems that regulate Gi heterotrimers, and in some cases are found in protein-protein complexes containing Gi subunits (Natarajan and Berk 2006; Kreuzer et al. 2004). The action of many SHR and RTK agonists to activate Gi signaling is sensitive to *Pertussis* toxin

scaffolding of G proteins and effectors also contributes to the specificity of Gi-mediated Gβγ signaling.

The complete list of effectors regulated by Gβγ subunits is expansive and beyond the scope of this Gαi topical essay (for comprehensive reviews of Gβγ signaling and the roles of Gi, see Clapham and Neer (1997), Smrcka (2008)). In brief, Gi-derived Gβγ activates the mitogen-activated protein kinase (MAPK) signaling cascade. Extracellular regulated kinases (ERKs) and downstream kinases are phosphorylated in response to cell treatment with agonists that stimulate Gi-coupled receptors (Gutkind 2000). Phospholipase Cβ (PLCβ) enzyme activity is co-modulated by Gαq-GTP and Gβγ subunits released from Gi heterotrimers to mediate phosphatidylinositol 3,4-bisphosphate (PIP_2) hydrolysis (Exton 1994). The produced inositol trisphosphate (IP_3) binds IP_3 receptors to activate Ca^{+2} release from intracellular stores. Gβγ directly regulates the activities of many ion channels. Two prominent examples are activation of G protein inwardly rectifying potassium (GIRK) channels that regulate cellular K^+ influx, and inhibition of N-type Ca^{+2} channels that convert neuronal action potentials to neurotransmitter release through cellular

Ca^{+2} influx (Nathan 1997; Tedford and Zamponi 2006). Gi heterotrimer activation potentiates signaling through the phosphatidyl-inositol-3 kinase (PI3Kγ) and protein kinase B/Akt signaling pathways. The 110 kDa PI3Kγcatalytic subunit is a direct effector of Gβγ (Stephens et al. 1994).

Gi-Family Regulation of Vesicle-Mediated Protein Transport

Gi family members impart multiple modes of regulation toward intracellular trafficking and secretory processes by influencing vesicle budding, priming, and fusion events. $G\alpha i_3$ overexpression or PTX treatment disrupted protein trafficking through the secretory pathway (Stow et al. 1991). The heterotrimeric G-protein activators AlF_4^-, mastoparan and related peptides, and compound 48/80 inhibited ER to Golgi transport (Beckers and Balch 1989; Schwaninger et al. 1992). PTX treatment also blocked vesicle budding from the trans-Golgi (Barr et al. 1991), and studies in model organisms revealed that Gαi/o proteins were important regulators of exocytosis (Ch'ng et al. 2008; Hajdu-Cronin et al. 1999; Miller et al. 1999; Lackner et al. 1999; Vashlishan et al. 2008).

One of the most well-studied trafficking pathways influenced by Gi family members is the regulation of insulin secretion by pancreatic β cells. Knowledge of PTX enhancement of glucose-stimulated insulin secretion (GSIS) predated the actual discovery of Gαi by at least a decade. PTX is known as islet activating protein (IAP) (Katada and Ui 1979, 1981a) and PTX treatment enhanced GSIS whether administered systemically or directly to primary cultures of pancreatic islets or β cell lines (Katada and Ui 1979, 1981a, b; Gulbenkian and Schobert 1968; Szentivanyi et al. 1963; Tabachnick and Gulbenkian 1969; Yajima et al. 1978). Subsequent reports showed that insulin secretion was inhibited by hormones that are Gi/o-coupled (reviewed in Sharp 1996). Consistent with these findings, selective ectopic expression of PTX in pancreatic β cells induced basal hyperinsulinemia and enhanced GSIS and glucose tolerance (Regard et al. 2007).

The specific Gαi family subunit(s) responsible for regulating insulin secretion has not been determined since the PTX effects would implicate $G\alpha i_1$, $G\alpha i_2$, or $G\alpha i_3$ and/or $G\alpha_o$. In fact, the regulation by Gαi family members appears to be redundant in part, as individual roles for Gαi, Gαo, and PTX-insensitive Gαz have been described. In the case of $G\alpha i_2$, chemically-induced diabetic rodent models exhibited decreased $G\alpha i_2$ expression in liver (Gawler et al. 1987) and adipose tissue (Baculikova et al. 2008), and antisense-mediated loss of $G\alpha i_2$ expression in liver and white adipose tissue lead to the development of insulin resistance (Moxham and Malbon 1996). Manipulation of the activation state of $G\alpha i_2$ in mice, either by conditional expression of GTPase-deficient $G\alpha i_2$-Q205L or homozygous knock-in of $G\alpha i_2$-G184S RGS-insensitive alleles (suggesting a prolonged time of $G\alpha i_2$ in the activated, GTP-bound state) resulted in enhanced glucose tolerance (Chen et al. 1997) or mice that were lean, resistant to high-fat diet-induced diabetes and had increased insulin sensitivity (Huang et al. 2008). These data suggest a key role for $G\alpha i_2$ in insulin secretion and signal regulation.

$G\alpha_o$ also participates in the regulation of insulin secretion by inhibiting secretory vesicle docking. A mouse with a conditional null $G\alpha_o$ allele in pancreatic islet cells had improved glucose tolerance and the effect of PTX to enhance insulin secretion was blocked, which led the authors to conclude that $G\alpha_o$ is the primary target of the PTX effect on insulin secretion (Zhao et al. 2010a). It was subsequently demonstrated that the $G\alpha o_B$ isoform was responsible for this effect (Wang et al. 2011).

Although most aspects of hormonal regulation of insulin secretion are PTX-sensitive, this is not always the case. Kimple and colleagues demonstrated that PGE1 inhibition of GSIS was insensitive to PTX and that this effect was blocked in a pancreatic β-cell line with reduced Gαz expression (Kimple et al. 2005). The same authors also demonstrated that Gαz-null mice are hyperinsulinemic and have increased glucose tolerance (Kimple et al. 2008). Collectively, Gαz appears to have an important role in hormonal regulation of insulin secretion apart from the roles ascribed to the PTX-sensitive Gαi and Gαo regulation.

Gi-family regulation of insulin vesicle secretion is manifested by liberated Gβγ subunits and shares a conserved mechanism with Gi regulation of neurotransmitter release. Gβγ subunits inhibit neurotransmitter release (Blackmer et al. 2005, 2001; Gerachshenko et al. 2005) by binding directly to the SNARE complex component SNAP-25 to disrupt synaptic vesicle fusion (Blackmer et al. 2005; Gerachshenko et al. 2005). SNARE complexes and

intracellular calcium levels similarly regulate insulin-containing secretory vesicle fusion and therefore insulin release from pancreatic β cells (Wang and Thurmond 2009). The secretory-promoting effects of elevated Ca^{+2} are blocked by noradrenaline, implicating Gi-family heterotrimers. Noradrenaline-inhibited insulin secretion was also blocked by Gβ antibodies, a Gβγ-activating peptide mSIRK, or *Botulinum A* toxin which cleaves the Gβγ binding site from the SNAP-25 carboxyl terminus (Zhao et al. 2010b). These results show that Gβγ released from Gi heterotrimers is the responsible G protein species that regulates secretory vesicle fusion and insulin/neurotransmitter release.

Noncanonical Gαi Signaling

As G-proteins became established as signal transducers for GPCRs at the cell surface, functional evidence suggested additional roles for Gi and Gαi in subcellular regions and contexts distinct from GPCR and classic effector signaling. Cell fractionation and immunofluorescence studies demonstrated populations of Gi proteins that did not reside on the plasma membrane, and in some cases Gαi was not always associated with Gβγ (Stow et al. 1991; Denker et al. 1996; Lin et al. 1998; Maier et al. 1995; Montmayeur and Borrelli 1994; Muller et al. 1994; Ogier-Denis et al. 1995; Pimplikar and Simons 1993; Schurmann et al. 1992; Wilson et al. 1993, 1994). Noncanonical roles for Gαi proteins include regulation of Golgi structure and function, (Jamora et al. 1999; Yamaguchi et al. 2000), signaling interactions with tyrosine kinase and steroid hormone receptors (Kreuzer et al. 2004; Kumar et al. 2007), and interactions with GPR/GoLoco proteins to regulate mitotic spindle positioning and asymmetric cell division (Yu et al. 2000; Gotta and Ahringer 2001; Parmentier et al. 2000; Schaefer et al. 2000).

Gαi Regulation of Golgi Function and Structure

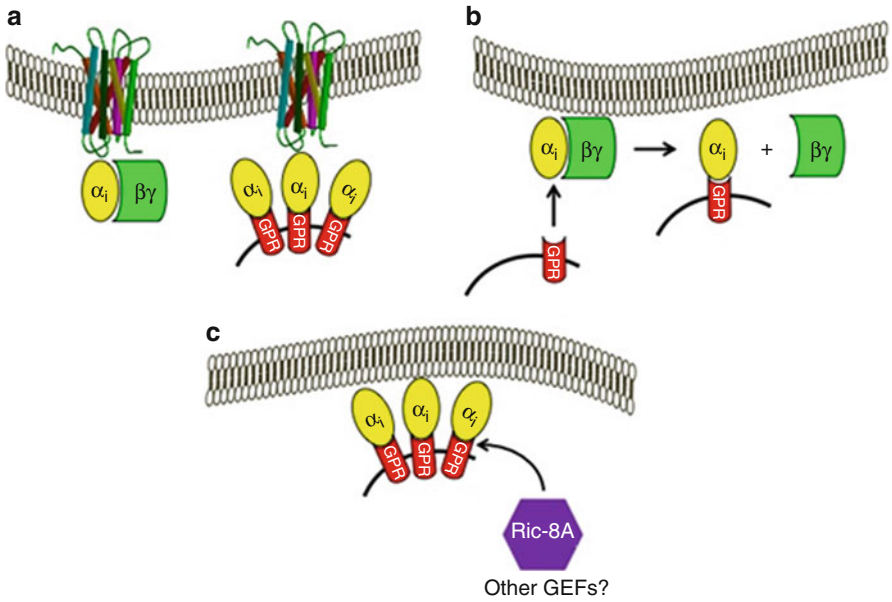
Gi-subunits, particularly Gαz and $G\alpha i_3$, are Golgi-localized (Stow et al. 1991; Wilson et al. 1993, 1994; Stow and de Almeida 1993). In addition to regulating vesicular protein transport through the Golgi, Gi/o-family proteins regulate overall Golgi structure. Chemically induced Golgi fragmentation was blocked by exogenous application of Gα subunits, including $G\alpha i_3$, to permeabilized NRK cells (Jamora et al. 1997) or by overexpression of Gαz or $G\alpha i_2$ (Yamaguchi et al. 2000). Expression of a putative dominant-negative Gαz mutant in HeLa cells disrupted Golgi structure (Nagahama et al. 2002). Golgi disruption was also observed upon overexpression of the Gαz-selective GTPase-activating protein RGSz (Nagahama et al. 2002). It will be important to determine which aspects of Gi regulation of vesicular transport and Golgi structure are related to each other (or not), and to discriminate those processes regulated through receptor-independent mechanisms and/or by Gi-coupled GPCRs.

Gαi Interactions with Steroid Hormone and Tyrosine Kinase Receptors

Gαi-GDP and Gβγ directly bind a variety of steroid hormone receptors in vitro, including estrogen receptor α (ERα). The effect of estrogen on ERα-dependent eNOS signaling and monocyte adhesion to endothelial cells was blocked by disruption of the ERα-Gαi interaction (Kumar et al. 2007). The mechanistic basis of this process awaits elucidation, but may involve a nontraditional, nucleotide-independent process of Gi heterotrimer activation by ERα. Gi heterotrimers functionally interact with a variety of tyrosine kinase receptors including the insulin receptor (IR), the epidermal growth factor receptor (EGFR), and the platelet-derived growth factor receptor (PDGFR) (Patel 2004). In most part, these functional interactions were established by demonstration that natural ligand (insulin, EGF, PDGF) effects on ▶ MAP kinase pathway activity were altered by cell pretreatment with PTX. $G\alpha i_2$ was actually shown to be recruited to the IR complex in a PTX- and guanine nucleotide-sensitive manner (Kreuzer et al. 2004). A role for Gαi proteins downstream of IR signaling was also supported by observation of PTX-sensitive antiautophagic responses to insulin in hepatocytes and insulin-sensitive localization of $G\alpha i_3$ to autophagic endomembranes (Gohla et al. 2007).

Gαi Regulation by Accessory Proteins – GPR Motif Proteins and non-receptor GEFs

Gαi family proteins are targets of many regulatory mechanisms and interacting proteins, perhaps more than any other family of Gα subunits. The identification of accessory proteins as regulatory factors included the use of protein-protein interaction screens,



G Protein α i/o/z, Fig. 3 *Schematic illustration of hypothetical modulation of alternative Gαi-mediated signaling pathways by Gαi-GPR/GoLoco modules*. (**a**) Receptor coupling to Gαiβγ (*left*) and Gαi-GPR (*right*) is hypothesized to either modulate distinct effector pathways or influence the strength, duration, or efficiency of GPCR-mediated signals. Direct interaction of Gαi-GPR complexes with GPCRs is a working hypothesis (Oner et al. 2010a, b; Vellano et al. 2011a). (**b**) Predicted influence of GPR motifs on Gαiβγ subunit interaction independent of nucleotide exchange. (**c**) Gαi-GPR complexes are substrates for receptor-independent GEFs such as Ric-8A and may be subject to a G-protein activation-deactivation cycle analogous to Gαiβγ, suggesting that the Gαi-GPR complex functions as a signaling entity that is distinct from Gαiβγ

purification of biochemical activities, forward genetic screens, and expression cloning methods (reviewed in Sato et al. 2006), also see Blumer & Lanier ESM Review of AGS Proteins). Among the Gαi-family accessory proteins identified are non-receptor GEFs (e.g., GAP-43, AGS1, ▶ Ric-8A, GIV), GAPs (e.g., RGS proteins), and guanine nucleotide dissociation inhibitors (GDIs) (e.g., proteins containing the GPR/GoLoco motif) (reviewed in Sato et al. 2006; Siderovski et al. 2005). Functional roles for each of these classes has perhaps been most clearly developed in model organisms, where the GPR-Gαi module appears to be involved in the integration of polarity cues with the orientation of the mitotic spindle during asymmetric cell division. Interestingly, a receptor-independent Gαi activation/deactivation cycle is implicated this process (Gonczy 2008; Knoblich 2010; Siderovski and Willard 2005). The discovery and overviews of each of these classes of accessory proteins are covered in depth elsewhere (Siderovski and Willard 2005; Blumer et al. 2011, 2007; Hollinger and Hepler 2002; McCudden et al. 2005; Ross and Wilkie 2000; Sato et al. 2006). The remainder of this subsection will serve to highlight recently reported key regulatory roles of these accessory proteins on Gαi/o function.

The GPR/GoLoco motif is a ~20 amino acid motif that binds Gαi/o/T-GDP independent of Gβγ and is found in at least seven mammalian proteins to date (Blumer et al. 2011). The GPR/GoLoco motif from RGS14 was cocrystallized with $G\alpha i_1$-GDP, revealing contacts along the switch II/α3 helix as well as the nucleotide binding pocket of $G\alpha i_1$ where an invariant Arg residue found in all GPR/GoLoco motifs directly contacts the α and β phosphates of GDP to inhibit its release (Kimple et al. 2002). By influencing Gαi–Gβγ subunit interactions independent of nucleotide exchange, GPR motifs may impart particular, system-dependent effects on Gβγ-sensitive pathways in some systems (Kinoshita-Kawada et al. 2004; Nadella et al. 2010; Regner et al. 2011; Sanada and Tsai 2005; Wiser et al. 2006) but not others (Webb et al. 2005). The GPR motif binding to Gαi-GDP is thus somewhat analogous to that of Gβγ subunits and recent evidence suggests

that Gαi-GPR complexes may actually interface with GPCRs (Oner et al. 2010a, b; Vellano et al. 2011a). Unlike the 1:1 stoichiometry of Gα:Gβγ, proteins with multiple GPR motifs bind more than one Gαi subunit simultaneously (Adhikari and Sprang 2003; Bernard et al. 2001; Kimple et al. 2004), which may have broad implications for signal processing through Gαi/o family subunits. Although the GPR-Gαi interaction can be regulated by GPCR activation (Oner et al. 2010a, b; Vellano et al. 2011a) and non-receptor GEFs (Garcia-Marcos et al. 2011; Tall and Gilman 2005; Thomas et al. 2008; Vellano et al. 2011b), the regulatory mechanisms controlling the GPR-Gαi cassette and the precise subcellular locations of GPR and Gαi interactions are not yet fully defined (Fig. 3).

The Gαi-GPR module is also a substrate for ▶ Ric-8A-catalyzed nucleotide exchange in a manner that may be analogous to GPCR-mediated regulation of Gαiβγ heterotrimers (Tall and Gilman 2005; Thomas et al. 2008; Vellano et al. 2011b). A non-GPCR-mediated Gαi-GPR activation (by Ric-8) and deactivation (by *C. elegans* RGS7) cycle and has been proposed to be the means by which the Gαi protein switch regulates mitotic spindle positioning processes during asymmetric cell division (Afshar et al. 2004; Couwenbergs et al. 2004; Hess et al. 2004; Wilkie and Kinch 2005). The non-receptor GEF, GIV/Girdin also appears to act on GPR-Gαi complexes in the regulation of autophagy (Garcia-Marcos et al. 2011).

Summary

The Gαi-class is one of four subfamilies of G protein α subunits. The Gαi subfamily has the largest number of individual members, and in most cases constitutes the bulk of expressed G protein α subunits in a given tissue or cell type. Gαi was discovered as a key component in the hormonal inhibition of ▶ adenylyl cyclase. GPCR signals that are transduced through Gi heterotrimers are propagated directly by the activated Gαi-GTP subunit, but most Gi signaling arguably stems from the Gβγ subunits of Gi heterotrimers. New roles for Gαi subunits have emerged more recently, in which Gαi acts independently of Gβγ. Gαi interaction with GPR/Goloco domain-containing proteins provides a means to regulate distinct signaling pathways including intracellular events that do not always occur at the cell periphery.

References

Adhikari A, Sprang SR. Thermodynamic characterization of the binding of activator of G protein signaling 3 (AGS3) and peptides derived from AGS3 with G alpha i1. J Biol Chem. 2003;278:51825–32.

Afshar K, Willard FS, Colombo K, Johnston CA, McCudden CR, Siderovski DP, Gonczy P. RIC-8 is required for GPR-1/2-dependent gα function during asymmetric division of *C. elegans* embryos. Cell. 2004;119:219–30.

Baculikova M, Fiala R, Jezova D, Macho L, Zorad S. Rats with monosodium glutamate-induced obesity and insulin resistance exhibit low expression of Galpha(i2) G-protein. Gen Physiol Biophys. 2008;27:222–6.

Barr FA, Leyte A, Mollner S, Pfeuffer T, Tooze SA, Huttner WB. Trimeric G-proteins of the trans-Golgi network are involved in the formation of constitutive secretory vesicles and immature secretory granules. FEBS Lett. 1991;294:239–43.

Beckers CJ, Balch WE. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. J Cell Biol. 1989;108:1245–56.

Berman DM, Gilman AG. Mammalian RGS proteins: barbarians at the gate. J Biol Chem. 1998;273:1269–72.

Bernard ML, Peterson YK, Chung P, Jourdan J, Lanier SM. Selective interaction of AGS3 with G-proteins and the influence of AGS3 on the activation state of G-proteins. J Biol Chem. 2001;276:1585–93.

Blackmer T, Larsen EC, Takahashi M, Martin TF, Alford S, Hamm HE. G protein betagamma subunit-mediated presynaptic inhibition: regulation of exocytotic fusion downstream of Ca^{2+} entry. Science. 2001;292:293–7.

Blackmer T, Larsen EC, Bartleson C, Kowalchyk JA, Yoon EJ, Preininger AM, Alford S, Hamm HE, Martin TF. G protein betagamma directly regulates SNARE protein fusion machinery for secretory granule exocytosis. Nat Neurosci. 2005;8:421–5.

Blumer JB, Smrcka AV, Lanier SM. Mechanistic pathways and biological roles for receptor-independent activators of G-protein signaling. Pharmacol Ther. 2007;113:488–506.

Blumer JB, Sadik Oner S, Lanier SM. Group II activators of G-protein signaling and proteins containing a G-protein regulatory motif. Acta Physiol. 2011;204:202–18.

Casey PJ, Fong HK, Simon MI, Gilman AG. Gz, a guanine nucleotide-binding protein with unique biochemical properties. J Biol Chem. 1990;265:2383–90.

Ch'ng Q, Sieburth D, Kaplan JM. Profiling synaptic proteins identifies regulators of insulin secretion and lifespan. PLoS Genet. 2008;4:e1000283.

Chen JF, Guo JH, Moxham CM, Wang HY, Malbon CC. Conditional, tissue-specific expression of Q205L G alpha i2 in vivo mimics insulin action. J Mol Med. 1997;75:283–9.

Chen-Goodspeed M, Lukan AN, Dessauer CW. Modeling of G alpha(s) and G alpha(i) regulation of human type V and VI adenylyl cyclase. J Biol Chem. 2005;280:1808–16.

Chisari M, Saini DK, Kalyanaraman V, Gautam N. Shuttling of G protein subunits between the plasma membrane and intracellular membranes. J Biol Chem. 2007;282:24092–8.

Clapham DE, Neer EJ. G protein βγ subunits. Annu Rev Pharmacol Toxicol. 1997;37:167–203.
Coleman D, Berghuis A, Lee E, Linder M, Gilman A, Sprang SR. Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. Science. 1994;265:1405–12.
Couwenbergs C, Spilker AC, Gotta M. Control of embryonic spindle positioning and Gα activity by *C. elegans* RIC-8. Curr Biol. 2004;14:1871–6.
Denker SP, McCaffery JM, Palade GE, Insel PA, Farquhar MG. Differential distribution of alpha subunits and beta gamma subunits of heterotrimeric G proteins on Golgi membranes of the exocrine pancreas. J Cell Biol. 1996; 133:1027–40.
Dessauer CW, Tesmer JJG, Sprang SR, Gilman AG. Identification of a Giα binding site on type V adenylyl cyclase. J Biol Chem. 1998;273:25831–9.
Didsbury JR, Snyderman R. Molecular cloning of a new human G protein evidence for two Giα like protein families. FEBS Lett. 1987;219:259–63.
Elliott MR. Coordinating speed and amplitude in G-protein signaling. Curr Biol. 2008;18:R777–83.
Exton JH. Phosphoinositide phospholipases and G proteins in hormone action. Annu Rev Physiol. 1994;56:349–69.
Farr GW, Scharl EC, Schumacher RJ, Sondek S, Horwich AL. Chaperonin-mediated folding in the eukaryotic cytosol proceeds through rounds of release of native and nonnative forms. Cell. 1997;89:927–37.
Gabay M, Pinter ME, Wright FA, Chan P, Murphy AJ, Valenzuela DM, Yancopoulos GD, Tall GG. Ric-8 proteins are molecular chaperones that direct nascent G protein α subunit membrane association. Sci Signal. 2011;4:ra79.
Garcia-Marcos M, Ear J, Farquhar MG, Ghosh P. A GDI and a GEF regulate autophagy by balancing g protein activity and growth factor signals. Mol Biol Cell. 2011;22:673–86.
Gawler D, Milligan G, Spiegel AM, Unson CG, Houslay MD. Abolition of the expression of inhibitory guanine nucleotide regulatory protein Gi activity in diabetes. Nature. 1987;327: 229–32.
Gerachshenko T, Blackmer T, Yoon EJ, Bartleson C, Hamm HE, Alford S. Gbetagamma acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. Nat Neurosci. 2005;8: 597–605.
Gilman AG. G proteins: transducers of receptor-generated signals. Annu Rev Biochem. 1987;56:615–49.
Gilman A. G proteins and regulation of adenylyl cyclase. Biosci Rep. 1995;15:65–97.
Gohla A, Klement K, Piekorz RP, Pexa K, von Dahl S, Spicher K, Dreval V, Haussinger D, Birnbaumer L, Nurnberg B. An obligatory requirement for the heterotrimeric G protein Gi3 in the antiautophagic action of insulin in the liver. Proc Natl Acad Sci USA. 2007;104:3003–8.
Gonczy P. Mechanisms of asymmetric cell division: flies and worms pave the way. Nat Rev Mol Cell Biol. 2008;9:355–66.
Gotta M, Ahringer J. Distinct roles for Galpha and Gbetagamma in regulating spindle position and orientation in caenorhabditis elegans embryos. Nat Cell Biol. 2001;3:297–300.
Gulbenkian A, Schobert L, Nixon C, Tabachnick II A. Metabolic effects of pertussis sensitization in mice and rats. Endocrinology. 1968;83:885–92.
Gutkind JS. Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. Sci STKE. 2000;2000:re1.
Hajdu-Cronin YM, Chen WJ, Patikoglou G, Koelle MR, Sternberg PW. Antagonism between G(o)alpha and G(q) alpha in Caenorhabditis elegans: the RGS protein EAT-16 is necessary for G(o)alpha signaling and regulates G(q)alpha activity. Genes Dev. 1999;13:1780–93.
Hausdorff WP, Pitcher JA, Luttrell DK, Linder ME, Kurose H, Parsons SJ, Caron MG, Lefkowitz RJ. Tyrosine phosphorylation of G protein alpha subunits by pp 60c-src. Proc Natl Acad Sci. 1992;89:5720–4.
Hess HA, Roper J-C, Grill SW, Koelle MR. RGS-7 completes a receptor-independent heterotrimeric G protein cycle to asymmetrically regulate mitotic spindle positioning in *C. elegans*. Cell. 2004;119:209–18.
Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacol Rev. 2002;54:527–59.
Huang X, Charbeneau RA, Fu Y, Kaur K, Gerin I, MacDougald OA, Neubig RR. Resistance to diet-induced obesity and improved insulin sensitivity in mice with a regulator of G protein signaling-insensitive G184S Gnai2 allele. Diabetes. 2008;57:77–85.
Jamora C, Takizawa PA, Zaarour RF, Denesvre C, Faulkner DJ, Malhotra V. Regulation of Golgi structure through heterotrimeric G proteins. Cell. 1997;91:617–26.
Jamora C, Yamanouye N, Van Lint J, Laudenslager J, Vandenheede JR, Faulkner DJ, Malhotra V. Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D. Cell. 1999;98:59–68.
Jones DT, Reed RR. Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. J Biol Chem. 1987;262:14241–9.
Katada T, Ui M. Islet-activating protein. Enhanced insulin secretion and cyclic AMP accumulation in pancreatic islets due to activation of native calcium ionophores. J Biol Chem. 1979;254:469–79.
Katada T, Ui M. Islet-activating protein. A modifier of receptor-mediated regulation of rat islet adenylate cyclase. J Biol Chem. 1981a;256:8310–7.
Katada T, Ui M. In vitro effects of islet-activating protein on cultured rat pancreatic islets. Enhancement of insulin secretion, adenosine 3′:5′-monophosphate accumulation and 45Ca flux. J Biochem. 1981b;89:979–90.
Kimple RJ, Kimple ME, Betts L, Sondek J, Siderovski DP. Structural determinants for GoLoco-induced inhibition of nucleotide release by Galpha subunits. Nature. 2002;416: 878–81.
Kimple RJ, Willard FS, Hains MD, Jones MB, Nweke GK, Siderovski DP. Guanine nucleotide dissociation inhibitor activity of the triple GoLoco motif protein G18: alanine-to-aspartate mutation restores function to an inactive second GoLoco motif. Biochem J. 2004;378:801–8.
Kimple ME, Nixon AB, Kelly P, Bailey CL, Young KH, Fields TA, Casey PJ. A role for G(z) in pancreatic islet beta-cell biology. J Biol Chem. 2005;280:31708–13.
Kimple ME, Joseph JW, Bailey CL, Fueger PT, Hendry IA, Newgard CB, Casey PJ. Galphaz negatively regulates insulin secretion and glucose clearance. J Biol Chem. 2008;283: 4560–7.

Kinoshita-Kawada M, Oberdick J, Xi Zhu M. A Purkinje cell specific GoLoco domain protein, L7/Pcp-2, modulates receptor-mediated inhibition of Cav2.1 Ca^{2+} channels in a dose-dependent manner. Brain Res Mol Brain Res. 2004;132:73–86.

Knoblich JA. Asymmetric cell division: recent developments and their implications for tumour biology. Nat Rev Mol Cell Biol. 2010;11:849–60.

Kreuzer J, Nurnberg B, Krieger-Brauer HI. Ligand-dependent autophosphorylation of the insulin receptor is positively regulated by Gi-proteins. Biochem J. 2004;380:831–6.

Kumar P, Wu Q, Chambliss KL, Yuhanna IS, Mumby SM, Mineo C, Tall GG, Shaul PW. Direct interactions with Gαi and Gβγ mediate nongenomic signaling by estrogen receptor α. Mol Endocrinol. 2007;21:1370–80.

Lackner MR, Nurrish SJ, Kaplan JM. Facilitation of synaptic transmission by EGL-30 Gqα and EGL-8 PLC[beta]: DAG binding to UNC-13 is required to stimulate acetylcholine release. Neuron. 1999;24:335–46.

Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE, Sigler PB. The 2.0 A crystal structure of a heterotrimeric G protein. Nature. 1996;379:311–9.

Lin HC, Duncan JA, Kozasa T, Gilman AG. Sequestration of the G protein beta gamma subunit complex inhibits receptor-mediated endocytosis. Proc Natl Acad Sci USA. 1998;95:5057–60.

Ma Y-C, Huang J, Ali S, Lowry W, Huang X-Y. Src tyrosine kinase is a novel direct effector of G proteins. Cell. 2000;102:635–46.

Maier O, Ehmsen E, Westermann P. Trimeric G protein alpha subunits of the Gs and Gi families localized at the Golgi membrane. Biochem Biophys Res Commun. 1995;208:135–43.

Marrari Y, Crouthamel M, Irannejad R, Wedegaertner PB. Assembly and trafficking of heterotrimeric G proteins. Biochemistry. 2007;46:7665–77.

McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS. G-protein signaling: back to the future. Cell Mol Life Sci. 2005;62:551–77.

Miller KG, Emerson MD, Rand JB. Goalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in *C. elegans*. Neuron. 1999;24:323–33.

Mixon MB, Lee E, Coleman DE, Berghuis AM, Gilman AG, Sprang SR. Tertiary and quaternary structural changes in Giα1 induced by GTP hydrolysis. Science. 1995;270:954–60.

Montmayeur JP, Borrelli E. Targeting of G alpha i2 to the Golgi by alternative spliced carboxyl-terminal region. Science. 1994;263:95–8.

Moxham CM, Malbon CC. Insulin action impaired by deficiency of the G-protein subunit G ialpha2. Nature. 1996;379:840–4.

Muller L, Picart R, Barret A, Bockaert J, Homburger V, Tougard C. Identification of multiple subunits of heterotrimeric G proteins on the membrane of secretory granules in rat prolactin anterior pituitary cells. Mol Cell Neurosci. 1994;5:556–66.

Mumby SM, Heukeroth RO, Gordon JI, Gilman AG. G-protein α-subunit expression, myristoylation, and membrane association in COS cells. Proc Natl Acad Sci. 1990;87:728–32.

Nadella R, Blumer JB, Jia G, Kwon M, Akbulut T, Qian F, Sedlic F, Wakatsuki T, Sweeney Jr WE, Wilson PD, et al. Activator of G protein signaling 3 promotes epithelial cell proliferation in PKD. J Am Soc Nephrol. 2010;21:1275–80.

Nagahama M, Usui S, Shinohara T, Yamaguchi T, Tani K, Tagaya M. Inactivation of Galpha(z) causes disassembly of the Golgi apparatus. J Cell Sci. 2002;115:4483–93.

Natarajan K, Berk BC. Crosstalk coregulation mechanisms of G protein-coupled receptors and receptor tyrosine kinases. Methods Mol Biol. 2006;332:51–77.

Nathan D. Signalling via the G protein-activated K^+ channels. Cell Signal. 1997;9:551–73.

Neer EJ, Lok JM, Wolf LG. Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. J Biol Chem. 1984;259:14222–9.

Ogier-Denis E, Couvineau A, Maoret JJ, Houri JJ, Bauvy C, De Stefanis D, Isidoro C, Laburthe M, Codogno P. A heterotrimeric Gi3-protein controls autophagic sequestration in the human colon cancer cell line HT-29. J Biol Chem. 1995;270:13–6.

Oner SS, An N, Vural A, Breton B, Bouvier M, Blumer JB, Lanier SM. Regulation of the AGS3.G{alpha}i signaling complex by a seven-transmembrane span receptor. J Biol Chem. 2010a;285:33949–58.

Oner SS, Maher EM, Breton B, Bouvier M, Blumer JB. Receptor-regulated interaction of activator of G-protein signaling-4 and Galphai. J Biol Chem. 2010b;285:20588–94.

Parmentier ML, Woods D, Greig S, Phan PG, Radovic A, Bryant P, O'Kane CJ. Rapsynoid/partner of inscuteable controls asymmetric division of larval neuroblasts in Drosophila. J Neurosci. 2000;20:RC84.

Patel TB. Single transmembrane spanning heterotrimeric G protein-coupled receptors and their signaling cascades. Pharmacol Rev. 2004;56:371–85.

Pimplikar SW, Simons K. Regulation of apical transport in epithelial cells by a Gs class of heterotrimeric G protein. Nature. 1993;362:456–8.

Regard JB, Kataoka H, Cano DA, Camerer E, Yin L, Zheng YW, Scanlan TS, Hebrok M, Coughlin SR. Probing cell type-specific functions of Gi in vivo identifies GPCR regulators of insulin secretion. J Clin Invest. 2007;117:4034–43.

Regner KR, Nozu K, Lanier SM, Blumer JB, Avner ED, Sweeney Jr WE, Park F. Loss of activator of G-protein signaling 3 impairs renal tubular regeneration following acute kidney injury in rodents. FASEB J. 2011;25:1844–55.

Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem. 2000;69:795–827.

Sadana R, Dessauer CW. Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies. Neurosignals. 2009;17:5–22.

Saini DK, Chisari M, Gautam N. Shuttling and translocation of heterotrimeric G proteins and Ras. Trends Pharmacol Sci. 2009;30:278–86.

Sanada K, Tsai LH. G protein betagamma subunits and AGS3 control spindle orientation and asymmetric cell fate of cerebral cortical progenitors. Cell. 2005;122:119–31.

Sato M, Blumer JB, Simon V, Lanier SM. Accessory proteins for G proteins: partners in signaling. Annu Rev Pharmacol Toxicol. 2006;46:151–87.

Schaefer M, Shevchenko A, Knoblich JA. A protein complex containing inscuteable and the galpha-binding protein pins

orients asymmetric cell divisions in Drosophila. Curr Biol. 2000;10:353–62.
Schurmann A, Rosenthal W, Schultz G, Joost HG. Characterization of GTP-binding proteins in Golgi-associated membrane vesicles from rat adipocytes. Biochem J. 1992;283(Pt 3):795–801.
Schwaninger R, Plutner H, Bokoch GM, Balch WE. Multiple GTP-binding proteins regulate vesicular transport from the ER to Golgi membranes. J Cell Biol. 1992;119:1077–96.
Sharp GW. Mechanisms of inhibition of insulin release. Am J Physiol. 1996;271:C1781–99.
Siderovski DP, Willard FS. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci. 2005;1:51–66.
Smrcka A. G protein βγ subunits: central mediators of G protein-coupled receptor signaling. Cell Mol Life Sci. 2008;65:2191–214.
Sprang SR. G protein mechanisms: insights from structural analysis. Annu Rev Biochem. 1997;66:639–78.
Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein βγ subunits. Cell. 1994;77:83–93.
Sternweis PC, Robishaw JD. Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J Biol Chem. 1984;259:13806–13.
Stow JL, de Almeida JB. Distribution and role of heterotrimeric G proteins in the secretory pathway of polarized epithelial cells. J Cell Sci Suppl. 1993;17:33–9.
Stow JL, de Almeida JB, Narula N, Holtzman EJ, Ercolani L, Ausiello DA. A heterotrimeric G protein, G alpha i-3, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. J Cell Biol. 1991;114:1113–24.
Suki WN, Abramowitz J, Mattera R, Codina J, Birnbaumer L. The human genome encodes at least three non-allellic G proteins with αi-type subunits. FEBS Lett. 1987;220:187–92.
Sunahara R, Dessauer C, Gilman AG. Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol. 1996;36:461–80.
Szentivanyi A, Fishel CW, Talmage DW. Adrenaline mediation of histamine and serotonin hyperglycemia in normal mice and the absence of adrenaline-induced hyperglycemia in pertussis-sensitized mice. J Infect Dis. 1963;113:86–98.
Tabachnick II, Gulbenkian A. Adrenergic changes due to pertussis: insulin, glucose and free fatty acids. Eur J Pharmacol. 1969;7:186–95.
Tall GG, Gilman AG. Resistance to inhibitors of cholinesterase 8A catalyzes release of Galphai-GTP and nuclear mitotic apparatus protein (NuMA) from NuMA/LGN/Galphai-GDP complexes. Proc Natl Acad Sci USA. 2005;102:16584–9.
Taussig R, Gilman AG. Mammalian membrane-bound adenylyl cyclases. J Biol Chem. 1995;270:1–4.
Taussig R, Tang WJ, Hepler JR, Gilman AG. Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. J Biol Chem. 1994;269:6093–100.
Tedford HW, Zamponi GW. Direct G protein modulation of Cav2 calcium channels. Pharmacol Rev. 2006;58:837–62.
Tesmer JJG, Berman DM, Gilman AG, Sprang SR. Structure of RGS4 bound to AlF4 − -activated Giα1: stabilization of the transition state for GTP hydrolysis. Cell. 1997;89:251–61.
Thomas CJ, Tall GG, Adhikari A, Sprang SR. Ric-8A catalyzes guanine nucleotide exchange on G alphai1 bound to the GPR/GoLoco exchange inhibitor AGS3. J Biol Chem. 2008;283:23150–60.
Vashlishan AB, Madison JM, Dybbs M, Bai J, Sieburth D, Ch'ng Q, Tavazoie M, Kaplan JM. An RNAi screen identifies genes that regulate GABA synapses. Neuron. 2008;58:346–61.
Vellano CP, Maher EM, Hepler JR, Blumer JB. G protein-coupled receptors and resistance to inhibitors of cholinesterase-8A (Ric-8A) both regulate the regulator of G protein signaling 14(RGS14):G{alpha}i1 complex in live cells. J Biol Chem. 2011a;286:38659–69.
Vellano CP, Shu FJ, Ramineni S, Yates CK, Tall GG, Hepler JR. Activation of the regulator of G protein signaling 14-Galphai1-GDP signaling complex is regulated by resistance to inhibitors of cholinesterase-8A. Biochemistry. 2011b;50:752–62.
Wall MA, Coleman DE, Lee E, Iñiguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR. The structure of the G protein heterotrimer Giα1β1γ2. Cell. 1995;83:1047–58.
Wang Z, Thurmond DC. Mechanisms of biphasic insulin-granule exocytosis – roles of the cytoskeleton, small GTPases and SNARE proteins. J Cell Sci. 2009;122:893–903.
Wang Y, Park S, Bajpayee NS, Nagaoka Y, Boulay G, Birnbaumer L, Jiang M. Augmented glucose-induced insulin release in mice lacking G(o2), but not G(o1) or G(i) proteins. Proc Natl Acad Sci USA. 2011;108:1693–8.
Webb CK, McCudden CR, Willard FS, Kimple RJ, Siderovski DP, Oxford GS. D2 dopamine receptor activation of potassium channels is selectively decoupled by Galpha-specific GoLoco motif peptides. J Neurochem. 2005;92:1408–18.
Wilkie TM, Kinch L. New roles for G alpha and RGS proteins: communication continues despite pulling sisters apart. Curr Biol. 2005;15:R843–954.
Wilson BS, Palade GE, Farquhar MG. Endoplasmic reticulum-through-Golgi transport assay based on O-glycosylation of native glycophorin in permeabilized erythroleukemia cells: role for Gi3. Proc Natl Acad Sci USA. 1993;90:1681–5.
Wilson BS, Komuro M, Farquhar MG. Cellular variations in heterotrimeric G protein localization and expression in rat pituitary. Endocrinology. 1994;134:233–44.
Wiser O, Qian X, Ehlers M, Ja WW, Roberts RW, Reuveny E, Jan YN, Jan LY. Modulation of basal and receptor-induced GIRK potassium channel activity and neuronal excitability by the mammalian PINS homolog LGN. Neuron. 2006;50:561–73.
Yajima M, Hosoda K, Kanbayashi Y, Nakamura T, Takahashi I, Ui M. Biological properties of islets-activating protein (IAP) purified from the culture medium of Bordetella pertussis. J Biochem. 1978;83:305–12.
Yamaguchi T, Nagahama M, Itoh H, Hatsuzawa K, Tani K, Tagaya M. Regulation of the golgi structure by the alpha subunits of heterotrimeric G proteins. FEBS Lett. 2000;470:25–8.
Yu F, Morin X, Cai Y, Yang X, Chia W. Analysis of partner of inscuteable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscuteable apical localization. Cell. 2000;100:399–409.
Zhao A, Ohara-Imaizumi M, Brissova M, Benninger RK, Xu Y, Hao Y, Abramowitz J, Boulay G, Powers AC, Piston D, et al.

Galphao represses insulin secretion by reducing vesicular docking in pancreatic beta-cells. Diabetes. 2010a;59:2522–9.
Zhao Y, Fang Q, Straub SG, Lindau M, Sharp GW. Noradrenaline inhibits exocytosis via the G protein betagamma subunit and refilling of the readily releasable granule pool via the alpha(i1/2) subunit. J Physiol. 2010b;588:3485–98.

G Protein α o

▶ G Protein α i/o/z

G Protein α z

▶ G Protein α i/o/z

G12α

▶ G Protein Alpha 12

G630042G04

▶ P-Rex

Gab1

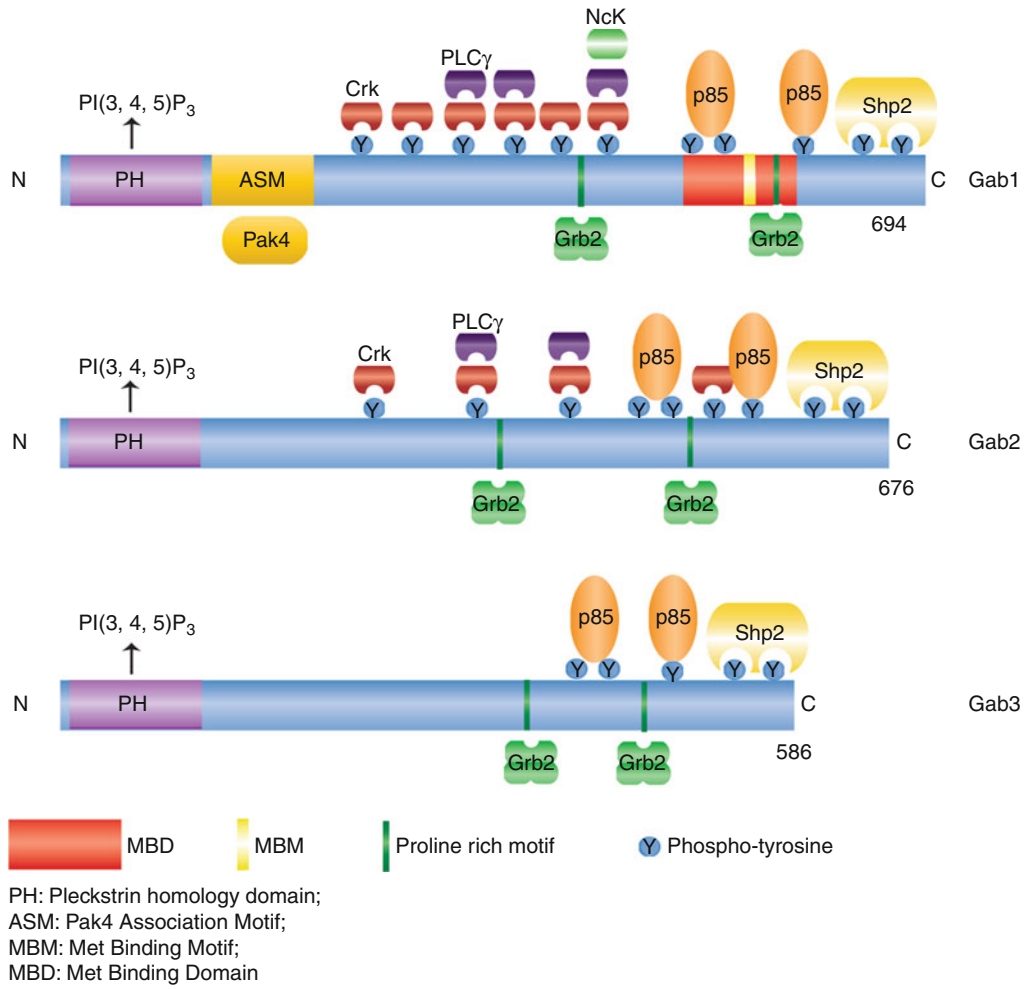
Richard Vaillancourt[1,2], Annina C. Spilker[1,2] and Morag Park[1,2,3,4]
[1]Goodman Cancer Research Centre, McGill University, Montréal, Canada
[2]Department of Biochemistry, McGill University, Montréal, Quebec, Canada
[3]Department of Medicine, McGill University, Montréal, Canada
[4]Department of Oncology, McGill University, Montréal, Canada

Historical Background

Gab proteins are scaffold proteins that are related to the insulin receptor substrates (IRS1/2/3), the fibroblast growth factor (▶ FGF) receptor substrate 2 (FRS2-α/β), the downstream of kinase (Dok), and the linker of T cell (▶ LAT). These proteins lack intrinsic enzymatic activity. Upon their recruitment to activated growth factor and cytokine receptors, they become tyrosine phosphorylated, providing binding sites for multiple proteins involved in signal transduction. By virtue of their ability to assemble multiprotein complexes, they act to modulate, amplify, and diversify the signals downstream from receptors (Pawson and Scott 1997; Schlessinger and Lemmon 2003).

The Gab family comprises three mammalian members, Gab1, Gab2, Gab3 (Fig. 1), the *Drosophila melanogaster* DOS (daughter of sevenless), and the *Caenorhabditis elegans* SOC-1. Gab1 was first identified in a screen for Grb2 binding proteins and was found to be a substrate for the EGF, TrkA, and insulin receptor tyrosine kinases (RTK). Gab1 was also independently identified as a hepatocyte growth factor (HGF) RTK, Met, interacting protein in a yeast two-hybrid screen (Birchmeier et al. 2003; Peschard and Park 2007). Gab2 was discovered as a phosphoprotein interacting with the Shp2 phosphatase (Gu and Neel 2003), and Gab3 was isolated based on its sequence similarity with Gab1 (Liu and Rohrschneider 2002). In addition, a fourth paralogue, Gab4, has been predicted based on sequence analysis (Wohrle et al. 2009). Although Gab family proteins are only moderately conserved at the level of the amino acid sequence, the domains and key sites for recruitment of signaling proteins downstream of activated receptors are highly conserved between Gab1 and Gab2 (Fig. 1).

Genetic and cell biology analyses have demonstrated that Gab proteins are crucial in RTK signaling and biology. *dos* and *soc-1* were found to be essential for RTK signaling in the developing fly and worm. *Gab1* null mice are embryonic lethal and show impaired migration of muscle precursor cells and muscle development, thinner placenta, impaired diaphragm formation, reduced liver size, defective heart development, skin problems, open eyelids, and liver regeneration defects (Schaeper et al. 2007). These phenotypes predominantly mimic *Hgf* null *and Met* null mice, but also include additional defects observed in *Pdgf* and *Egf* null mice, demonstrating the important role played by Gab1 in transduction of signals from these growth factor signals during development. In the adult, Gab1 regulates glucose uptake in the liver, bone homeostasis, and differentiation of myocytes and B cells.



Gab1, Fig. 1 Domain structure of the Gab family of scaffold proteins. Schematic representation of the mammalian Gab1, 2, and 3 proteins. Gab proteins all share a conserved N-terminal PH domain involved in phospholipid binding. In Gab1, tyrosine phosphorylation creates binding sites for signaling molecules including Crk, Nck, PLCγ, p85, and Shp2. Proline rich regions mediate the interaction with the SH3 domain of Grb2. The Met Binding Domain (MBD), which includes the Met Binding Motif (MBM), mediates the direct recruitment of Gab1 to the Met receptor

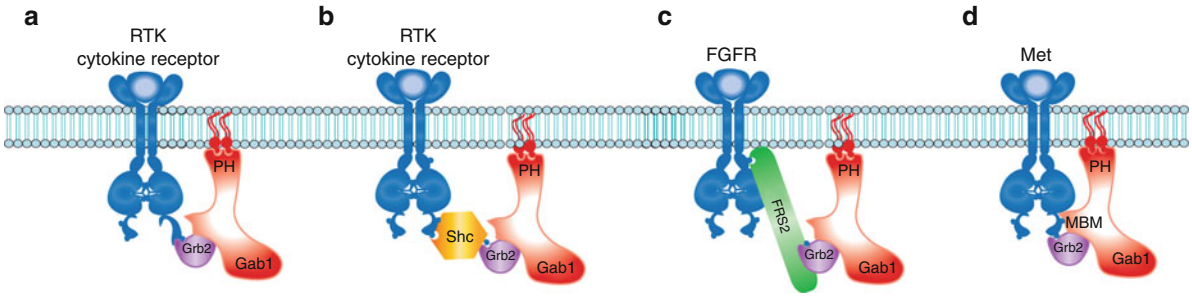
Despite having similar overall structure and signaling capacity, *Gab2* null mice are viable, have a normal life span, yet show severe defects in mast cell lineages (Gu and Neel 2003), whereas *Gab3* null mice have no apparent phenotype. The marked differences in knockout phenotypes suggest that despite high similarity in their domain structure organization, Gab proteins play different roles in RTK signaling during development in mammals.

This entry will focus on the Gab1 protein, its role in signal transduction, and its implication in development and cancer initiation and progression.

Gab1 in Signal Transduction

Membrane Targeting

Following receptor activation, Gab1 translocates from the cytosol to the plasma membrane. At least three mechanisms regulate this translocation (Fig. 2). Gab1 is indirectly recruited to cell surface receptors by the small adaptor Grb2 (Peschard and Park 2007). All mammalian Gab proteins contain a canonical PXXP motif and an atypical PXXXR motif responsible for the recruitment of the C-terminal SH3 domain of Grb2. The SH2 domain of Grb2 can interact with



Gab1, Fig. 2 Mode of Gab1 recruitment to activated receptors. Gab1 is recruited to activated receptors via different mechanisms. (**a**) The SH2 domain of the Grb2 adaptor binds to a consensus tyrosine phosphorylated motif YVNV. Gab1 is recruited to this complex by interaction with the SH3 domain of Grb2. (**b**) and (**c**) Through a ternary complex, where Grb2 couples Gab1 to the Shc adaptor or FRS2 scaffolds via its SH2 domain. In this model, a Gab1/Grb2 complex binds the Shc/Receptor or FRS2/FGFR complex. (**d**) Gab1 is recruited by a dual mechanism to the Met receptor. The MBM of Gab1 directly binds Met and requires tyrosine phosphorylation of Met. Gab1 is also indirectly recruited to Met by the Grb2 adaptor

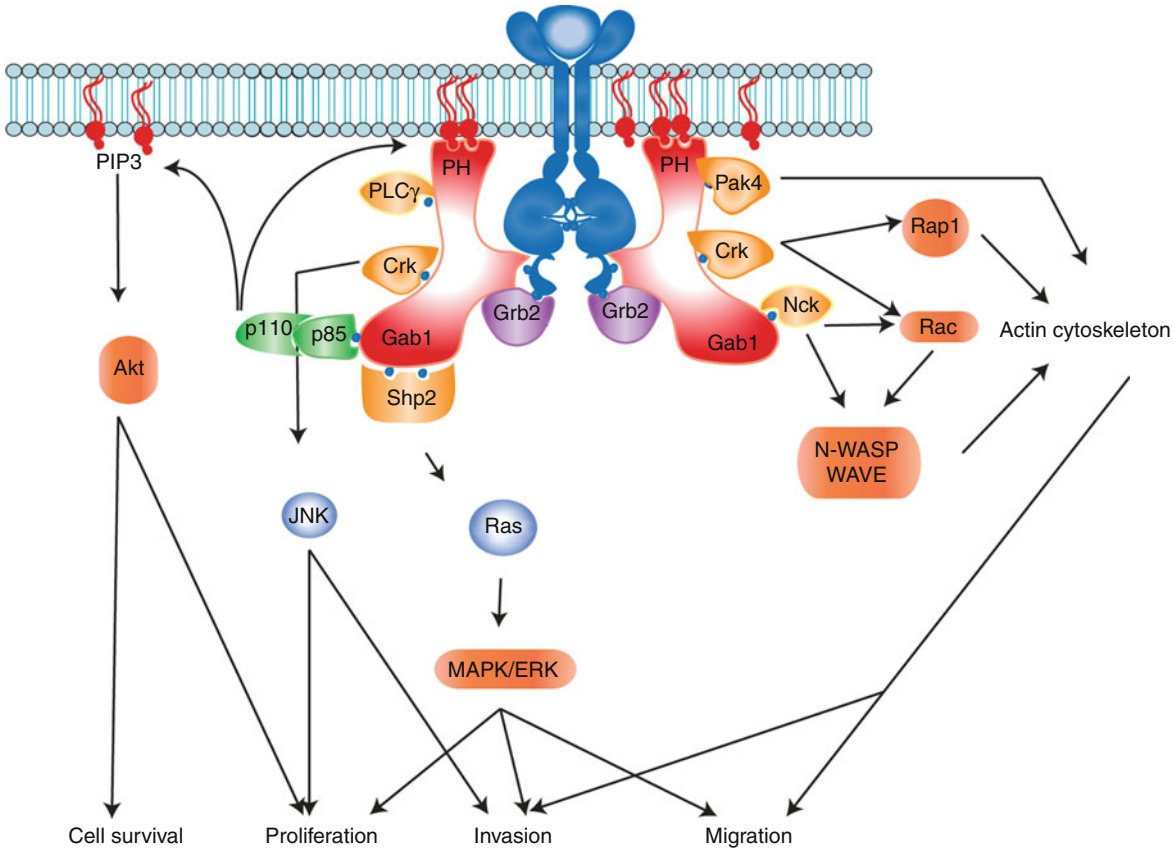
a phosphotyrosine motif (YVNV) on activated receptors, thus creating a bridge between the receptor and Gab proteins. Alternatively, following activation of the FGF receptor, the FRS2α and β scaffolds are phosphorylated on 5 Grb2 binding sites and mediates the recruitment of a Grb2–Gab1 complex to the FGFR. This ternary complex plays an important role in ▶ PI3K activation downstream of an ▶ FGF signal (Eswarakumar et al. 2005). Similarly, Gab1 is recruited to cytokine and immune receptors through a Shc–Grb2–Gab1 complex.

In addition to the indirect recruitment via Grb2, Gab1 is also directly recruited to the Met receptor. This direct interaction is mediated by the Met Binding Motif (MBM) on Gab1 and requires the activated kinase domain of Met (Birchmeier et al. 2003; Peschard and Park 2007). The Gab1 MBM does not resemble classical SH2 or PTB domains and is not conserved in other members of the Gab family. The direct recruitment of Gab1 correlates with sustained phosphorylation of Gab1, Erk and Akt downstream from Met, and is required for the invasive morphogenic program induced by HGF (Peschard and Park 2007).

The N-terminal region of all Gab proteins contains a conserved, phosphoinositide-binding, pleckstrin homology (PH) domain that is important for both membrane targeting and protein function. The PH domain of Gab1 predominantly binds phosphoinositol-3,4,5-phosphate ($PI_{3,4,5}P3$), which is required for its translocation from the cytoplasm to the cell cortex, lamellipodia, membrane ruffles, and cell–cell junctions in sheets of epithelial cells in response to receptor activation (Peschard and Park 2007). Gab1 mutants unable to bind $PI_{3,4,5}P3$ or lacking the PH domain are poorly phosphorylated and defective in transducing signals and biological responses downstream from the EGF and Met RTKs (Peschard and Park 2007; Rodrigues et al. 2000). This is overcome by targeting Gab1 to the plasma membrane using a ▶ Src myristoylation sequence (Peschard and Park 2007), highlighting the importance of membrane localization for Gab1 signaling.

Tyrosine Phosphorylation

Gab1 is a key intermediate required for biological responses downstream of RTKs, cytokine and immune receptors, including cell proliferation, cell survival, cell migration, and epithelial morphogenesis (Fig. 3). Tyrosine phosphorylation of Gab1 on specific tyrosine residues is a fundamental mechanism for Gab1-mediated signal transduction. This phosphorylation is a consequence of the recruitment of Gab1 to activated growth factor, cytokine, and immune receptors. Met, EGFR, PDGFR, VEGFR2, c-Kit, IGF1R, TrkA, RET, Insulin-R, EPO-R, TPO-R, gp130, IL-3-R, INFα/γ-R, and T and B cell receptors all induce tyrosine phosphorylation of Gab1 following activation (Liu and Rohrschneider 2002; Nishida and Hirano 2003; Sarmay et al. 2006). Whereas RTKs can directly phosphorylate Gab1, other types of receptor use JAK or Src family kinases. Tyrosine phosphorylation of Gab1 provides docking sites for SH2 domain containing molecules, leading to the recruitment of enzymes (Shp2, PLCγ) or adaptor molecules (Crk, Nck, p85). In the following section, we describe the signaling pathways activated downstream of Gab1.



Gab1, Fig. 3 Gab1 signaling. Gab1 regulates cell survival by activating the PI3K/Akt pathway through the recruitment of p85. Shp2 binding to Gab1 activates the Ras/MAPK pathway to regulate cell proliferation, invasion, and migration. The recruitment of Crk, Pak4, and Nck to Gab1 regulates actin cytoskeleton remodeling, membrane dynamics, and cell migration

Gab1 Signaling

Shp2 and MAPK Activation (pY627, 659)

Gab1 binds to the phosphatase Shp2 in response to multiple growth factors and cytokines including EGF, insulin, FGF, PDGF, or HGF (Gu and Neel 2003; Liu and Rohrschneider 2002; Wohrle et al. 2009). Recruitment of Shp2 SH2 domains leads to the activation of Shp2 enzymatic activity and is required for full activation of the MAPK pathway by the EGFR (Birchmeier et al. 2003; Peschard and Park 2007). Knockin mice expressing a Gab1ΔShp2 mutant are embryonic lethal and display impaired migration of muscle progenitor cells to the limb buds and placental defects, a phenotype similar to *Gab1*, *Met* and *Hgf* null animals (Schaeper et al. 2007), highlighting the importance of the Gab1–Shp2 interaction during development. Different models for the function of the Gab–Shp2 complex in Ras signaling have been proposed; however, the exact mechanisms of this regulation still need to be defined. One model supported by data from *D. melanogaster* proposes that recruitment of $p120^{Ras\text{-}GAP}$ on tyrosines 307 and 317 of Gab1 promotes the return of Ras to its inactive, GDP-bound state. Shp2 recruitment to Gab1 promotes dephosphorylation of these $p120^{Ras\text{-}GAP}$ sites thus decreasing the negative regulation of Ras, resulting in sustained activation (Gu and Neel 2003; Wohrle et al. 2009).

p85 and PI3K Signaling (pY 447, 472, 589)

Gab1 plays a crucial role in the activation and amplification of PI3K signaling downstream of RTKs and cytokine receptors. The majority of RTK-dependent PI3K activity is associated with Gab1 and depends on its ability to recruit the p85 regulatory subunit of PI3K through three YXXM motifs that include tyrosines 447, 472, and 589. Gab1-dependent PI3K activation

is critical for cell survival and proliferation through the Akt pathway in many biological contexts. This is particularly important for RTKs that are unable to directly recruit p85, including EGFR, VEGFR-2, and FGFR. The ability of Gab1 to activate the PI3K pathway is also required for actin remodeling and formation of membrane ruffles, cell migration, and tubulogenesis downstream from several RTKs. This possibly occurs through generation of $PI_{3,4,5}P3$-rich membranes following localized Gab1-dependent PI3K activation and subsequent activation of guanine exchange factors (GEFs) for the Rac GTPase. A Gab1-p85 interaction is required for cell scatter and epithelial morphogenesis induced by HGF (Peschard and Park 2007), to induce Rac activation and lamellipodia formation downstream of the Ret RTK (Maeda et al. 2004), and for the formation of HGF-induced circular dorsal ruffles (Abella et al. 2010). During development, Gab1/p85 interaction is also required for keratinocyte migration as well as EGF-induced eyelid closure (Schaeper et al. 2007).

Crk (pY242, 259, 307, 317, 373, 406)

The interaction between Gab1 and Crk relies on six YxxP motifs in Gab1 and the SH2 domain of Crk. The Gab1–Crk interaction controls cytoskeleton rearrangement, cell motility, adhesion, morphology and polarity downstream of RTKs, antigen and cytokine receptors. These biological outcomes possibly depend on the ability of Crk to regulate the activation of the small GTPases Rac and Rap1 via the recruitment of the Rac GEF Dock180 and the Rap1 GEF C3G to their SH3 domains (Peschard and Park 2007).

Gab1 overexpression also enhances HGF- and EGF-induced JNK activation. Downstream of Met, this effect is abrogated by co-expression of a dominant negative Crk mutant, suggesting that Met activates JNK through a Gab1–Crk complex. In addition, Gab1–Crk signaling to JNK is correlated with anchorage independent growth, and MMP-1 secretion, supporting a role for Gab1–Crk signaling in cell transformation and invasion (Lamorte et al. 2000).

PLCγ (pY307, 373, 406)

Structure-function analysis identified that the SH2 domain of PLCγ was recruited to three of the Crk binding sites (pY307, 373, 406). These studies have proposed a requirement for the Gab1–PLCγ interaction for branching morphogenesis in response to HGF/Met signaling (Birchmeier et al. 2003) and for endothelial cell migration (Laramee et al. 2007) downstream from the VEGFR-2.

Nck (pY406)

Nck1 SH2 domain directly interacts with tyrosine 406 on Gab1 downstream from Met, EGF, and PDGF RTKs activation. This interaction is required for induction of dorsal ruffles downstream of EGF, PDGF, and Met RTKs, and for Met-dependent Rac activation and for epithelial morphogenesis in MDCK cells and provides a mechanism through which Gab1 binding to RTKs can signal to the actin cytoskeleton (Abella et al. 2010). Although these biological outcomes were originally attributed to Crk and/or PLCγ, which also bind Gab1 on tyrosine 406, it is now important to assess the relative contribution of each signaling molecule to these biological processes.

Pak4 (ASM, aa. 116–234)

In addition to proteins containing SH2 domains, the P21-activated serine/threonine kinase 4 (Pak4) is recruited to Gab1 downstream from the Met RTK. This interaction has been mapped to amino acids 116 to 234 of Gab1 (Fig. 1) and requires phosphorylation. If this association is direct, however, remains to be addressed. Pak4 association with Gab1 is required for HGF-induced epithelial cell dispersion, migration, and invasive growth, likely through the ability of Pak4 to phosphorylate cofilin and promote remodeling of the actin cytoskeleton (Paliouras et al. 2009).

Feedback Loops Regulating Gab1 Signaling

Cross Talk Between PI3K and Shp2

The most important arms of Gab1 signaling network are the Shp2/MAPK arm and the PI3K/Akt arm. Activation of these pathways can influence each other through negative and positive feedback loops. As described earlier, Gab1 is a substrate for the Shp2 phosphatase. Recruitment of Shp2 to Gab1 leads to the dephosphorylation of the p85 binding sites on Gab1, which attenuates PI3K signaling.

Activation of PI3K potentiates activation of the MAPK pathway by increasing Shp2 recruitment to Gab1. p85 binding to Gab1 leads to an increase in local $PI_{3,4,5}P3$ formation, resulting in increased Gab1 membrane targeting and tyrosine phosphorylation. Downstream of EGFR, VEGFR, and EPO-R, a Gab1Δp85 mutant has a reduced ability to activate

the MAPK pathway due to a decreased ability to recruit Shp2 (Liu and Rohrschneider 2002; Nishida and Hirano 2003; Wohrle et al. 2009). These data indicate the existence of Gab1-dependent cross talk between the PI3K and MAPK signaling pathways.

Serine/Threonine Phosphorylation

Growth factor and cytokine stimulation induces a decrease in the mobility of Gab1 on an SDS PAGE that is attributed to increased serine and threonine phosphorylation. The biological role of this Ser/Thr phosphorylation depends on the cell type and receptor activated. Insulin and EGF stimulation induces Erk-dependent phosphorylation of Gab1 on Ser454, Ser581, Ser597, and Thr476, which are located in the vicinity of the p85 binding sites. This Ser/Thr phosphorylation correlates with reduced Tyr phosphorylation and attenuated PI3K signaling. In contrast, Met activation induces Erk-dependent phosphorylation of Thr476 and is associated with an increase in PI3K signaling (Wohrle et al. 2009). In melanocytes, however, HGF induces a PKCβII-dependent Ser/Thr phosphorylation of Gab1 associated with attenuated PI3K activation, as well as a decrease in cell migration and invasion (Oka et al. 2008). Furthermore, MAPK-dependent phosphorylation of Ser551 positively regulates the function of the PH domain of Gab1 in response to IL-6, and therefore enhances its recruitment to the plasma membrane (Wohrle et al. 2009). Taken together, these data suggest that Gab1 Tyr and Ser/Thr phosphorylation can influence each other by creating feedback loops allowing fine-tuned responses by modulating protein–protein interactions and subcellular localization.

Gab1 in Cancer

Even though Gab1 is essential in embryonic development, it has not been strongly linked to specific disease. Disease-linked phenotypes due to germline mutations in *GAB1* may not be visible because Gab1 is essential during development. Somatic mutations of *GAB1* have been described in cancers; however, their significance for tumorigenesis remains to be addressed. Nevertheless, in vitro and in vivo data have identified a role for Gab1 protein in cellular transformation and cancer progression in various systems due its critical role in RTK signaling. Expression of a mutant EGFR unable to efficiently phosphorylate Gab1 delays the onset of ErbB2-driven mammary tumor in mouse model (Gillgrass et al. 2003). In addition, expression of a Gab1 mutant uncoupled from Shp2, and Shp2 knock down impairs invasion and migration of Met overexpressing colorectal cancer cells in matrigel and prevents tumor formation when these cells are subcutaneously injected into nude mice (Seiden-Long et al. 2008). Furthermore, recent studies demonstrated that Gab1 plays a role in resistance to EGFR tyrosine kinase inhibitors induced by HGF and Met in NSCLC patients and cell lines by promoting cell survival (Turke et al. 2010).

In contrast to Gab1, Gab2 is often over-expressed in breast, ovarian, gastric, and skin cancer. In breast epithelial cell lines, over-expression of Gab2 cooperates with ErbB2 over-expression to promote cell survival and proliferation (Gu and Neel 2003; Wohrle et al. 2009). The differences between the roles of Gab1 and Gab2 in tumor initiation and progression may reflect that despite having similar signaling capacity, these two proteins have nonredundant functions as supported by the different phenotypes observed in the respective knockout mice. Alternatively, it may reflect the genetic instability of the *GAB2* locus located on chromosome 11q14.1 that is often amplified in human cancers.

Summary

The Gab1 scaffold protein is a critical node in signaling networks initiated by RTKs, cytokine and immune receptors. Recruitment of signaling molecules to Gab1 leads to two different outcomes. First, activation of PI3K/Akt, Shp2/MAPK, and JNK pathways engages a transcriptional program regulating cell survival, proliferation, morphogenesis, and invasion. Second, the recent discoveries that Pak4, Nck, and Crk are recruited to Gab1 downstream of RTKs indicate that Gab1 is a crucial scaffold for the regulation of the actin cytoskeleton and cell motility.

It is becoming evident that the role of Gab1 in signal transduction is more complex and dynamic than the classic definition of adaptor molecules. Recent data demonstrated that Gab1 does not only amplify and diversify receptor signals, but spatially organizes these signals in microdomains such as dorsal ruffles (Abella et al. 2010).

Gab1 associates with numerous receptors, binds many different downstream signaling molecules, and shows dynamic relocalization upon stimulation. Gab1 therefore likely engages in complexes of different composition and localization over time. In the future, it will be important to dissect the spatial and temporal regulation of these signaling complexes.

References

Abella JV, Vaillancourt R, Frigault MM, Ponzo MG, Zuo D, Sangwan V, Larose L, Park M. The Gab1 scaffold regulates RTK-dependent dorsal ruffle formation through the adaptor Nck. J Cell Sci. 2010;123:1306–19.

Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4:915–25.

Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 2005;16:139–49.

Gillgrass A, Cardiff RD, Sharan N, Kannan S, Muller WJ. Epidermal growth factor receptor-dependent activation of Gab1 is involved in ErbB-2-mediated mammary tumor progression. Oncogene. 2003;22:9151–5.

Gu H, Neel BG. The "Gab" in signal transduction. Trends Cell Biol. 2003;13:122–30.

Lamorte L, Kamikura DM, Park M. A switch from p130Cas/Crk to Gab1/Crk signaling correlates with anchorage independent growth and JNK activation in cells transformed by the Met receptor oncoprotein. Oncogene. 2000;19:5973–81.

Laramee M, Chabot C, Cloutier M, Stenne R, Holgado-Madruga M, Wong AJ, Royal I. The scaffolding adapter Gab1 mediates vascular endothelial growth factor signaling and is required for endothelial cell migration and capillary formation. J Biol Chem. 2007;282:7758–69.

Liu Y, Rohrschneider LR. The gift of Gab. FEBS Lett. 2002;515:1–7.

Maeda K, Murakami H, Yoshida R, Ichihara M, Abe A, Hirai M, Murohara T, Takahashi M. Biochemical and biological responses induced by coupling of Gab1 to phosphatidylinositol 3-kinase in RET-expressing cells. Biochem Biophys Res Commun. 2004;323:345–54.

Nishida K, Hirano T. The role of Gab family scaffolding adapter proteins in the signal transduction of cytokine and growth factor receptors. Cancer Sci. 2003;94:1029–33.

Oka M, Kikkawa U, Nishigori C. Protein kinase C-betaII represses hepatocyte growth factor-induced invasion by preventing the association of adapter protein Gab1 and phosphatidylinositol 3-kinase in melanoma cells. J Invest Dermatol. 2008;128:188–95.

Paliouras GN, Naujokas MA, Park M. Pak4, a novel Gab1 binding partner, modulates cell migration and invasion by the Met receptor. Mol Cell Biol. 2009;29:3018–32.

Pawson T, Scott JD. Signaling through scaffold, anchoring, and adaptor proteins. Science. 1997;278:2075–80.

Peschard P, Park M. From Tpr-Met to Met, tumorigenesis and tubes. Oncogene. 2007;26:1276–85.

Rodrigues GA, Falasca M, Zhang Z, Ong SH, Schlessinger J. A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. Mol Cell Biol. 2000;20:1448–59.

Sarmay G, Angyal A, Kertesz A, Maus M, Medgyesi D. The multiple function of Grb2 associated binder (Gab) adaptor/scaffolding protein in immune cell signaling. Immunol Lett. 2006;104:76–82.

Schaeper U, Vogel R, Chmielowiec J, Huelsken J, Rosario M, Birchmeier W. Distinct requirements for Gab1 in Met and EGF receptor signaling in vivo. Proc Natl Acad Sci USA. 2007;104:15376–81.

Schlessinger J, Lemmon MA. SH2 and PTB domains in tyrosine kinase signaling. Sci STKE. 2003;2003:RE12.

Seiden-Long I, Navab R, Shih W, Li M, Chow J, Zhu CQ, Radulovich N, Saucier C, Tsao MS. Gab1 but not Grb2 mediates tumor progression in Met overexpressing colorectal cancer cells. Carcinogenesis. 2008;29:647–55.

Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, Toschi L, Rogers A, Mok T, Sequist L, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell. 2010;17:77–88.

Wohrle FU, Daly RJ, Brummer T. Function, regulation and pathological roles of the Gab/DOS docking proteins. Cell Commun Signal. 2009;7:22.

Gab2

Yongping Wang and Haihua Gu
Department of Pathology, University of Colorado Denver, Aurora, CO, USA

Synonyms

P97

Historical Background

Grb2-associated binder 2, Gab2, belongs to the Gab/DOS family of scaffolding adaptors that include mammalian ▶ Gab1, Gab3, *Drosophila Daughter of Sevenless* (DOS), and *Caenorhabditis elegans Suppressor of Clear* 1 (SOC1) (Gu and Neel 2003; Wohrle et al. 2009). Gab1, the first identified member of this family of adaptors, was discovered in search of protein ligands for Grb2 SH3 domain (Holgado-Madruga et al. 1996). DOS was identified as a potential substrate for Corkscrew (Csw), the *Drosophila* ortholog of the SH2 domain containing protein tyrosine phosphatase 2 (Shp2) (Herbst et al. 1996). Gab2, the third member

identified in this family, was initially cloned as a binding protein and substrate of Shp2 (Gu et al. 1998). Gab2 gene is located on human chromosome 11q14.1. Two other groups later cloned Gab2 by searching DNA database for protein with sequence homology to Gab1 (Nishida et al. 1999; Zhao et al. 1999). SOC1 was uncovered in a screen for suppressors of hyperactive signaling from Egl-15, an FGF receptor ortholog (Schutzman et al. 2001). Gab3 was identified by sequence similarity to Gab1 and Gab2 (Wolf et al. 2002). Gab1 and Gab2 are expressed ubiquitously. However, Gab2 is expressed at relatively higher level in myeloid cells and low in lymphoid tissues and fibroblasts (Gu et al. 1998). Gab3 also has a widespread expression pattern, although it is highly expressed in lymphoid tissue (Wolf et al. 2002). GenBank database also contains a human cDNA clone (Acession Number AB076978), termed Gab4. However, it is unclear how this "Gab4" cDNA sequence information was obtained. Although mRNAs for parts of the "Gab4" cDNA are found as ESTs in testis specifically, no report has shown that the full-length "Gab4" protein is expressed 10 years after the sequence was deposited into the GenBank. Thus, it is unclear whether this "Gab4" clone represents a *bona fide* Gab protein.

Gab2 Domain Structures

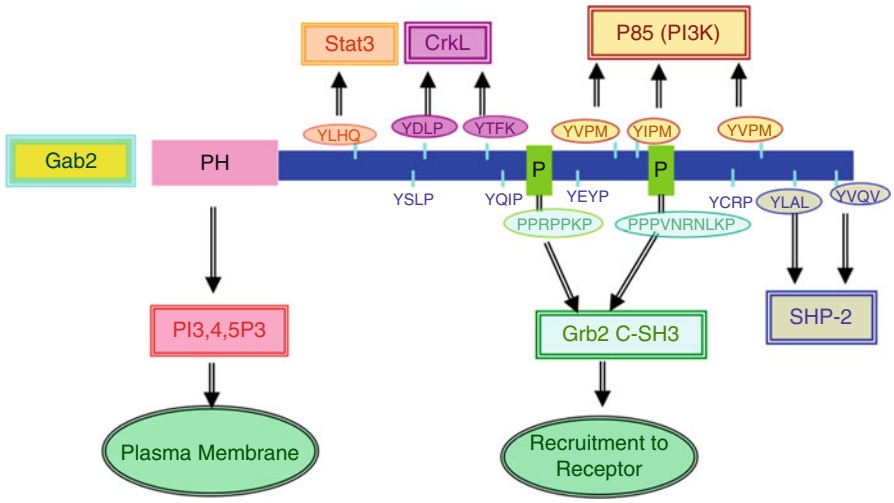
Gab2 and its family members have a similar topology although the overall amino acid sequence identity among the Gab/DOS family members is about 20–40%. Gab2 contains an N-terminal pleckstrin homology (PH) domain (~120 aa) and the C-terminal portion of the molecule (Fig. 1). Gab2 PH domain has the highest sequence identity with the PH domains of the other Gab/DOS family members except the SOC1 PH domain, ranging from ~45% to 80% (Gu et al. 1998; Wohrle et al. 2009). The main function of the Gab2 PH domain is to preferentially bind phosphatidylinositol (*3,4,5*)-trisphosphate (PI3,4,5P3), the lipid product of ▶ phosphoinositide 3-kinase (PI3K) (Yu et al. 2006a). Although the C-terminal portion of Gab2 does not have significant sequence homology to other Gab/DOS members, it contains multiple conserved tyrosine phosphorylation and serine/threonine phosphorylation sites, and proline-rich sequences. Tyrosyl phosphorylation in Gab2 results in the recruitment of various SH2 domain-containing signaling molecules including Shp2 and p85 (the regulatory subunit of PI3K) and activation of key cell signaling pathways (Figs. 1 and 2). Serine/threonine phosphorylation negatively modulates Gab2 interactions with SH2 domain-containing signaling molecules (Gu and Neel 2003; Wohrle et al. 2009). Gab2 contains two proline-rich motifs that are required for binding to the Grb2 C-SH3 domain (Lock et al. 2000). However, these two proline-rich motifs do not conform to the typical SH3 domain ligand (i.e., PXXP) that is mainly involved in hydrophobic interaction with the SH3 domain. Both of the Gab2 SH3-domain binding sites contain the atypical SH3 domain-binding motif, RXXK (Fig. 1), which engages electrostatic interaction with the Grb2 C-SH3 domain (Harkiolaki et al. 2009). All of these structural features play important roles in mediating Gab2 function in cell signaling.

Gab2 in Cell Signaling

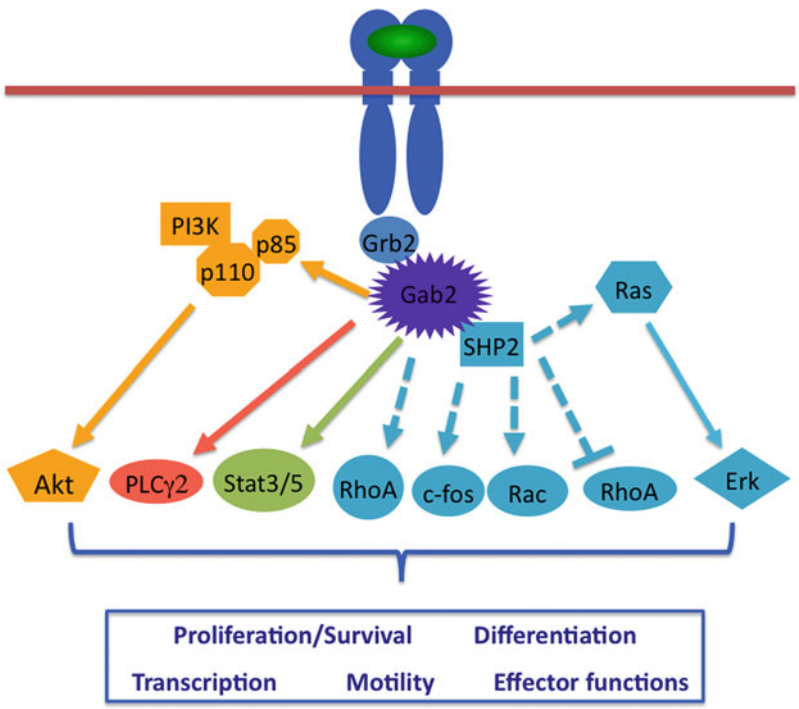
Gab2 functions to mediate the activation of critical cell signaling pathways by multiple cell surface receptors for growth factors, cytokines, antigens, and immunoglobulins (Fig. 2). The current model is that upon receptor activation, Gab2 is recruited to the activated receptor, becomes tyrosyl phosphorylated, binds SH2 domain-containing signaling molecules, and activates key downstream signaling pathways. Gab2-activated signaling events can be turned off at least by serine and threonine phosphorylation in Gab2 (Gu and Neel 2003; Wohrle et al. 2009).

Gab2 Recruitment to the Receptor

Gab2 is recruited to the activated receptors through other signaling intermediates because Gab2 does not contain modular domain that can directly interact with the receptor. The main route of Gab2-receptor interaction is mediated through Grb2. Grb2 has one SH2 domain flanked by two SH3 domains. The two RXXK motifs in Gab2 (Harkiolaki et al. 2009) lead to constitutive interaction of Gab2 with the Grb2 C-SH3 domain (Lock et al. 2000). The Grb2 SH2 domain mediates the targeting of the Grb2-Gab2 complex to the activated receptor (i.e., Flt3-ITD) or upstream activator (i.e., BCR-ABL) that contains



Gab2, Fig. 1 Schematic structure of Gab2. Gab2 has an N-terminal pleckstrin homolog (*PH*) domain that binds preferentially *PI3,4,5P3*, and the C-terminal half of the molecule containing multiple tyrosine phosphorylation sites (YXXX) that recruit different SH2 domain-containing signaling proteins including Shp2, p85, Stat3, and CrkL, and two proline-rich motifs (*P*) that bind Grb2 C-SH3 domain. The specific interactions between Gab2 and its ligands are color-coded. For details, see text



Gab2, Fig. 2 Signaling pathways regulated by Gab2. Gab2 activates the Ras/Erk pathway, via its ability to bind to and activate the SH2 domain-containing protein tyrosine phosphatase Shp2, and the PI3K/Akt pathway, by virtue of its ability to bind the p85 regulatory subunit of PI3K, thereby activating the associated p110 catalytic subunit. Studies also reveal that Gab2 can activate other signaling pathways such as members of RhoGTPase family, Stat3/5, PLCγ2, and immediate early gene transcription. All of these pathways play important roles in regulating the growth, differentiation, migration, and effector functions of different cell types. *Dashed lines* mean that the detailed mechanisms are still lacking. For details, see text

Grb2-SH2 domain binding sites (YXNX) (Masson et al. 2009; Sattler et al. 2002). However, in cell signaling initiated by receptors for IL3/IL5/GM-CSF/IL2 and FcεRI that lacks direct Grb2 binding to their cytoplasmic tails, another adapter protein Shc, brings the Grb2-Gab2 complex to these receptors that contain binding sites for the Shc PTB or SH2 domain (Gu et al. 2000; Yu et al. 2006a). Shc also has three Grb2-SH2 binding sites which, when phosphorylated, recruit the Grb2-Gab2 complex to the receptor (Gu and Neel 2003). A Gab2 mutant with the two RXXK mutated cannot be tyrosyl phosphorylated efficiently and is impaired in its ability to mediate receptor signaling (Brummer et al. 2006; Yu et al. 2006a).

The PH domain provides another route for Gab2 recruitment. PI3,4,5P3 produced by the receptor-activated ▶ PI3K recruits Gab2 via its PH domain to the subcellular compartment where the activated receptor is located. The importance of Grb2 C-SH3 binding sites and PH domain in mediating Gab2 recruitment and functions depends on the receptor involved. The Grb2 C-SH3 binding sites play a dominant role in Gab2 function in EGFR and Flt3 signaling (Brummer et al. 2006; Masson et al. 2009) whereas in the FcγR-induced signaling response, the PH domain exerts a major role (Yu et al. 2006a). However, both Grb2 C-SH3 binding sites and PH domain contribute to Gab2 action in FcεRI-induced signaling (Yu et al. 2006a).

Protein Tyrosine Kinases That Phosphorylate Gab2

Upon recruited to the activated receptors or receptor signaling complexes, Gab2 undergoes tyrosyl phosphorylation in a receptor-dependent manner. Although it is likely that receptor tyrosine kinase may directly phosphorylate on certain tyrosine residues in Gab2 (Lee and States 2000), this scenario has not been carefully examined. JAK2, the receptor-associated protein tyrosine kinase, plays an essential role in cytokine receptor signaling. However, JAK2 is not required for the G-CSF-induced Gab2 tyrosyl phosphorylation (Zhu et al. 2004). Instead, most of the studies show that the cytoplasmic tyrosine kinases are responsible for Gab2 tyrosine phosphorylation. In T cell antigen receptor (TCR) signaling, ▶ ZAP-70 forms a complex with Gab2 and phosphorylates it (Yamasaki et al. 2001). Syk is required for Gab2 tyrosyl phosphorylation and is associated with Gab2 upon FcεRI activation (Yu et al. 2006a). Members of ▶ Src Family protein tyrosine Kinase (SFK) such as Src and Lyn are also responsible for Gab2 tyrosyl phosphorylation upon activation of CSF1R (Lee and States 2000) and G-CSFR (Zhu et al. 2004).

Signaling Pathways Activated by Gab2

Tyrosyl-phosphorylated Gab2 can activate multiple downstream signaling pathways by recruiting SH2 domain-containing signaling proteins. The major Gab2-induced signaling pathways include Shp2 and ▶ PI3K (Fig. 2). Recent studies also reveal additional signaling pathways activated by Gab2.

Role in Shp2 pathway. Gab2 contains two tyrosine motifs VDYXXV/L (where X = any amino acid) (Fig. 1), when phosphorylated, which that bind Shp2. Shp2 has low basal activity due to allosteric inhibition of its PTP domain by the N-terminal SH2 domain. Upon binding to tyrosyl-phosphorylated Gab2, the basal inhibition is relieved, resulting in strong activation of Shp2 (Neel et al. 2003). Vertebrates express another SH2 domain containing PTP, Shp1. The SH2 domains of Shp1 and Shp2 recognize similar phosphorylated tyrosine containing motifs. However, the interaction between the tyrosyl-phosphorylated Gab2 and Shp1 has not been observed. It is not well understood why Gab2 binds preferentially to Shp2. Nevertheless, this deferential binding ability helps explain the distinct biological functions of Shp1 and Shp2 (Neel et al. 2003).

Gab2, acting via Shp2, is required for full activation of Erk downstream of many receptors. Expression of a Gab2 mutant with Y->F in the VDYXXV/L motifs impairs Erk activation in response to CSF-1 (Liu et al. 2001), IL2 (Arnaud et al. 2004b), and EGF stimulation (Brummer et al. 2006) as well as under three-dimensional culture condition (Bentires-Alj et al. 2006). In addition, SCF-induced full activation of Ras and Erk requires Gab2 interaction with Shp2 (Yu et al. 2006b), indicating that Gab2/Shp2 contribute to the efficient Erk activation at the level of Ras. Gab2/Shp2 complexes may serve as "amplifiers" of initial Ras/Erk pathway activation (Gu and Neel 2003).

Gab2/Shp2 complexes also have additional signaling roles besides activation of the Ras/Erk pathway. Expression of Gab2 mutants that cannot bind Shp2 fails to activate IL3-induced immediate early gene (i.e., fos) transcription without affecting Erk activation (Gu et al. 1998). The Gab2/Shp2 complex appears to activate the Rac-JNK pathway in response

to SCF in mast cells (Yu et al. 2006b). Gab2 via Shp2 promotes migration and invasion of mammary epithelial cells by recruiting p190RhoGAP and inhibiting RhoA activation (Abreu et al. 2011).

Role in PI3K pathway. Gab2 has three potential binding sites (YXXM) for the SH2 domain of p85. By binding p85, Gab2 mediates the activation of the ▶ PI3K pathway for receptors that lack the p85 binding sites. For example, Gab2 is implicated in PI3K activation from the receptor for IL-3/GM-CSF (Gu et al. 2000), EGFR (Kong et al. 2000), FcγR (Gu et al. 2003), and FcεRI (Gu et al. 2001). Gab2 also recruits PI3K in response to stimulation of receptor systems, such as the TCR (Nishida et al. 1999; Pratt et al. 2000) and BCR (Nishida et al. 1999), in which co-receptors recruit PI3K. In these systems, Gab2/p85 probably serves to amplify receptor-evoked PI3K activity. Gab2-induced activation of PI3K can have different signaling effects depending on the cell type. Recruitment of PI3K to Gab2 is critical for FcεRI-induced degranulation of mast cell (Gu et al. 2001). In contrast, Gab2/PI3K complexes inhibit TCR-induced IL2 production (Pratt et al. 2000; Yamasaki et al. 2001).

Role in other signaling pathways. Gab2 has been implicated in the activation of the Stat pathway. Gab2 has been shown in complexes with Stat5 (Nyga et al. 2005). However, it is still unclear how Stat5 interacts with Gab2 and whether Gab2 is required for Stat5 activation. It appears that the constitutively activated mutant of Stat5 via interaction with Gab2 activates the PI3K-Akt and Erk pathways in BaF3 cells (Nyga et al. 2005). A recent study also reveals that Stat3 interacts with the phosphorylated tyrosine residue in $_{195}$YLHQ motif of Gab2. Gab2 is required for Stat3 activation, which is important for Gab2-dependent transformation of primary hematopoietic cells by Stk/Ron in response to Friend virus infection (Ni et al. 2007). Gab2 association with PLCγ2 has been observed in osteoclasts upon RANK activation (Mao et al. 2006). It appears that PLCγ2 acts as an adaptor mediating Gab2 tyrosyl phosphorylation (Mao et al. 2006). Gab2 contributes to efficient activation of ▶ NF-κB induced by RANKL (Wada et al. 2005). However, it is unknown whether PLCγ2 association with Gab2 is required for RANK signaling. Although Gab2 via Shp2 can inhibit RhoA activation in mammary epithelial cells, a report shows that Gab2 activates RhoA, which is important for microtubule-dependent granule transport to plasma membrane in mast cells. It is unclear how Gab2 activates RhoA in mast cells, which is likely independent of Shp2 (Nishida et al. 2005). Yeast two-hybrid screens have identified CrkL (Crouin et al. 2001) that can bind Gab2. The functional significance of this interaction remains unclear.

Turning Off Gab2 Signaling Activity

Gab2 activates cell signaling pathways through phosphotyrosine interaction with SH2 domains. One way to terminate Gab2-initiating signaling is to dephosphorylate tyrosine residues in Gab2 by protein tyrosine phosphatases. Although Gab2 was identified as potential substrate for Shp2 (Gu et al. 2000), the functional consequence of Gab2 dephosphorylation by Shp2 is still unclear. Recent studies reveal that serine and threonine phosphorylation of Gab2 function as negative feedback mechanism to inhibit Gab2 signaling activities. Phosphorylation of three amino acid residues contributes to negative regulation of Gab2 tyrosyl phosphorylation and Gab2-involved responses. Ser159, phosphorylated by Akt, is involved in reducing Gab2 tyrosyl phosphorylation by ErbB2 (Lynch and Daly 2002). Gab2 Ser210 and Thr391, phosphorylated partially by Akt or an Akt-dependent kinase, recruit 14-3-3 protein, which reduces tyrosyl phosphorylation of Gab2 and Gab2-initiated signaling responses (Brummer et al. 2008). In addition, Ser623 phosphorylation by Erk results in reduced Gab2 association with Shp-2 and Erk activation induced by IL2 (Arnaud et al. 2004a). However, the mechanism by which Ser/Thr phosphorylation inhibits Gab2 signaling is still not well understood.

Biological Functions

Gab2 mainly plays positive roles in regulating cell proliferation, survival, differentiation, and migration in a cell type-specific manner. While Gab2 activation of PI3K contributes to proliferation of hematopoietic cells (Gu et al. 2000), and hepatocytes (Kong et al. 2000), Gab2 via Shp-2 is required for the proliferation of mammary epithelial cells in three-dimensional culture (Bentires-Alj et al. 2006) and mast cells (Yu et al. 2006b). Likewise, while Gab2 activation of PI3K plays a critical role in differentiation and survival of neuronal cells (Mao and Lee 2005), Gab2 via association with Shp-2 and

activation of Erk promotes differentiation of macrophages (Liu et al. 2001) and myeloid cells (Dorsey et al. 2002). In addition, Gab2 expression also contributes to cell migration in different cell types (Horst et al. 2009; Meng et al. 2005). Despite its major role as a positive signal transducer in regulating cell responses, some studies also suggest that Gab2 plays a negative role in cell signaling. Gab2 mediates the suppression of TCR-evoked IL2 gene expression in T lymphocytes (Pratt et al. 2000; Yamasaki et al. 2001).

Studies using mice with targeted disruption of the Gab2 gene (Gab2−/−) help elucidate the functions of Gab2 in vivo. Gab2 is not required for the normal development of mice since Gab2−/− mice are viable and healthy (Gu et al. 2001; Nishida et al. 2002). However, detailed analyses of the Gab2−/− mice reveal that Gab2 expression is required for the growth and functions of a variety of hematopoietic cells. Gab2−/− mast cells are defective in FcεRI-evoked degranulation and cytokine gene expression (Gu et al. 2001), which contributes to the impaired allergy response of the Gab2−/− mice (Gu et al. 2001). In addition, mast cell development is comprised in Gab2−/− mice due to impaired c-Kit-evoked signaling of Gab2−/− mast cells (Nishida et al. 2002; Yu et al. 2006b). Although differentiation of the Gab2−/− macrophage seems to be normal, FcγR-induced phagocytosis is diminished in Gab2−/− macrophages (Gu et al. 2003). Eosinophil number in the peripheral blood from Gab2−/− mice is also significantly reduced (Gu and Neel 2003). Gab2−/− mice also display mild osteopetrosis and decreased bone resorption due to defective RANK-evoked signaling in Gab2−/− osteoclasts and osteoclast differentiation from Gab2−/− progenitor cells (Wada et al. 2005). Hematopoietic stem cells from Gab2−/− mice show reduced survival and self-renewal capability (Zhang et al. 2007). Further studies of mutant mice that lack the expression of both Gab1 and Gab2 in cardiomyocytes uncover a role of Gab2 in contributing to the normal cardiac function in adult mice through mediating neuregulin1/ErbB signaling (Nakaoka et al. 2007).

Gab2 in Cancer and Alzheimer's Disease

Gab2 has been implicated in different types of cancers including solid tumors and leukemia. Gab2 protein overexpression has been reported in breast cancer cells and breast tumors (Bentires-Alj et al. 2006; Daly et al. 2002) and melanoma (Horst et al. 2009). In breast tumors, Gab2 overexpression is associated with early stage of breast cancer (Fleuren et al. 2010). Gab2 gene amplification is at least one mechanism contributing to Gab2 protein overexpression in breast cancer (Bentires-Alj et al. 2006) and melanoma (Horst et al. 2009). Gab2 overexpression is associated with metastatic melanoma (Horst et al. 2009). Studies by manipulating the expression of Gab2 in cell lines and mice indicate that Gab2 expression alone or in combination with other oncogenes promote breast tumor cell growth (Bentires-Alj et al. 2006; Brummer et al. 2006), migration, invasion, and metastasis in vitro and in mice (Bentires-Alj et al. 2006; Ke et al. 2007). Gab2-regulated Shp2-Erk pathway contributes to the proliferation, invasion, and metastasis of breast cancer cells (Bentires-Alj et al. 2006; Ke et al. 2007). In contrast, Gab2-regulated PI3K pathway promotes migration, invasion, and metastasis of melanoma cells (Horst et al. 2009). Although Gab2 protein overexpression or gene amplification has been reported in gastric carcinomas (Lee et al. 2007), acute myeloid leukemia (Zatkova et al. 2006), and ovarian cancer (Brown et al. 2008), the functional roles of Gab2 in these cancers remain unexplored.

Besides Gab2 overexpression, endogenous level of Gab2 plays critical roles in leukemogenesis induced by BCR-ABL and juvenile myelomonocytic leukemia (JMML)-associated Shp2 mutants. BCR-ABL is the causative oncogene for chronic myelogenous leukemia (CML). Gab2 via Grb2 interacts with BCR-ABL through Y177 (a Grb2 SH2 domain binding site) and mediates BCR-ABL-evoked PI3K-Akt and Erk activation, and oncogenic transformation of myeloid progenitor cells (Sattler et al. 2002). Importantly, Gab2 is required for BCR-ABL-induced CML-like disease in mice (Gu and Neel 2003). Further supporting a role for Gab2 in CML, a recent study shows that Gab2 protein expression is significantly enhanced in bone marrow samples of CML patients with accelerated phase and blast crisis diseases (Aumann et al. 2011). About 35% of JMML has somatic Shp2 mutations. Transplantation of wild type, not Gab2−/−, bone marrow progenitor cells transduced with retroviruses expressing the JMML-associated Shp2 mutants induces JMML-like disease in mice, indicating that Gab2 is required for leukemogenesis induced by these Shp2 mutants (Mohi et al. 2005). Lastly, only Gab2, not Gab1, mediates

oncogenic transformation of fibroblasts by the V-Sea oncogene (Ischenko et al. 2003) and induction of erytholeukemia by the Stk receptor tyrosine kinase in response to Friend virus infection (Teal et al. 2006).

A recent study using genome-wide association analysis reveals that several Gab2 polymorphic alleles can increase the risk of Alzheimer's disease (AD) onset in patients carrying the APOE epsilon4 allele (Reiman et al. 2007). Subsequent studies by more than ten different groups indicate that the association of Gab2 polymorphic alleles with increase AD onset depends on patient cohorts, with about half of those studies supporting Gab2 polymorphic allele as a risk factor for AD in APOE epsilon4 carriers whereas roughly the other half of the studies fail to demonstrate significant association of Gab2 polymorphic allele with increased risk of AD. It is not understood how the polymorphic alleles of Gab2 contribute to Alzheimer disease onset. These polymorphic alleles are located in the intron regions of Gab2 gene. They are more likely to change the expression of Gab2, not the protein structure of Gab2. Knockdown of Gab2 expression by siRNA increases ▶ GSK-3 and Tau phosphorylation in neuronal cell culture, suggesting that polymorphic alleles of Gab2 may involve in Alzheimer disease onset by modulating Tau phosphorylation (Reiman et al. 2007). Future study by changing the expression level of Gab2 in the brain of mouse model of AD should help clarify the role of Gab2 in AD.

Summary

Since the discovery of Gab2 13 years ago, results from molecular, biochemical, cellular, and genetic studies have revealed Gab2 as a critical regulator of multiple important cell signaling pathways including PI3K-Akt and Shp2-Ras-Erk. One important question needs to be addressed is how Gab2 via Shp2 regulates the activation of the Ras-Erk pathway. In addition, it will also be important to understand the detailed mechanism of the negative regulation on Gab2 signaling by serine/threonine phosphorylation. Gab2 overexpression or expression is functionally implicated in various diseases including cancer, allergy, and Alzheimer's. Because Gab2 belongs to the so-called undruggable target, it will be a challenge to find agents or small molecule inhibitors that can decrease Gab2 expression or blocking Gab2 activation of downstream signaling pathways. However, with the new technological advances in the drug discovery field, drugging Gab2 should provide potential new therapeutics for treating human diseases with minimal side effects since mice lacking Gab2 expression are generally healthy.

References

Abreu MT, Hughes WE, Mele K, Lyons RJ, Rickwood D, Browne BC, et al. Gab2 regulates cytoskeletal organization and migration of mammary epithelial cells by modulating RhoA activation. Mol Biol Cell. 2011;22:105–16.

Arnaud M, Crouin C, Deon C, Loyaux D, Bertoglio J. Phosphorylation of Grb2-associated binder 2 on serine 623 by ERK MAPK regulates its association with the phosphatase SHP-2 and decreases STAT5 activation. J Immunol. 2004a; 173:3962–71.

Arnaud M, Mzali R, Gesbert F, Crouin C, Guenzi C, Vermot-Desroches C, et al. Interaction of the tyrosine phosphatase SHP-2 with Gab2 regulates Rho-dependent activation of the c-fos serum response element by interleukin-2. Biochem J. 2004b;382:545–56.

Aumann K, Lassmann S, Schopflin A, May AM, Wohrle FU, Zeiser R, et al. The immunohistochemical staining pattern of Gab2 correlates with distinct stages of chronic myeloid leukemia. Hum Pathol. 2011;42:719–26.

Bentires-Alj M, Gil SG, Chan R, Wang ZC, Wang Y, Imanaka N, et al. A role for the scaffolding adapter GAB2 in breast cancer. Nat Med. 2006;12:114–21.

Brown LA, Kalloger SE, Miller MA, Shih Ie M, McKinney SE, Santos JL, et al. Amplification of 11q13 in ovarian carcinoma. Genes Chromosomes Cancer. 2008;47:481–9.

Brummer T, Schramek D, Hayes VM, Bennett HL, Caldon CE, Musgrove EA, et al. Increased proliferation and altered growth factor dependence of human mammary epithelial cells overexpressing the Gab2 docking protein. J Biol Chem. 2006;281:626–37.

Brummer T, Larance M, Herrera Abreu MT, Lyons RJ, Timpson P, Emmerich CH, et al. Phosphorylation-dependent binding of 14-3-3 terminates signalling by the Gab2 docking protein. EMBO J. 2008;27:2305–16.

Crouin C, Arnaud M, Gesbert F, Camonis J, Bertoglio J. A yeast two-hybrid study of human p97/Gab2 interactions with its SH2 domain-containing binding partners. FEBS Lett. 2001; 495:148–53.

Daly RJ, Gu H, Parmar J, Malaney S, Lyons RJ, Kairouz R, et al. The docking protein Gab2 is overexpressed and estrogen regulated in human breast cancer. Oncogene. 2002;21:5175–81.

Dorsey JF, Cunnick JM, Mane SM, Wu J. Regulation of the Erk2-Elk1 signaling pathway and megakaryocytic differentiation of Bcr-Abl(+) K562 leukemic cells by Gab2. Blood. 2002;99:1388–97.

Fleuren ED, O'Toole S, Millar EK, McNeil C, Lopez-Knowles E, Boulghourjian A, et al. Overexpression of the oncogenic signal transducer Gab2 occurs early in breast cancer development. Int J Cancer. 2010;127:1486–92.

Gu H, Neel BG. The "Gab" in signal transduction. Trends Cell Biol. 2003;13:122–30.

Gu H, Pratt JC, Burakoff SJ, Neel BG. Cloning of p97/Gab2, the major SHP-2 binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. Mol Cell. 1998;2:729–40.

Gu H, Maeda H, Moon JJ, Lord JD, Yoakim M, Nelson BH, et al. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. Mol Cell Biol. 2000;20:7109–20.

Gu H, Saito K, Klaman LD, Shen J, Fleming T, Wang Y, et al. Essential role for Gab2 in the allergic response. Nature. 2001; 412:186–90.

Gu H, Botelho RJ, Yu M, Grinstein S, Neel BG. Critical role for scaffolding adapter Gab2 in Fc gamma R-mediated phagocytosis. J Cell Biol. 2003;161:1151–61.

Harkiolaki M, Tsirka T, Lewitzky M, Simister PC, Joshi D, Bird LE, et al. Distinct binding modes of two epitopes in Gab2 that interact with the SH3C domain of Grb2. Structure. 2009;17:809–22.

Herbst R, Carroll PM, Allard JD, Schilling J, Raabe T, Simon MA. Daughter of sevenless is a substrate of the phosphotyrosine phosphatase Corkscrew and functions during sevenless signaling. Cell. 1996;85:899–909.

Holgado-Madruga M, Emlet DR, Moscatello DK, Godwin AK, Wong AJ. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. Nature. 1996;379:560–4.

Horst B, Gruvberger-Saal SK, Hopkins BD, Bordone L, Yang Y, Chernoff KA, et al. Gab2-mediated signaling promotes melanoma metastasis. Am J Pathol. 2009;174:1524–33.

Ischenko I, Petrenko O, Gu H, Hayman MJ. Scaffolding protein Gab2 mediates fibroblast transformation by the SEA tyrosine kinase. Oncogene. 2003;22:6311–8.

Ke Y, Wu D, Princen F, Nguyen T, Pang Y, Lesperance J, et al. Role of Gab2 in mammary tumorigenesis and metastasis. Oncogene. 2007;26:4951–60.

Kong M, Mounier C, Wu J, Posner BI. Epidermal growth factor-induced phosphatidylinositol 3-kinase activation and DNA synthesis. Identification of Grb2-associated binder 2 as the major mediator in rat hepatocytes. J Biol Chem. 2000; 275:36035–42.

Lee AW, States DJ. Both src-dependent and -independent mechanisms mediate phosphatidylinositol 3-kinase regulation of colony-stimulating factor 1-activated mitogen-activated protein kinases in myeloid progenitors. Mol Cell Biol. 2000; 20:6779–98.

Lee SH, Jeong EG, Nam SW, Lee JY, Yoo NJ. Increased expression of Gab2, a scaffolding adaptor of the tyrosine kinase signalling, in gastric carcinomas. Pathology. 2007; 39:326–9.

Liu Y, Jenkins B, Shin JL, Rohrschneider LR. Scaffolding protein Gab2 mediates differentiation signaling downstream of Fms receptor tyrosine kinase. Mol Cell Biol. 2001; 21:3047–56.

Lock LS, Royal I, Naujokas MA, Park M. Identification of an atypical Grb2 carboxy-terminal SH3 domain binding site in Gab docking proteins reveals Grb2-dependent and independent recruitment of Gab1 to receptor tyrosine kinases. J Biol Chem. 2000;275(40):31536–45.

Lynch DK, Daly RJ. PKB-mediated negative feedback tightly regulates mitogenic signalling via Gab2. EMBO J. 2002; 21:72–82.

Mao Y, Lee AW. A novel role for Gab2 in bFGF-mediated cell survival during retinoic acid-induced neuronal differentiation. J Cell Biol. 2005;170:305–16.

Mao D, Epple H, Uthgenannt B, Novack DV, Faccio R. PLCgamma2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. J Clin Invest. 2006; 116:2869–79.

Masson K, Liu T, Khan R, Sun J, Ronnstrand L. A role of Gab2 association in Flt3 ITD mediated Stat5 phosphorylation and cell survival. Br J Haematol. 2009;146:193–202.

Meng S, Chen Z, Munoz-Antonia T, Wu J. Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor. Biochem J. 2005; 391:143–51.

Mohi MG, Williams IR, Dearolf CR, Chan G, Kutok JL, Cohen S, et al. Prognostic, therapeutic, and mechanistic implications of a mouse model of leukemia evoked by Shp2 (PTPN11) mutations. Cancer Cell. 2005;7:179–91.

Nakaoka Y, Nishida K, Narimatsu M, Kamiya A, Minami T, Sawa H, et al. Gab family proteins are essential for postnatal maintenance of cardiac function via neuregulin-1/ErbB signaling. J Clin Invest. 2007;117:1771–81.

Neel BG, Gu H, Pao L. The "Shp"ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci. 2003;28:284–93.

Ni S, Zhao C, Feng GS, Paulson RF, Correll PH. A novel Stat3 binding motif in Gab2 mediates transformation of primary hematopoietic cells by the Stk/Ron receptor tyrosine kinase in response to Friend virus infection. Mol Cell Biol. 2007; 27:3708–15.

Nishida K, Yoshida Y, Itoh M, Fukada T, Ohtani T, Shirogane T, et al. Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. Blood. 1999;93:1809–16.

Nishida K, Wang L, Morii E, Park SJ, Narimatsu M, Itoh S, et al. Requirement of Gab2 for mast cell development and KitL/c-Kit signaling. Blood. 2002;99:1866–9.

Nishida K, Yamasaki S, Ito Y, Kabu K, Hattori K, Tezuka T, et al. Fc{epsilon}RI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. J Cell Biol. 2005;170:115–26.

Nyga R, Pecquet C, Harir N, Gu H, Dhennin-Duthille I, Regnier A, et al. Activated STAT5 proteins induce activation of the PI 3-kinase/Akt and Ras/MAPK pathways via the Gab2 scaffolding adapter. Biochem J. 2005;390:359–66.

Pratt JC, Igras VE, Maeda H, Baksh S, Gelfand EW, Burakoff SJ, et al. Cutting edge: gab2 mediates an inhibitory phosphatidylinositol 3'-kinase pathway in T cell antigen receptor signaling. J Immunol. 2000;165:4158–63.

Reiman EM, Webster JA, Myers AJ, Hardy J, Dunckley T, Zismann VL, et al. GAB2 alleles modify Alzheimer's risk in APOE epsilon4 carriers. Neuron. 2007;54:713–20.

Sattler M, Mohi MG, Pride YB, Quinnan LR, Malouf NA, Podar K, et al. Critical role for Gab2 in transformation by BCR/ABL. Cancer Cell. 2002;1:479–92.

Schutzman JL, Borland CZ, Newman JC, Robinson MK, Kokel M, Stern MJ. The *Caenorhabditis elegans* EGL-15 signaling pathway implicates a DOS-like multisubstrate adaptor protein in fibroblast growth factor signal transduction. Mol Cell Biol. 2001;21:8104–16.

Teal HE, Ni S, Xu J, Finkelstein LD, Cheng AM, Paulson RF, et al. GRB2-mediated recruitment of GAB2, but not GAB1, to SF-STK supports the expansion of Friend virus-infected erythroid progenitor cells. Oncogene. 2006;25:2433–43.
Wada T, Nakashima T, Oliveira-dos-Santos AJ, Gasser J, Hara H, Schett G, et al. The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis. Nat Med. 2005;11:394–9.
Wohrle FU, Daly RJ, Brummer T. Function, regulation and pathological roles of the Gab/DOS docking proteins. Cell Commun Signal. 2009;7:22.
Wolf I, Jenkins BJ, Liu Y, Seiffert M, Custodio JM, Young P, et al. Gab3, a new DOS/Gab family member, facilitates macrophage differentiation. Mol Cell Biol. 2002;22:231–44.
Yamasaki S, Nishida K, Hibi M, Sakuma M, Shiina R, Takeuchi A, et al. Docking protein Gab2 is phosphorylated by ZAP-70 and negatively regulates T cell receptor signaling by recruitment of inhibitory molecules. J Biol Chem. 2001;276:45175–83.
Yu M, Lowell CA, Neel BG, Gu H. Scaffolding adapter Grb2-associated binder 2 requires Syk to transmit signals from FcepsilonRI. J Immunol. 2006a;176:2421–9.
Yu M, Luo J, Yang W, Wang Y, Mizuki M, Kanakura Y, et al. The scaffolding adapter Gab2, via Shp-2, regulates kit-evoked mast cell proliferation by activating the Rac/JNK pathway. J Biol Chem. 2006b;281:28615–26.
Zatkova A, Schoch C, Speleman F, Poppe B, Mannhalter C, Fonatsch C, et al. GAB2 is a novel target of 11q amplification in AML/MDS. Genes Chromosomes Cancer. 2006;45:798–807.
Zhang Y, Diaz-Flores E, Li G, Wang Z, Kang Z, Haviernikova E, et al. Abnormal hematopoiesis in Gab2 mutant mice. Blood. 2007;110:116–24.
Zhao C, Yu DH, Shen R, Feng GS. Gab2, a new pleckstrin homology domain-containing adapter protein, acts to uncouple signaling from ERK kinase to Elk-1. J Biol Chem. 1999;274:19649–54.
Zhu QS, Robinson LJ, Roginskaya V, Corey SJ. G-CSF-induced tyrosine phosphorylation of Gab2 is Lyn kinase dependent and associated with enhanced Akt and differentiative, not proliferative, responses. Blood. 2004;103:3305–12.

GABA$_A$ Receptor

Mikko Uusi-Oukari
Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku, Turku, Finland

Synonyms

γ-Aminobutyric acid type A receptor; γ-Aminobutyrate$_A$ receptor

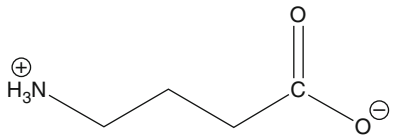
Historical Background

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain, where it was first discovered in 1950. It is a small zwitterionic γ-amino acid with molecular weight of 103 g/mol (Fig. 1). Such a hydrophilic molecule cannot cross the blood brain barrier. It is produced in the brain by decarboxylation of L-glutamate by the enzyme glutamic acid decarboxylase (GAD). GABA was recognized as an inhibitory transmitter in 1967. It interacts with two types of receptors: ionotropic GABA$_A$ receptors (GABA$_A$Rs) that are fast-acting ligand-gated chloride channels, and metabotropic GABA$_B$ receptors that are coupled indirectly via G Proteins to either Ca^{2+} or K^+ channels to produce slow and prolonged inhibitory responses. The first GABA$_A$R subunits were cloned in 1987. Since then, molecular biological, electrophysiological, and pharmacological studies have revealed the highly heterogeneous nature of GABA$_A$Rs (Olsen and Sieghart 2008; Froestl 2011).

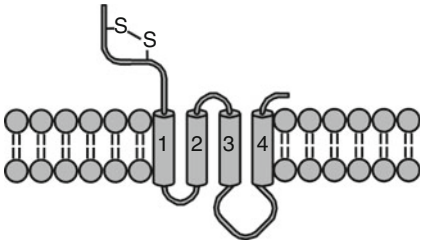
Structure of GABA$_A$ Receptors

GABA$_A$Rs belong to Cys-loop superfamily of ligand-gated ion channels. In addition, the Cys-loop receptor superfamily comprises the Nicotinic Acetylcholine Receptors, the Glycine Receptors, the 5-Hydroxytryptamine$_3$ Receptor, and Zinc-Activated Cation Channel. The subunits of Cys-loop receptors share a common primary structure consisting of large extracellular domain with a "signature" disulfide, four transmembrane segments (TM), and a large variable cytoplasmic domain (cytoplasmic loop) between TM3 and TM4 (Fig. 2). The secondary and three-dimensional structures of the subunits and the quaternary pentameric assembly of the subunits are also well conserved within the superfamily.

Mammalian GABA$_A$Rs are assembled from 19 subunits that belong in 8 subunit classes according to sequence similarity: α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ, and ρ1–ρ3 (Olsen and Sieghart 2008). Each subunit is encoded by a homologous but separate gene. The subunits produce heteropentameric receptor complexes (Fig. 3). Most GABA$_A$Rs consist of α, β, and γ subunits with a subunit stoichiometry of 2α:2β:1γ (Olsen and Sieghart 2008). The γ2 subunit is the γ isoform

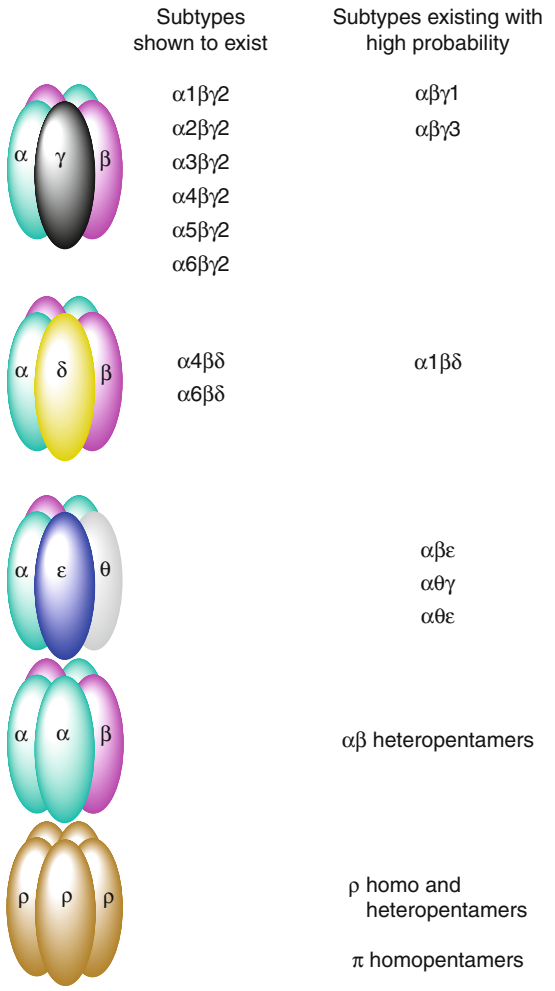


GABA$_A$ Receptor, Fig. 1 γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian brain



GABA$_A$ Receptor, Fig. 2 Secondary structure of a GABA$_A$ receptor subunit. The subunit consists of a large extracellular domain containing the signature disulfide loop, four transmembrane domains, and a large intracellular domain between TM3 and TM4

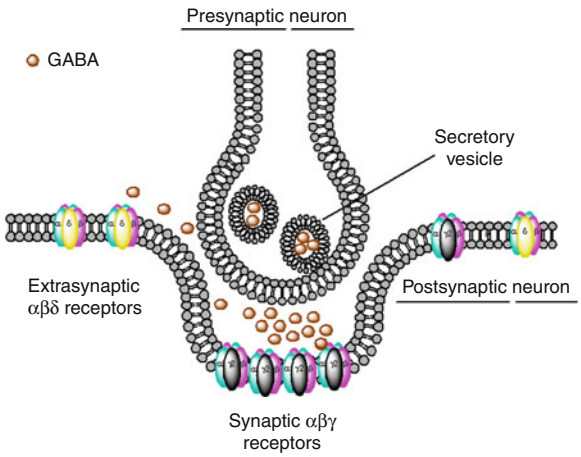
present in more than 90% of receptors. Thus, 75–80% of GABA$_A$Rs contain γ2 (Whiting 2003). γ2 subunit in the receptor complex confers sensitivity to benzodiazepines. The αβγ receptor subtypes clearly identified in the brain thus far consist of each α subunit isoform in combination with β and γ2 subunits (Fig. 3). The α1 is the most abundant α subunit; it is present in most brain regions and its expression colocalizes with those of β2 and γ2. Thus, the α1β2γ2 receptor subtype comprises 40–50% of brain GABA$_A$Rs (Olsen and Sieghart 2008). Subunits α4 and α6 combine with β and δ subunits to form α4βδ and α6β2δ receptor subtypes that are localized extrasynaptically (Fig. 4). Receptor subtypes existing with high probability include αβγ receptors containing either the γ1 or γ3 subunit; receptors containing only α and β subunits (αβ); and αβγ or αβδ receptors containing two different α or β variants (Olsen and Sieghart 2008). Rho subunits form homomeric and heteromeric ρ pentamers. Epsilon and θ are believed to combine with other classes of GABA$_A$R subunits to form receptors, but the native receptor combinations are currently not known. The π subunit is expressed outside CNS and forms homo-oligomeric complexes (Fig. 3) (Olsen and Sieghart 2008; Uusi-Oukari and Korpi 2010).



GABA$_A$ Receptor, Fig. 3 GABA$_A$ receptor subunits combine to form various GABA$_A$ receptor subtypes. A part of the combinations has been shown to exist in native brain, while the presence of other combinations have been deduced from similarities between the subunits in mRNA expression patterns and in immunochemical experiments showing similar localization of the subunits

Regulation of GABA$_A$ Receptor Expression

The large number of GABA$_A$R genes and the various types of neurons and glial cells in the brain with different patterns of subunit expression suggest a complex system regulating their transcription (Laurie et al. 1992a; Wisden et al. 1992; Olsen and Sieghart 2008). Major changes occur during development in the subunit expression patterns (Laurie et al. 1992b). Changes in receptor subunit expression also take place in adult brain. The changes are often suggested



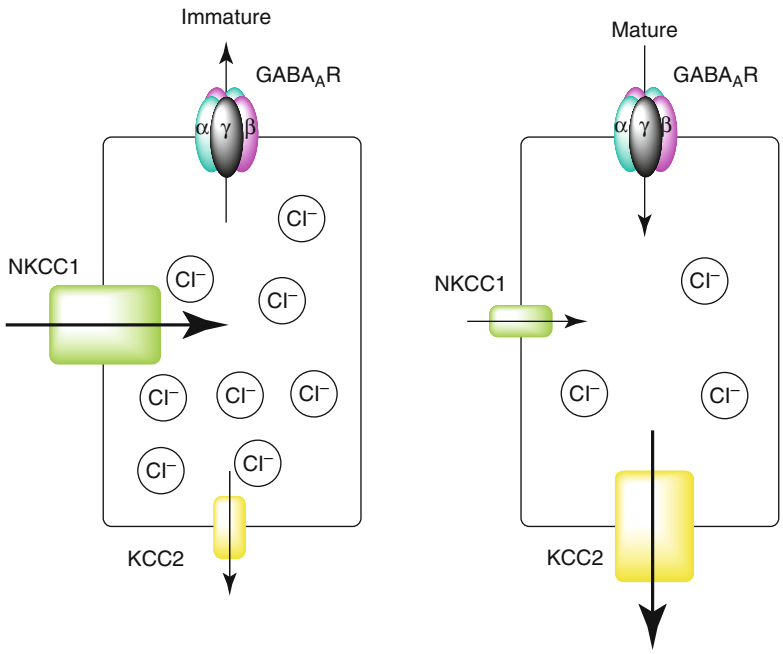
GABA$_A$ Receptor, Fig. 4 Synaptic GABA$_A$ receptors form synaptic clusters in the postsynaptic membrane. Extrasynaptic receptors are localized in extrasynaptic parts of the cell membrane. Synaptic receptors are activated by high concentration of presynaptically released GABA, while extrasynaptic receptors are activated by ambient GABA present in the interstitial space

to reflect changes in neuronal activity. Activity-dependent signaling pathways modulate the function of both transcriptional activators and repressors, but the transcription factors responsible for the developmental and brain region/cell-specific expression of GABA$_A$R subunits are presently unknown. Calcium is a crucial second messenger in the transduction of synaptic activity into gene expression, and it is involved in the mechanisms of GABA$_A$R up- and downregulation (Gault and Siegel 1998; Lyons et al. 2001). Some mechanisms regulating α1 mRNA transcription have been revealed recently. The transcription factor cAMP Response Element Binding Protein (CREB) is induced in response to stimulation with neurotransmitters, neuromodulators, and neurotrophic factors. Activation of Protein Kinase C (PKC) in primary rat neocortical cultures increases transcription of α1 mRNA via phosphorylation of CREB that is bound to the *GABRA1* promoter (Hu et al. 2008). In contrast, activation of Protein Kinase A (PKA) represses α1 mRNA transcription via Inducible cAMP Early Repressor (ICER) that forms inactive heterodimers with CREB (Hu et al. 2008). Brain-Derived Neurotrophic Factor (BDNF) decreases α1 transcription via activation of the Janus Kinase/► Signal Transducer and Activator of Transcription (STAT) pathway (Lund et al., 2008). BDNF-dependent phosphorylation of STAT3 induces the synthesis of ICER that binds with phosphorylated CREB at the *GABRA1* promoter CRE site, thereby repressing transcription (Lund et al. 2008). BDNF has been shown to regulate transcription and cell surface expression of many GABA$_A$R subunits, the effects being brain region- and subunit-specific (Uusi-Oukari and Korpi 2010).

Cell surface expression of GABA$_A$Rs includes various interacting proteins affecting receptor cell surface expression and postsynaptic accumulation. Heterodimers of α and β subunits are initially formed in a process involving interaction with endoplasmic reticulum (ER) – associated chaperones calnexin and binding immunoglobulin protein (BiP). Heteropentameric GABA$_A$Rs assembled in the ER are stabilized with ubiquitin-like protein Plic-1 that interacts with α and β subunits and facilitates the exit of assembled receptors from ER to the Golgi. GABA$_A$R γ2 subunit is palmitoylated at cytoplasmic cysteine residues by the Golgi resident palmitoyltransferase GODZ, thus promoting translocation of receptors through the Golgi apparatus to the plasma membranes and to synapses. BIG2, a GTP exchange factor (GEF) is implicated in facilitating exit of GABA$_A$Rs by interacting with β subunits. Translocation of GABA$_A$Rs to the cell surface is further facilitated by several proteins including GABARAP (interacts with GABA$_A$R γ subunits), *N*-ethylmaleimide-sensitive factor (NSF), and glutamate receptor interacting protein (GRIP). Postsynaptic clustering of GABA$_A$R subtypes α2βγ2 and α3βγ2 is facilitated by interaction of α2 and α3 subunits with gephyrin, a multifunctional protein that serves as a subsynaptic scaffold organizing the spatial distribution of receptors and other proteins in inhibitory postsynaptic membranes. The interaction of α1-3βγ2 GABA$_A$Rs with the postsynaptic cytoskeleton is regulated by the activity-dependent and calcineurin-regulated phosphorylation state of γ2 subunit. Transmembrane domain 4 and intracellular domain of the γ2 subunit have been shown to be necessary for recruiting gephyrin to the synapse. The role of gephyrin is to stabilize clustered GABA$_A$Rs at the cell surface (Luscher et al. 2011; Vithlani et al. 2011).

GABA$_A$ergic Signaling Is Developmentally Shifted from Depolarizing (Excitatory) to Hyperpolarizing (Inhibitory)

In prenatal and early postnatal stage of the development, the intracellular Cl^- concentration ($[Cl^-]_i$) of immature



GABA$_A$ Receptor, Fig. 5 In immature neurons the $[Cl^-]_i$ is higher than that in extracellular space. The chloride efflux via GABA$_A$ receptors along chloride concentration gradient results in depolarization of the cell membrane. In mature neurons the $[Cl^-]_i$ is lower than $[Cl^-]$ in extracellular space. Thus, chloride influx via GABA$_A$ receptors along chloride concentration gradient results in hyperpolarization of the cell membrane. High intracellular $[Cl^-]_i$ in immature neurons is due to high and low expression levels of NKCC1 and KCC2 transporters, respectively. In mature neurons, the expression levels of NKCC1 and KCC2 are opposite to that in immature cells resulting in low $[Cl^-]_i$

neurons is higher than that of the extracellular space. This is due to high expression of the cation-chloride cotransporter NKCC1 that accumulates Cl^- inside the cells by an energy-dependent transport process (Fig. 5). Thus, GABA$_A$R activation at an early stage of development results in Cl^- efflux via the receptor-associated anion channel and in subsequent depolarization of the cell membrane. Excitatory GABA$_A$R activity regulates neuronal proliferation, migration, differentiation, and neuronal network formation and remodeling. The depolarizing activity of GABA activates Voltage-Dependent Ca^{2+} Channels, relieves Mg^{2+} blockade of *N*-Methyl-D-Aspartate Receptors (NMDAR), and can lead to generation of action potentials (Ben-Ari et al. 2007).

During brain development, GABA signaling is established before glutamatergic transmission, suggesting that GABA is the principal excitatory transmitter during early development. GABA$_A$Rs are expressed well before synapses are formed. GABA is released at an early developmental stage and acts as a trophic factor to modulate several essential developmental processes including neuronal proliferation, migration, differentiation, synapse formation, neuronal growth, and network construction. This early intercellular communication is based on diffusion and distal paracrinic actions that contrasts with the local fast communication provided by synaptic currents. GABA tonically reduces the speed of cell migration via GABA$_A$R activation. Astrocytes may generate a microenvironment that controls the degree of GABA$_A$R activation and the migration of neuronal precursors (Ben-Ari et al. 2007).

Proliferation of neocortical progenitors in ventricular and subventricular zones of the developing cortex is downregulated by GABA$_A$R activation that leads to depolarization of plasma membrane and increase in intracellular Ca^{2+}. The interneuronal precursors synthesize and release GABA, and express GABA$_A$Rs to respond to the secreted GABA. Cortical entry of tangentially migrating interneuronal precursors arriving from the medial ganglionic eminence is enhanced

by GABA and GABA$_A$Rs. This enhanced motility of interneurons is dependent on GABA$_A$R-mediated depolarization and downstream activation of L-type calcium channels. However, soon after interneurons enter the cortex, their spontaneous calcium oscillations and their migration terminate. This is due to an increase in the expression of KCC2 transporter which reduces the $[Cl^-]_i$ and terminates depolarizing activity of GABA$_A$Rs (Jovanovic and Thomson 2011).

Developmental changes in GABA signaling are determined by the progressive negative shift in E_{GABA} that in turn reflects the developmental reduction of intracellular $[Cl^-]_i$. KCC2 is the principal transporter for Cl^- extrusion from neurons. KCC2 extrudes K^+ and Cl^- using the electrochemical gradient for K^+. Cl^- extrusion is weak in immature neurons and increases with neuronal maturation. The KCC2 is strongly expressed in mature neurons (while the expression of NKCC1 is strongly downregulated), thus underlying the developmental changes in Cl^- extrusion. K^+-Cl^- cotransport also contributes to the low $[Cl^-]_i$ in mature neurons. Developmental expression of KCC2 is pivotal for development of hyperpolarizing GABA$_A$R-mediated inhibition (Ben-Ari et al. 2007).

Synaptic (Phasic) and Extrasynaptic (Tonic) GABA$_A$ Receptor-Mediated Inhibition

The receptor subtypes α1-3βγ2 form clusters in synapses. They are activated with a very high local concentration of presynaptically released GABA. A large number of clustered synaptic receptors are activated very quickly and their desensitization is also fast. In contrast to α1-3βγ2 combinations that are clustered in synapses, α5βγ2 receptor subtype is predominantly clustered extrasynaptically by interaction with phospho-activated radixin, which links these receptors to submembrane microfilaments (Luscher et al. 2011). Part of αβγ2 receptors are freely moving in extrasynaptic portion of plasma membrane. In addition to α4βδ and α6βδ receptors that are localized exclusively extrasynaptically, part of each αβγ2 receptor subtypes contribute, in addition to αβδ receptors, to the production of the continuous tonic inhibition (Fig. 4). α4/6βδ receptors are particularly suited for tonic activity, because they possess high affinity for GABA and are thus activated by the ambient GABA leaking out from synapses or released extrasynaptically. In addition, α4/6βδ receptors desensitize very slowly, therefore being tonically active (Luscher et al. 2011; Belelli et al. 2009).

Summary

In addition to production of receptor subtype-selective drugs with minimal adverse effects, one of the major challenges in GABA$_A$R research is to resolve the signaling molecules and pathways responsible for developmental and brain region/cell-specific regulation of GABA$_A$R subunit and receptor subtype expression. The work is already in progress and new challenges are arising from the progression.

References

Belelli D, Harrison NL, Maguire J, Macdonald RL, Walker MC, Cope DW. Extrasynaptic GABAA receptors: form, pharmacology, and function. J Neurosci. 2009;29:12757–63.

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev. 2007;87:1215–84.

Froestl W. An historical perspective on GABAergic drugs. Future Med Chem. 2011;3:163–75.

Gault LM, Siegel RE. NMDA receptor stimulation selectively initiates GABA(A) receptor delta subunit mRNA expression in cultured rat cerebellar granule neurons. J Neurochem. 1998;70:1907–15.

Hu Y, Lund IV, Gravielle MC, Farb DH, Brooks-Kayal AR, Russek SJ. Surface expression of GABAA receptors is transcriptionally controlled by the interplay of cAMP-response element-binding protein and its binding partner inducible cAMP early repressor. J Biol Chem. 2008;283:9328–40.

Jovanovic JN, Thomson AM. Development of cortical GABAergic innervation. Front Cell Neurosci. 2011;5:14.

Laurie DJ, Seeburg PH, Wisden W. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. J Neurosci. 1992a;12:1063–76.

Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci. 1992b;12:4151–72.

Lund IV, Hu Y, Raol YH, Benham RS, Faris R, Russek SJ, Brooks-Kayal AR. BDNF selectively regulates GABAA receptor transcription by activation of the JAK/STAT pathway. Sci Signal. 2008;1:ra9.

Luscher B, Fuchs T, Kilpatrick CL. GABAA receptor trafficking-mediated plasticity of inhibitory synapses. Neuron. 2011;70:385–409.

Lyons HR, Land MB, Gibbs TT, Farb DH. Distinct signal transduction pathways for GABA-induced GABA(A) receptor down-regulation and uncoupling in neuronal culture: a role

for voltage-gated calcium channels. J Neurochem. 2001;78:1114–26.
Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev. 2008; 60:243–60.
Uusi-Oukari M, Korpi ER. Regulation of GABA(A) receptor subunit expression by pharmacological agents. Pharmacol Rev. 2010;62:97–135.
Vithlani M, Terunuma M, Moss SJ. The dynamic modulation of GABAA receptor trafficking and its role in regulating the plasticity of inhibitory synapses. Physiol Rev. 2011; 91:1009–22.
Whiting PJ. GABA-A receptor subtypes in the brain: a paradigm for CNS drug discovery? Drug Discov Today. 2003; 8:445–50.
Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci. 1992;12:1040–62.

Gadd45

Joshua D. Brown-Clay[1] and Albert J. Fornace Jr. [1,2]
[1]Department of Biochemistry and Molecular and Cellular Biology, Georgetown University, Washington, DC, USA
[2]Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

Gadd45 Family Members

Gadd45a: Gadd45α; DDIT1 (DNA-damage inducible transcript 1)
Gadd45b: Gadd45β; Myd1118
Gadd45g: Gad45γ; Cytokine responsive 6 (CR6)

Historical Background

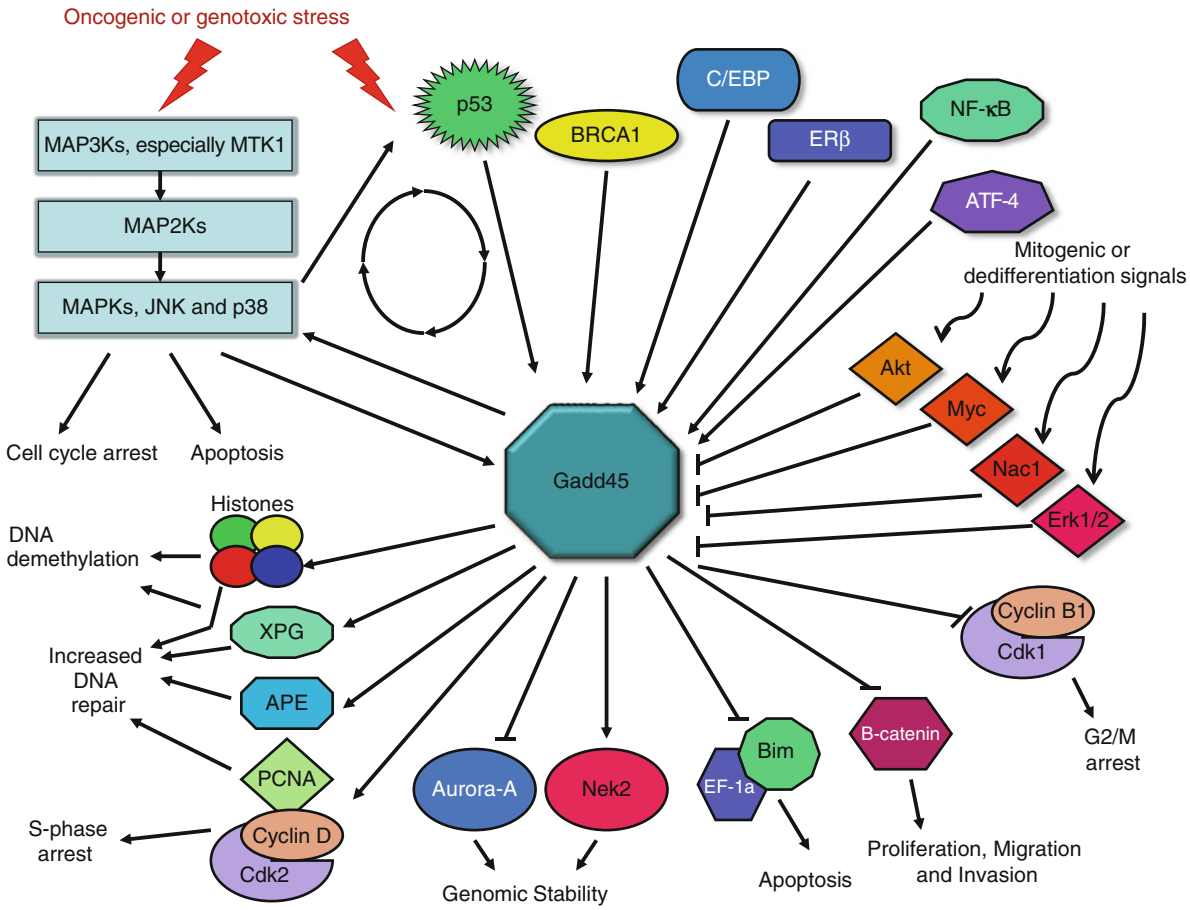
The *Gadd* genes were first cloned from Chinese hamster ovarian cells in 1988 as a subset of transcripts that were consistently upregulated after exposure to ultraviolet (UV) radiation and in many cases to other DNA-damaging agents, including methyl methane sulfonate (MMS), hydrogen peroxide, and *N*-acetoxy-2-acetylaminofluorene (Fornace et al. 1988). They were also found to be induced by other growth cessation signals, such as medium depletion or hydroxyurea. This class of transcripts was termed *Gadd*, for *g*rowth *a*rrest and *D*NA *d*amage inducible. The Gadd45 protein, now referred to as Gadd45a, was peculiar among the radiation-response genes in that it could be induced in an ATM-dependent and protein kinase C-independent manner after exposure of human cells to ionizing radiation (IR) and this induction was subsequently found to be ▶p53-regulated; indeed, Gadd45 was the first stress gene discovered that was transcriptionally regulated by p53 (Hollander and Fornace 2002).

There are three Gadd45 proteins, encoded by three different genes: Gadd45a, Gadd45b, and Gadd45g. Gadd45b was originally cloned as a gene expressed after terminal differentiation and growth arrest of M1D + myeloid precursor cells induced by IL-6. Gadd45g was similarly originally cloned as an early IL-2 response gene in T cells. These three proteins are highly conserved among metazoa, although insects have only a single *GADD45* gene, which is more similar to Gadd45g, indicating this may be the ancestral gene. They are all small (18 kDa), highly negatively charged (in the top two percentile of proteins in the ratio of negative charge to amino acids) and localize to the nucleus (Cretu et al. 2009). Gadd45a is the most well-characterized isoform and will be the focus of this entry; the other two members will be covered briefly.

Regulation of Gadd45

A myriad of factors regulate *Gadd45a* expression in the cell at the transcriptional, posttranscriptional, and posttranslational levels, frequently in response to genotoxic stress; these are illustrated in Fig. 1 and also in Table 1. *Gadd45* is one of the very few genes that has been shown to be consistently upregulated after IR, across numerous conventional and gene expression profiling studies in p53 wild-type cells (Snyder and Morgan 2004). Although ubiquitously expressed, basal *Gadd45* expression is very low, but its expression varies through the cell cycle, with levels highest during G_1 phase and lowest during S-phase (Kearsey et al. 1995).

A well-characterized mechanism of induction of Gadd45a expression is binding of p53 to a conserved site within the third intron of the *Gadd45a* gene, stimulating its transcription. This binding is induced by



Gadd45, Fig. 1 Schematic representation of representative upstream regulators of Gadd45 and its downstream inducers. *Arrows* indicate positive regulation while *blocked lines* indicate negative regulation. This figure is by no means a complete list of all of Gadd45's regulators and inducers

genotoxic stress but has been found to be necessary only in the case of IR exposure and not in the Gadd45a response to UV radiation or MMS, although loss of p53 does attenuate subsequent Gadd45a induction. WT1, a transcription factor that is mutated in various tumors and congenital defects, can also induce *Gadd45* transcription in a p53-dependent manner but in the absence of direct p53-DNA binding in response to nonionizing radiation (Gao et al. 2009).

A number of other tumor suppressor genes induce Gadd45a expression. BRCA1 induces Gadd45a expression indirectly by interacting with the transcription factors Oct-1 and NF-YA. The CCAAT/enhancer-binding protein-α (C/EBPα) activates Gadd45 expression, and other members of the C/EBP family can induce Gadd45g expression as well (Gao et al. 2009).

Mitogen-activated protein kinase (MAPK) signaling, via c-Jun N-terminal Kinases (JNK) and p38 kinases, induces expression of Gadd45a. Specifically, they activate c-Jun, which, similar to p53, binds to the third intron of *Gadd45a* and activates its transcription. However, it is of interest that transient ERK signaling induces Gadd45a expression while sustained expression has been shown to repress it (Gao et al. 2009); this Gadd45a induction may be due to transient activation of the other MAPK pathways through cross talk. ▶ Estrogen receptor β (ERβ) can bind to the promoter of Gadd45a in a ligand-independent manner and recruits c-Jun and NCOA2 to stimulate transcription and subsequent G_2/M arrest (Paruthiyil et al. 2011).

The mammalian forkhead family transcription factor FoxO3A also directly binds to the *Gadd45a* promoter and induces its transcription. The activating

Gadd45, Table 1 *Upstream regulators*. While by no means comprehensive, this table lists the regulators of Gadd45 and whether they positively (P) or negatively (N) affect a particular Gadd45 protein's expression. *Green* letters indicate a direct transcriptional activation or repression while *orange* characters indicate an indirect, upstream protein-protein interaction. *Red* indicates posttranscriptional regulation

	Gadd45a	Gadd45b	Gaddg
Upstream Inducers			
BRCA1/2	P	–	–
p53	P	P	–
NF-kB	P/N	P	P
C/EBP	P	–	P
c-Myc	N	–	–
AKT	N	–	–
WT1	P	–	–
Egr-1	P	P	–
Oct-1	P	–	–
NF-YA	P	–	–
p38	P	–	–
JNK	P	–	–
Erk1/2	N	–	–
Nac-1	–	–	N
AUF1	N	–	–
TIAR1	N	–	–
Estrogen Receptor β	P	–	–
Foxo3a	P	–	–
Pregnane X receptor	–	P	–

transcription factor-4 (ATF-4) plays a central role in cellular stress responses and induces *Gadd45a* transcription in response to arsenite exposure, leucine deprivation, inhibition of the proteasome, and endoplasmic reticulum stress; however, only after arsenite exposure or proteasome inhibition did Gadd45a protein levels rise, showing that there is a sophisticated regulation of Gadd45a that responds differentially to various cellular stressors (Gao et al. 2009).

Transcriptional repression also takes place. The proto-oncogenes c-▶ Myc and Akt repress *Gadd45a* expression (Gao et al. 2009), highlighting the role of Gadd45a in arresting cell growth. Akt inhibition of Gadd45a is most likely through Akt-mediated FoxO3a inactivation.

Gadd45a is also regulated at the posttranscriptional level. In unstressed cells, AUF1 destabilized *Gadd45a* mRNA and TIAR1 hindered its translation, potently inhibiting expression of the Gadd45a protein. After exposure of cells to MMS or UV radiation, these proteins quickly dissociated from *Gadd45a* mRNA through an unknown mechanism and allowed robust expression of the protein. Conversely, the mRNA stabilizing protein, nucleolin, bound *Gadd45a* mRNA after cellular stimulation with arsenic chloride or inhibition of NF-κB and potently increased both mRNA and protein levels (Lal and Gorospe 2006). It was also recently discovered that MAP2Ks upstream of p38 can phosphorylate three proteins involved in RNA regulation, hnRNPA0, TIAR, and PARN, stabilizing *Gadd45a* mRNA (Reinhardt et al. 2010).

▶ NF-κB activation of Egr-1 leads to direct Egr-1-mediated transcriptional activation of Gadd45a. The NF-κB-activating kinases, IKKα and IKKβ, are also able to induce Gadd45 expression through a NF-κB-independent mechanism. The p65 (RelA) subunit of NF-κB binds directly to three κB elements in the promoter of Gadd45b and activates its transcription. However, NF-κB also inhibits Gadd45a expression through activation of c-Myc and downregulation of nucleolin. Therefore, NF-κB's differential regulation of Gadd45a may contribute to the observed pro- and anti-oncogenic actions of NF-κB, although the mechanisms governing this switch are not well understood (Yang et al. 2009).

The final level of Gadd45a regulation is at the posttranslational level. Arsenite stimulation of cells induces the formation of an IκB-kinase-β (IKKβ)/NF-κB p50 subunit complex that decreases ubiquitinated Gadd45a levels and its subsequent proteasomal degradation (Yang et al. 2009).

Gadd45b- and Gadd45g-specific mechanisms of transcriptional regulation also exist. The p65 (RelA) subunit of NF-κB binds directly to three κB elements in the promoter of Gadd45b and activates its transcription (Yang et al. 2009). Nucleus accumbens-1 (Nac1) is a transcription factor that is associated with embryonic stem cell self-renewal and pluripotency; it is also found to be upregulated in several cancer types, and particularly chemoresistant, recurring ovarian carcinomas. Nac1-mediated downregulation of Gadd45g was found to contribute to paclitaxel resistance in ovarian cancer cells (Jinawath et al. 2009).

The Effects and Consequences of Gadd45a Expression

As can be expected from a protein that is predominantly induced after genotoxic and many other

Gadd45, Table 2 *Downstream effectors*. While by no means comprehensive, this table lists the downstream effectors of Gadd45

Downstream effectors			
PCNA	DNA repair and demethylation; S-phase arrest	DNA repair and demethylation	S-phase arrest
Cdk1	G2/M arrest	G2/M arrest	G2/M arrest
APE	Stimulates DNA base excision repair	–	–
Aurora-A protein kinase	Maintenance of genomic stability	–	–
Nek2	Maintenance of genomic stability	–	–
p38	Cell cycle arrest, apoptosis, negative regulation of T-cell activation, full activation of innate immune cells, induction of senescence	Cell cycle arrest, apoptosis (or survival?), negative regulation of T-cell activation, full activation of innate and adaptive immune cells	Cell cycle arrest, apoptosis, negative regulation of T-cell activation, full activation of innate and adaptive immune cells
JNK	Cell cycle arrest and apoptosis	Cell cycle arrest and apoptosis	Cell cycle arrest and apoptosis
EF-1a	Release of Bim, apoptosis	–	–
Apc	Destruction of b-catenin	–	–
β-catenin	Inhibition of its pro-invasion program, increased b-catenin plasma membrane localization and cell-cell adhesion	–	–
p21	Negative regulation of p21 in keratinocytes, allowing NER	Positive regulation in senescing chondrocytes	Interacts but outcome unknown
XPG	Stimulates DNA nucleotide excision repair; perhaps mediates DNA demethylation		

stresses, the most well-characterized functions of Gadd45a are to induce growth arrest and stimulate DNA repair. Although few direct biochemical mechanisms have been shown for Gadd45a, it has been repeatedly found to form complexes with other proteins and even chromatin. Thus, it seems likely that the ability to both facilitate protein-protein interactions and sequester proteins may be important; these interactions and their subsequent effects are also illustrated in Fig. 1 as well as in Table 2 for select proteins.

Gadd45a plays a role in both S-phase and G_2/M arrest (Smith et al. 1994) (Hollander and Fornace 2002); it can displace proliferating cell nuclear antigen (PCNA) from the cyclin D1 complex, possibly inhibiting DNA replication during S-phase (Smith et al. 1994). Likewise, Gadd45a can also bind cyclin-dependent kinase 1 (Cdk1), most likely preventing its association with cyclin B1, inhibiting Cdk1 activity and arresting the cell at the G_2/M checkpoint (Hollander and Fornace 2002).

Gadd45a has the ability to stimulate DNA repair, seemingly through its ability to interact with PCNA and DNA repair complexes. In vitro and cell culture assays have shown that recombinant Gadd45a can stimulate nucleotide excision repair (NER) (Smith et al. 1994; Tran et al. 2002), while loss of Gadd45a expression in ex vivo assays of lymphoblasts resulted in a substantially reduced NER ability (Gao et al. 2009). More recently, Gadd45a deficiency was linked with reduced base excision repair (BER), cytoplasmic localization of apurinic endonuclease (APE), a key enzyme in the BER pathway, and decreased APE interaction with PCNA, as well as delayed removal of apurinic sites. Gadd45a's capability to interact with acetylated or UV radiation-exposed (which leads to histone acetylation) mononucleosomes and increase DNA accessibility locally may facilitate its stimulation of DNA repair (Ma et al. 2009).

Gadd45a works by multiple mechanisms to maintain genomic stability throughout mitosis. Mouse embryonic fibroblasts (MEFs) and mice with a Gadd45a$^{-/-}$ genotype were much more likely to exhibit centrosome amplification and incomplete chromosomal condensation during mitosis. This would result in defective chromosomal segregation, likely leading to the chromosomal and chromatid aberrations often seen in this genotype (Hollander and Fornace 2002), which is quite similar to the p53$^{-/-}$ phenotype. Gadd45a physically associates with Aurora-A protein kinase, the deregulated expression of which similarly

produces centrosome abnormality, and strongly inhibits its activity. Conversely, Gadd45a and Brca1 are both required for the full, physiological transcriptional upregulation of Nek2, the proper concentration of which has been found to be essential for timely centrosome separation (Gao et al. 2009).

Gadd45a has been repeatedly associated with apoptotic induction after genotoxic stress. Its level rises notably in apoptotic mammalian cells and inhibition of Gadd45a expression reduces apoptosis in response to DNA damage. The p38 and c-Jun N-terminal kinases (JNKs) mediate most of Gadd45a's proapoptotic effects. These two MAPKs act to induce cell cycle arrest and the apoptotic response. All three Gadd45 proteins were found to be able to bind the N-terminus of MTK1, a mitogen-activated protein kinase kinase kinase (MAP3K) that exclusively activates p38 and JNK signaling, inducing a conformational change that resulted in its autophosphorylation, activation, and a strong apoptotic response. Gadd45a activation of p38 and JNK signaling, which are upstream activators of Gadd45a (as well as of p53, which also induces Gadd45a expression), forms the basis for a positive feedback loop to raise the levels of these tumor suppressive signaling molecules in the event of genotoxic stress and unresolved DNA damage; this positive feedback loop is illustrated in Fig. 1. Furthermore, Gadd45a expression is necessary for sustained p38 and JNK signaling and consequent growth arrest or apoptosis in keratinocytes after UV radiation exposure (Gao et al. 2009).

Gadd45a also effects its pro-apoptosis program through interaction with the cytoskeleton. Elongation factor 1α (EF-1α) is a microtubule-severing protein that binds, bundles, and promotes microtubule assembly, playing a key role in cytoskeletal stability. Induction of Gadd45a expression leads to interaction of Gadd45a with EF-1α and inhibits its bundling of microtubules, destabilizing the cytoskeleton. This causes release of Bim, a ▶ Bcl-2 family pro-apoptotic protein, from microtubule-associated complexes and allows its translocation to the mitochondria, releasing cytochrome c into the cytoplasm and initiating apoptosis (Gao et al. 2009).

Gadd45a inhibits tumor cell invasion and migration induced by high levels of ▶ β-catenin. After UV radiation induction, Gadd45a stimulates p38 promotion of dephosphorylation of glycogen synthase kinase 3β (GSK3β), activating the adenomatous polyposis coli (APC) destruction complex, which increases β-catenin phosphorylation and degradation. Similarly, Gadd45a increases p38 positive regulation of both nuclear translocation of APC, an important step in degradation of β-catenin, and plasma membrane localization of β-catenin, preventing activation of its pro-invasion transcriptional program, and increasing its interaction with Caveolin-1, strengthening cell-cell adhesion (Gao et al. 2009).

Gadd45a interacts with tumor suppressor cyclin-dependent kinase inhibitor 1a (encoded by *CDKN1A*), also known as $p21^{Cip1/Waf1}$. The exact nature of this interaction and the outcome remain unclear, however. The two proteins compete for interaction with PCNA and Gadd45a seems to negatively regulate CDKN1A expression in keratinocytes, allowing NER repair after UV radiation exposure (Gao et al. 2009).

Deficiency of Gadd45a was found to sensitize cells to cisplatin and UV radiation, implying subtleties to the proapoptotic effects of this protein or more likely reduced DNA repair in the absence of Gadd45a. In hematopoietic cells exposed to UV radiation, Gadd45a was involved in a NF-κB-p38 survival pathway (Cretu et al. 2009). Additionally, Gadd45a protects neurons from apoptotic cell death after withdrawal of nerve growth factor in spinal cord ligation (Lin et al. 2011). The first two examples may be explained as Gadd45a enhancing survival by mitigating the effects of genotoxic stress, e.g., arresting cell replication and stimulating DNA repair. However, the last example is clearer evidence for a pro-survival function of Gadd45a and for dramatic tissue specificity in Gadd45a action.

Oncogene-induced senescence (Bulavin et al. 2003) and establishment of the senescent phenotype in response to DNA damage requires Gadd45a expression (Passos et al. 2010). In both cases, Gadd45 signaling through p38 was essential for induction of this phenotype and also for full transactivation of p53, the activity of which was shown elsewhere to be essential for entry of cells into a senescent state. In senescent human fibroblast cells, p53 preferentially occupied the promoters of Gadd45 and p21 and this was associated with a unique combination of phosphorylated p53 sites (Gao et al. 2009). Therefore, the positive feedback loop between Gadd45, p38, and p53 is essential for induction and maintenance of the senescent phenotype after oncogenic overexpression or high DNA damage

in fibroblasts and keratinocytes, and most likely in other cell types as well.

Immunologically, Gadd45a is a negative regulator of T-cell activation and proliferation. Gadd45a-deficient mice, particularly females, develop a lupus-like syndrome with high titers of anti-DNA and –histone antibodies (Lu 2006). Surprisingly, T cells from Gadd45$^{-/-}$ mice showed constitutive p38 activation, which functions via an alternative pathway to stimulate T-cell activation, and recombinant Gadd45a inhibited p38 in isolated Gadd45$^{-/-}$ T cells (Salvador et al. 2005). Despite this heightened T-cell activity, Th1 differentiation in Gadd45$^{-/-}$ mice is impaired due to reduced expression of IL-12 and CD40 co-stimulatory molecule by dendritic cells (Jirmanova et al. 2007).

Finally, there is accumulating evidence that a NER or BER-like process is involved in removal of DNA methylation, an epigenetic marker associated with repression of transcriptional initiation. Acetylation is a requisite step for DNA demethylation, as is general RNA transcription (indicating that transcription of additional factors not normally present at high levels, such as Gadd45, is necessary). Gadd45a is able to interact directly with the four core histones and increase DNase accessibility to DNA with hyperacetylated mononucleosomes in vitro, perhaps allowing access of demethylation/DNA repair complexes to DNA in the cell. In vivo studies showed significant specificity of Gadd45b-mediated DNA demethylation and Gadd45a- and Gadd45b-null mice displayed conserved global genomic methylation patterns, indicating that Gadd45 is more likely to be involved in demethylation and transcriptional activation of specific genes. A role for Gadd45 in DNA demethylation was not observed in two studies, but a number of recent reports do emphasize the highly cell-type and context-specific nature of this mechanism; this fact, together with differing experimental conditions, may explain the observed discrepancies (Ma et al. 2009). Recently, TATA-binding protein-associated factor 12 (TAF12) was found to recruit Gadd45a and the nucleotide excision repair complex to the promoter of ribosomal DNA and induce its transcription in a demethylation-dependent manner (Schmitz et al. 2009). The Gadd45 protein interacts directly with various nuclear hormone receptors, including constitutive active/androstane receptor (CAR) (Yamamoto et al. 2010), RXR α, RAR α, ER α, PPAR α, PPAR β, and PPARγ2, perhaps mediating or facilitating transcriptional initiation of their target genes (Ma et al. 2009). Gadd45a- and Gadd45b-mediated DNA demethylation has also been found to be necessary for full expression of epidermal differentiation-inducing genes during calcium-induced differentiation of epidermal stem cells (Sen et al. 2010). Lastly, Gadd45a is significantly overexpressed in CD4+ T cells from systemic lupus erythematous patients and mediates demethylation, with subsequent increased transcription, of the promoter regions of CD11a and CD70, both of which contribute to autoimmunity and therefore progression or maintenance of the disease (Li et al. 2010), despite the role of Gadd45a in T cells as a negative regulator.

Gadd45b and Gadd45g

The other two Gadd45 proteins are less well characterized compared to Gadd45a. Gadd45g is clearly defined as a pro-apoptotic, cell cycle arrest–inducing protein, similar to Gadd45a, and Gadd45b as well to a lesser extent, although there is some controversy surrounding it. Gadd45b and Gadd45g also inhibit Cdk1 activity and play a role in S and G_2/M checkpoints. Gadd45b and Gadd45g activate MTK1 in order to trigger JNK signaling (Yang et al. 2009). Both Gadd45b and Gadd45g interact with p21$^{Cip1/Waf1}$; Gadd45b does positively regulate its expression in senescing chondrocytes but the outcome of this interaction is otherwise unclear in other tissues and contexts (Gao et al. 2009). Gadd45b facilitates p38-mediated retinoblastoma tumor suppressor protein (Rb) activation by enhancing their interaction after Fas stimulation in murine hepatocytes (Cho et al. 2010). It also mediates TGF-induced apoptosis in murine hepatic cells in a p38- and Smad-dependent manner and both Gadd45b and Gadd45g overexpression induced apoptosis in HeLa cells. Gadd45g has been associated with neuronal cell death and Gadd45b with the apoptotic response in neural ischemia (Cretu et al. 2009). Gadd45g levels were found to be significantly lower in anaplastic thyroid cancer cells compared to primary cultured thyrocytes and its reintroduction by viral expression significantly inhibited proliferation (Yang et al. 2009).

However, the role of Gadd45b in apoptosis and cell growth arrest has been controversial. Gadd45b has also

been found to mediate ▶ TNFα-induced NF-κB suppression of JNK-induced apoptosis by binding directly to MKK7 and inhibiting its catalytic activity. It also suppresses JNK signaling in hematopoietic cells in response to UV treatment (although this was later challenged) (Yang et al. 2009). Similarly, stimulation of CAR induces it to interact with Gadd45b and cause Gadd45b-mediated repression of both JNK signaling and subsequent cell death in mouse hepatocytes (Yamamoto et al. 2010). The role for Gadd45b in TGFβ-mediated apoptosis has been shown with a genetic approach in Gadd45b-/- mouse hepatocytes where this Gadd45 protein was required for p38 activation (Yoo et al. 2003). Gadd45b promoted liver regeneration in vivo (Papa et al. 2008) and was protective of retinal ganglion cells in the response to different neuronal injuries, such as oxidative stress, and TNFα and glutamate cytotoxicity (Liu et al. 2009).

Contrary to Gadd45a, Gadd45b and Gadd45g potentiate p38 signaling in Th1 and CD8+ cytotoxic T cells, which is necessary for full effector function, but, similar to Gadd45a, are negative regulators of T-cell activation and proliferation (Lu 2006; Ju et al. 2009). Additionally, Gadd45b is necessary for full expression of the Th1 lineage-inducing proteins, T-bet and Eomes (Ju et al. 2009); thus, the Gadd45 family members seem to synergistically work together to promote full maturation and function of Th1 and CD8+ cells, but they also prevent inappropriate overexpression, except under certain pathological conditions.

Finally, Gadd45b has been found to be required for specific DNA demethylation of factors critical for activity-induced adult neurogenesis (Ma et al. 2009). Thus, there appears to be a wide variety of roles for these two proteins and further characterization is necessary to determine the extent of their roles in cellular biology.

The Gadd45-Null Phenotype

Gadd45a-knockout mice display genomic instability in a significant proportion of cells, immunological deficiencies, low levels of fetal exencephaly and, despite wild-type basal levels of carcinogenesis, treatment of Gadd45a$^{-/-}$ mice with carcinogens such as dimethylbenzanthracene or IR results in increased rates of tumorigenesis with a shorter latency period (Hollander and Fornace 2002; Lu 2006). This indicates that Gadd45a is more important in stress signaling and is the response to DNA damage than constitutive suppression of carcinogenesis. Although most mouse models using a genetic approach indicate tumor suppressor features for Gadd45a, a recent report showed that it can function as a promoter or suppressor of mammary cancer dependent on the oncogenic stress. It's tumor promoting activity seems to be an extension of Gadd45a's ability to repress beta-catenin activity, the negative regulation of which is normally associated with tumor suppression. However, this appears to have tumorigenic consequences in a Myc-driven breast cancer mouse model (Tront et al. 2010).

Additionally, although in vitro studies have implicated Gadd45a in a large number of different signaling pathways, in vivo studies seem to show that the most important downstream effector of Gadd45a signaling is the p38 MAP kinase. Mouse models of Gadd45a deficiency show that Gadd45a-dependent protection against UV irradiation-induced skin tumors requires functional p38 (Hildesheim et al. 2002) and abolition of either Gadd45a or p38 activity resulted in compromised negative regulation of β-catenin via the adenomatous polyposis coli destruction complex (Gao et al. 2009). Notably, p53-signaling in the sunburn response has been shown to require Gadd45a for effective p38 activation which then signals to p53 (Hildesheim et al. 2002). The tissue-specific Gadd45a regulation of p38 signaling in dendritic and T cells is discussed above and highlights the importance of p38 as a target of Gadd45. Although the genomic instability exhibited by Gadd45a$^{-/-}$ mice may be due to additional mechanisms, much of the observed phenotype seem to be mostly due to altered p38 signaling.

Involvement of Gadd45 in Cancer

Gadd45 has been implicated in a number of studies of cancer. Gadd45a deficiency in mice resulted in increased rates of IR- or dimethylbenzanthracene-induced tumors with a shorter latency period, as discussed above. Deletion of Gadd45a in an XPC$^{-/-}$ mouse model of lung cancer led to an increase in lung tumor malignancy, and allelic deletion of Gadd45a is associated with multiple tumor types including lung (Hollander et al. 2005). The Gadd45a promoter is methylated in a majority of breast cancers and a significant fraction of prostate cancers, while the

Gadd45b promoter was likewise hypermethylated in several human hepatocellular carcinomas, in both cases with subsequent downregulation of expression. Sustained ERK1/2 signaling in an acute myeloid leukemia model cell line was found to downregulate Gadd45a, the reintroduction of expression of which induced S-phase arrest and apoptosis (Cretu et al. 2009). Mechanistically, simultaneous overexpression of H-ras and knockout of Gadd45a were sufficient to transform cells, indicating that Gadd45a knockout may serve as one of the "two hits" in oncogenic transformation (Bulavin et al. 2003).

Despite the apparent tumor suppressor role of Gadd45, it also appears to offer malignant cells survival advantages, in line with its roles in cell growth arrest and DNA repair and beyond. In one study, point mutations were found in exon 4 of the *Gadd45a* gene in 14% of studied pancreatic cancer samples and Gadd45a expression in p53-positive tumors was significantly associated with a lower patient survival rate. Gadd45a induction was also found to protect melanoma cells from UV radiation–induced death. Lack of Gadd45a induction in cervical carcinomas correlated significantly with a good clinical response to radiotherapy (Gao et al. 2009). Additionally, despite decreased FOXO3A transcriptional activity, Gadd45a expression was found to be upregulated in thyroid cancers (Karger et al. 2009). The pregnane X receptor can activate Gadd45b/p38 MAPK signaling to induce change of morphology and migration in a hepatocellular carcinoma cell line (Kodama and Negishi 2011). However, given the higher reported rate of promoter hypermethylation or upregulation of Gadd45 transcription-repressing proteins, multiple Gadd45 functions may be important such that alteration of a single function may be insufficient to induce or intensify the tumor phenotype.

Gadd45g is also deficient in several tumors. Its promoter region was found to be hypermethylated and its transcription repressed in a significant number of non-small cell lung cancers (Na et al. 2010), lymphomas, nasopharyngeal carcinomas, cervical carcinomas, esophageal carcinomas, pituitary adenomas (Yang et al. 2009), and gastric, colorectal, and pancreatic cancers (Zhang et al. 2010); however, genetic mutation and inactivation was very rare. Exogenous reintroduction of Gadd45g resulted in G_2/M arrest in a number of tumor cell lines, including prostate carcinoma and pituitary adenoma (Yang et al. 2009).

Summary

In summary, the Gadd45 proteins are mostly characterized as classical tumor suppressors that induce cell cycle arrest and apoptosis in response to DNA damage or oncogenic stimuli. Moreover, they play important roles in a range of other physiological processes, including DNA demethylation and repair, maintenance of genomic stability through mitosis and immunological regulation and activation, although the details and exact mechanisms of Gadd45 involvement in these are still under investigation. Equally intriguing is the accumulating evidence, after initial negative findings, for a role for Gadd45 in both suppressing and promoting cancer. Gadd45 occupies a key role as a hub between different signaling pathways, which no doubt contributes to the difficulty elucidating its function and the contradictory reports; this is in addition to significant tissue specificity in its expression and downstream effectors. That its overexpression has been linked to cancer cell survival, chemoresistance, and migration marks it as a possibly valuable therapeutic target. However, given its tumor suppressor and pleiotropic aspects, research mindful of the difficulties inherent in analyzing a key signaling molecule involved in multiple processes would be necessary to determine the most efficacious manner of exploiting it for clinical benefit.

References

Bulavin DV, Kovalsky O, Hollander MC, Fornace Jr AJ. Loss of oncogenic H-ras-induced cell cycle arrest and p38 mitogen-activated protein kinase activation by disruption of Gadd45a. Mol Cell Biol. 2003;23:3859–71.

Cho HJ, Park SM, Hwang EM, et al. Gadd45b mediates Fas-induced apoptosis by enhancing the interaction between p38 and retinoblastoma tumor suppressor. J Biol Chem. 2010; 285:25500–5.

Cretu A, Sha X, Tront J, Hoffman B, Liebermann DA. Stress sensor *Gadd45* genes as therapeutic targets in cancer. Cancer Ther. 2009;7:268–76.

Fornace Jr AJ, Alamo IJ, Hollander MC. DNA damage-inducible transcripts in mammalian cells. Proc Natl Acad Sci USA. 1988;85:8800–4.

Gao M, Guo N, Huang C, Song L. Diverse roles of GADD45alpha in stress signaling. Curr Protein Pept Sci. 2009;10:388–94.

Hildesheim J, Bulavin DV, Anver MR, et al. Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signaling via MAPK and p53. Cancer Res. 2002;62:7305–15.

Hollander MC, Fornace Jr AJ. Genomic instability, centrosome amplification, cell cycle checkpoints and Gadd45a. Oncogene. 2002;21:6228–33.
Hollander MC, Philburn RT, Patterson AD, et al. Deletion of XPC leads to lung tumors in mice and is associated with early events in human lung carcinogenesis. Proc Natl Acad Sci USA. 2005;102:13200–5.
Jinawath N, Vasoontara C, Yap KL, et al. NAC-1, a potential stem cell pluripotency factor, contributes to paclitaxel resistance in ovarian cancer through inactivating Gadd45 pathway. Oncogene. 2009;28:1941–8.
Jirmanova L, Jankovic D, Fornace Jr AJ, Ashwell JD. Gadd45alpha regulates p38-dependent dendritic cell cytokine production and Th1 differentiation. J Immunol. 2007;178:4153–8.
Ju S, Zhu Y, Liu L, et al. Gadd45b and Gadd45g are important for anti-tumor immune responses. Eur J Immunol. 2009; 39:3010–18.
Karger S, Weidinger C, Krause K, et al. FOXO3a: a novel player in thyroid carcinogenesis? Endocr Relat Cancer. 2009; 16:189–99.
Kearsey JM, Coates PJ, Prescott AR, Warbrick E, Hall PA. Gadd45 is a nuclear cell cycle regulated protein which interacts with $p21^{Cip1}$. Oncogene. 1995;11:1675–83.
Kodama S, Negishi M. Pregnane X receptor PXR activates the *GADD45beta* gene, eliciting the p38 MAPK signal and cell migration. J Biol Chem. 2011;286:3570–8.
Lal A, Gorospe M. Egad, more forms of gene regulation: the gadd45a story. Cell Cycle. 2006;5:1422–5.
Li Y, Zhao M, Yin H, et al. Overexpression of the growth arrest and DNA damage-induced 45alpha gene contributes to autoimmunity by promoting DNA demethylation in lupus T cells. Arthritis Rheum. 2010;62:1438–47.
Lin CR, Yang CH, Huang CE, et al. GADD45A protects against cell death in dorsal root ganglion neurons following peripheral nerve injury. J Neurosci Res. 2011;89:689–99.
Liu B, Suyeoka G, Papa S, Franzoso G, Neufeld AH. Growth arrest and DNA damage protein 45b (Gadd45b) protects retinal ganglion cells from injuries. Neurobiol Dis. 2009; 33:104–10.
Lu B. The molecular mechanisms that control function and death of effector CD4+ T cells. Immunol Res. 2006;36:275–82.
Ma DK, Guo JU, Ming GL, Song H. DNA excision repair proteins and Gadd45 as molecular players for active DNA demethylation. Cell Cycle. 2009;8:1526–31.
Na YK, Lee SM, Hong HS, Kim JB, Park JY, Kim DS. Hypermethylation of growth arrest DNA-damage-inducible gene 45 in non-small cell lung cancer and its relationship with clinicopathologic features. Mol Cells. 2010;30:89–92.
Papa S, Zazzeroni F, Fu YX, et al. Gadd45beta promotes hepatocyte survival during liver regeneration in mice by modulating JNK signaling. J Clin Invest. 2008;118:1911–23.
Paruthiyil S, Cvoro A, Tagliaferri M, Cohen I, Shtivelman E, Leitman DC. Estrogen receptor beta causes a G2 cell cycle arrest by inhibiting CDK1 activity through the regulation of cyclin B1, GADD45A, and BTG2. Breast Cancer Res Treat. 2011;129(3):777–84.
Passos JF, Nelson G, Wang C, et al. Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol Syst Biol. 2010;6:347.
Reinhardt HC, Hasskamp P, Schmedding I, et al. DNA damage activates a spatially distinct late cytoplasmic cell-cycle checkpoint network controlled by MK2-mediated RNA stabilization. Mol Cell. 2010;40:34–49.
Salvador JM, Mittelstadt PR, Belova GI, Fornace Jr AJ, Ashwell JD. The autoimmune suppressor Gadd45alpha inhibits the T cell alternative p38 activation pathway. Nat Immunol. 2005;6:396–402.
Schmitz KM, Schmitt N, Hoffmann-Rohrer U, Schafer A, Grummt I, Mayer C. TAF12 recruits Gadd45a and the nucleotide excision repair complex to the promoter of rRNA genes leading to active DNA demethylation. Mol Cell. 2009;33: 344–53.
Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA. DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature. 2010;463:563–7.
Smith ML, Chen I, Zhan Q, et al. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science. 1994;266:1376–80.
Snyder AR, Morgan WF. Gene expression profiling after irradiation: clues to understanding acute and persistent responses? Cancer Metastasis Rev. 2004;23:259–68.
Tran H, Brunet A, Grenier JM, et al. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science. 2002;296:530–4.
Tront JS, Huang Y, Fornace Jr A, Hoffman B, Liebermann DA. Gadd45a functions as a promoter or suppressor of breast cancer dependent on the oncogenic stress. Cancer Res. 2010;70:9671–81.
Yamamoto Y, Moore R, Flavell RA, Lu B, Negishi M. Nuclear receptor CAR represses TNFalpha-induced cell death by interacting with the anti-apoptotic GADD45B. PLoS One. 2010;5:e10121.
Yang Z, Song L, Huang C. Gadd45 proteins as critical signal transducers linking NF-kappaB to MAPK cascades. Curr Cancer Drug Targets. 2009;9:915–30.
Yoo J, Ghiassi M, Jirmanova L, et al. Transforming growth factor-beta-induced apoptosis is mediated by Smad-dependent expression of GADD45b through p38 activation. J Biol Chem. 2003;278:43001–7.
Zhang W, Li T, Shao Y, et al. Semi-quantitative detection of GADD45-gamma methylation levels in gastric, colorectal and pancreatic cancers using methylation-sensitive high-resolution melting analysis. J Cancer Res Clin Oncol. 2010;136:1267–73.

Galanin Receptor

► GALR, Galanin Receptor

GALNR

► GALR, Galanin Receptor

GalR

▶ GALR, Galanin Receptor

GAL-R

▶ GALR, Galanin Receptor

GALR, Galanin Receptor

Zhenhui Liu[1], Linfang Li[2] and Min Zhang[2]
[1]Department of Marine Biology, Key Laboratory of Marine Genetics and Breeding, Ocean University of China, Qingdao, PR, China
[2]Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao, China

Synonyms

Galanin receptor; GALNR; GalR; GAL-R

Historical Background

Galanin is a 29 amino acids (30 amino acids in human) long neuroendocrine peptide, originally isolated from porcine upper intestine in 1983 (Tatemoto et al. 1983). Subsequently, galanin was found to be expressed widely in brain and peripheral tissues. Galanin triggers cellular responses by binding to specific galanin receptors, and then the signals are transduced into intracellular effectors via G proteins. So far, there are three receptors (GalR1, GalR2, GalR3) were cloned (Branchek et al. 2000), and all of them are G-protein-coupled receptors (GPCR). The three receptors have substantial difference in their functional coupling and subsequent signaling activities, which contributes to the diversity of possible physiological effects of galanin. Galanin receptors can be found throughout the peripheral and central nervous systems (CNS) and the endocrine system, and they can regulate numerous physiological process such as sleeping regulation, feeding, nociception, nerve regeneration, learning and memory, neuroendocrine release, and gut secretion and contractility. However, the specific function of each subtype of galanin receptor remains to be fully elucidated, although great progress is being made in recent studies which newly available subtype selective agonists and antagonists and transgenic mouse models were established (Lu et al. 2008).

Agonists and Antagonists

Molecular cloning of galanin receptor subtypes has allowed design and screening for receptor subtype-specific agonists and antagonists in order to study the molecular basis of galanin actions and to develop potential therapeutic compounds. Selective galanin agonists are anticonvulsant, while antagonists produce antidepressant and anxiolytic effects in animals (Lu et al. 2005), so either agonist or antagonist ligands for the galanin receptors may be potentially therapeutic compounds in humans.

Agonists

Galanin: Human galanin is a 30 amino acids long, non-C-terminally amidated peptide, while galanin from all the other species is 29 amino acids long, C-terminally amidated (Tatemoto et al. 1983). Endogenous galanin is a high-affinity agonist for all three galanin receptors, GalR1, GalR2, and GalR3. The N-terminal 14 amino acids of galanin are fully conserved between different species (Lu et al. 2005). Deletion of first 16 amino acids of galanin causes the complete loss of its affinity for galanin receptors, suggesting the N-terminal 16 amino acids of galanin are critical for receptor binding.

Galanin 1–15 fragment: Galanin (1–15) have been shown to be highly efficacious galanin receptor agonists in vitro and in vivo, but with slightly different pharmacological profiles from that of galanin in the dorsal hippocampus.

Galmic and Galnon: As two small rationally designed galanin receptor agonists, both compounds have been synthesized using a tripeptidomimetic scaffold (galnon) and an oxazole scaffold (Galmic) (Saar et al. 2002). They are systemically active and substantially more resistant to degradation than the endogenous agonist galanin, making it possible to examine galanin receptor roles by systemic application intraperitoneally. The drawbacks of galnon and galmic are that they are low affinity (micromolar affinities), nonreceptor subtype selective, and interacting with other pharmacologically important targets.

Galanin 2–11 fragment: It was also called AR-M1896. Galanin (2–11) has been introduced as a GalR2 selective agonist. However, it also binds to GalR3 receptors with an affinity that is similar to that for GalR2, which has higher than for GalR1 receptors. Its effects have been demonstrated in the spinal cord and locus coerules.

Galanin-like peptide (GALP): GALP is a 60 amino acids long endogenously occurring peptide, which shares amino acid sequence homology with galanin (1–13) in position 9–21. GALP is a high-affinity agonist for both GalR1 and GalR2 receptors with slight preference for GalR2 over GalR1 (18-fold), but not GalR3.

Antagonists

M15, M32, M35, M40, and C7 peptides: They are synthesized chimeric high-affinity ligands, consisting of mammalian galanin (1–13) conjugated to other bioactive molecules. They act as antagonists by mediating a decrease in cyclic AMP production.

Spirocoumaranon (Sch 202596): It is fungal metabolite, belonging to non-peptide galanin receptor ligand. This compound was reported to act as an inhibitor on human GalR1. In addition, Sch 202596 is a low-affinity compound with additional problems precluding their further optimization.

3-[(3,4-dichlorophenyl)imino]-1-(6-methoxy-3-pyridinyl)-1,3-dihydro-2Hindol-2-one: The synthesized compound has 15 nM affinity for GalR3 and shows receptor subtype selectivity and low affinity to other pharmacological targets.

The Distribution of the Three Galanin Receptors, GalR1, GalR2, and GalR3

The first known galanin receptor GalR1 was isolated from the human Bowes melanoma cell in 1994 (Habert-Ortoli et al. 1994). GalR1 was subsequently cloned from rat and mouse. Human GalR1 contains 349 amino acids, and the gene has been mapped to chromosome 18q23. GalR1 mRNA is widely expressed in the mammalian CNS, including brain and spinal cord, as well as in the gut and pancreas-derived cells such as RIN-4b. GalR2 and GalR3 were first cloned from rat hypothalamus in 1997 (Howard et al. 1997). Human GalR2 receptor contains 387 amion acids and has been mapped to chromosome 17q25.3. Unlike GalR1, GalR2 is widely distributed in almost all tissues, including in the hypothalamus, hippocampus, amygdala, and pyriform cortex, as well as in the dentate gyrus, mammillary nuclei and cerebellar cortex, and peripheral tissues such as the vas deferens, prostate, uterus, ovary, stomach, large intestine. GalR3 mRNA is relatively abundant in peripheral tissues, but expression in the CNS is more limited, been largely confined to the hypothalamus and the midbrain and hindbrain. The GalR3 is the least abundantly expressed of the galanin receptor subtypes.

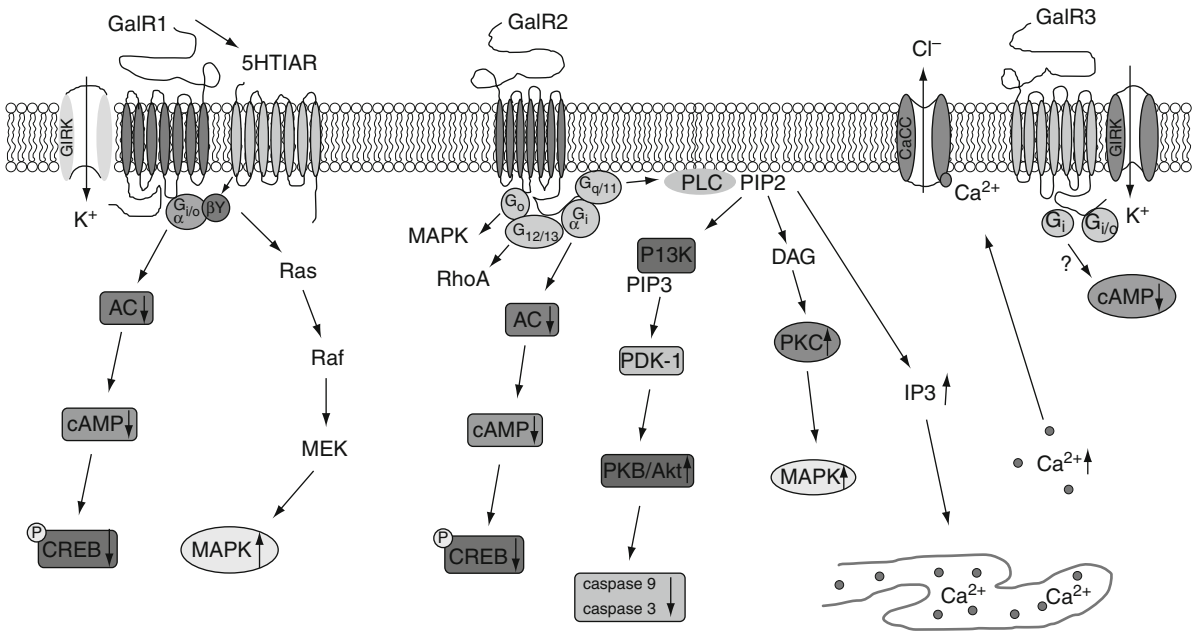
In areas of the mouse brain involved in drug addiction, including the ventral tegmental area (VTA), substantia nigra (SN), nucleus accumbens (NA), and locus coeruleus (LC), all three galanin receptors were found, with the most highly expressed of GalR1 protein in the VTA, NA, and SN, suggesting that GalR1 may play a predominant role in galanin-mediated regulation of dopamine neurotransmission. GalR1 and GalR3 protein levels are high in the LC, indicating that these isoforms may be important for galanin-mediated regulation of noradrenergic transmission during opiate withdrawal.

In the areas of gastrointestinal tract, GalR1 and GalR2 mRNAs were detected in all segments with the highest levels in the large intestine and stomach, respectively. GalR3 mRNA levels were quite low and mostly confined to the colon. The differential distribution of GalRs supports the hypothesis that the complex effects of galanin in the gastrointestinal tract result from the activation of multiple receptor subtypes (Anselmi et al. 2005).

Galanin Receptor Signaling

The galanin receptors are GPCR; therefore, cells devoted to the binding of galanin share common signal transduction components triggered by G proteins. Generally, the first step is that ligands interact with their specific galanin receptors on the membrane. Then the signal pathway mediated by the G protein is activated that initiates intracellular signal cascades. However, the three subtypes of the galanin receptors have differences in their functional coupling and subsequent signaling activities (Fig. 1, Lang et al. 2007).

GalR1 is coupled to the G_i proteins and therefore capable of inhibiting the intracellular cAMP signaling pathway upon ligand binding. It has demonstrated that rat or human GalR1 expressed in transfected cell lines inhibits forskolin-stimulated cAMP production in a pertussis toxin (PTX)-sensitive manner. GalR1 is specifically upregulated in the locus coeruleus



GALR, Galanin Receptor, Fig. 1 Signaling pathways of galanin receptor subtypes. *AC* adenylate cyclase, *CaCC* Ca^{2+}-dependent chloride channel, *cAMP* 3′,5′-cyclic adenosine monophosphate, *(p)CREB* (phosphorylated) cAMP response element-binding protein, *DAG* diacylglycerol, *IP3* inositol triphosphate, *MEK* mitogen-induced extracellular kinase, *PDK-1* phosphoinosotide-dependent protein kinase 1, *PIP2* phosphatidylinositol bisphosphate, *PIP3* phosphatidylinositol trisphosphate, *PI3K* phosphatidylinositol 3-kinase, *PKB* protein kinase B, *PLC* phospholipase C, *5HT1AR* 5-hydroxtryptamine-1A receptor, *GIRK* G protein-coupled inwardly rectifying potassium channel (Adapted from Lang et al. (2007))

(LC)-like Cath, a cell line in a cyclic AMP-dependent manner. GalR1 protein and mRNA levels are also upregulated in the LC of galanin knockout mice. Also, GalR1 activates G proteins via $G_{\beta\gamma}$-subunits. Activation of GalR1 expressed in squamous carcinoma cells induces a marked and prolonged ERK1/2 activation, in this case via $G_{\alpha i}$-subunits, leading to induction of the cell cycle arrest and suppresses proliferation in a p53-independent manner. In addition, galanin binding to GalR1 led to receptor internalization in the transfected Chinese hamster ovary (CHO) cells, which may be a mechanism for regulating the endogenous signaling cascade in native cells. Recently, Borroto-Escuela et al. (2010) have examined the possible existence of GalR1-5HT1AR (5-hydroxtryptamine-1A receptor) heteromers, indicating the existence of GalR1-5HT1A receptor–receptor interactions in the discrete brain regions. This would give rise to explore possible novel therapeutic strategies for treatment of depression by targeting the GalR1-5HT1A heteromers.

GalR2 can be coupled to different classes of G proteins, initiating two second-messenger signal cascades: the cyclic adenosine monophosphate (cAMP) and phosphoinositide signals, which are two parallel streams of intracellular events. In the pathway of phosphoinositide signal, phospholipase C (PLC) is activated. Thus, inositol phosphate hydrolysis is increased, mediating the release of Ca^{2+} into the cytoplasm and opening Ca^{2+}-dependent chloride channels. In this case, GalR2 may act through $G_{q/11}$-type G proteins since the intracellular effects are not affected by PTX. In addition, it is suggested that neuronal survival enhanced by galanin is mediated by the AKT signaling pathway leading to suppression of caspase-3 and caspase-9 activity. In the pathway of cAMP signal, the forskolin-stimulated cAMP production was inhibited in CHO cells transfected with rat GalR2 and HEK-293 cells transfected with human GalR2 after galanin stimulation, and the G_i-type G proteins were suggested to be used. Furthermore, both GalR1 and GalR2 activations inhibit cyclic AMP-responsive element-binding (CREB) protein. However, there are somewhat controversial points on this pathway. It is also supported that GalR2 is coupled to

a G_o-type G-protein, activating MAPK in a PKC-dependent fashion or to a $G_{12/13}$-type G-protein, activating RhoA.

GalR3 appears to couple to a $G_{i/o}$-type G-protein to stimulate the activation of an inward K^+ current. In addition, a G_i-type G-protein was also reported to be involved in GalR3 signaling. However, the detail signaling pathways mediated by GalR3 are still poorly understood. New molecular tools, such as application of subtype-specific antisense reagents and gene knockout approaches, will clarify our understanding of the role of galanin receptor subtypes in galaninergic signaling.

Effects of GalRs in NS

Galanin and its receptors are widely expressed in the CNS and peripheral nervous system (PNS), indicating the numerous physiological effects of GalRs. The three galanin receptors have been found to be involved in the control of feeding, alcohol intake, seizure threshold, cognitive performance, and mood and pain threshold (Mitsukawa et al. 2008).

Learning and Memory

The effects of galanin participating in the learning and memory have been shown in many studies. It is generally accepted that the ability to perform learning and memory tasks will be impaired if too much galanin in the hippocampus or lateral ventricles of rats. This effect can be blocked by GalR2 antagonist M40. However, we should also be aware that the results have some controversy. In some recent testing of a GalR2-KO strain (Lang et al. 2007), the mutant mice were not significantly different to wild-type littermates on the cognition tests (Ogren et al. 1996). This suggests that GalR2 may be not centrally involved in learning and memory. But it cannot rule out the possibility that galanin levels have not reached sufficient levels during the behavioral tasks, or compensatory developmental changes in the other galanin receptors (Ogren et al. 1996). Further experiments should be able to address these possibilities.

Mood Regulation and Alcohol Intake

Galanin was found to be coexpressed with noradrenaline in the noradrenergic neurons in the locus coeruleus (LC) and with serotonin in the serotonergic neurons in the dorsal raphe nucleus (DRN) (Lu et al. 2005). The overactivity of the LC noradrenergic neurons leads to suppression of the firing of the DRN serotonergic neurons, thus causing depression. It was suggested by Lu et al. (2005) that GalR2 agonists, like blockers of serotonin (SSRIs), may be effective in the treatment of major depression. Also, GalR3 antagonists have anti-depressant-like activity. The GalR3 subtype selective antagonists had been synthesized, and they were confirmed to be active in some anxiety or acute antidepressant models. In addition, GalR3 has been shown a significant association with alcoholism, but there was no effect of GalR1 or GalR2 haplotypes on alcoholism risk. Thus, Mitsukawa et al. (2008) indicated that development of galanin receptor antagonists, in particular GalR3 antagonists, might be a breakthrough in the addiction relevant field.

Feeding

It is reported that galanin had the ability to induce food intake strongly (Leibowitz 2005). The choice of food preference – if protein, carbohydrates, and fat are available – is fat first and carbohydrate second. It also suggested that the food preference was caused by activating the process of ingestion rather than controlling satiety feeling. Although all three galanin receptor subtypes are present in brain regions important for galanin-stimulated feeding, it is indicated that the rat feeding response was attributed to either GalR1 or an unidentified GalR rather than to GalR2 and GalR3. Furthermore, the studies on GalR1 null mutation carrying mice showed that GalR1 mediated the important effects that are required for glycemic control and body weight control (Zorrilla et al. 2007).

Pain

Galanin and its three receptors are expressed in both sensory and spinal cord interneurons. Also, nerve injury such as axotomy can lead to a rapid high-level expression of galanin in the sensory ganglia. The expression profiles of galanin and the three receptors indicate that they may play a key gatekeeper role in pain signaling (Wiesenfeld-Hallin and Xu 2001). Galanin has double-eged function in many pain models. It can both act as inhibitory or excitatory mediator, depending on the circumstance such as the nature of stimulus of the nociceptive (thermal, mechanical, chemical), the acute or chronic state, and the concentration of galanin available to act on the nociceptive afferent nerves. It is suggested that low galanin doses can escalate and high doses suppress

pain. The GalR2-mediated depolarizing effects may contribute to pain sensation, while GalR1-mediated hyperpolarization of the sensory and interneurons are responsible for the analgesic effect. Therefore, GalR1 agonists may be the potential candidates for systemic or intrathecal use in pain therapy.

Neuronal Injury

The expression of galanin is strongly increased in mRNA or protein levels after neuronal injury, suggesting a trophic role for galanin. Indeed, galanin acts as a survival- and growth-promoting factor for different types of neurons in the peripheral and central nervous system. For example, the decrease of galanin level was observed in many damage models of different brain region, including the cut or extrusion of motor and sensory neurons, traverse of central nerve, focal cerebral ischemia. Also, GalR2 was suggested to be related to the survival of hippocampal neuron and injured brain (O'Meara et al. 2000). It is reported that the selective GalR2 peptide agonist AR-M1896 counteracts a number of morphological alterations induced by glutamate toxicity in neuronal hippocampal cells.

Anxiety

Galanin and receptors were found to be expressed in brain areas relevant to emotional behavior, such as the amygdala and the BNST (the bed nucleus of the stria terminalis). Its coexistence with noradrenaline (NE) and serotonin in relevant neural pathways were also discovered. Thus, the roles of galanin and its receptors in animal models of anxiety were concerned. Behavioral studies of both GalR1- and GalR2-KO mouse strains suggested a role for galanin in regulating anxiety-related behaviors. In addition, it is reported that galanin express the activity of anti-anxiety via GalR1 under the relatively high pressure. Also, there are other experiments indicated that GalR2 and GalR3 have been involved in the process of anti-anxiety. However, the concrete process concerning the three receptors has to be further research.

Effects of Galanin and GalRs for Many Pathological Diseases

Epilepsy

During the past decade, the growing evidences have been suggesting that galanin is in fact a powerful inhibitor of seizure activity. In the hippocampus, galanin inhibits both acetylcholine release and postsynaptic cholinergic functions. It was reported that both acetylcholine release and postsynaptic effects of pilocarpine and acetylcholine were increased in GalR1 null mutation mouse, thus promoting seizures (Fetissov et al. 2003). The duration of epilepticus can be significantly shortened by the pretreatment of galanin, but this effect can be reversible by the injection of a GalR1 antagonist, M35. Moreover, M35 alone promotes the establishment of seizures and prolongs their duration. Collectively, galanin can affect the maintenance phase of seizures possibly via GalR1. Pharmacological application of galanin agonists should be a potently useful antiepileptic.

Alzheimer's Disease

Alzheimer's disease is characterized by the progressive degeneration of the cholinergic/galaninergic neurons and the cognitive function losing gradually. Learning and cognitive performance of rodents were shown to be impaired after intrahippocampal- or intracerebroventricularly (i.c.v)-injected galanin (Rustay et al. 2005). This is in line with the overexpression of galanin in Alzheimer's disease. Also, the expression of galanin receptor was found to be higher in Alzheimer's disease afflicted brains. GalR1 antagonists, as a cognitive enhancer, might help the treatment of the Alzheimer's disease since they would disinhibit the release of acetylcholine. In addition, GalR2 agonists were suggested to run roles in neuroprotection and neurogenesis. Thus, GalR2 agonists are also expected to be useful to cognitive disorders of neurodegenerative etiology.

Tumors

The expressions of galanin and galanin receptors have been detected in several tumors such as pancreatic, hypothalamic, and pituitary tumors; small cell lung carcinoma; and colon cancer isolates (Mitsukawa et al. 2008), which prompts that galanin can be involved in many tumor pathophysiological process. For example, galanin has mitogenic effects on human pancreatic cancer cells and stimulated the proliferation and prolactin secretion of rat pituitary tumor cells in vitro. Therefore, galanin and galanin receptor expressions are becoming increasingly used markers for certain tumors. In head and neck squamous cell carcinoma (HNSCC), signal mechanism has also been studied, which GalR1 and GalR2 were revealed to act as tumor suppressors in a p53-independent manner.

GalR1 or GalR2 induces cell cycle arrest and suppresses proliferation in HNSCC, and GalR2 can also induce apoptosis (Kanazawa et al. 2010). Indeed, galanin has been clinically used in pancreatic tumor therapy.

Diabetes

Galanin has been detected in pancreatic islet cells in several species. There are a large number of galanin-like immunoreactive (galanin-LI) cells in both the peripheral and central regions of the islet of Langerhans of normal rat pancreas, but the galanin-LI cells are significantly less in diabetic ones. In addition, the concentration of insulin in humans was shown to be suppressed after galanin injection. Therefore, the positive correlation of galanin with diabetes is expected. It was suggested that the specific mechanism for galanin effect was involved in GalR1 since the GalR1 agonist M40 can suppress glucose-stimulated insulin release. GalR3 was also reported to be involved in this process.

The Evolution of GalR

To obtain insights into the evolution of GalRs, Liu et al. (2010) have searched the genomes of the deuterostomes by extensive BLAST survey and phylogenetic analyzes. Typical GalR1 and GalR2 genes were found from fish to human; however, GalR3 were only found in some mammalian. Interestingly, two GalR1 genes were found in fish, including *F. rubripes*, *T. nigroviridis*, and *D. rerio*, and two GalR2 genes were presence in *G. gallus*. These indicate that the GalR1 and GalR2 gene duplication events occurred in fish and chick, respectively. GalR2 and GalR3 share similar genomic structures, which most of them are composed of two exons and one intron. However, most of GalR1 are composed of three extrons and two introns. Typical GalR genes were not detected in the genomic databases of invertebrate deutserotomes, including *S. purpuratus*, *C. intestinalis*, and amphioxus, but three GalR1/Alstr homologs and two GalR1/Gpr151 homologs in amphioxus, two GalR1/Gpr151 homologs in sea squirt, and one GalR1/Gpr151 homolog in sea urchin were identified. It is highly possible that the GalR genes in vertebrates may evolve from the homologous genes of GalR1/Alstr/Gpr151 in invertebrate deuterostomes. Also, GalR3 genes may be the products of GalR2 duplication during evolution, while GalR2 genes may evolve from GalR1.

Summary

The widely distribution of galanin and GalRs in the CNS and in the PNS and various organs demonstrates that the galanin and GalRs play key roles in numerous physiological functions such as seizure, learning, nociception, nerve regeneration, food intake, and reproduction. However, there is controversy concerning some certain physiological functions of galanin and GalR, such as learning and memory, anxiety behavior modulation. With more galanin receptor knockout mice are got, the biological effects mediated by activation of distinct galanin receptor subtypes in various organ systems would be more clear. The search for agonists and antagonists of specific GalRs will promote the research and application of GalRs in the drug target.

Acknowledgements This work was supported by Program for New Century Excellent Talents in University to Zhenhui Liu (No. NCET-08-0501), the Ministry of Science and Technology (MOST) of China (No.2008AA092603 and 2008AA09Z409) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (No. 2009–1001).

References

Anselmi L, Stella Jr SL, Lakhter A, Hirano A, Tonini M, Sternini C. Galanin receptors in the rat gastrointestinal tract. Neuropeptides. 2005;39:349–52.

Borroto-Escuela DO, Narvaez M, Marcellino D, Parrado C, Narvaez JA, Tarakanov AO, Agnati LF, Díaz-Cabiale Z, Fuxe K. Galanin receptor-1 modulates 5-hydroxtryptamine-1A signaling via heterodimerization. Biochem Biophys Res Commun. 2010;393:767–72.

Branchek TA, Smith KE, Gerald C, Walker MW. Galanin receptor subtypes. Trends Pharmacol Sci. 2000;21:109–17.

Fetissov SO, Jacoby AS, Brumovsky PR, Shine J, Iismaa TP, Hokfelt T. Altered hippocampal expression of neuropeptides in seizure-prone GALR1 knockout mice. Epilepsia. 2003;44:1022–33.

Habert-Ortoli E, Amiranoff B, Loquet I, Laburthe M, Mayaux JF. Molecular cloning of a functional human galanin receptor. Proc Natl Acad Sci USA. 1994;91:9780–3.

Howard AD, Tan C, Shiao LL, Palyha OC, McKee KK, Weinberg DH, Feighner SD, Cascieri MA, Smith RG, Van Der Ploeg LH, Sullivan KA. Molecular cloning and characterization of a new receptor for galanin. FEBS Lett. 1997;405:285–90.

Kanazawa T, Misawa K, Carey TE. Galanin receptor subtypes 1 and 2 as therapeutic targets in head and neck squamous cell carcinoma. Expert Opin Ther Targets. 2010;14:289–302.

Lang R, Gundlach AL, Kofler B. The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. Pharmacol Ther. 2007;115:177–207.

Leibowitz SF. Regulation and effects of hypothalamic galanin: relation to dietary fat, alcohol ingestion, circulating lipids and energy homeostasis. Neuropeptides. 2005;39:327–32.
Liu Z, Xu Y, Wu L, Zhang S. Evolution of galanin receptor genes: insights from the deuterostome genomes. J Biomol Struct Dyn. 2010;28:97–106.
Lu X, Barr AM, Kinney JW, Sanna P, Conti B, Behrens MM, Bartfai T. A role for galanin in antidepressant actions with a focus on the dorsal raphe nucleus. Proc Natl Acad Sci USA. 2005;102:874–9.
Lu X, Ross B, Sanchez-Alavez M, Zorrilla EP, Bartfai T. Phenotypic analysis of GalR2 knockout mice in anxiety- and depression-related behavioral tests. Neuropeptides. 2008;42:387–97.
Mitsukawa K, Lu X, Bartfai T. Galanin, galanin receptors and drug targets. Cell Mol Life Sci. 2008;65:1796–805.
O'Meara G, Coumis U, Ma SY, Kehr J, Mahoney S, Bacon A, Allen SJ, Holmes F, Kahl U, Wang FH, Kearns IR, Ove-Ogren S, Dawbarn D, Mufson EJ, Davies C, Dawson G, Wynick D. Galanin regulates the postnatal survival of a subset of basal forebrain cholinergic neurons. Proc Natl Acad Sci USA. 2000;97:11569–74.
Ogren SO, Kehr J, Schött PA. Effects of ventral hippocampal galanin on spatial learning and on in vivo acetylcholine release in the rat. Neuroscience. 1996;75:1127–40.
Rustay NR, Wrenn CC, Kinney JW, Holmes A, Bailey KR, Sullivan TL, Harris AP, Long KC, Saavedra MC, Starosta G, Innerfield CE, Yang RJ, Dreiling JL, Crawley JN. Galanin impairs performance on learning and memory tasks: findings from galanin transgenic and GAL-R1 knockout mice. Neuropeptides. 2005;39:239–43.
Saar K, Mazarati AM, Mahlapuu R, Hallnemo G, Soomets U, Kilk K, Hellberg S, Pooga M, Tolf BR, Shi TS, Hökfelt T, Wasterlain C, Bartfai T, Langel U. Anticonvulsant activity of a nonpeptide galanin receptor agonist. Proc Natl Acad Sci USA. 2002;99:7136–41.
Tatemoto K, Rökaeus Å, Jornvall H, McDonald TJ, Mutt V. Galanin- a novel biologically active peptide from porcine intestine. FEBS Lett. 1983;164:124–8.
Wiesenfeld-Hallin Z, Xu XJ. Neuropeptides in neuropathic and inflammatory pain with special emphasis on cholecystokinin and galanin. Eur J Pharmacol. 2001;429:49–59.
Zorrilla EP, Brennan M, Sabino V, Lu X, Bartfai T. Galanin type 1 receptor knockout mice show altered responses to high-fat diet and glucose challenge. Physiol Behav. 2007;91:479–85.

Gamma-Pak

▶ Pak2

Gardner-Rasheed Feline Sarcoma Viral (v-fgr) Oncogene

▶ FGR (Gene Name)

Gastric Inhibitory Polypeptide Receptor

▶ Glucose-Dependent Insulinotropic Polypeptide Receptor (GIPR)

GAT (GABA Transporters)

Sergei Krirschuk and Werner Kilb
Institute of Physiology & Pathophysiology, University Medical Center Mainz, Mainz, Germany

Synonyms

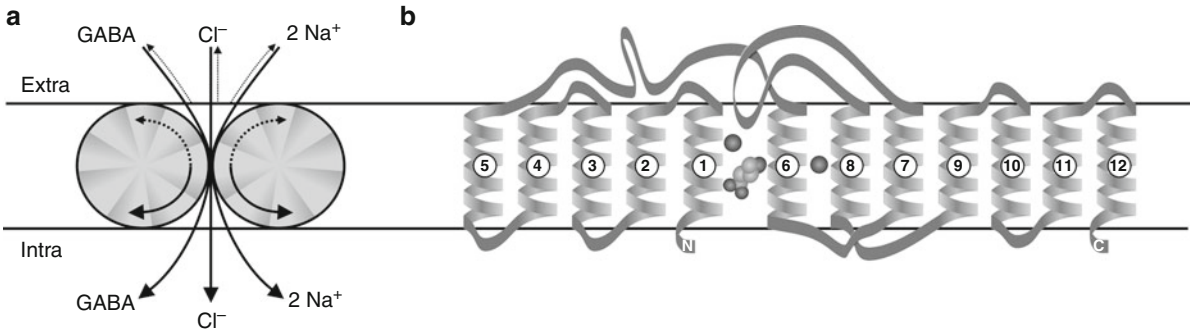
BGT-1; GAT-1; GAT-2; GAT-3

Historical Background

The amino acid γ-amino butyric acid (GABA) is the main inhibitory neurotransmitter in the adult brain. The actions of GABA are mediated via two distinct classes of receptors: ionotropic ($GABA_A$ and $GABA_c$) and metabotropic ($GABA_B$) receptors (Farrant and Kaila 2007). For adequate synaptic transmission, the released GABA has to be rapidly sequestered from the synaptic cleft by high affinity GABA transporters. Experiments performed in neuronal and astroglial cell cultures in the 1970s revealed that a number of GABA analogues selectivity inhibited GABA uptake in one or the other culture type. Based on these results, it was concluded that neurons and astrocytes express distinct GABA transporters (Krogsgaard-Larsen et al. 1987). Molecular biological techniques revealed that most probably four distinct GABA transporters exist (Borden 1996).

Properties and Subtypes of GABA Transporters

GABA transporters (GATs) belong to the superfamily of solute carriers and the class of Na^+-Cl^--dependent neurotransmitter transporters (Nelson 1998). All GATs described so far mediate the symport of 1 molecule GABA with 1 Cl^- ion and 2 Na^+ ions (Fig. 1a).



GAT (GABA Transporters), Fig. 1 Transport properties and topology of GABA-transporters. (**a**) Schematic diagram illustrating that GATs mediate the symport of 1 molecule GABA with 1 Cl^- and 2 Na^+ ions. Although the transport direction is inward directed under most physiological conditions (*bold arrows*), the direction of GABA transport can be reversed under various physiological and pathophysiological conditions (*dashed arrows*). (**b**) Proposed membrane topology of Na^+-Cl^--dependent transporters according to Yamashita et al. (2005). GABA transporters contain presumably 12 transmembrane helices. GABA and one Na^+ ion (*gray circle*) were probably bound to transmembrane helices 1 and 6, while the second Na^+ ion interacts with transmembrane helices 6 and 8. All binding sites were located in a central pocket formed by the transmembrane helices and 1, 6, 8, and 10 and part of the extracellular loop 4 (between TMH 7 and 8)

GATs share great homology to other classes of the Na^+-Cl^--dependent neurotransmitter transporters, like glycine-, dopamine-, serotonin-, or choline-transporters, but are structurally and functionally distinct from excitatory amino acid transporters (EAAT1-5) and vesicular neurotransmitter transporters (e.g., VGAT, VGluT1-3, VAChT) (Masson et al. 1999). Like other Na^+-Cl^--dependent transporters, GATs contain presumably 12 transmembrane helices (Fig. 1b). The GABA and the Na^+ binding sites are located on the transmembrane helices 1, 6, and 8, which together with the transmembrane helix 10 and part of the extracellular loop 4 form a central cavity with alternating contact to the extra- and intracellular compartment (Yamashita et al. 2005).

Four different GATs have been identified in rodents and humans so far (Borden 1996). These four GATs have different GABA affinities, distinct pharmacological profiles and show a specific cellular distribution. There are two nomenclatures for GABA transporters. In the rat/human nomenclature GABA transporters are called GAT-1, betaine/GABA transporter-1 (BGT-1), GAT-2, and GAT-3 (Borden et al. 1992). In the mouse nomenclature GATs are called GAT1, GAT2, GAT3, and GAT4 (without hyphen, Liu et al. 1993). They were encoded by the genes SLC6A1 (for GAT-1) and SLC6A11-13 (for GAT-3, BGT-1, and GAT-2, respectively). In this review the rat/human nomenclature will be used.

GAT-1 was the first GABA transporter to be cloned. Data obtained in different expression system (Xenopus oocytes, COS-7 cells, etc.) revealed that GAT-1 has an IC50 of 5–30 μM and is Na^+ and Cl^- dependent. The expression of GAT-1 mRNA is restricted to the central nervous system, where it was found to be present in all brain regions examined. Immunohistochemical studies showed that all GABAergic neurons (as identified by the expression of GAD67) express GAT-1. In addition, some non-GABAergic neurons and glial cells (for instance, Bergmann glial cells in the cerebellum) also express GAT-1. GAT-1 immunoreactivity is almost exclusively associated with punctate structures resembling axon terminals and fibers. These observations indicate that GAT-1 is a preferentially neuronal GABA transporter and is predominantly synaptically located, suggesting that the main physiological function of GAT-1 is the fast removal of GABA from the synaptic cleft, i.e., termination of GABAergic transmission, and the recycling of GABA to the presynaptic terminal.

GAT-2 shows about 52% amino acid identity with GAT-1. GAT-2 has an IC50 of 17 μM and is also Na^+ and Cl^- dependent. GAT-2 mRNA is present both in the central nervous system and in a number of nonneural tissues such as kidney, liver, or hearth. Although GAT-2 immunoreactivity was mainly detected in the leptomeninges (pia and arachnoid) surrounding the brain, GAT-2 staining was also

observed in some neuronal and glial cells. Because GAT-2 positive punctae are mainly localized to glial limitans and outline blood vessels, GAT-2 may regulate GABA levels in the cerebrospinal fluid, thereby regulating GABAergic transmission indirectly.

GAT-3 displays 52% amino acid identity with GAT-1 and 67% amino acid identity with GAT-2. GAT-3 has an IC50 of 33 μM and is also Na^+ and Cl^- dependent. The expression of GAT-3 mRNA is restricted to the central nervous system. Immunohistochemical studies show that GAT-3 is predominantly expressed in astrocytes. Moreover, GAT-3 is preferentially located in distal astrocytic processes including perivascular end-feet. GAT-3 positive punctae are adjacent to axon terminals making both symmetrical and asymmetrical synapses. These observations suggest that the main physiological function of GAT-3 is a removal of GABA that escaped from the synaptic cleft, i.e., prevention of synaptic crosstalk.

BGT-1 was firstly identified in kidney, where it mediates the accumulation of the osmolyte betaine in renal medullary epithelial cells to make them resistant to hypertonicity. Since this transporter is able to utilize also GABA as substrate, it was termed BGT-1 (for Betaine/GABA transporter). Surprisingly, BGT-1 has a higher affinity for GABA ($IC_{50} = 42$ μM) than for betaine ($IC_{50} = 400$ μM). This is, however, in line with BGT-1 function in the kidney because the concentration of betaine in plasma is about 180 μM, while GABA concentration in plasma is <1 μM. BGT-1 shows 68% and 65% degrees of identity with GAT-2 and GAT-3. BGT-1 was also expressed in the CNS, most probably on astrocytes, where it contributes to the regulation of extrasynaptic GABA levels (Clausen et al. 2006).

Despite these evident differences in the cellular and subcellular localization of GATs in the adult CNS, the situation seems to be less clear in the immature brain, where a substantial abundance of GAT-1 in astrocytes and of GAT-3 in neurons was reported (Conti et al. 2004). In rodent neocortex, GAT-1 expression is rather low at birth and reaches adult levels approximately by the end of third postnatal week (Conti et al. 2004). In contrast, GAT-2 and GAT-3 expression is relatively high at birth and reaches the adult levels the first and second first postnatal weeks, respectively (Conti et al. 2004).

Stoichiometry and Kinetics of GABA Transporters

GATs mediate the symport of one uncharged GABA molecule, two positively charged Na^+ ions, and one negatively charged Cl^- ion, thus generating an electrogenic transport process (Attwell et al. 1993). Therefore, the thermodynamic equilibrium provides a reversal potential for GABA transport (Richerson and Wu 2003):

$$E_m = \frac{RT}{(2z_{Na} + z_{Cl})F} \times \ln\left(\frac{[GABA]_i}{[GABA]_a} \times \left(\frac{[Na^+]_o}{[Na^+]_i}\right)^2 \times \frac{[Cl^-]_o}{[Cl^-]_i}\right)$$

For neuronal cells, the concentrations of molecules involved in GABA transport can be found in the literature: $[Na^+]_i = 15$ mM, $[Cl^-]_i = 7$ mM, $[GABA]_i = 2$ mM, $[Na^+]_o = 150$ mM, $[Cl^-]_i = 135$ mM, $[GABA]_i = 0.1$ μM. Surprisingly, these values predict the reversal potential for GABA transport of about −60 mV, i.e., unexpectedly close to the resting potential (Attwell et al. 1993; Richerson and Wu 2003). Only if membrane potential is more negative than reversal potential, GABA transporters will operate in the inward direction, i.e., will take up GABA. But when membrane potential is more positive than the reversal potential, which can, e.g., occur during excitatory synaptic transmission, GABA transporters will operate in the reverse mode and will release GABA. Moreover, elevations of either $[Na^+]_i$ (for instance, as consequence of AMPA/NMDA receptor activation) or $[Cl^-]_i$ (for instance, as consequence of $GABA_A$ receptor activation) will increase the reversal potential of GABA transport, therefore favoring its switch to reversed mode. This suggests that synaptic activity can modulate GAT-mediated uptake/release and thus extracellular GABA levels. However, such a synaptic modulation is only possible if GATs are fast enough to follow these dynamic processes. Although GATs expressed in Xenopus oocytes demonstrated rather slow kinetics (one cycle per about 100 ms), recent data indicate that the turnover rate is faster than previously believed, taking about 10 ms per translocation at 37°C. Moreover, recent study in both artificial expression system and

in hippocampal neurons showed that GAT-1 is fast enough to be reversed during an action potential (Wu et al. 2007).

Modulation of GABA Transport

In addition to the modulation via membrane voltage and intracellular ions, functional modulation of GATs occurs through a variety of second messengers such as kinases, arachidonic acid, and pH (Beckman and Quick 1998). These factors may act directly on the transporter protein or by modulating the interaction with other proteins. Interestingly, not only GAT kinetics is regulated but also the number of GATs expressed on the surface. GATs could be internalized and reinserted into the plasma membrane within minutes and, moreover, GAT-1 trafficking resembles the cycling of neurotransmitter-filled synaptic vesicles (Deken et al. 2003). Such redistribution of GAT-1 protein between the plasma membrane and the cytoplasm is regulated by different G-protein coupled receptors via protein kinase C (PKC). PKC activation leads to GAT-1 internalization, while PKC inhibition increases the surface expression of GAT-1 (Beckman et al. 1999). The surface expression of GAT-1 is also dependent on the availability of its substrate. GAT-1 stimulation by GABA or other transported substrates increases GAT-1 surface expression, whereas GAT-1 inhibitors that are not substrates induce GAT-1 internalization. Thus, the number of GAT-1 on the cell surface and/or synapse is fine-tuned by GABA itself (Bernstein and Quick 1999).

GABA Transporters in Diseases

A variety of reports suggest an essential function of GATs in the etiology of neuronal diseases. There is a strong link between GAT function and epilepsy, although the functional implications of GAT in epilepsy are far from being completely understood. During epileptic activity, the enhanced neuronal activity promotes the reversal of GATs and thus a nonvesicular GABA release that can dampen neuronal excitation. Enhanced GAT-1 expression increases the susceptibility for epileptic seizures, most probably by reducing extracellular GABA levels and shortening GABA availability in the synaptic cleft. Accordingly, inhibition of GATs attenuates epileptic seizures; therefore, GAT inhibitors are used for antiepileptic medication. In addition, alterations in the expression of GATs, in particular of GAT-1, have been reported in epileptic animals, where an up- and downregulation of different GATs has been described. The alteration in the GAT expression levels may either serve to enhance nonvesicular GABA release under epileptic conditions, thus contributing to homeostatic inhibition, but can also aggravate seizures. In any way, these complex alterations in epileptic tissue complicate a pharmacological interaction with GATs (Allen et al. 2004; Conti et al. 2004). A downregulation of GAT-1 has also been shown in schizophrenic patients (Schleimer et al. 2004).

A beneficial effect of GATs has been suggested for brain ischemia. Under ischemic condition, the GATs reversed due to a massive increase in the intracellular Na^+ concentration under this condition, with lead to the nonsynaptic release of GABA. Although this increased GABA concentration may promote a neuroprotective effect, it can also contribute to cell swelling, thus aggravating excitotoxic insults. Recent reports indicate that this GABA release occurred mainly via neuronal GAT-1, while glial GAT-3 does not contribute. In this respect, it is also interesting that ischemic conditions stimulate the synthesis of GABA, thus additionally increasing the driving force for reversed GABA transport (Allen et al. 2004).

Summary

GABA transporters belong to the family of Na^+-Cl^--dependent neurotransmitter transporters and mediate the electrogenic symport of 1 molecule GABA with 1 Cl^- and 2 Na^+ ions. Due to its transport stochiometry, the regular inward transport direction can be easily reversed under various physiological and pathophysiological conditions, thereby mediating nonvesicular GABA release. Four different GATs have been described so far: GAT-1 is mainly expressed in GABAergic neurons of all brain regions and mediates the fast removal of GABA from the synaptic cleft. GAT-2 is expressed in neuronal and nonneuronal tissues and may regulate GABA levels in the cerebrospinal fluid. GAT-3 expression was restricted to the central nervous system where it was described predominantly in

astrocytes. GAT-3 is most probably involved in the regulation of GABA at extrasynaptic sites. The betaine/GABA transporter BGT-1 was expressed in nonneuronal and neuronal tissues. In the kidney, it mediates the accumulation of the osmolyte betaine.

References

Allen NJ, Karadottir R, Attwell D. Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy. Pflugers Arch. 2004;449:132–42.

Attwell D, Barbour B, Szatkowski M. Nonvesicular release of neurotransmitter. Neuron. 1993;11:401–7.

Beckman ML, Quick MW. Neurotransmitter transporters: regulators of function and functional regulation. J Membr Biol. 1998;164:1–10.

Beckman ML, Bernstein EM, Quick MW. Multiple G protein-coupled receptors initiate protein kinase C redistribution of GABA transporters in hippocampal neurons. J Neurosci. 1999;19:RC9.

Bernstein EM, Quick MW. Regulation of gamma-aminobutyric acid (GABA) transporters by extracellular GABA. J Biol Chem. 1999;274:889–95.

Borden LA. GABA transporter heterogeneity: pharmacology and cellular localization. Neurochem Int. 1996;29:335–56.

Borden LA, Smith KE, Hartig PR, Branchek TA, Weinshank RL. Molecular heterogeneity of the gamma-aminobutyric acid (GABA) transport system. Cloning of two novel high affinity GABA transporters from rat brain. J Biol Chem. 1992;267:21098–104.

Clausen RP, Frolund B, Larsson OM, Schousboe A, Krogsgaard-Larsen P, White HS. A novel selective gamma-aminobutyric acid transport inhibitor demonstrates a functional role for GABA transporter subtype GAT2/BGT-1 in the CNS. Neurochem Int. 2006;48:637–42.

Conti F, Minelli A, Melone M. GABA transporters in the mammalian cerebral cortex: localization, development and pathological implications. Brain Res Brain Res Rev. 2004;45:196–212.

Deken SL, Wang D, Quick MW. Plasma membrane GABA transporters reside on distinct vesicles and undergo rapid regulated recycling. J Neurosci. 2003;23:1563–8.

Farrant M, Kaila K. The cellular, molecular and ionic basis of GABA(A) receptor signalling. Prog Brain Res. 2007;160:59–87.

Krogsgaard-Larsen P, Falch E, Larsson OM, Schousboe A. GABA uptake inhibitors: relevance to antiepileptic drug research. Epilepsy Res. 1987;1:77–93.

Liu QR, Lopez-Corcuera B, Mandiyan S, Nelson H, Nelson N. Molecular characterization of four pharmacologically distinct gamma-aminobutyric acid transporters in mouse brain [corrected]. J Biol Chem. 1993;268:2106–12.

Masson J, Sagne C, Hamon M, El Mestikawy S. Neurotransmitter transporters in the central nervous system. Pharmacol Rev. 1999;51:439–64.

Nelson N. The family of Na^+/Cl^- neurotransmitter transporters. J Neurochem. 1998;71:1785–803.

Richerson GB, Wu Y. Dynamic equilibrium of neurotransmitter transporters: not just for reuptake anymore. J Neurophysiol. 2003;90:1363–74.

Schleimer SB, Hinton T, Dixon G, Johnston GA. GABA transporters GAT-1 and GAT-3 in the human dorsolateral prefrontal cortex in schizophrenia. Neuropsychobiology. 2004;50:226–30.

Wu Y, Wang W, Diez-Sampedro A, Richerson GB. Nonvesicular inhibitory neurotransmission via reversal of the GABA transporter GAT-1. Neuron. 2007;56:851–65.

Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E. Crystal structure of a bacterial homologue of Na+/Cl–dependent neurotransmitter transporters. Nature. 2005;437:215–23.

GAT-1

► GAT (GABA Transporters)

GAT-2

► GAT (GABA Transporters)

GAT-3

► GAT (GABA Transporters)

GATA-3 (GATA Binding Protein 3)

Anuradha Ray, Anupriya Khare, Nandini Krishnamoorthy and Prabir Ray
Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, and Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Synonyms

GATA-binding protein 3

Historical Background

Cellular fate during development, differentiation, and function is tightly regulated and orchestrated in

a hierarchical fashion by transcriptional activators and repressors. GATA-3 is one such master regulator of cellular fate, which was identified in 1990 along with two other GATA family members and was found to be abundantly expressed in T lymphocytes and the brain (Yamamoto et al. 1990). GATA-3 was first described as a transcription factor that interacts with the TCR-α gene enhancer (Ho et al. 1991). It belongs to the GATA family of transcription factors that are conserved proteins containing one or two C2-C2-type zinc fingers and a highly conserved C4 zinc finger that recognizes a consensus DNA sequence A/TGATAA/G from which the name of the family originated (Hosoya et al. 2010). The mammalian GATA family of transcription factors consists of six members: GATA-binding protein 1 (GATA-1)–GATA-6. These proteins are highly homologous, conserved among species, have distinctive tissue-specific expression patterns, and play essential roles during vertebrate development. Based on their profile of tissue-specific expression, the GATA proteins can be classified as hematopoietic (GATA-1–GATA-3) or non-hematopoietic (GATA-4–GATA-6) (Hosoya et al. 2010). In contrast to GATA-1 and GATA-2 that are primarily expressed in hematopoietic cells, or GATA-4, -5, and -6 whose expression is restricted to mesoderm- and endoderm-derived tissues, such as the heart, liver, and intestines, GATA-3 is present in both hematopoietic (e.g., T cells) and non-hematopoietic tissues.

Similar to other GATA proteins, GATA-3 contains a distinct amino-terminal region that contains two trans-activation domains followed by two highly conserved zinc-finger domains in which the C-terminal finger and the immediately adjacent conserved basic region together constitute the DNA-binding domain (Hosoya et al. 2010). The laboratory of Engel was the first to clone the GATA-3 gene and further dissected important biochemical properties of the GATA-3 protein (George et al. 1994). The entire coding region of the murine GATA-3 locus is approximately 23-kilo base pairs. The gene is composed of six exons. Exon 1 is untranslated and exon 2 contains the initiation codon for murine GATA-3. The amino and carboxy zinc fingers are encoded by exons 4 and 5, respectively. Exon 6 comprises the 3′ untranslated region and the polyadenylation signal (George et al. 1994). For GATA-3 to regulate gene expression, it must translocate from the cytoplasm into the nucleus to access its target genes. GATA-3 contains a classical nuclear import signal, and is transported into the nucleus by importin-α. The affinity of GATA-3 to importin-α is regulated by phosphorylation, which is mediated by p38 mitogen-activated protein kinase (MAPK) (Maneechotesuwan et al. 2007). The GATA-3 protein is unstable with a short half-life (1 h) in developing Th2 cells. GATA-3 is ubiquitinated in vitro and in vivo and degraded by the 26s proteasome pathway. The deletion of the ubiquitination sites lends stability to the protein.

GATA-3 is the main GATA family member that is expressed in immune cells and can be easily detected in developing and mature T cells, natural killer (NK) cells, and CD1-restricted natural killer T (NKT) cells. Beyond the immune system, GATA-3 is expressed in many embryonic and adult tissues, including the adrenal glands, kidneys, central nervous system, inner ear, hair follicles, skin and breast tissue (mammary glands), and important functions for GATA-3 in several of these tissues have been shown in knockout and conditional knockout mouse models (Hosoya et al. 2010). In immune cells, GATA-3 is best known to function as a master regulator of T-helper-2 (Th2) cell differentiation (Zhang et al. 1997, 1999; Zheng and Flavell 1997). However, in recent years, GATA-3 has been found to have additional crucial functions in early T cell commitment, β-selection, and CD4+ T cell development (Hosoya et al. 2010).

GATA-3 is a critical regulator in both mouse and human development. The expression pattern of *GATA-3* during embryonic development, at least at the tissue level, is highly conserved among different vertebrates (George et al. 1994). In human embryos, *GATA-3* expression can be detected from the beginning of the fourth week of gestation (Debacker et al. 1999). From then on, *GATA-3* transcripts are observed in various developing embryonic and fetal tissues, including the developing kidney, the parathyroids, and the inner ear. GATA-3 null embryos die between E11 and E12 due to internal bleeding, and display growth retardation, deformities in the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis, suggesting that this gene is important in the development of various systems (Pandolfi et al. 1995). Haploinsufficiency of GATA-3 results in Barakat syndrome in humans, characterized by familial hypoparathyroidism, sensorineural deafness, and renal dysplasia (also known as HDR syndrome), and can be caused by mutations in GATA-3 that render it physically or functionally

inactive. Interestingly, mutations that abrogate the DNA-binding ability of GATA-3 are also found in human breast cancer specimens.

GATA-3: Essential for T Cell Development and Th2 Differentiation

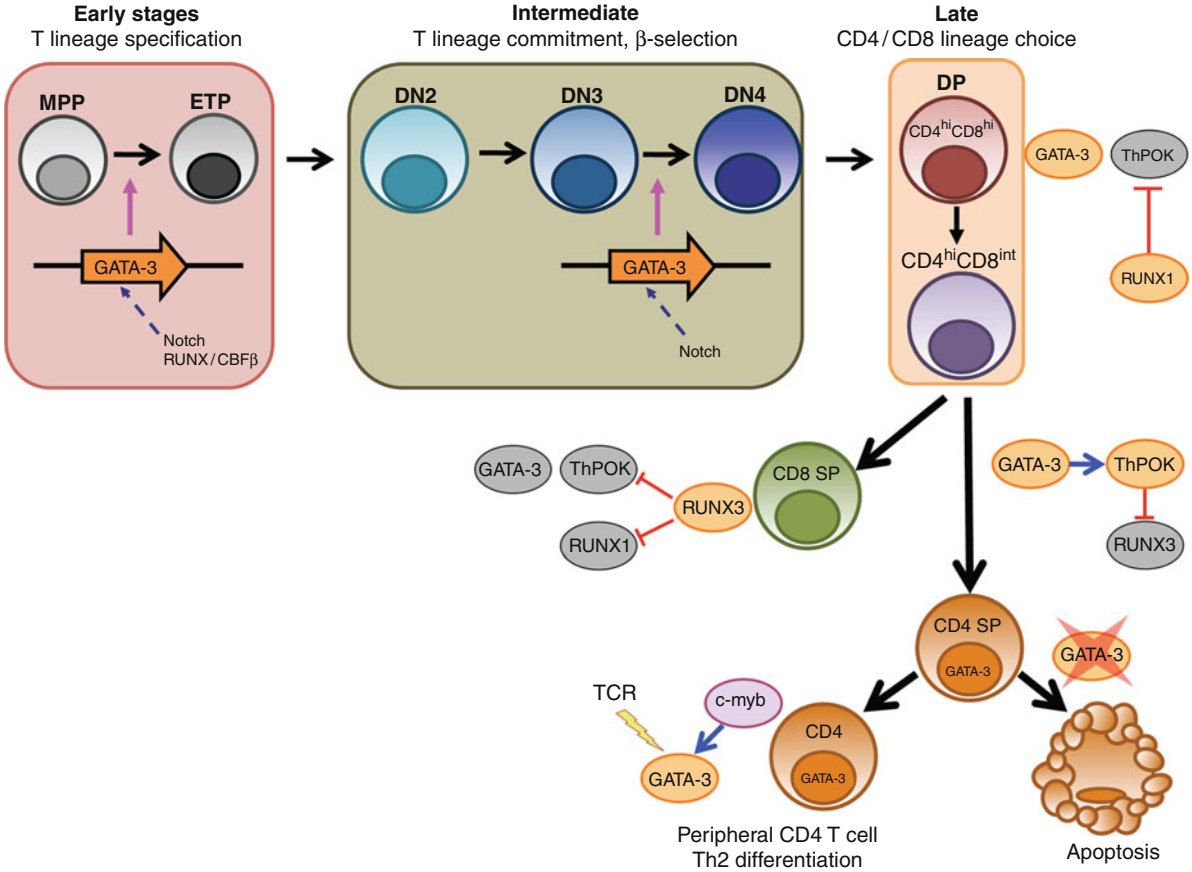
GATA-3 is one of the first genes that are transcriptionally activated in hematopoiesis as early as hematopoietic stem cells (HSCs) stage (Debacker et al. 1999). GATA-3 mRNA is also expressed in the multipotent progenitors (MPPs) and at the pre-pro-B cell stage in the bone marrow. Its involvement during early stages of thymopoiesis was first proposed in 1996 and poorer Thy1+ cell development was shown in fetal thymus organ culture following the introduction of antisense oligonucleotide to GATA-3 (Hattori et al. 1996). More definite information about the expression and role of GATA-3 in early stages of lymphopoiesis was provided by studies using various GATA-3 mutant mice. Collectively, these studies suggest that the major role of GATA-3 in thymopoiesis is to regulate the cellular differentiation of thymic stromal progenitors (TSPs) to ETPs, and in turn, downstream T cell development stages as well. Most likely the underlying mechanism does not involve cellular survival or proliferation although more conclusive investigations are required to support or dismiss the proposed hypothesis (Hosoya et al. 2010). Overexpression strategies, used to explore the role of GATA-3 in thymopoiesis also demonstrated that the expression level of GATA-3 decides the fate of lineage differentiation from early progenitors (Hosoya et al. 2010). It appears that a regulated low expression level of GATA-3 in pre-thymic progenitors is required to initiate thymopoiesis and induce T cell development.

Similar to early stages of thymopoiesis, GATA-3 has been shown to contribute to intermediate stages, especially the DN2-DN3-DN4 transition, of T cell development (Hosoya et al. 2010). Conditional ablation of GATA-3 in DN2-DN3 thymocytes was shown to result in abnormal accumulation of DN3 cells and reduced frequency of DN4 and later stages of T cells. On further analysis of the DN3 and DN4 thymocytes, it was observed that even though DN3 thymocytes from the GATA-$3^{flox/flox}$ Lck cre mice expressed rearranged Tcrβ gene, the intracellular expression of TCR β protein in DN4 cells was reduced as compared to that in the wild type. Also the frequency of Annexin V + apoptotic cells was higher in mutant mice as compared to control mice, suggesting that deficiency of GATA-3 affected DN3-DN4 transition and results in poorer expression of pre-TCR complex on DN4 thymocytes causing their elimination by apoptosis. GATA-3 has been also shown to be expressed in γδ T cells but its function and necessity in thymic γδ T cells is unknown (Hosoya et al. 2010). After β-selection, GATA-3 expression has been shown to be repressed and then later re-induced between early DP and late DP stages that continues to be expressed through CD4 SP stage, while decreasing in CD8 SP T cells (Hosoya et al. 2010). Figure 1 illustrates the central role of GATA-3 in T cell development.

Role of GATA-3 in the Development and Function of Other Hematopoietic Cells

In addition to T cells, GATA-3 is also expressed in other hematopoietic cell lineages such as natural killer (NK) cells and CD1-restricted natural killer T (NKT) cells (Hosoya et al. 2010). Recent studies have proposed a potential role for GATA-3 in development, maturation, and function of invariant NKT (iNKT) cells. These cells are a unique subset of T cells expressing Vα14Jα18 (mouse) or Vα24Jα18 (human) T cell receptors, which often dimerize with Vβ8, Vβ7, or Vβ2. Similar to the conventional α/β T cells, iNKT cells also originate from thymic DP cells but they are positively selected by CD1d molecules. Subsequent to successful positive selection, iNKT cells differentiate into either CD4+ or CD4-CD8- mature iNKT cells that constitute approximately 5% of the peripheral T cell pool (Kinjo and Kronenberg 2005). Following antigen encounter, this unique T cell subset has been shown to rapidly produce large amounts of various Th1 and Th2 cytokines including IFN-γ, IL-4, and IL-13, a phenomenon called cytokine storm. Over the years, multiple studies have demonstrated that iNKT cells play a crucial immunomodulatory role not only against infections, but are also involved in autoimmunity, allergy, and cancer.

As in the case of α/β T cells, the role of GATA-3 in iNKT cell development, differentiation, and function was also studied by either loss-of-function approach or by enforced expression of GATA-3 in the system. Mice in which *GATA-3* was deleted using *Cd4*-Cre were found to have near normal numbers of thymic



GATA-3 (GATA Binding Protein 3), Fig. 1 Role of GATA-3 in various stages of T cell development. GATA-3 is expressed at different stages (early, intermediate, and late) of T cell lymphopoiesis and regulates various cell fate decisions involving T lineage commitment and development. In the thymus, GATA-3 (induced by transcription factors like Notch and RUNX/CBFβ) plays a crucial role in cellular differentiation of MPPs to ETPs, DN3-DN4 transition, and β-selection. It also regulates CD4 versus CD8 lineage choice following positive selection. The commitment of $CD4^{hi}CD8^{int}$ cells to the CD4 SP lineage requires the expression of T-helper-inducing POZ/Kruppel-like factor (ThPOK), whereas commitment to the CD8 SP lineage requires the expression of runt-related transcription factor (RUNX). In $CD4^{hi}CD8^{int}$ cells, Runx1 contributes to Thpok repression. In CD4-differentiating cells, Runx1-mediated Thpok repression is relieved and further promoted by GATA-3. Thpok prevents Runx3 upregulation and CD8 differentiation. In CD8-differentiating cells, Thpok repression is maintained, presumably through Runx3. GATA-3 also regulates additional developmental events required for CD4 cell differentiation and further maturation of committed CD4 SP thymocytes

iNKT cells, but interestingly the majority of these were CD4-CD8- with a selective loss of CD4 iNKT cells which was similar to the loss of conventional CD4+ T cell in the absence of GATA-3 (Hosoya et al. 2010). Unlike in the thymus, there was a sixfold reduction of iNKT cells in peripheral lymphoid organs such as the spleen and almost complete lack in the liver suggestive of apoptosis of iNKT cells that did not develop or mature properly in the absence of GATA-3 indicated by failure to upregulate CD69 expression subsequent to their thymic egress. Lastly, GATA-3-deficient iNKT cells failed to respond to the NKT cell agonist α-galactosylceramide in vivo and mount a cytokine storm. This unresponsiveness is possibly due to defects in TCR signal transduction and was probably upstream of protein kinase C and calcium influx. This is because GATA-3-deficient iNKT cells were still capable of producing IFN-γ but not Th2 cytokines like IL-4, IL-5, or IL-13 that suggests that GATA-3 has a similar role in iNKT cells as in conventional T cells.

GATA-3 and Th2 Differentiation

The role of GATA-3 as a key transcription factor that is essential for Th2 differentiation is without doubt the most studied function of this protein. The role of this transcription factor as a master regulator of Th2 differentiation was independently codiscovered in the laboratories of Ray and Flavell (Zhang et al. 1997; Zheng and Flavell 1997). However, the initial clue for the involvement of GATA-3 in Th2 differentiation was provided by a prior study by Ray and colleagues that identified GATA-3 binding to the IL-5 promoter that was crucial for cyclic AMP-induced expression of the cytokine gene in T cells (Siegel et al. 1995). Subsequently, using Th1 or Th2 cells generated from naive $CD4^+$ T cells and representational differential analysis (RDA), the role of GATA-3 in Th2 differentiation was established (Zhang et al. 1997; Zheng and Flavell 1997). Although GATA-3 was identified as a key factor for Th2 development, a careful analysis of regulation of the individual Th2 cytokine genes showed that unlike the IL-5 promoter, the IL-4 promoter, which lacked high affinity GATA-binding sites, was not directly regulated by GATA-3 (Zhang et al. 1998). Furthermore, in Th2 cells, antisense GATA-3 RNA inhibited IL-5 but not IL-4 promoter activation (Zhang et al. 1998). If GATA-3 was only responsible for IL-5 activation, how could it be a master Th2 regulator? Rao and colleagues showed that a distal enhancer in the IL-4 gene binds GATA-3 to induce its expression (Agarwal et al. 2000). In light of this study, it was not surprising when conditional deletion of GATA-3 from CD4 T cells resulted in global suppression of all Th2 cytokines that included IL-4, IL-5, and IL-13 (Zhu et al. 2004). Furthermore, deletion of GATA-3 resulted in Th1 differentiation without the requirement for IL-12 and IFN-γ and inhibition of GATA-3 in differentiated Th2 cells caused a dramatic decrease in IL-5 and IL-13 expression although IL-4 expression was better preserved possibly due to the ability of other factors such as c-Maf that can directly regulate IL-4 but not IL-5 or IL-13 promoter activity (Zhu et al. 2004). These data taken together reiterated the dominant role of GATA-3 in Th2 differentiation. The expression of GATA-3 is significantly upregulated in human cells that express Th2 cytokine genes (Nakamura et al. 1999). Inhibiting its expression using siRNA strategy compromised Th2 differentiation and similar data was obtained in individuals with the loss of one functional GATA-3 allele.

One of the important attributes of GATA-3 that makes it a dominant Th2 transcription factor is its ability to autoactivate itself. This property of GATA-3 was a serendipitous finding in the laboratory of Murphy when they were investigating the mechanism underlying the ability of GATA-3 to promote Th2 differentiation in a STAT6-independent fashion (Ouyang et al. 2000). These investigators found that activation of naïve CD4 + T cells results in upregulation of endogenous GATA-3 expression, which was also evident in IL-4/STAT6-deficient T cells indicating that this property was not dependent on the initial secretion of IL-4 (Ouyang et al. 2000). Thus the maintenance and amplification of the Th2 loop is efficiently promoted by autoactivation of endogenous GATA-3 expression. p38 MAPK was shown to be important for cAMP-mediated phosphorylation of GATA-3 that promotes Th2 cytokine gene expression (Chen et al. 2000). Interestingly, corticosteroids, which are commonly used to treat allergic diseases, have a potent inhibitory effect on GATA-3 in T cells by competing for importin-α, and by inducing the expression of a p38 MAPK inhibitor (Maneechotesuwan et al. 2009).

GATA-3 in the Context of Other T Cell Transcription Factors

The induction and sustenance of a Th2 response is an intricately orchestrated process involving the concerted inhibition of select transcription factors while promoting others.

NF-κB is required for GATA-3 expression in differentiating Th2 cells. ▶ Nuclear factor kappa B (NF-κB) is a transcription factor involved in TCR-induced activation signals in peripheral T cells. Mice lacking the p50 subunit of NF-κB showed impaired GATA-3 induction that blunted Th2 responses (Das et al. 2001). This defect was specific to Th2 cells since p50-deficient mice showed normal T-bet expression and secretion of the Th1 cytokine, IFN-γ. Importantly, this defect was restricted to the initial differentiation of Th2 cells, and inhibition of p50 activity in differentiated Th2 cells did not affect GATA-3 expression or secretion of Th2 cytokines (Das et al. 2001).

STAT5 and GATA-3 synergize to promote Th2 phenotype. Naïve T cells require IL-2 for their differentiation and maintenance. IL-2 utilizes ▶ STAT5a and

▶ STAT5b for downstream signaling. Naïve T cells from STAT5a knockout mice are handicapped in their ability to differentiate into Th2 cells. In fact the constitutive expression of STAT5a in naïve T cells can induce the differentiation of Th2 cells even in the absence of IL-2 (Zhu et al. 2003). Furthermore, low TCR stimulation causes early IL-4 production, which is dependent on IL-2-mediated STAT5 signaling and also GATA-3. However, the early expression of GATA-3 can be achieved in an IL-4-independent fashion and is linked to autoactivation of GATA-3 expression (Ouyang et al. 2000). Also, neutralization of IL-2 cripples this early burst of IL-4 cytokine without compromising GATA-3 expression. Importantly, while the induction of a Th2 response has been shown to be dependent on both IL-2/STAT5 and GATA-3, these pathways operate independent of each other. GATA-3 is not considered to be downstream of IL-2 since the addition of IL-2 to in vitro culture can be delayed by 2 days without affecting the frequency of IL-4 producers. From these observations it became obvious that even if GATA-3 and STAT5 operate via independent pathways, these molecules synergize to promote Th2 responses. In fact, this hypothesis was proven correct in experiments in which forced expression of GATA-3 and STAT5 in naïve T cells or in Th1 cells induced a higher frequency of IL-4 producers when compared to cells expressing either factor alone (Zhu et al. 2006). While emphasizing the role of IL-2 in promoting Th2 responses it is also important to discuss the cooperation between GATA-3 and growth factor independent-1 (Gfi-1), which was initially cloned from an IL-2-independent cell line. The expression of this transcription factor is induced by IL-4 signaling in activated T cells and is dependent on STAT6. Deletion of Gfi-1 was shown to cause reduced expression of GATA-3 and IL-4. The few cells expressing GATA-3 in Gfi-1-deficient T cells were found to be unable to upregulate GATA-3 expression even with IL-2 supplementation. Conversely, forced expression of Gfi-1 in cells expressing high levels of GATA-3 resulted in a proliferative advantage of these cells. In summary, Gfi-1 is able to amplify the proliferation of high GATA-3-expressing CD4 T cells in response to IL-2 (Zhu et al. 2006). Taken together, these studies showed that Gfi-1, STAT5, and GATA-3 form the triad of Th2-promoting factors.

Notch and GATA-3. Notch signaling has been implicated in Th2 differentiation by directly regulating GATA-3 expression (Amsen et al. 2007) although more studies are needed to determine the importance of this regulation in vivo.

GATA-3 dominates STAT6 in Th2 induction. STAT6 is closely associated with Th2 responses and IL-13 and IL-4 trigger the phosphorylation of STAT6. Also, the kinetics of GATA-3 expression parallels STAT6 activation. The polarization of STAT6-deficient T cells under in vitro conditions does lead to the production of Th2 cytokines although less than that in WT cells, but more than in WT cells skewed under Th1 condition (Ouyang et al. 2000). Interestingly, analysis of DNaseI hypersensitivity sites in STAT6-deficient T cells reconstituted with GATA-3 reveals a pattern similar to that seen in WT cells, indicating that the chromatin remodeling fingerprint of a Th2 response is independent of STAT6 (Ouyang et al. 2000). Also, data from CTLA-4/STAT6 double knockout mice clearly show that in the absence of inhibitory signals, GATA-3 expression is sufficient to drive the differentiation of Th2 cells, illustrating the transcriptional dominance of GATA-3 in this process (Bour-Jordan et al. 2003). Although studies document evidence of STAT6-independent mechanisms of GATA-3 induction, the consensus is that STAT6 ensures optimal GATA-3 expression.

GATA-3 suppresses Th1 induction via STAT4. GATA-3 actively represses IL-12 and IFN-γ signaling both of which are critical for Th1 differentiation, implying a possible negative function of GATA-3 in Th1 development. Retroviral expression of GATA-3 during Th1 development results in the induction of Th2 phenotype with high levels of IL-4, IL-5, and IL-13 (Ferber et al. 1999). It was further revealed that the promotion of the Th2 phenotype could not simply be attributed to the high level of Th2 cytokines since the same results could be recapitulated in T cells deficient in both IL-4 and STAT6 and programmed toward a Th1 phenotype. GATA-3-mediated inhibition of Th1 differentiation is remarkable only when GATA-3 is introduced as early as day 1 in the Th1 differentiation program. If this "early development window" is missed then the effect of IL-12-mediated STAT4 signaling becomes dominant and expression of GATA-3 in committed Th1 cells results only in a modest decrease in IFN-γ and does not promote IL-4 production (Usui et al. 2003). Surprisingly, unlike in CD4 T cells, in NK cells, GATA-3 appears to be required for the production of IFN-γ (Samson et al. 2003). While there is no direct evidence that GATA-3

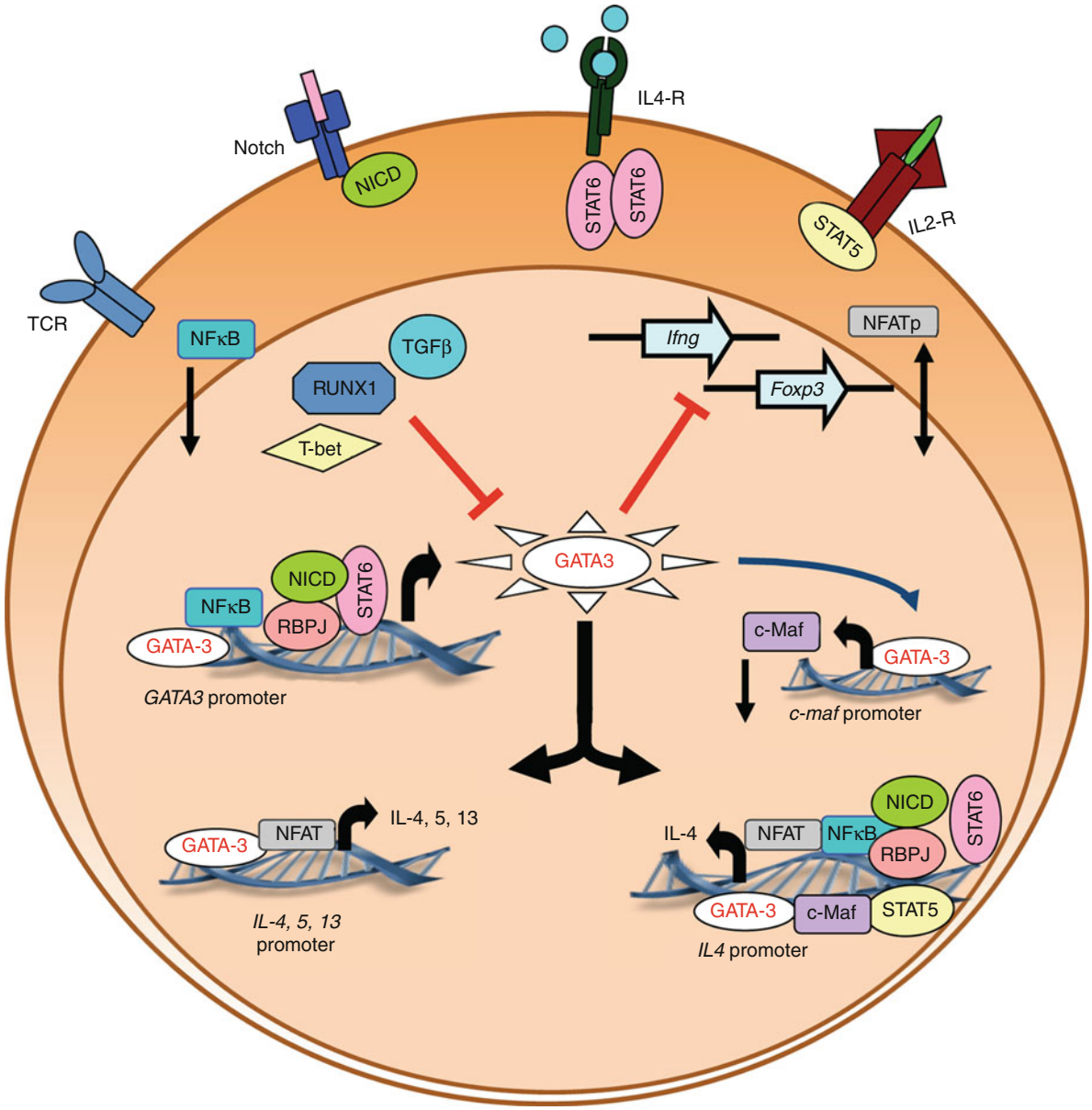
represses the promoter activity of IFN-γ, GATA-3 represses IL-12R β2 mRNA expression, thereby preventing IL-12 signaling during the early Th1 developmental window (Ouyang et al. 1998). Also GATA-3 expression significantly blunts ▶ STAT4 phosphorylation and promotes Th2 response. The mechanism behind GATA-3-mediated STAT4 suppression is unknown, but clearly the suppression of STAT4 phosphorylation is not mediated through effects on T-bet (Usui et al. 2003).

GATA-3 and NFAT1 bind to an enhancer region in the IL-4 gene. The NFAT family of transcription factors is activated by receptors involved in calcium mobilization, and their activation is blocked by cyclosporin A (CsA) and FK506. Rao and colleagues identified an enhancer region in the 3′ end of the IL-4 gene, designated V_A, and showed that both NFAT and GATA-3 bind to this enhancer region suggesting an active cooperation between these transcription factors in enhancing the expression of IL-4 (Agarwal et al. 2000).

GATA-3 derepresses RUNX3 silencing of IL-4. RUNX (runt-related transcription factor) proteins dictate important T cell lineage choices and consist of three family members, RUNX1, 2, and 3. These proteins have unique DNA-binding subunits that complex with the non-DNA-binding subunit core-binding factor β. It has been recently shown that RUNX3 can bind to a silencer region in the IL-4 gene and repress expression of the IL-4 gene (Naoe et al. 2007). Interestingly, the expression of RUNX3 is similar in Th1 and Th2 cells, which suggests that another molecule is responsible for derepressing the suppressive effect mediated by RUNX3. In fact, forced overexpression of GATA-3 in Th1 cells results in the dissociation of RUNX3 from the IL-4 silencer region (Naoe et al. 2007). It is interesting to note how GATA-3 promotes IL-4 secretion without directly influencing its promoter activity.

T-bet represses GATA-3 activity. T-bet, also called TBX21, is a member of the T-box gene family and its role in Th1 commitment was discovered by the Glimcher laboratory shortly after the role of GATA-3 in Th2 development was identified (Szabo et al. 2000). With the discovery of T-bet and GATA-3 it seemed highly probable that cross-regulation between Th1 and Th2 cells might underlie some form of antagonism between these two master regulators. The first clue to T-bet-mediated suppression of GATA-3 came from T-bet knockout mice, which expressed high levels of endogenous GATA-3 (Finotto et al. 2002). Also, retroviral expression of T-bet in developed Th2 cells not only promoted IFN-γ production, but also suppressed IL-4 and IL-5 production (Usui et al. 2006). This effect was found to be partly independent of STAT4. Furthermore, T-bet-deficient CD4 T cells produced IFN-γ if endogenous GATA-3 was suppressed that derepressed GATA-3-mediated inhibition of STAT4 lifted (Finotto et al. 2002; Usui et al. 2006). The possibility of T-bet directly inhibiting GATA-3 was formally explored by Glimcher and colleagues (Hwang et al. 2005). It was shown that the phosphorylation of T-bet occurs as early as day 2 during Th1 skewing and is mediated by IL-2 inducible T cell kinase ITK, which results in the tyrosine phosphorylation of T-bet at position 525 (Hwang et al. 2005). Mutation of this tyrosine residue prevented the physical association between GATA-3 and T-bet. In fact, T-bet was shown to physically interact with the N-terminal region of GATA-3 once it was phosphorylated and this sequestration of GATA-3 by T-bet prevented its binding to the IL-5 promoter. Thus an important function of T-bet is repression of GATA-3 binding to its target (Hwang et al. 2005).

GATA-3 and Tregs. Regulatory T cells (Tregs) promote tolerance and these cells express the transcription factor FOXP3, which is required for their suppressive activity. Interestingly, GATA-3 seems to be an important molecular switch which inhibits the expression of FOXP3 by directly binding to its promoter (Mantel et al. 2007). This repression is delicately balanced by the availability of IL-4. Low concentration of IL-4, which is not sufficient to induce robust expression of GATA-3, is important for triggering the expression of FOXP3 resulting in the conversion of naïve CD4 T cells into regulatory T cells. On the other hand, high concentration of IL-4 rapidly promotes GATA-3 expression, which in turn represses induction of FOXP3 (Mantel et al. 2007). Interestingly, there seems to be an innate mechanism, which predisposes cells expressing low levels of FOXP3 to convert into GATA-3-expressing Th2 cells (Wang et al. 2010). These data collectively show that the balance between tolerance and allergic inflammation mediated by Th2 cells is extremely fine-tuned in which GATA-3 plays an important regulatory role. The roles of multiple transcription factors with respect to GATA-3 function and their influence on Th2 differentiation are shown in Fig. 2.



GATA-3 (GATA Binding Protein 3), Fig. 2 GATA-3 in Th2 cell differentiation. GATA-3 is essential for Th2 differentiation. Shown are various other transcription factors that are involved in transcriptional regulation of Th2 cytokine genes along with GATA-3. GATA-3 also autoactivates its own expression and blocks Th1 development

GATA-3 and Disease

GATA-3 and Asthma. Asthma is a pathological condition associated with an overzealous Th2 response in response to innocuous antigens resulting in mucus production and eosinophilic infiltration. Studies have documented that asthmatics have a higher percentage of $CD4^+$ T cells producing IL-4, IL-5, and IL-13 which are secreted in the airways of patients with asthma (Walker et al. 1992; Robinson et al. 1992). The association of elevated Th2 responses and asthma underscores an important role for GATA-3 in the disease process. In fact, GATA-3 mRNA expression is significantly increased in the airways of asthmatic subjects compared with that in normal control subjects and positively correlates with IL-5 expression (Nakamura et al. 1999).

Mice expressing a dominant negative mutant of GATA-3 demonstrate reduced inflammation, low IgE levels and blunted eosinophilia (Zhang et al. 1999). Similarly, delivering antisense GATA-3 reduced airway inflammation (Finotto et al. 2001). The dominant effect of GATA-3 in driving an asthmatic response is further evident from studies analyzing the repressive effects mediated by other transcription molecules on GATA-3. For example, mice deficient in ROG display enhanced airway hyperresponsiveness and inflammation in response to Th2-specific antigenic stimulation (Hirahara et al. 2008). T-bet-deficient mice demonstrate spontaneous induction of asthma even in the absence of immunological challenge with enhanced eosinophilia and airway hyperresponsiveness (Finotto et al. 2002).

GATA-3 and breast cancer. In recent years, a dominant role for GATA-3 in development of mammary cells has come to light. Deletion of GATA-3 driven by K14-Cre resulted in the failure to develop mammary placodes. GATA-3 has been shown to have a dominant function in controlling the development of luminal cells in the mammary gland and the maintenance of the differentiated phenotype of the luminal cells in the adult mammary gland. More recently, bioinformatic analysis of breast cancer specimens revealed loss of expression of GATA-3 in poorly differentiated and highly metastatic tumors (Chou et al. 2010).

Summary

Since its cloning 20 years ago, a treasure trove of information on GATA-3 has been unearthed that encompasses its divergent role in the development and function of both hematopoietic and non-hematopoietic cells. Undoubtedly, it is best known as the master regulator of Th2 cell differentiation, which implicates it in allergic diseases such as asthma. Rather unexpectedly, GATA-3 plays a regulatory role in a completely different setting, which is the luminal cell that is essential for mammary gland development. Low GATA-3 expression has been associated with poor prognosis in breast cancer and is being considered as a better prognostic marker than the estrogen receptor status of the tumors. Clearly, GATA-3 casts a wide net and identification of its partners in different cell types in homeostasis and in disease may provide new opportunities for therapeutic intervention of immune- and nonimmune-mediated diseases.

Acknowledgments This work was supported by National Institutes of Health grants AI048927 and HL077430 (to A.R.) and HL048932 (to A.R. and P.R.).

References

Agarwal S, et al. Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. Immunity. 2000;12(6):643–52.

Amsen D, et al. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. Immunity. 2007;27(1):89–99.

Bour-Jordan H, et al. CTLA-4 regulates the requirement for cytokine-induced signals in T(H)2 lineage commitment. Nat Immunol. 2003;4(2):182–8.

Chen CH, et al. Cyclic AMP activates p38 mitogen-activated protein kinase in Th2 cells: phosphorylation of GATA-3 and stimulation of Th2 cytokine gene expression. J Immunol. 2000;165(10):5597–605.

Chou J, et al. GATA3 in development and cancer differentiation: cells GATA have it. J Cell Physiol. 2010;222(1):42–9.

Das J, et al. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat Immunol. 2001;2(1):45–50.

Debacker C, et al. Embryonic expression of the human GATA-3 gene. Mech Dev. 1999;85(1–2):183–7.

Ferber IA, et al. GATA-3 significantly downregulates IFN-gamma production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. Clin Immunol. 1999;91(2):134–44.

Finotto S, et al. Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression. J Exp Med. 2001;193(11):1247–60.

Finotto S, et al. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. Science. 2002;295(5553):336–8.

George KM, et al. Embryonic expression and cloning of the murine GATA-3 gene. Development. 1994;120(9):2673–86.

Hattori N, et al. Involvement of transcription factors TCF-1 and GATA-3 in the initiation of the earliest step of T cell development in the thymus. J Exp Med. 1996;184(3):1137–47.

Hirahara K, et al. Repressor of GATA regulates TH2-driven allergic airway inflammation and airway hyperresponsiveness. J Allergy Clin Immunol. 2008;122(3):512–20 e11.

Ho IC, et al. Human GATA-3: a lineage-restricted transcription factor that regulates the expression of the T cell receptor alpha gene. EMBO J. 1991;10(5):1187–92.

Hosoya T, et al. From the cradle to the grave: activities of GATA-3 throughout T-cell development and differentiation. Immunol Rev. 2010;238(1):110–25.

Hwang ES, et al. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science. 2005;307(5708):430–3.

Kinjo Y, Kronenberg M. V alpha14 i NKT cells are innate lymphocytes that participate in the immune response to diverse microbes. J Clin Immunol. 2005;25(6):522–33.

Maneechotesuwan K, et al. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. J Immunol. 2007;178(4):2491–8.

Maneechotesuwan K, et al. Suppression of GATA-3 nuclear import and phosphorylation: a novel mechanism of corticosteroid action in allergic disease. PLoS Med. 2009;6(5):e1000076.
Mantel PY, et al. GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. PLoS Biol. 2007;5(12):e329.
Nakamura Y, et al. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. J Allergy Clin Immunol. 1999;103(2 Pt 1):215–22.
Naoe Y, et al. Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. J Exp Med. 2007;204(8):1749–55.
Ouyang W, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity. 1998;9(5):745–55.
Ouyang W, et al. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity. 2000;12(1):27–37.
Pandolfi PP, et al. Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nat Genet. 1995;11(1):40–4.
Robinson DS, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med. 1992;326(5):298–304.
Samson SI, et al. GATA-3 promotes maturation, IFN-gamma production, and liver-specific homing of NK cells. Immunity. 2003;19(5):701–11.
Siegel MD, et al. Activation of the interleukin-5 promoter by cAMP in murine EL-4 cells requires the GATA-3 and CLE0 elements. J Biol Chem. 1995;270(41):24548–55.
Szabo SJ, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655–69.
Usui T, et al. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. Immunity. 2003;18(3):415–28.
Usui T, et al. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. J Exp Med. 2006;203(3):755–66.
Walker C, et al. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. Am Rev Respir Dis. 1992;146(1):109–15.
Wang Y, et al. An intrinsic mechanism predisposes Foxp3-expressing regulatory T cells to Th2 conversion in vivo. J Immunol. 2010;185(10):5983–92.
Yamamoto M, et al. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. Genes Dev. 1990;4(10):1650–62.
Zhang DH, et al. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem. 1997;272(34):21597–603.
Zhang DH, et al. Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. J Immunol. 1998;161(8):3817–21.
Zhang DH, et al. Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. Immunity. 1999;11(4):473–82.
Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell. 1997;89(4):587–96.
Zhu J, et al. Stat5 activation plays a critical role in Th2 differentiation. Immunity. 2003;19(5):739–48.
Zhu J, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol. 2004;5(11):1157–65.
Zhu J, et al. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res. 2006;16(1):3–10.

GATA-Binding Protein 3

▶ GATA-3 (GATA Binding Protein 3)

Gb

▶ G Protein Beta/Gamma

GC1L

▶ ADAP1

GC-A

▶ Guanylyl Cyclase Receptors

GCAP (Guanylate Cyclase–Activating Protein)

Karl-Wilhelm Koch
Department of Biology and Environmental Sciences, University of Oldenburg, Oldenburg, Germany

Synonyms

Guanylyl cyclase–activating protein

Historical Background

The primary processes of vertebrate visual excitation are located in the rod and cone photoreceptor cells of the retina. Illumination of the rod and cone cells

GCAP (Guanylate Cyclase–Activating Protein), Fig. 1 Domain topography of a GCAP molecule highlighting the four EF-hand Ca^{2+}-binding motifs and the myristoyl group at the amino terminus

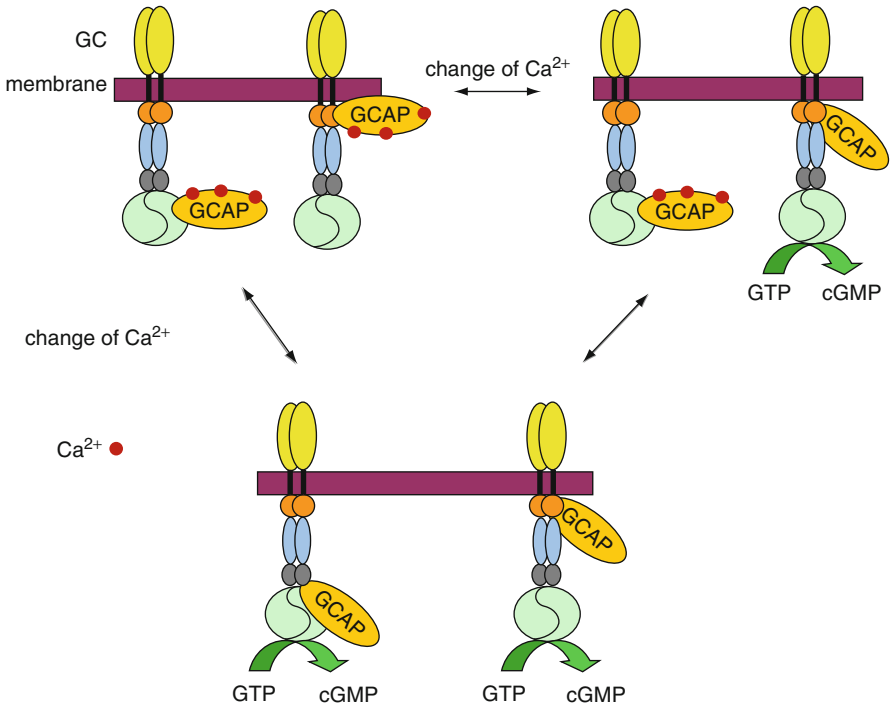
triggers a biochemical cascade leading to hyperpolarization of the cell. This conversion of a light signal into an electrical signal is called phototransduction, and in the 1970s the concept of an intracellular second messenger mediating this process was developed. Two competing hypotheses were intensively discussed, the "calcium hypothesis" and the "cGMP hypothesis" to reconcile different lines of experimental results. With the identification of a cGMP-gated cation channel (cyclic nucleotide–gated channel, CNG-channel) in the plasma membrane of rod and cone cells, the second messenger of light excitation was finally identified (for a historical overview see Luo et al. 2008). Calcium on the other hand was found to be important for the sensitivity regulation (light adaptation) of photoreceptor cells (Fain et al. 2001)and was recognized as an essential part of negative feedback loops operating in photoreceptor cells ("calcium feedback"). In the current picture of phototransduction, light is absorbed by visual pigments thereby triggering a G protein–mediated signaling cascade and leading to the hydrolysis of cGMP by a phosphodiesterase. CNG-channels are open at the high concentration of cGMP in the dark and close after removal of cGMP. Synthesis of cGMP is catalyzed by retina-specific guanylate cyclases (GCs) according to the reaction scheme GTP → cGMP + pyrophosphate (PP_i). This step is under control of a calcium feedback, low Ca^{2+}-concentrations increase and high Ca^{2+}-concentration decrease GC activity. However, this regulatory step can only be of physiological relevance, if the cytoplasmic Ca^{2+}-concentration changes over the time course of a light response. Indeed this was observed, since the cytoplasmic concentration of Ca^{2+} decreases after illumination due to the halted influx of Ca^{2+} through the CNG-channel and the continuous extrusion via a Na^+/K^+, Ca^{2+}-exchanger. Thus a decrease in cytoplasmic Ca^{2+} accelerates cGMP synthesis. While this concept was developed in the 1980s, it became clear that the Ca^{2+}-sensitive regulation of photoreceptor GC activity is mediated by a soluble Ca^{2+}-binding protein (Koch and Stryer 1988). The consequent search for this protein led to the identification of a novel class of Ca^{2+}-binding proteins, named guanylate cyclase–activating proteins (GCAPs) (Stephen et al. 2008; Dizhoor et al. 2010). Soon after their discovery and the determination of their amino acid sequence, it became apparent that GCAPs belong to the subfamily of neuronal calcium sensor (NCS) proteins, a group of EF-hand Ca^{2+}-binding proteins mainly found in neurons and sensory cells (Burgoyne 2007).

GCAP Isoforms

First discovered in mammals, GCAP isoforms have been identified in many vertebrates including human, bovine, monkey, mice, chicken, fish, and amphibians. The different isoforms are classified on the basis of their amino acid sequences and are given a number like in GCAP1, GCAP2, etc. So far mammalian rod and cone cells express two or three GCAP isoforms, which are GCAP1, GCAP2, and GCAP3, but a larger variety was found in teleost fish, where the expression of eight isoforms was predicted in pufferfish (*Fugu rubripes*) and six isoforms were described in zebrafish (*Danio rerio*) and carp (*Cyprinus carpio*). The physiological meaning of variable GCAP expression is currently under investigation (Rätscho et al. 2010).

Main Features of Protein Structure

GCAPs are small Ca^{2+}/Mg^{2+}-binding proteins of approximately 200 amino acid length with acidic and hydrophobic properties. They harbor four EF-hand Ca^{2+}-binding motifs in their primary structure, of which the first one (EF-hand1) seems to be nonfunctional based on amino acid sequence comparison with canonical EF-hand structures (Fig. 1). GCAPs are modified at the amino terminus by acyl groups mainly myristoyl (so far this has not been verified for all GCAP isoforms). In this aspect they are similar to other NCS proteins that are myristoylated at the amino terminus. However, the myristoyl group confers different functions to different NCS proteins. High-resolution structures of GCAPs (GCAP1, GCAP2, and GCAP3 either by x-ray crystal analysis or nuclear magnetic resonance) showed



GCAP (Guanylate Cyclase–Activating Protein),
Fig. 2 Mode of GC activation and regulation by GCAPs. In the dark state of the photoreceptor cell, GCAPs with three Ca^{2+} bound interact with the GC dimer. GCAP isoforms might have different interactions sites on GC as this was demonstrated for bovine GCAP1 and GCAP2. Decrease of cytoplasmic Ca^{2+} triggers conformational changes in GCAPs, but at different Ca^{2+}-concentrations for each GCAP. This leads to a sequence of activation steps and a differential mode of GC regulation (Ca^{2+}-relay model)

a general match to other known three-dimensional structures of NCS proteins (the best investigated example is ▶ recoverin, another Ca^{2+}-sensor protein operating in vision). In the Ca^{2+}-bound state GCAPs are compact proteins with the four EF-hand motifs arranged in two domains. These two domains separate the protein in an amino-terminal and a carboxy-terminal part (Stephen et al. 2008).

Tissue Distribution

Tissue distribution of GCAPs had been investigated by in situ hybridization and/or immunocytochemistry. All GCAPs show a prominent transcription and/or expression in the outer vertebrate retina, presumably in the outer and inner segments of photoreceptor cells, but GCAP-specific staining was also observed in cone somata, cell bodies, axons, axon terminals, and synaptic pedicles. A comparative study on several mammalian retinas showed a species-dependent labeling pattern for GCAP1 and GCAP2. Furthermore, for some species as human and zebrafish (but not mice) GCAP3 appeared as a cone-specific isoform of GCAP. Other teleost fish–specific isoforms like for example GCAP4, GCAP5, and GCAP7 are also cone specific (Imanishi et al. 2004; Rätscho et al. 2009; Takemoto et al. 2009).

Regulation of Guanylate Cyclase Activity

GCAPs regulate the activity of sensory GCs, in particular, the photoreceptor cell–specific forms. A triggering step of GCAP function is the light-induced decrease of the intracellular messenger cGMP and the consequent decrease of the cytoplasmic Ca^{2+}-concentration in a rod or cone cell. Since GCAPs operate as Ca^{2+}-sensors, they can detect changing concentrations of Ca^{2+} and thereby undergo a conformational change. GCAPs form a complex with the target GC in the Ca^{2+}-free and Ca^{2+}-bound state and it is assumed that the Ca^{2+}-induced conformational change is transferred to the GC by protein–protein interaction. This in turn is thought to lead to an increase of catalytic activity at the cyclase catalytic domain (Fig. 2). Binding of ATP to the intracellular kinase homology domain is known to enhance the GC activity (Koch et al. 2010). The switch of a GCAP molecule from an inhibitor to an activator state does critically depend on the binding of Ca^{2+} at submicromolar and binding of Mg^{2+} at submillimolar concentrations. In the Ca^{2+}-bound state (three Ca^{2+} bound) GCAPs suppress GC activity, in some cases below the basal level. Dissociation of Ca^{2+} from GCAP facilitates the exchange of Ca^{2+} for Mg^{2+} at two EF-hands, which is important for binding to the target GC and increase of GC activity (Dizhoor et al. 2010).

Although GCAPs have several general properties (see above) in common, they differ in many aspects particularly concerning their regulatory properties. One characteristic distinguishing feature is the different Ca^{2+}sensitivity, by which GCAP1 and GCAP2 regulate GC activity. Both proteins are present in rod and cone photoreceptor cells and overlap in their expression profile. A physiological meaning of this observation could be a so-called Ca^{2+}-relay model of GC activation (Koch 2006; Burgoyne 2007). It is based on the observations that GCAP1 and GCAP2 are present in almost equal concentrations and that they equal in total the concentration of a GC dimer in rod cells. In the dark state of the cell, when the cytoplasmic Ca^{2+}-concentration is high (Fig. 2, upper part) both GCAPs bind to a GC dimer (for simplicity every dimer is associated with one GCAP molecule). In this state, the GC activity is very low and may just maintain the cytoplasmic dark concentration of cGMP by keeping a balance with the low dark activity of the cGMP hydrolyzing *phosphodiesterase*. Illumination of rod or cone cells leads to a decrease in cGMP and consequently to a fall in Ca^{2+}. Depending on the light conditions and on the bleaching protocol, the cytoplasmic Ca^{2+}–concentration reaches an intermediate level, which transforms only GCAP1 into an activator of the GC and keeps GCAP2 still in an inactive state (Fig. 2, upper part, right side). This differential activating modus is in accordance with the observed differences in Ca^{2+}-sensitivity. Stronger illumination then causes a further decrease of intracellular Ca^{2+}, which induces the transition of GCAP2 into the activator state (Fig. 2, lower part). Changing light intensity of light flashes and background light might establish a different steady state of cytoplasmic Ca^{2+} causing a switch between different GCAP modes. By this mechanism a photoreceptor cell could expand its dynamic range of responses to Ca^{2+}.

GCAPs in Inherited Retinal Diseases

Dysfunction or progressive visual impairment leading to blindness is often associated with inherited retinal diseases. Many proteins in photoreceptor cells with key functions for cell excitation and adaptation are known to be mutated in patients suffering from these diseases. Mutations in the GCAP gene are very often located within or near the third or fourth EF-hand and correlate with diseases like autosomal dominant cone or cone-rod dystrophies (Stephen et al. 2008; Behnen et al. 2010). Disease-related GCAP1 mutants show in almost all cases an incomplete suppression of GC activity at physiological Ca^{2+}-concentration in the dark leading to a constitutive activation of the target enzyme. In turn this causes an elevated cGMP level in the cell and a distortion of the Ca^{2+}-homeostasis.

Summary

The second messenger cGMP and its fine-tuned regulation of hydrolysis and synthesis are keystones of the molecular events in phototransduction. Regulation of sensory GCs by GCAPs represents a concept of cellular signaling different from the more classical case of cGMP synthesis control via hormone binding to a receptor GC (e.g., natriuretic peptide receptor GCs). Instead GCAPs are a specialized group of neuronal Ca^{2+}-binding proteins that detect changes in cytoplasmic Ca^{2+} and control GC activity on the cytoplasmic part of the membrane-bound GC. The diversity of GCAPs in fishes, in particular in cones of the fish retina could further indicate that they are an essential part of a "Ca^{2+}-relay model" operating in cone vision under changing illumination conditions. Finally, GCAPs might participate in regulatory protein–protein interactions involving other target proteins, as this has already been shown for GCAP2. The variety of GCAPs found in teleost fishes could also hint to a wider diversity of GCAP target molecules.

References

Behnen P, Dell'Orco D, Koch KW. Involvement of the calcium sensor GCAP1 in hereditary cone dystrophies. Biol Chem. 2010; 391:631–7.

Burgoyne RD. Neuronal calcium sensor proteins: generating diversity in neuronal $Ca2^{+}$ signaling. Nat Rev Neurosci. 2007; 8:182–93.

Dizhoor AM, Olshevskaya EV, Peshenko IV. Mg^{2+}/Ca^{2+} cation binding cycle of guanylyl cylase activating proteins (GCAPs): role in regulation of photoreceptor guanylyl cyclase. Mol Cell Biochem. 2010;334:117–24.

Fain GL, Matthews HR, Cornwall C, Koutalos Y. (Adaptation in vertebrate photoreceptors. Physiol Rev. 2001;81:117–51.

Imanishi Y, Yang L, Sokal I, Filipek S, Palczewski K, Baehr W. Diversity of guanylate cyclase-activating proteins (GCAPs) in teleost fish: characterization of three novel GCAPs (GCAP4, GCAP5, GCAP7) from zebrafish (Danio rerio) and prediction of eight GCAPs (GCAP1-8) in pufferfish (Fugu rubripes). J Mol Evol. 2004;59:204–17.

Koch KW. GCAPs, the classical neuronal calcium sensors in the retina – a Ca^{2+}-relay model of guanylate cyclase activation. Calcium Bind Proteins. 2006;1:3–6.

Koch KW, Stryer L. Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. Nature. 1988;334:64–6.
Koch KW, Duda T, Sharma RK. Ca^{2+}-modulated vision-linked ROS-GC guanylate cyclase transduction machinery. Mol Cell Biochem. 2010;334:105–15.
Luo DG, Xue T, Yau KW. How vision begins: an odyssey. Proc Natl Acad Sci USA. 2008;105:9855–62.
Rätscho N, Scholten A, Koch KW. Expression profiles of three novel sensory guanylate cyclases and guanylate cyclase-activating proteins in the zebrafish retina. Biochim Biophys Acta. 2009;1793:1110–4.
Rätscho N, Scholten A, Koch KW. Diversity of guanylate cyclases in teleost fishes. Mol Cell Biochem. 2010;334:207–14.
Stephen R, Filipek S, Palczewski K, Sousa MC. Ca^{2+}-dependent regulation of phototransduction. Photochem Photobiol. 2008; 84:903–10.
Takemoto N, Tachibanaki S, Kawamura S. High cGMP synthetic activity in carp cones. Proc Natl Acad Sci USA. 2009; 106:11788–93.

GC-B

► Guanylyl Cyclase Receptors

GC-C

► Guanylyl Cyclase Receptors

GC-D

► Guanylyl Cyclase Receptors

GC-G

► Guanylyl Cyclase Receptors

GCS1L

► ADAP1

GDA1

► E-NTPDase Family

GEFT, p63RhoGEF

► ARHGEF25

gep

► G Protein Alpha 12

Gi Protein Alpha Subunit

► G Protein α i/o/z

Gie1

► Arl8b

gIP-10

► CXCL10

Glucose-Dependent Insulinotropic Polypeptide Receptor (*GIPR*)

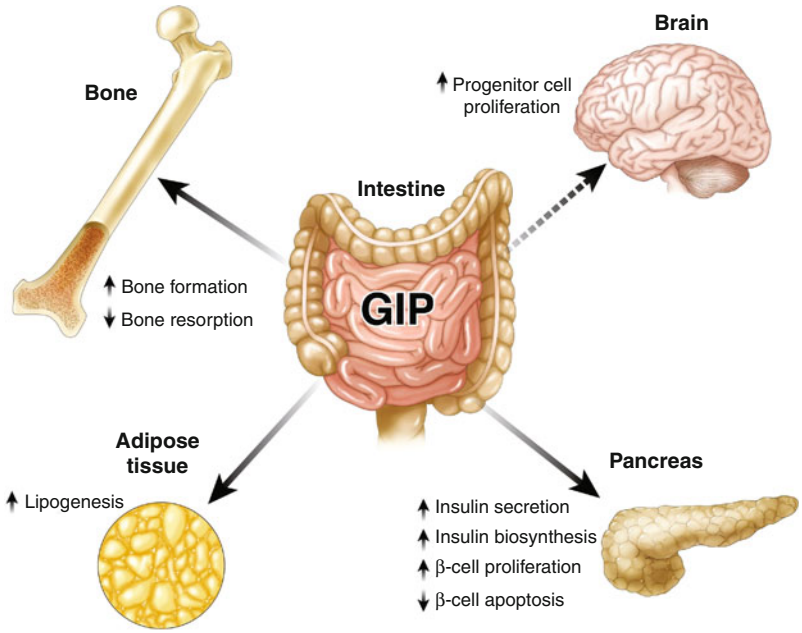
Rakesh Chandarana[1], Jacinta S. D'Souza[2] and Evans C. Coutinho[1]
[1]Bombay College of Pharmacy, Mumbai, India
[2]UM-DAE-Centre for Excellence in Basic Sciences, Kalina campus, Santacruz (E), Mumbai, India

Synonyms

Gastric inhibitory polypeptide receptor

Historical Background

Way back in 1900, duodenal mucous extract was shown to inhibit gastric acid secretion. It was in 1930 that Kosaka and Lim observed that intravenous



Glucose-Dependent Insulinotropic Polypeptide Receptor (*GIPR*), Fig. 1 GIP actions in peripheral tissues (Reproduced with permission from Elsevier-L no. 2731900143478)

infusion of intestinal extracts led to inhibition of gastric acid secretion and gastric emptying; the term "enterogastrone" was subsequently floated (Kosaka and Lim 1930; Meier et al. 2002). Brown and coworkers purified this extract to homogeneity and reported the amino acid sequence of this newly found hormone naming it as "Gastric Inhibitory Polypeptide" (GIP) since the latter also inhibited gastric acid secretion. In parallel, Moore and coworkers had for the first time (1906) found antidiabetogenic effect of duodenal mucous membrane extract (Moore 1906). The development of immunoassay for insulin in 1960 revealed that the duodenal extract stimulated secretion of insulin from pancreas in humans. It was also observed that orally administered glucose instigated higher insulin secretion as compared to glucose administered intravenously. This established a hormonal link between intestine and pancreas which was later termed as enteroinsular axis. Subsequent experimentation led to the finding that two peptides (commonly termed as incretins) were released from intestine upon oral food intake that led to the increased insulin secretion from the pancreas. Interestingly, one of the peptides was found to be same as GIP and since it was involved in glucose-mediated insulin secretion, it was renamed by Brown and Pederson as glucose-dependent insulinotropic polypeptide (GIP) maintaining the same acronym; the other peptide being termed as glucagon like polypeptide-1(GLP-1). The insulinotropic effect on pancreatic islet β-cells was then recognized to be the principal physiologic action of GIP. Together with glucagon-like peptide-1, GIP is largely responsible for the secretion of insulin postprandially. In the course of trying to elucidate the molecular mechanism of insulin secretion and GIP effect, functionally relevant and high affinity-binding sites for GIP as GIP receptors were identified on the surface of hamster β-cells by radioisotopic assay using ^{125}I-labeled GIP (Amiranoff et al. 1984, 1985, 1986). Subsequently, the expression of the mRNA for GIP receptor was observed in pancreatic cells followed by its cloning, functional expression, sequencing, and chromosomal localization (Takeda et al. 1987; Gremlich et al. 1995; Yamada et al. 1995).

Physiological Actions of GIP

GIP is known to exert myriad of physiological effects on tissues such as pancreas, central nervous system, bone, adipose tissue, stomach, and liver (Fig. 1). In the pancreas, it potentiates the glucose-dependent insulin secretion from the β-cells. It also positively influences insulin biosynthesis and increases β-cell survival

(Drucker 2006). In the CNS, it enhances the proliferation of hippocampal progenitor cells and enhances sensorimotor coordination and memory recognition. In adipose tissue, it increases the deposition of fat via increased synthesis of lipoprotein lipase, stimulates fatty acids synthesis and re-esterification, enhances incorporation of fatty acid into triglycerides and reduction of lipolysis. It increases the density of bone and enhances new bone formation. It is also shown to exert an inhibitory effect on gastric acid secretion and upregulates intestinal hexose transport. In the liver, it reduces the glucagon-stimulated hepatic glucose production. By and large, the physiological effects of GIP are exerted via the receptor that is a G-protein coupled receptor (namely, GIPR).

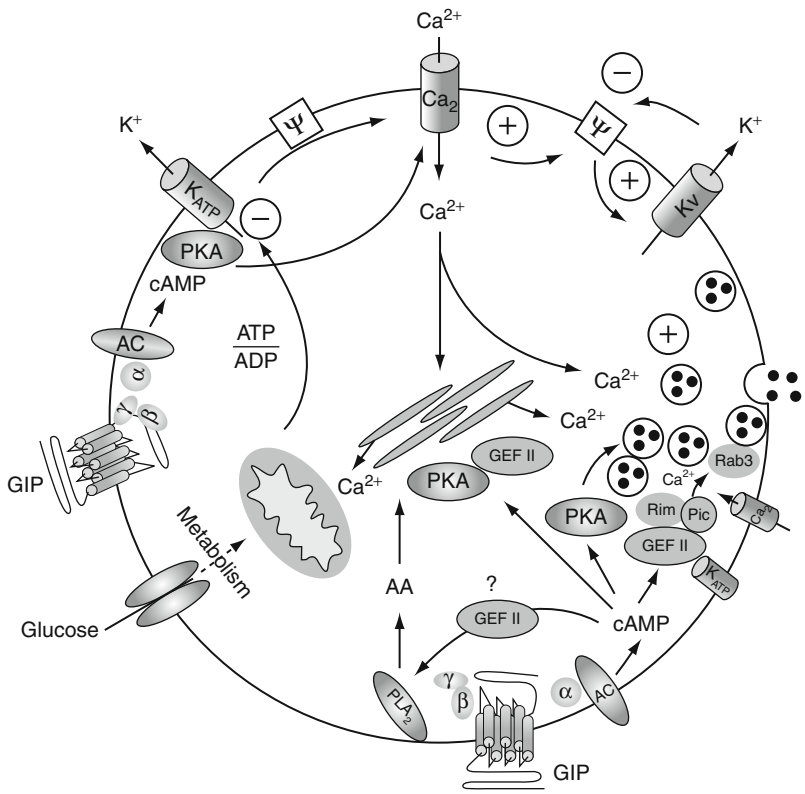
Glucose-Dependent Insulinotropic Polypeptide Receptor (GIPR)

The human GIPR (hGIPR) is a 466 amino acid, seven transmembrane (heptahelical) receptor belonging to class B of the G-protein-coupled receptor (GPCR) family. It is a glycoprotein, well conserved across all mammalian cells. However, GIP receptors have not been identified in non-mammalian species to date. The gene encoding hGIPR is located on chromosome 19q13.3 and comprises of 14 exons and 12 introns (Gremlich et al. 1995). GIPR is abundantly expressed on the surface of the β-cells of the pancreas and those of the adipose tissue, bone, and nerves. It is also known as a secretin receptor since it belongs to the same family that contains secretin, glucagon, glucagon-like peptide-1 (GLP-1), vasoactive intestinal polypeptide (VIP), growth-hormone-releasing hormone (GHRH), and pituitary adenylate cyclase-activating polypeptide (PACAP). It is involved in the transmission of vital secretory and mitogenic signals to the inside of the cell and activates the intracellular signal transduction pathways (discussed later). GIPR is activated by the binding of GIP, secreted by the K-cells of the duodenum. GIP comes into blood circulation in response to nutrient (fatty acid, glucose) absorption in proportionate amount and is involved in the secretion of insulin from the β-cells of pancreas and thus it is known to be glucose dependent. The insulinotropic action of the peptide is known as incretin effect and due to this GIP-GIPR has immense antidiabetogenic potential (Gault et al. 2003; Ranganath 2008).

Structural Organization and Dynamics of GIP–GIPR Interaction

Like any GPCR, the GIPR assembly on the cell membrane has five domains – the extracellular N-terminus domain (residues 22–138), intracellular C-terminus domain (residues 399–466), seven transmembrane domains (TM) (TM1 residues139–161, TM2 residues 170–189, TM3 residues 218–242, TM4 residues 255–278, TM5 residues 294–319, TM6 residues 342–362, TM7 378–398), three extracellular loops (ECl) (ECl1 residues 190–217, ECl2 residues 279–293, ECl3 residues 363–377) and three intracellular loops (ICl) (ICl1 residues 162–169, ICl2 residues 243–254, ICl3 residues 320–341). The transmembrane domains are linked to each other by intracellular loops on the inside of the cell and by the extracellular loops on the outside. The C-terminus of the receptor is coupled to a heterotrimeric GTP-binding protein also called G-protein, which is made up of three subunits Gα, Gβ, and Gγ. The Gα subunit harbors a catalytic site for binding GTP and in an inactive state of the receptor it is bound to GDP. Upon activation by the receptor, GTP displaces GDP at the Gα subunit, thereby activating it. The GβGγ dimer dissociates from the trimeric form and Gα activates its membrane bound effector, adenylyl cyclase, causing a cascade of reactions in the downstream signaling pathway (Baggio and Drucker 2007; McIntosh et al. 2009).

According to the receptoral dynamics suggested by Hoare, the N-terminus of the receptor is responsible for high affinity binding to the C-terminus of GIP (Hoare 2005). Once bound, the N-terminus of GIP is projected onto the extracellular loops of GIPR for its activation. Upon activation, the signal is transmitted within the cell through one of the transmembrane domains to the intracellular C-terminus domain. A recently solved crystal structure (Parthier et al. 2007) of GIP bound to the extracellular N-terminus domain reveals the N-terminus domain of the receptor as a α-helical structure spanning amino acids Ala32 to Ala52 and the two antiparallel β sheets (β1a: Ser64 to Phe65; β1b: Cys70 to Trp71; β2a: Ala78 to Ser83; β2b: Phe98 to Cys103) and two short helices at the C–terminus end (His91 to Val94 and Thr116 to Cys118). There are three disulfide bonds, a typical characteristic of family B GPCR, these are between the N-terminus and the first β sheet (Cys46 and Cys70), between the two β sheets (Cys61 and Cys103) and



Glucose-Dependent Insulinotropic Polypeptide Receptor (*GIPR*),

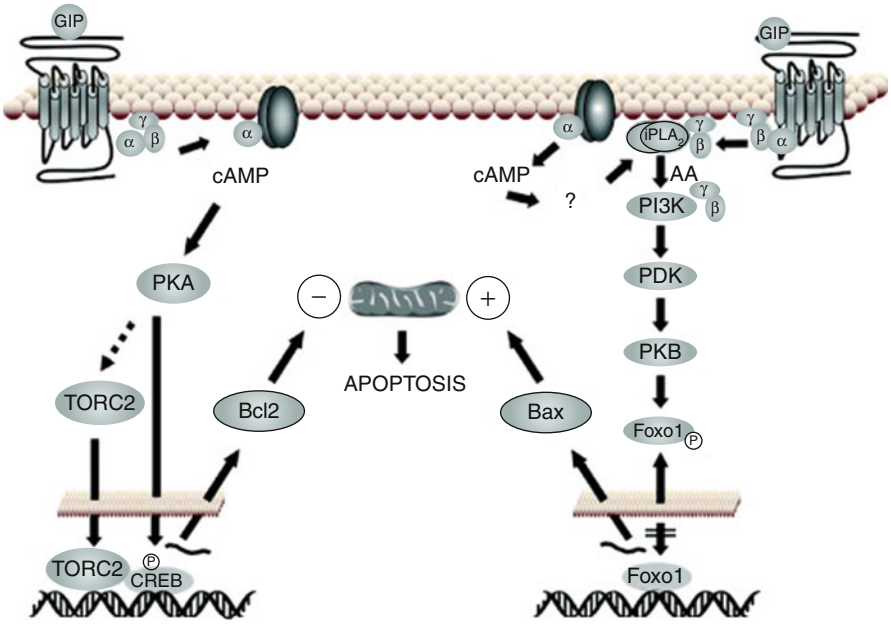
Fig. 2 Representation of the main signaling pathways by which glucose and GIP are proposed to stimulate insulin secretion. *cAMP* cyclic AMP, *PKA* protein kinase A, *GEFII* guanine nucleotide exchange factor II, *Pic* piccolo; Rim2, regulating synaptic membrane exocytosis 2; Rab2 (member RAS oncogene family); PLA_2, Ca^{2+}-independent phospholipase A_2; K_{ATP}, ATP-dependent K^+ channel; Ca_v, voltage-dependent Ca^{2+} channel; K_v, voltage-dependent K^+ channel (Reproduced with permission from Elsevier-L no. 2731910996902)

between Cys84 and Cys118. GIP is also seen to be composed of an α helix that spans Phe6 to Ala28. The helix is partly amphipathic in nature between Gln20 and Ala28 with all the hydrophobic amino acids (Phe22, Val23, Leu26, and Leu27) aligned on one side and projected towards the N-terminus domain of the receptor; this suggests that the interaction between GIP and its receptor is primarily hydrophobic in nature. Apart from hydrophobic interactions, hydrogen bonding also occurs between the peptide and the receptor domain.

The Signal Transduction Pathways of GIP-GIPR

Insulin Secretory Pathway

Insulin secretion is primarily mediated by signals originating from two sources – one by glucose and the other by incretins (Fig. 2). When the postprandial blood glucose concentration increases, it is taken up into the pancreatic cells via Glut2 uniporter. Inside the cell, glucose undergoes glycolysis and mitochondrial oxidation by Krebs cycle, thus generating ATP. This leads to an increase in the ATP/ADP ratio and closure of the K^+ (K_{ATP}) channel. As a result, depolarization of the cell membrane occurs, leading to opening of the voltage-gated calcium channel with an influx of Ca^{2+} inside the cells. The increased cytoplasmic Ca^{2+} mobilizes the vesicle-containing insulin to the surface of the cell membrane; exocytosis of its content releases insulin. GIPR is shown to have a potentiating effect on the insulin secretion. Upon activation, it stimulates the ▶ adenylyl cyclase enzyme via G-protein and catalyzes the formation of cAMP. The resultant increase in cAMP further activates the protein kinase A (PKA)-dependent as well as protein kinase A (PKA)-independent pathways (Baggio and Drucker 2007; McIntosh et al. 2009). The PKA-dependent pathway involves GIPR-mediated activation of PKA which phosphorylates a number of downstream proteins including Glut2, K^+ (K_{ATP}) channel and the voltage-gated Ca^{2+} channel leading to exocytosis of insulin from the secretory vesicles. On the other hand, the PKA-independent pathway involves cAMP-specific guanine nucleotide exchange factor (GEF) II also



Glucose-Dependent Insulinotropic Polypeptide Receptor (*GIPR*),
Fig. 3 Diagram of proposed pathways by which GIP increases expression of Bcl2 and decreases expression of bax. See test for details. *cAMP* cyclic AMP, *PKA* protein kinase A, *TORC2* cAMP-responsive ▶ CREB coactivator 2, ▶ *CREB* cAMP-response element-binding protein, *Bcl2* B-cell leukemia/lymphoma 2, *Bax* Bcl2-associated X protein, *PI3K* phosphoinositide-3-kinase, *Foxo1* forkhead box O1, *iPLA*$_2$ Ca^{2+}-independent phospholipase A_2 (Reproduced with permission from Elsevier-L no. 2731910996902)

known as Epac, which under inactive state is associated with Kir6.2. The increased cAMP produced due to GIPR stimulation dissociates the GEFII from Kir6.2, which leads to calcium-dependent dimerization of Rim2 and Piccolo. This in turn interacts with Rab3 leading to exocytosis of insulin granules and release of insulin.

GIPR is also known to stimulate insulin secretion by another pathway which leads to increased arachidonic acid production through Group VIA islet Ca^{2+}-independent phospholipase A_2 (iPLA$_2$).

β-Cell Growth and Survival Pathways

Apart from insulin secretion, GIPR signaling is also known to play a vital role in β-cell survival and proliferation. The dominant negative mutant of human GIPR in β-cells generated by transgenic method showed a diminished islet size and the development of diabetes in mice (McIntosh et al. 2009). Also, mice with the receptor knockout failed to respond to a high fat diet with hyperinsulinemia. This suggests the role of GIP signaling in regulation of β-cell mass in insulin resistance. GIPR serves as a mitogenic and anti-apoptotic factor for β-cells by pleiotropic activation of several interlinked pathways involving PKA/▶ CREB, MAPK, and PI3-kinase (Fig. 3). The major effectors of these pathways are kinases, enzymes that require ATP for their functioning; hence linked to glucose metabolism and Ca^{2+} signaling.

- The PKA/▶ CREB pathway involves activation of PKA by GIPR signaling and inhibition of phosphorylation of the cytoplasmic enzyme adenosine mono phosphate kinase (AMPK), which is responsible for phosphorylation of TORC2. TORC2 is a coactivator of the nuclear regulating factor ▶ CREB, present inside the nucleus. Under the reduced state of phosphorylation, the nuclear localization of TORC2 increases and within the nucleus it interacts with phospho-▶ CREB. The union of the TORC2-phospho-▶ CREB binds to the CRE-1 element of the Bcl-2 promoter and upregulates the expression of the downstream *Bcl-2* gene. The Bcl-2 protein so formed prevents the apoptosis of β-cells (Kim et al. 2008).
- The growth and cell survival effect of GIPR signaling also occurs via a PKA-independent mechanism. GIPR activation stimulates phosphatidylinositol 3-kinase (PI3K), which activates the downstream protein kinase B (▶ PKB) by phosphorylation. The so activated ▶ PKB in turn phosphorylates various components of the apoptotic machinery such as caspase 9, Bad, glycogen synthase kinase 3B, and a member of the forkhead/winged helix/Foxo family response element (FHRE) in β-cells and downregulates *Bax* gene. The product of the *Bax* gene plays an important role as a mediator of apoptotic cell death (Kim et al. 2005).

- GIPR is also known to exert a mitogenic effect on β-cells by activating the mitogen-activated protein kinase (MAPK) or the extracellular signal-regulated kinase1/2 (▶ MEK1/2 – ERK1/2) modules and the PI3K/▶ PKB pathway. The ▶ MEK1/2 and ERK1/2 are activated by phosphorylation and they in turn phosphorylate downstream substrates of ERK1/2, ▶ p38MAPK and ▶ PKB. This further inactivates caspase-3 and DNA fragmentation and prevents apoptosis (Trumper et al. 2002).

GIPR has a potential influence on increased insulin biosynthesis as well. The rat insulinoma cell line showed increased expression of insulin mRNA upon treatment with GIP. Once the action is accomplished, GIPR signaling is terminated rapidly by physiological cleavage of GIP by a dipeptidyl peptidase-IV (DPP-IV) enzyme at the N-terminus (McIntosh et al. 2009).

GIPR and Antidiabetic Drug Design

Since the emergence of the incretin concept, there has been increasing efforts directed toward designing novel antidiabetic drugs. The structure of the N-terminus domain of the receptor in complex with the peptide has been solved and the key amino acids involved in this interaction are known; this knowledge can help in designing GIPR agonists. There have been attempts to enhance the action of incretins on the receptor by designing peptide analogs that are resistant to DPP-IV and small molecules that are inhibitors of the enzyme. The design of incretin receptor agonists is in the state of infancy and so far no small molecule has been developed that can activate the GIPR. However, several peptide analogs resistant to DPP-IV and inhibitors of DPP-IV have been introduced into therapeutics (Green and Flatt 2007).

Summary

GIPR has emerged as a major signaling molecule on the enteroinsular axis with a key role in glucose homeostasis. As a GPCR, it is predominantly expressed on the surface of β-cells of islets of pancreas, adipose tissue, central nervous system, bone, and to some extent in stomach and liver cells. Along with glucose, it is known to potentiate insulin secretion from pancreas, increase insulin biosynthesis, and enhance β-cell survival by exerting mitogenic effects. It is also known to increase fat deposition in adipose tissue, enhance memory recognition in CNS, and inhibit gastric acid secretion in stomach. The past two decades of research have exhaustively delineated various pathways involved in GIPR signaling in pancreas with PI3K, PKA, ▶ PKB, and MAPK being major ones. Due to its role in insulin secretion, GIPR has become an attractive target for studying its role in pathophysiology underlying diabetes and for designing novel antidiabetics. Though GIPR agonists are yet to see the light of the day, inhibitors of DPP-IV, which enhance the action of GIP and prolong GIPR signaling, have been successfully designed and put to practice.

References

Amiranoff B, Vauclin-Jacques N, Laburthe M. Functional GIP receptors in a hamster pancreatic beta cell line, In 111: specific binding and biological effects. Biochem Biophys Res Commun. 1984;123:671–676. doi:0006-291X(84)90281-X [pii].

Amiranoff B, Vauclin-Jacques N, Laburthe M. Interaction of gastric inhibitory polypeptide (GIP) with the insulin-secreting pancreatic beta cell line, In lll: characteristics of GIP binding sites. Life Sci. 1985;36: 807–13.

Amiranoff B, Couvineau A, Vauclin-Jacques N, Laburthe M. Gastric inhibitory polypeptide receptor in hamster pancreatic beta cells. Direct cross-linking, solubilization and characterization as a glycoprotein. Eur J Biochem. 1986;159:353–8. doi:10.1111/j.1432-1033.1986.tb09875.

Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. Gastroenterology. 2007;132:2131–57. doi:10.1053/j.gastro.2007.03.054.

Drucker DJ. The biology of incretin hormones. Cell Metab. 2006;3:153–65. doi:10.1016/j.cmet.2006.01.004.

Gault VA, O'Harte FP, Flatt PR. Glucose-dependent insulinotropic polypeptide (GIP): anti-diabetic and anti-obesity potential? Neuropeptides. 2003;37:253–63. doi:S0143417903000891 [pii].

Green BD, Flatt PR. Incretin hormone mimetics and analogues in diabetes therapeutics. Best Pract Res Clin Endocrinol Metab. 2007;21:497–516. doi:10.1016/j.beem.2007. 09.003.

Gremlich S, Porret A, Hani EH, Cherif D, Vionnet N, Froguel P, Thorens B. Cloning, functional expression, and chromosomal localization of the human pancreatic islet glucose-dependent insulinotropic polypeptide receptor. Diabetes. 1995;44:1202–8. doi:10.2337/diabetes.44.10.1202.

Hoare SR. Mechanisms of peptide and nonpeptide ligand binding to class B G-protein-coupled receptors. Drug Discov Today. 2005;10:417–27. doi:10.1016/S1359-6446(05)03370-2.

Kim SJ, Winter K, Nian C, Tsuneoka M, Koda Y, McIntosh CH. Glucose-dependent insulinotropic polypeptide (GIP) stimulation of pancreatic beta-cell survival is dependent upon phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling, inactivation of the forkhead transcription factor Foxo1, and down-regulation of bax expression. J Biol Chem. 2005;280:22297–307. doi:10.1074/jbc.M500540200.

Kim SJ, Nian C, Widenmaier S, McIntosh CH. Glucose-dependent insulinotropic polypeptide-mediated up-regulation of beta-cell antiapoptotic Bcl-2 gene expression is coordinated by cyclic AMP (cAMP) response element binding protein (CREB) and cAMP-responsive CREB coactivator 2. Mol Cell Biol. 2008;28:1644–56. doi:10.1128/MCB.00325-07.
Kosaka T, Lim RKS. Demonstration of the humoral agent in fat inhibition of gastric secretion. Proc Soc Exp Biol Med (New York, NY). 1930;27:890–1. doi:10.3181/00379727-27-5024.
McIntosh CH, Widenmaier S, Kim SJ. Glucose-dependent insulinotropic polypeptide (gastric inhibitory polypeptide; GIP). Vitam Horm. 2009;80:409–71. doi:10.1016/S0083-6729(08)00615-8.
Meier JJ, Nauck MA, Schmidt WE, Gallwitz B. Gastric inhibitory polypeptide: the neglected incretin revisited. Regul Pept. 2002;107:1–13. doi:S0167011502000393 [pii].
Moore B. On the treatment of diabetus mellitus by acid extract of duodenal mucous membrane. Biochem J. 1906;1:28–38.
Parthier C, Kleinschmidt M, Neumann P, Rudolph R, Manhart S, Schlenzig D, Fanghanel J, Rahfeld JU, Demuth HU, Stubbs MT. Crystal structure of the incretin-bound extracellular domain of a G protein-coupled receptor.Proc Natl Acad Sci USA. 2007;104:13942–7. doi:10.1073/pnas.0706404104.
Ranganath LR. Incretins: pathophysiological and therapeutic implications of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1. J Clin Pathol. 2008;61:401–9. doi:10.1136/jcp. 2006.043232.
Takeda J, Seino Y, Tanaka K, Fukumoto H, Kayano T, Takahashi H, Mitani T, Kurono M, Suzuki T, Tobe T, et al. Sequence of an intestinal cDNA encoding human gastric inhibitory polypeptide precursor. Proc Natl Acad Sci USA. 1987;84:7005–8.
Trumper A, Trumper K, Horsch D. Mechanisms of mitogenic and anti-apoptotic signaling by glucose-dependent insulinotropic polypeptide in beta(INS-1)-cells. J Endocrinol. 2002;174:233–46. doi:JOE04661 [pii].
Yamada Y, Hayami T, Nakamura K, Kaisaki PJ, Someya Y, Wang CZ, Seino S, Seino Y. Human gastric inhibitory polypeptide receptor: cloning of the gene (GIPR) and cDNA. Genomics. 1995;29:773–6. doi:10.1006/geno.1995.9937.

GluD1

► Delta Glutamate Receptor (GluD1, GluD2)

GluD2

► Delta Glutamate Receptor (GluD1, GluD2)

GluRδ1

► Delta Glutamate Receptor (GluD1, GluD2)

GluRδ2

► Delta Glutamate Receptor (GluD1, GluD2)

Glutamate Receptors

Elek Molnár
MRC Centre for Synaptic Plasticity, School of Physiology and Pharmacology, University of Bristol, Medical Sciences Building, University Walk, Bristol, UK

Synonyms

Ionotropic glutamate receptors (AMPA, kainate and NMDA receptors); Metabotropic glutamate receptors

Nomenclature of Glutamate Receptor Proteins

International Union of Basic and Clinical Pharmacology (IUPHAR) recommended and previous nomenclatures of glutamate receptor proteins

1. Ionotropic glutamate receptors (Collingridge et al. 2009)
   - AMPA receptor subunits GluA1-4 (previously: GluR1-4; GluRA-D; GLU_{A1-4})
   - Kainate receptor subunits GluK1-5 (previously: GluR5-7, KA-1/2; EAA3-5, EAA1/2; GLU_{K5-7}, $GLU_{K1/2}$)
   - NMDA receptor subunits GluN1, GluN2A-D, GluN3A-B (previously: NR1, NR2A-D, NR3A/B; GLU_{N1}, GLU_{N2A-D}, $GLU_{N3A/B}$)
   - δ receptor subunits GluD1-2 (previously: GluRδ1/2)
2. Metabotropic glutamate receptors (Nicoletti et al. 2011)
   - mGlu1-8 (previously: mGluR1-8)

Historical Background

The excitatory neurotransmitter role of L-glutamate gradually emerged in the 1950s–1960s. Early studies

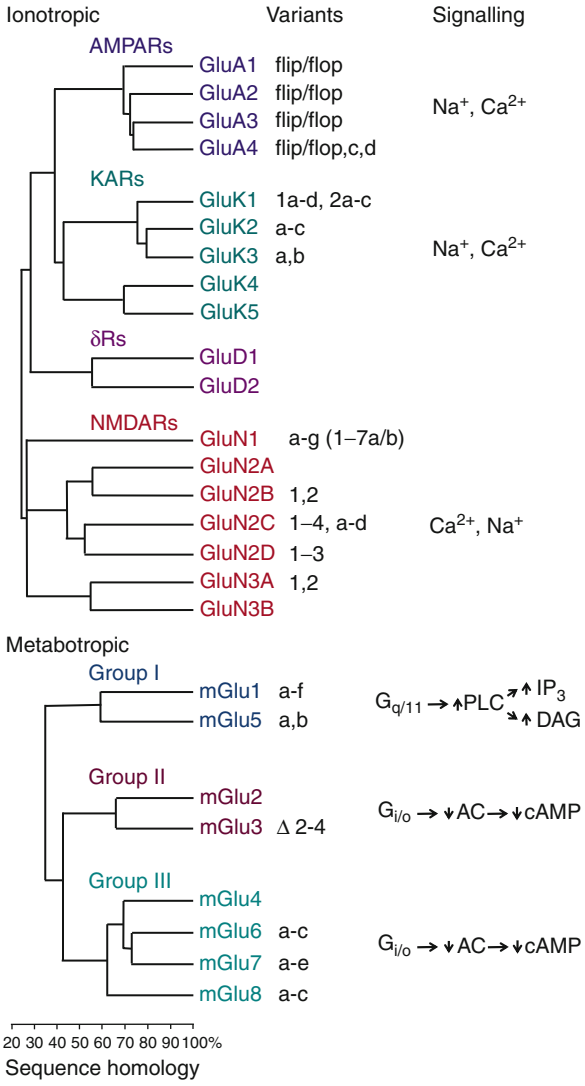
indicated that L-glutamate: (1) was present in high concentrations throughout the mammalian central nervous system (CNS), (2) produced convulsions, and (3) excited single neurons (reviewed in Lodge 2009). The development of increasingly specific pharmacological tools during the 1970s started to reveal considerable functional diversity. The family of glutamate-activated cation channels (ionotropic glutamate receptors [iGluRs]) was classified into three major pharmacological subfamilies, defined by their most selective agonists: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) receptors (Dingledine et al. 1999; Lodge 2009). In the mid-1980s, evidence began to appear of the existence of another glutamate receptor (GluR) group termed metabotropic GluRs (mGluRs) that are directly coupled to second-messenger systems via GTP-binding proteins (Conn and Pin 1997; Nicoletti et al. 2011). However, iGluR and mGluR proteins remained elusive until the late 1980s. The application of the newly emerging expression cloning approach led to a breakthrough and provided the original sequence information for the first iGluR subunits GluA1 and GluN1 (Lodge 2009). The first member of the family of mGluRs (mGlu1a) was also discovered by expression cloning (Nicoletti et al. 2011). Successive cloning by sequence homology led to the identification of additional iGluR subunits, mGluR isoforms, and their splice variants (Dingledine et al. 1999; Niswender and Conn 2010). Receptor localization studies in the 1990s–2000s, together with the development of a range of transgenic animals and more selective pharmacological tools, started to reveal the mechanisms of glutamatergic signaling in the CNS. The availability of X-ray crystal structures, molecular modeling and site-directed mutagenesis studies provided much improved understanding of the molecular organization of iGluRs (Traynelis et al. 2010) and mGluRs (Nicoletti et al. 2011). A very diverse range of receptor phosphorylation–dephosphorylation events, protein–protein interactions, and receptor trafficking have been identified as important regulators of GluR function and synaptic plasticity (Collingridge et al. 2004; Henley et al. 2011).

Key Features of the Glutamate Receptor Family

The amino acid L-glutamate is the predominant neurotransmitter of the vast majority of excitatory synapses in the mammalian CNS. This ubiquitous neurotransmitter acts via the activation of a number of ionotropic (ligand-gated ion channels) and metabotropic (G-protein-coupled) GluRs. These receptors are involved in nearly all aspects of nervous system development and function including many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), mechanisms that are involved in learning and memory (Collingridge et al. 2004). GluRs are also involved in a very wide range of neurological and psychiatric disorders including chronic neurodegenerative conditions (Lau and Tymianski 2010). They are thus also potential targets for therapies for CNS disorders.

Ionotropic Glutamate Receptors

The related subunit proteins of glutamate-gated ion channels (iGluRs) are encoded by 18 genes in mammals. Based on their sequence homology, electrophysiological properties, and pharmacological selectivity they are subdivided into four subtypes: AMPA, kainate, NMDA, and δ receptors (Fig. 1). While the role of δ receptor subunits is unclear and they seem to be incapable of forming receptors that can be activated by any known agonists, properties of other iGluRs are extensively studied. All iGluRs share a common basic structure, which differs from other ligand-gate ion channels. iGluRs are formed from the tetrameric assembly of homologous subunits around a central ion pore (Dingledine et al. 1999; Traynelis et al. 2010). The membrane topology of the iGluR subunits consists of a large extracellular N-terminal domain and four hydrophobic membrane-associated domains (M1-4; Fig. 2a). M2 is a re-entrant loop in the phospholipid bilayer and represents the channel pore-forming region (Dingledine et al. 1999). Each subunit carries its own ligand-binding site, which consists of residues that are distributed throughout both the distal N-terminal domain (called S1) and the extracellular loop between M3 and M4 (called S2; Fig. 2a; Dingledine et al. 1999). The S1S2 ligand-binding domain regions of some of the iGluR subunits have been isolated and crystalized with and without bound ligands (Traynelis et al. 2010). X-ray analysis of these proteins and protein-ligand complexes elucidated fine molecular details of the binding site, and the conformational changes of different agonists and

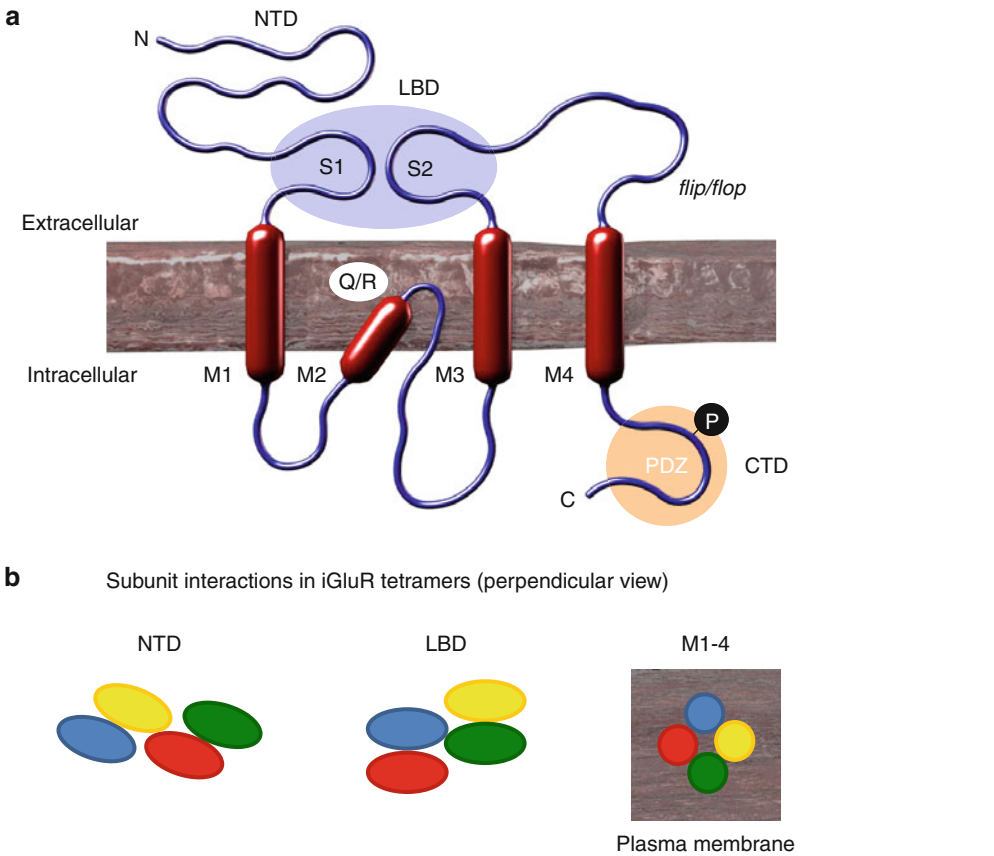


Glutamate Receptors, Fig. 1 IUPHAR recommended nomenclature, classification and sequence homology dendrograms of iGluR and mGluR proteins. The known splice variants and main signaling mechanisms are indicated next to the code name of the GluR proteins

antagonists induce to open or block the ion channels or modulate the manifestation of their activity (Traynelis et al. 2010). Recent crystallographic studies have provided the first detailed structure of the antagonist-bound tetrameric GluA2 AMPARs (Sobolevsky et al. 2009), which started to reveal complex subunit domain interactions within iGluRs (Fig. 2b). Oligomerization of iGluR subunits plays a major role in their surface expression (Greger et al. 2007). Nonfunctional receptors are retained intracellularly, suggesting that glutamate binding and associated conformational changes are prerequisites for the forward trafficking of intracellular KARs following multimeric assembly (Fleck 2006). Functional and immunolocalization studies indicate that segregated populations of iGluRs are localized at diverse subcellular compartments (Molnár 2008). All members of the iGluR family bind to a variety of intracellular and transmembrane proteins (Traynelis et al. 2010). These interactions allow local signaling to proceed, providing the possibility of spatial and temporal specificity to receptor regulation.

AMPA receptors (AMPARs) mediate fast synaptic transmission in the CNS and they are key components of the modifiable synaptic response. AMPARs are formed by co-assembly of the GluA1-4 subunits (Fig. 1), each of which can form functional homomeric receptors, although most native AMPARs contain both the GluA2 subunit and either GluA1, GluA3, or GluA4 (Dingledine et al. 1999). All AMPAR subunits exist as two splice variants termed *flip* and *flop*. The alternatively spliced region is in the extracellular loop (S2) adjacent to the last transmembrane domain (M4; Fig. 2a). The *flip* splice variants of GluA1-4 desensitize more slowly and to a lesser extent than the *flop* variants, which can influence the amplitude of the total AMPAR current (Dingledine et al. 1999). The C-terminal domain of AMPAR subunits also undergo alternative splicing to yield short and long forms of the intracellular domain (Dingledine et al. 1999). GluA2 undergoes editing in the M2 channel pore-forming region at the functionally significant Q/R site (Isaac et al. 2007; Fig. 2a). The GluA2 subunit in the edited form is responsible for the Ca^{2+} impermeability of AMPARs (Isaac et al. 2007).

AMPARs are highly mobile proteins that undergo constitutive and activity-dependent translocation to, and removal from, synapses (Henley et al. 2011). Increases in synaptic AMPAR function through changes in their number, subunit composition, and/or properties result in the long-term potentiation (LTP) of synaptic efficacy. Conversely, removal of synaptic AMPARs provides a mechanism for long-term depression (LTD) (Collingridge et al. 2004; Molnár 2008). Several functionally important phosphorylation sites have been identified in the C-terminal domains of AMPAR subunits (Molnár 2008). The effects of these phosphorylation/dephosphorylation events together



Glutamate Receptors, Fig. 2 (**a**) Schematic representation of iGluR subunit membrane topology. iGluR subunits composed of two extracellular domains: the N-terminal domain (NTD) and the ligand-binding domain (LBD) formed by the S1 and S2 segments. Unlike other ligand-gated ion channels (e.g., nicotinic acetylcholine receptors, $GABA_A$ receptors), iGluRs contain three transmembrane domains (M1, M3, and M4). A membrane re-entrant loop (M2) forms the pore of the channel in iGluRs and contains the Q/R editing site in GluA2, GluK1, and GluK2 subunits. The intracellular C-terminal domain (CTD) contains phosphorylation sites and binds to intracellular proteins. See text for details. Schematic image was provided by Dr. Andrew Doherty. (**b**) Assembled iGluR subunits have an overall twofold symmetry perpendicular to the membrane plane; the extracellular N-terminal domains (NTDs) and ligand-binding domains (LBDs) are organized as dimmers of dimmers, and the ion channel forming membrane-associated domains (M1-4) exhibit a fourfold symmetry (Sobolevsky et al. 2009)

with protein–protein interactions (e.g., α-actinin, AP2, GRIP, GRIP2, IQGAP1, mLIN-10, NSF, PICK1, PSD95, RIL, SAP97, Shank3, syntenin, TARPs, 4.1; Traynelis et al. 2010) are crucial in functional changes, localization and trafficking of these receptors so that they can fulfill their roles in synaptic transmission and plasticity (Collingridge et al. 2004; Molnár 2008; Henley et al. 2011).

Kainate receptors (KARs) are key players in the modulation of neuronal-network activity throughout the CNS (Lerma 2003). While other iGluRs (AMPA and NMDA receptors) mainly operate at postsynaptic sites, KARs are located at both presynaptic and postsynaptic sites where they modulate neurotransmitter release or mediate excitatory neurotransmission, respectively (Lerma 2003; Jane et al. 2009). KARs are also involved in neuronal differentiation, synaptic plasticity, epileptogenesis, chronic pain, neurodegeneration, and neuronal cell death (Jane et al. 2009). Some of the functions of KARs involve metabotropic action through coupling with a G-protein, which does not require an ionotropic action.

For instance, KARs regulate neuronal excitability by inhibition of Ca^{2+}-dependent K^+ channels (Rodríguez-Moreno and Sihra 2007).

Molecular cloning has identified five KAR subunits, named GluK1-5 (Fig. 1; Lerma 2003). KAR subunits are subdivided into low-affinity (GluK1-3) and high-affinity (GluK4/5) kainate-binding subunits (Jane et al. 2009). Electrophysiological and biochemical analysis of recombinant KARs indicate that functional KAR channels are formed by both homomeric and heteromeric expression of GluK1-3 subunits. In contrast, the GluK4 and GluK5 subunits do not form functional homomeric channels, but they co-assemble with the GluK1-3 subunits (Jane et al. 2009). The diversity of KARs is increased by the existence of splice variants for GluK1 (1a-d, 2a-c), GluK2 (a-c), and GluK3 (a,b) subunits (Fig. 1; Jane et al. 2009). GluK1 and GluK2 are subject to mRNA editing at the functionally significant Q/R site in the channel pore forming domain (Fig. 2a). Q/R editing of GluK1 and GluK2 subunits reduces Ca^{2+}-permeability of KARs (Lerma 2003).

Studies with recombinant receptors in cell lines and cultured neurons have started to define rules for the trafficking of KARs to the plasma membrane. The relative level of their surface expression depends on subunit composition, alternative splicing of their C-terminal domains, and editing of the Q/R site in the pore forming M2 domain. Some subunits (GluK2a and GluK3a) contain a forward trafficking motif, whereas others (GluK1a, GluK1b, GluK2b, GluK3b, and GluK5) are retained in the endoplasmic reticulum due to retention signals (Jane et al. 2009).

KAR subunits and splice variants show great divergence in their C-terminal cytoplasmic domain, which has been identified as a region of interaction with a number of protein partners (Traynelis et al. 2010). Many KAR-interacting proteins have been identified (e.g., actifilin, calmodulin, CASK, COPI, cortactin, dynamin-1, dynamitin, G-α(q/11), GRIP/GRIP2, NETO1/2, NSF, PICK1, profiling, PSD95, SAP97, SAP102, SNAP25, spectrin, SUMO, syntenin, VILIP1/3, 14-3-3). Some of these proteins have been implicated in trafficking, synaptic localization and modulation of the properties of KARs (Traynelis et al. 2010).

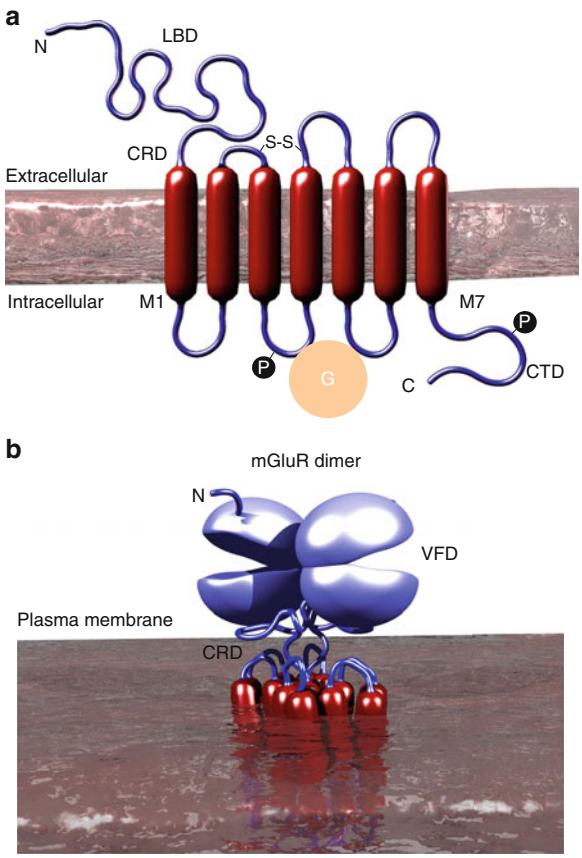
NMDA receptors (NMDARs) function as modulators of synaptic response and coincidence detectors. At resting membrane potentials, NMDARs are inactive. This is due to a voltage-dependent block of the channel pore by Mg^{2+}, preventing ion flows through it. Sustained activation of AMPARs by, for instance, a train of impulses arriving at a presynaptic terminal, depolarizes the postsynaptic cell, releasing the channel inhibition and thus allowing NMDAR activation. In addition to Na^+ and K^+, NMDARs are also permeable to Ca^{2+}. Thus, NMDAR activation leads to a Ca^{2+} influx into the postsynaptic cells, a signal that is instrumental in the activation of a number of signaling cascades. Depending on the pattern of synaptic plasticity, NMDARs are responsible for a wide range of postsynaptic functions, including LTP and LTD (Collingridge et al. 2004). Excessive activation of NMDARs leads to pathological processes (e.g., excitotoxic injury) in a number of acute and chronic neurological disorders (Lau and Tymianski 2010).

NMDARs are obligate heterotetramers formed from assemblies of GluN1 subunits with GluN2A-D and GluN3A/B (Fig. 1). In addition, GluN3A can assemble with GluN1 (without other GluN2 subunits) to form excitatory, Ca^{2+}-impermeant glycine receptors. Eight possible variations of the GluN1 subunit arise by alternative splicing of a single gene transcript. The insertion of one splice cassette at the N-terminal region of GluN1 and the deletion of two independent consecutive splice variants at the C terminus have been identified. Therefore, a large number of different NMDARs with differing functional and pharmacological properties exist in different parts of the brain or at different stages in development (Molnár 2008). Unusually for the iGluR, L-glutamate is not the only agonist for the NMDAR. Glycine, another amino acid, is a co-agonist and both transmitters must bind in order for the receptor to function. The binding sites for glutamate and glycine are found on different subunits – glycine binds to the GluN1 subunit while glutamate binds to the GluN2 subunit. This is one reason why both subunit types are required to generate a fully functioning receptor. The GluN2B subunit also possesses a binding site for polyamines, regulatory molecules that modulate the functioning of NMDARs. Like other iGluRs, NMDARs also interact with a wide range of cytoskeletal, scaffolding and signaling proteins (e.g., α-actin-2, AP2, calmodulin, CaMKII, CARPI, COPII, GPS2, LIN7, MAP1S, PACSIN1, plectin, PSD95, RACK1, SALM1, SAP97, SAP102, S-SCAM; Traynelis et al. 2010).

Metabotropic Glutamate Receptors

mGluRs play important roles in regulating the activity of many synapses in the CNS. These receptors activate a multitude of signaling pathways; therefore, they are involved in a wide number of physiological and pathological processes: modulation of slow excitatory and inhibitory responses; the regulation of Ca^{2+}, K^{+}, and nonselective cation channels; the inhibition and facilitation of transmitter release; the induction of LTP/LTD; the formation of various types of memory; the regulation of iGluR trafficking; modification of NMDAR-mediated synaptic transmission; the regulation of neuronal development; and signaling between neurons and glial cells (Ferraguti and Shigemoto 2006; Gladding et al. 2009; Niswender and Conn 2010). mGluRs are also implicated in various diseases such as epilepsy, anxiety and stress disorders, depression, schizophrenia, fragile X mental retardation, Parkinson's disease, chronic pain, drug addiction, hypoxic brain damage, and excitotoxic neuronal death (Nicoletti et al. 2011).

The seven transmembrane-domain proteins mGluRs (Fig. 3) are coupled to heterotrimeric GTP-binding proteins which link the receptors to downstream signaling pathways (Conn and Pin 1997; Nicoletti et al. 2011). The family of mGluRs comprises eight different subtypes (mGlu1-8 receptors; Fig. 1) classified into three groups on the basis of sequence similarities, pharmacological properties, and intracellular signal transduction mechanisms (Conn and Pin 1997; Niswender and Conn 2010). Group I includes mGlu1 and mGlu5 receptors, which couple to G_q and activate phospholipase C (PLC). In group II (mGlu2, mGlu3) and group III (mGlu4, mGlu6, mGlu7, and mGlu8) receptors couple to G_i/G_o and inhibit ▶ adenylyl cyclise (AC) (Niswender and Conn 2010). Many of these receptors (mGlu1, mGlu3, mGlu5-8) exist as various isoforms with different intracellular C-termini generated by alternative splicing (Fig. 1; Ferraguti and Shigemoto 2006; Niswender and Conn 2010). The large N-terminal domain of the mGluRs contains the ligand-binding site, which is formed by two-hinged globular domains – the so-called Venus fly trap domain (Fig. 3a, b; Nicoletti et al. 2011). Binding of glutamate causes the two domains to close, providing the structural change in the transmembrane domains that triggers intracellular G-protein activation. mGluRs



Glutamate Receptors, Fig. 3 Schematic representation of mGluR membrane topology (**a**) and diagram of the mGluR dimer (**b**). mGluRs contain large extracellular domains called the Venus flytrap domains (VFDs), which bind L-glutamate and other ligands. The cysteine-rich domain (CRD) links the VFD to the seven transmembrane domains (M1-7). The intracellular C-terminal domain (CTD) is often subject to alternative splicing to generate different C-terminal tails. The open state of the VFD is the inactive state and can be stabilized by antagonists. Schematic images were provided by Dr. Andrew Doherty

form dimers stabilized by an inter-subunit disulfide bond (Fig. 3b). Only mGluR subtypes coupled to the same G-protein can form heterodimers (Nicoletti et al. 2011). The binding of a single agonist per dimer is sufficient for receptor activation (Nicoletti et al. 2011). mGluRs are expressed by neurons and glia, where they locate in the proximity of the synaptic cleft. In neurons, mGlu1/5 are mostly localized postsynaptically, mGlu4/7/8 at the presynapse, while mGlu2/3 do not show any preference. mGlu6 is only expressed in retinal

bipolar cells postsynaptic to photoreceptors (Ferraguti and Shigemoto 2006). Proteins interacting with mGluRs include: 4.1 G, adenosine receptor A1, $Ca_v2.1$, Ca^{2+}-sensing receptor, calmodulin, caveolin-1/-2β, $GABA_{B1}$ receptor, filamin-A, G-protein βγ, GRIP, GRK2, ▶ homer, optineurin, pias1, PICK1, PKA, PKC, PP1γ1, PP2α, siah-1A, syntenin, tamalin, α/β-tubulin (Enz 2006).

Summary

While iGluRs are responsible for fast synaptic transmission, mGluRs modulate slow synaptic transmission through intracellular second messengers. Both iGluRs and mGluRs are critically important signaling molecules for normal brain function. They transduce the vast majority of excitatory neurotransmission and regulate the strength of both excitatory and inhibitory transmission in the CNS via complex interactions. Glutamatergic systems are dysfunctional in most neuropathologies, and aberrant receptor function appears to have causative roles in many neurological disorders. In addition to neurons, iGluRs and mGluRs have been identified in nonneuronal cells where they are involved in a broad range of signaling processes (Julio-Pieper et al. 2011). The full functional significance of these nonneuronal GluRs remains to be established. A confound in the study of GluRs has been the lack of congruence between the properties of recombinant receptors expressed in heterologous systems and those of native receptors studied in the brain tissue. This mismatch suggests that heterologously expressed receptors lack modulatory components that can influence essential properties. The discovery of auxiliary subunits for iGluRs (e.g., TARPS, NETO1/2; Traynelis et al. 2010), dimerization of mGluRs (Niswender and Conn 2010), various interaction partners (Enz 2006; Traynelis et al. 2010), phosphorylation/dephosphorylation (Molnár 2008), and other covalent modifications of GluRs has started to provide answers for many of these discrepancies. A better understanding of the specific functions and molecular interactions of native GluR subtypes and the development of more subtype/subunit-specific pharmacological tools are needed for future breakthroughs in the treatment of several neurological and psychiatric disorders.

References

Collingridge GL, Isaac JTR, Wang YT. Receptor trafficking and synaptic plasticity. Nat Rev Neurosci. 2004;5:952–62.

Collingridge GL, Olsen RW, Peters J, Spedding M. A nomenclature for ligand-gated ion channels. Neuropharmacology. 2009;56:2–5.

Conn PJ, Pin J-P. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol. 1997;37:205–37.

Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. Pharmacol Rev. 1999;51:7–61.

Enz R. The trick of the tail: protein-protein interactions of metabotropic glutamate receptors. Bioessays. 2006;29:60–73.

Ferraguti F, Shigemoto R. Metabotropic glutamate receptors. Cell Tissue Res. 2006;326:483–504.

Fleck MW. Glutamate receptors and endoplasmic reticulum quality control: looking beneath the surface. Neuroscientist. 2006;12:232–44.

Gladding CM, Fitzjohn SM, Molnár E. Metabotropic glutamate receptor-mediated long-term depression: molecular mechanisms. Pharmacol Rev. 2009;61:395–412.

Greger IH, Ziff EB, Penn AC. Molecular determinants of AMPA receptor subunit assembly. Trends Neurosci. 2007;30:407–16.

Henley JM, Barker EA, Gledov OO. Routes, destinations and delays: recent advances in AMPA receptor trafficking. Trends Neurosci. 2011;34:258–68.

Isaac JTR, Ashby MC, McBain CJ. The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. Neuron. 2007;54:859–71.

Jane DE, Lodge D, Collingridge GL. Kainate receptors: pharmacology, function and therapeutic potential. Neuropharmacology. 2009;56:90–113.

Julio-Pieper M, Flor PJ, Dinan TG, Cryan JF. Exciting times beyond the brain: metabotropic glutamate receptors in peripheral and non-neural tissues. Pharmacol Rev. 2011;63:35–58.

Lau A, Tymianski M. Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch Eur J Physiol. 2010;460:525–42.

Lerma J. Roles and rules of kainate receptors in synaptic transmission. Nat Rev Neurosci. 2003;4:481–95.

Lodge D. The history of the pharmacology and cloning of ionotropic glutamate receptors and the development of idiosyncratic nomenclature. Neuropharmacology. 2009;56:6–21.

Molnár E. Molecular organization and regulation of glutamate receptors in developing and adult mammalian central nervous systems. In: Lajtha A, Vizi ES, editors. Handbook of neurochemistry and molecular neurobiology: neurotransmitter systems. 3rd ed. New York: Springer; 2008. p. 415–41.

Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD, Wroblewski JT, Pin JP. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology. 2011;60:1017–41.

Niswender CM, Conn PJ. Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol. 2010;50:295–322.

Rodríguez-Moreno A, Sihra TS. Kainate receptors with a metabotropic modus operandi. Trends Neurosci. 2007;30:630–7.
Sobolevsky AI, Rosconi MP, Gouaux E. X-ray structure, symmetry and mechanism of an AMPA-subtype glutamate receptor. Nature. 2009;462:745–56.
Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev. 2010;62:405–96.

Glutathione-*S*-Transferases: As Signaling Molecules

Thyaga Raju Kedam, Pallavi Chittoor and Divya Kurumala
Department of Biochemistry, Sri Venkateswara University College of Sciences, Tirupati, AP, India

Synonyms

GST; Gsto 1

Historical Background

The Glutathione-*s*-transferases exist as cytosolic, mitochondrial, and microsomal can participate in signal transduction by not phosphorylating any factor that is directly involved in cell growth and death. This signal transduction is considered to be a new way of implication in cell metabolic pathways due to the influence of external, such as xenobiotics and UV radiation, and internal, such as oxidative stress, free radicals, etc., agents. The GST binding assay studies revealed that they participate in the inhibition of various proteins, for example, phosphoproteins, AP-1, JNK, etc., in the systems to regulate cell mechanisms during cell synthesis.

Biomembranes and Signal Transduction

A biomembrane is an enclosing or separating membrane that acts as a selective barrier, within or around a cell. It consists of a lipid bilayer with embedded proteins that may constitute close to 50% of membrane content (Mark Latash 2007). It has a selective permeable structure, which is essential for effective separation of a cell or organelle from its surroundings. Biomembrane has certain mechanical or elastic properties. These membranes contain receptors on their surface and receive signals from various influences. During this process an extracellular signaling molecule activates a membrane receptor that in turn alters intracellular molecules creating a response (Silverthorn 2006) called signal trasduction. In signal transduction, the chemical signal binds to the outer portion of the transmembrane receptor, changing its shape and conveying another signal inside the cell. Some chemical messengers, like testosterone, can pass through the cell membrane, and bind directly to receptors in the cytoplasm or nucleus. Sometimes these chemicals can have cascade of amplified signals, so a small signal can result in a large response (Reece and Campbell 2002). Eventually, the signal creates a change in the cell, either in the expression of the DNA in the nucleus or in the activity of enzymes in the cytoplasm. These processes can take milliseconds (for ion flux), minutes (for protein and lipid mediated kinase cascades), hours, or days (for gene expression). In 1980, Martin Rodbell examined the effects of glucagon on a rat's hepatocyte membrane receptor and noted that guanosine triphosphate disassociated glucagon from this receptor, and stimulated the G-protein, which heavily influenced the cell's metabolism. Thus, he deduced that the G-protein was a transducer to accept glucagon molecules and affect the cell (Rodbell 1980). For this, he shared the 1994 Nobel Prize in Physiology/Medicine with Alfred G. Gilman. The current understanding of signal transduction processes reflects contributions made by Rodbell and many other research groups throughout the years.

Signal transduction involves the binding of extracellular signaling molecules, and intracellular signaling cascades can be started through cell–substratum interactions (Beato et al. 1996). Most of the chemicals have receptors within the cytoplasm and act by stimulating the binding of their receptors to the promoter region of their responsive genes (Hammes 2003). Examples of signaling molecules include the hormone melatonin (Sugden et al. 2004), the neurotransmitter acetylcholine (Kistler et al. 1982), the cytokine interferon γ (Schroder et al. 2004), environmental stimuli, and certain microbial molecules. All of these may be independent of signal transduction stimulation by other molecules, as is the case for the

toll-like receptor. It may occur with help from stimulatory molecules located at the cell surface of other cells, as with T-cell receptor signaling. Single-celled organisms may respond to environmental stimuli through the activation of signal transduction pathways. For example, slime molds secrete cyclic adenosine monophosphate upon starvation, stimulating individual cells in the immediate environment to aggregate (Hanna et al. 1984), and yeast cells use mating factors to determine the mating types of other cells and participate in sexual reproduction (Sprague 1991).

Receptors

Receptors can be roughly divided into two major classes: extracellular receptors and intracellular receptors.

Extracellular receptors are integral transmembrane proteins and make up most receptors. They span the plasma membrane of the cell, with one part of the receptor on the outside of the cell and the other on the inside. Signal transduction occurs as a result of a ligand binding to the outside; the molecule does not pass through the membrane. This binding stimulates a series of events inside the cell; different types of receptor stimulate different responses and receptors typically respond to only the binding of a specific ligand. Upon binding, the ligand induces a change in the chemical confirmation of the inside part of the receptor. These result in either the activation of an enzyme in the receptor or the exposure of a binding site for other intracellular signaling proteins within the cell, eventually propagating the signal through the cytoplasm.

Intracellular receptors include nuclear receptors and cytoplasmic receptors, and are soluble proteins localized within the nucleoplasm or the cytoplasm, respectively. The typical ligands for nuclear receptors are lipophilic hormones, with steroid hormones (e.g., testosterone, progesterone, and cortisol) and derivatives of vitamin A and D among them. To reach its receptor and initiate signal transduction, the hormone must pass through the plasma membrane, usually by passive diffusion. The nuclear receptors are ligand-activated transcription activators; on binding with the ligand (the hormone), the ligands will pass through the nuclear membrane into the nucleus and enable the transcription of a certain gene and, thus, the production of a protein. Intracellular signal transduction is by and large carried out by second messenger molecules.

Ca^{2+} concentration is usually maintained at a very low level in the cytosol by sequestration in the smooth endoplasmic reticulum and the mitochondria. The Ca^{2+} release from endoplasmic reticulum into the cytosol results in the binding of the released Ca^{2+} to signal proteins, which is then activated. Ca^{2+} is used in a multitude of processes, among them muscle contraction, release of neurotransmitter from nerve endings, vision in retina cells, proliferation, secretion, cytoskeleton management, cell migration, gene expression, and metabolism. The three main pathways that lead to Ca^{2+} activation are:

1. G-protein-regulated pathways
2. Pathways regulated by receptor-tyrosine kinases
3. Ligand- or current-regulated ion channels

There are two different ways by which Ca^{2+} can regulate proteins:

1. A direct recognition of Ca^{2+} by the protein
2. Binding of Ca^{2+} in the active site of an enzyme

One of the best-studied interactions of Ca^{2+} with a protein is the regulation of calmodulin by Ca^{2+}. Calmodulin itself can regulate other proteins, or be part of a larger protein (e.g., phosphorylase kinase). The Ca^{2+}–calmodulin complex plays an important role in proliferation, mitosis, and neural signal transduction. The response of cells to extracellular stimuli is in part mediated by a number of intracellular kinase and phosphatase enzymes (Hunter 1995). The mitogen-activated protein (MAP) kinases are members of discrete signaling cascades, which are focal points for diverse extracellular stimuli, and function to regulate fundamental cellular processes. Four distinct subgroups within the MAP kinase family have been described. These include (1) extracellular signal-regulated kinases (ERKs), (2) c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), (3) ERK5/big MAP kinase 1 (BMK1), and (4) the p38 group of protein kinases. The JNK group of protein kinases is activated in response to a number of cellular stresses, including high osmolarity and oxidation (Ip and Davis 1998). The ERK5/BMK1 MAP kinase signaling pathway regulates serum-induced early gene expression (Kato et al. 1997). The p38 group kinases have been found to be involved in inflammation, cell growth, cell differentiation, the cell cycle, and cell death (New and Han 1998).

The JNK Signal Transduction Pathway

The c-Jun NH_2-terminal kinase (JNK) is a member of an evolutionarily conserved subfamily of mitogen-activated protein (MAP) kinases. Recent studies have led to progress toward understanding the physiological function of the JNK signaling pathway. Mitogen-activated protein (MAP) kinase signaling pathways relay, amplify, and integrate signals from a diverse range of extracellular stimuli, thereby controlling the genomic and physiological response of a cell to changes in the environment. In mammalian systems, these responses include cellular proliferation, differentiation, development, the inflammatory response, and apoptosis. The c-Jun NH_2-terminal kinase (JNK) represents one subgroup of MAP kinases that is activated primarily by cytokines and exposure to environmental stress. A major target of the JNK signaling pathway is the activation of the AP-1 (Activator protein-1) transcription factor that is mediated, in part, by the phosphorylation of c-Jun and related molecules. The JNK proteins are encoded by three genes such as *JNK1*, *JNK2*, and *JNK3*. Recent mutation study on mice has revealed that the JNK proteins are required for cell viability; however, they are required for cellular physiology.

Two protein kinases that activate JNK have been identified (▶ MKK4 and MKK7). During embryo development the modification of MKK4 alone causes death to hepatic cells but MKK7 is necessary for embryo viability (Davis 2000). Disruption of either MKK4 or MKK7 was found to cause partial defects in stress-stimulated JNK activation (e.g., ultraviolet light). In contrast, disruption of both genes prevented JNK activation by these stimuli (Tournier et al. 2001). Interestingly, MKK4 and MKK7 preferentially phosphorylate JNK on Tyr and Thr, respectively (Lawler et al. 1998) and both protein kinases are activated in response to environmental stress (Davis 2000). As dual phosphorylation of JNK on Tyr and Thr is required for full activation, it is likely that MKK4 and MKK7 cooperate to activate JNK in response to environmental stress. However, the roles of MKK4 and MKK7 are different in cells stimulated with cytokines. Treatment of cells with tumor necrosis factor (TNF) causes activation of MKK7, but not MKK4. Gene-knockout studies demonstrate that MKK7 is essential for TNF-stimulated JNK activation and that MKK4-deficiency causes some reduction in JNK activation (Tournier et al. 2001). Together, these data indicate that TNF stimulates MKK7 activity, that JNK activation is triggered by MKK7, and that the basal activity of MKK4 is required for full activation of JNK in response to TNF. In yeast, four groups of potential scaffold proteins that may coordinate JNK signaling modules have been reported: CrkII; filamin; β-arrestin; and JIP (JNK interacting protein). This can occur due to binary complex formation of JNK proteins. In Drosophila, the JNK signaling pathway is required for the morphogenetic process of dorsal closure (Davis 2000) with the help of the TGF-β family member Decapentaplegic (Dpp)-initiated elongation and migration of dorsal epithelial cells during dorsal closure.

The JNK signaling pathway has been implicated in many pathological conditions, including cancer, stroke, heart disease, and inflammatory diseases (Davis 2000). Drugs that inhibit JNK signaling may, therefore, be therapeutically beneficial. Furthermore, such drugs will facilitate research on JNK function. In addition, the JNK pathway participates in activating transcription factor 2, ELK-1, and the SAP-1a transcription factor, and JNK may influence p53 and NF-κB pathways (Beuckmann et al. 2000). Extracellular signals such as growth factors, transforming oncoproteins, and UV irradiation stimulate phosphorylation of c-Jun at ser-63/73 and activate c-Jun-dependent transcription. The binding of JNK to the N-terminal region of c-Jun permits substrate phosphorylation. This pathway has also been shown to be important in the control of cell survival and death pathways by the induction of apoptosis.

The p38 Signal Transduction Pathway

The p38 signaling transduction pathway, a Mitogen-activated protein (MAP) kinase pathway, plays an essential role in regulating many cellular processes including inflammation, cell differentiation, and cell growth and death. Activation of p38 often through extracellular stimuli, such as bacterial pathogens and cytokines, mediates signal transduction into the nucleus to turn on the responsive genes. p38 also transduces signals to other cellular components to execute different cellular responses.

Protein p38α (or simply p38) was first isolated as 38 kDa protein, which has specificity to phosphorylate

tyrosine in response to LPS stimulation (Han et al. 1994). Three p38 homologues of alpha, p38β, p38γ (or ERK6, SAPK3), and p38 δ (or SAPK4) are cloned in mammals. The p38α and p38β genes are ubiquitously expressed. However, p38γ and δ are differentially expressed in different tissues. p38γ is predominantly expressed in skeletal muscle (Lechner et al. 1996), and p38δ is enriched in lung, kidney, testis, pancreas, and small intestine. p38γ expression was reported to be induced during muscle differentiation and p38β expression was shown to be developmentally regulated (Lechner et al. 1996). An upregulation of the expression of p38 isoforms was observed in the inflammatory cell lineages. Sequence comparisons revealed that each p38 isoform has more than 60% identity within this group, but only 40–45% to the other MAP kinase family members.

p38 homologues have been identified and cloned in both low and high eukaryotic species, including fly, frog, and yeast. Their role has been implicated in osmoregulation, responses to extracellular stress stimuli, and cell cycle events. Mammalian p38δ are also activated by environmental stresses. Since mammalian p38 was identified in studies designed to understand signaling pathways during inflammation (Han et al. 1994), extensive data of p38 regulation have been developed in immune systems. p38 activation has been observed in inflammatory responses, as in LPS-treated macrophages (Han et al. 1994), TNF-stimulated endothelial cells, IL-17-stimulated chondrocytes, IL-18-stimulated U1 monocytic cell line, human platelets stimulated with thrombin, and chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-treated or phorbol myristate acetate (PMA)-treated human neutrophils.

MAP kinase–activated protein kinase 2 (MAPKAPK2 or M2) was the first identified p38α substrate. Subsequently, a closely related protein kinase, M3 (or 3pk), was also found to be a substrate of p38α. Moreover, activated M2 and 3 phosphorylate various substrates including small heat shock protein 27 (HSP27), lymphocyte-specific protein 1 (LSP1), cAMP response element-binding protein (CREB), ATF1, and tyrosine hydroxylase. The expression of many cytokines, transcription factors, and cell surface receptors was found to be coordinated by p38. The activation of the p38 pathway plays an essential role in: (1) Production of proinflammatory cytokines such as IL-1b, TNF-a, and IL-6; (2) induction of enzymes such as COX-2, which controls connective tissue remodeling in pathological condition; (3) expression of an intracellular enzyme such as iNOS, which regulates oxidation; and (4) induction of adherent proteins such as VCAM-1 and many other inflammatory-related molecules. In addition to these, the p38 pathway plays a regulatory role in the proliferation and differentiation of cells of the immune system.

Protein Kinase C (PKC)

Protein kinase C was originally identified as a serine/threonine kinase that was maximally active in the presence of diacylglycerols (DAG) and calcium ion. It is now known that there are at least ten proteins of the PKC family. Each of these enzymes exhibits specific patterns of tissue expression and activation by lipid and calcium. PKCs are involved in the signal transduction pathways initiated by certain hormones, growth factors, and neurotransmitters. The phosphorylation of various proteins, by PKC, can lead to either increased or decreased activity. Of particular importance is the phosphorylation of the EGF receptor by PKC, which downregulates the tyrosine kinase activity of the receptor. This effectively limits the length of the cellular responses initiated through the EGF receptor.

Glutathione-*S*-Transferase

Glutathione transferases (EC 2.5.1.18; GSTs) are members of a multigene family of isoenzymes ubiquitously expressed in most living organisms. GSTs are a family of Phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a wide variety of xenobiotics. This detoxification ability plays a role in cellular protection from environmental and oxidative stress, yet is also implicated in cellular resistance to drugs. Advances in the molecular biology of the GSTs over the past several years have revealed a broader role for these enzymes. Indeed, GSTs have been found to be involved in the biosynthesis and metabolism of prostaglandins (Beuckmann et al. 2000), steroids, and leukotrienes; in the management of toxic products of lipid oxidation and *S*-glutathiolated proteins generated by oxidative stress (Ruxana Begum and Kedam 2010); and in the acquisition of resistance to chemotherapeutic agents (Tew 1994).

The GST proteins are also extensively involved in the control of ►ROS species, which can cause

oxidative stress. Excess free radicals are formed in many ways, for example, trauma, medications, metabolism of lipids, etc., and can create a potentially dangerous and unstable cellular environment, linked to pathology of tissue damage, degenerative diseases such as Parkinson's, and accelerated aging. Steroid hormone synthesis has also been linked to GST activity, where GST catalysis is in part responsible for progesterone and testosterone synthesis. Membrane bound GSH transferases (mitochondrial) are more active and specific for leukotriene synthesis.

Three major families of GST proteins are: (1) cytosolic, (2) mitochondrial, and (3) microsomal (also referred to as membrane-associated proteins in eicosanoid and glutathione (MAPEG)), of which the cytosolic GSTs constitute the largest family (Hayes et al. 2005). On the basis of amino acid sequence similarities, substrate specificity, and immunological cross-reactivity, seven classes of cytosolic GSTs have been identified in mammals (Board et al. 2000). These classes are designated by the names of the Greek letters α (*alpha*), μ (*mu*), π (*pi*), σ (*sigma*), θ (*theta*), ω (*omega*), and ζ (*zeta*), and abbreviated in Roman capitals A, M, P, S, T, O, and Z. The mammalian GSTs, such as cytosolic, mitochondrial, and microsomal forms, each of which displays distinct catalytic as well as non-catalytic binding properties are given below:

Cytosolic	Soluble, predominantly found in the cytosol of hepatocytes. Highly polymorphic
Mitochondrial	Soluble
Microsomal	Come to be known as "MAPEG" – membrane-associated proteins in eicosanoid and glutathione metabolism. GST1-3. These enzymes form part of a larger super family of small membrane proteins, additional members being leukotriene C_4 synthase and 5-lipoxygenase-activating protein. Largely involved in eicosanoid synthesis. Six identified to date

Cytosolic and mitochondrial forms are soluble enzymes sharing similarities in 3D structure, but share no resemblance with microsomal forms. Cytosolic GSTs, the most studied of the GSTs, were originally defined based on their substrate/inhibitor specificity.

Most GST classes show a high degree of polymorphism and include several subunits. Each subunit with 199–244 amino acids in length, and 22–29 kDa mass, contains a catalytically independent active site that consists of a GSH-binding site ("G-site") in the amino-terminal domain and a site that binds the hydrophobic substrate ("H-site") in the carboxy-terminal domain. More than a dozen cytosolic GST subunits have been identified in humans. As the functional enzymes are dimeric, and those of α and μ classes, in addition to homodimers, can also form heterodimers, the number of isoenzymes that can be generated from these subunits is significantly larger. The isoenzymes are named according to their class and subunit composition, with each subunit designated by an Arabic numeral (e.g., GSTA1-2 denotes the enzyme composed of subunits 1 and 2 of α class). Expression of the different classes of GSTs varies among tissues and with developmental stage. For example, α-class GSTs are predominantly expressed in liver, testis, and kidney and their expression levels are similar in both adult and fetal tissues. In contrast, GSTπ (GSTP1-1), originally isolated from placenta, is found mainly in brain, lung, and heart; its expression in liver decreases during embryonic development, becoming very low in adult tissue and expressed on phenobarbitol treatment in hepatocyte of rat.

Alpha Class

The GSTα isoform is mainly expressed in the liver and is encoded by a gene cluster localized on chromosome 6p12. This cluster contains five genes encoding proteins belonging to GSTA1–A5. Human tissues widely express transcripts for GSTA1, A2 and A4, whereas expression of GSTA3 is rare and GSTA5 has yet to be detected in human tissues. Epidemiological results show that aberrant expression of GSTα has been linked to an increased risk in colorectal cancer, ovarian cancer, and clear cell renal cell carcinoma. The GSTA1 gene contains seven exons and is ~12 kb in length. A genetic polymorphism of GSTA1 is characterized by two alleles, GSTA1*A and GSTA1*B. These alleles differ in promoter regions based on three linked single nucleotide substitutions at positions −567, −69, and −52. Specifically, the −52 substitution has been shown to increase promoter activity in GSTA1*A, thus making it predominantly expressed. Coles et al. (2001) previously showed a correlation between GSTA1*B expression and an increased risk of colorectal cancer. However, as with many such published accounts, other groups have recently reported conflicting evidence

finding no association between GST expression and susceptibility to colorectal cancer. GSTA2 has not been extensively studied, but it is known to have several variants (GSTA2*A–E). The catalytic properties of GSTA2 variants A–D do not seem to differ; however, the novel variant GSTA2E shows reduced rates of catalysis when compared to A–D. This may be due to substitution of the highly conserved Pro residue. GSTA3 is selectively expressed in steroidogenic tissues and plays a role in steroid hormone biosynthesis. Three GSTA3 transcripts have been identified and found exclusively in African populations. Polymorphisms in GSTA3 may affect steroidogenesis through altered protein levels or function, and it has recently been hypothesized that alterations in genes involved in steroidogenesis and sex steroid metabolism could potentiate risk factors for the development of ovarian cancer.

Omega Class

The Omega class of GSTs contains two functional members, GSTO1 and GSTO2, and a pseudogene (GSTO3p). Based on two defining features, the Omega class is both structurally and functionally distinct from other eukaryotic GSTs (Townsend et al. 2003). This protein on X-ray crystallography shows a unique 19-residue N-terminus extension that forms a structural unit quite unlike any found in other classes. At present, its function remains undefined (Board et al. 2000) and known substrates of other GSTs are not catalyzed by GSTO.

GSTO1 is a single gene located on chromosome 10 that codes for GSTO1 proteins expressed abundantly in the liver, macrophages, glial and endocrine cells. To date, four polymorphisms have been identified, GSTO1*A–D. Among the Australian, African, and Chinese populations, GSTO1*A was the most prevalent haplotype with a frequency ranging from 0.6 to 0.9, whereas GSTO1*B*A was the least common, with a frequency of 0.01–0.05. GSTO1*A demonstrated a GSH-dependent reduction of dehydroascorbate, a function characteristic of glutaredoxins rather than GSTs (Board et al. 2000). This allele was first described as the human monomethylarsenic acid reductase, (MMA [V]), and is the rate-limiting enzyme of inorganic arsenic metabolism. GSTO2, while separated from GSTO1 by 7.5 KB on chromosome 10, shares 64% amino-acid identity. GSTO2, like GSTO1, is ubiquitously expressed and shares GSH-dependent dehydroascorbate reductase activity. However, GSTO2 has a high catalytic activity toward CDNB, and its over expression induced apoptosis, suggesting a possible role in cell signaling (Wang et al. 2005). This is the first report made on cell signaling by GSTs.

Zeta Class

The Zeta class (GSTZ1) is a single 10.9 KB gene located on chromosome 14 that codes for a 29-kDa protein. GSTZ1 was independently characterized as maleylacetoacetate isomerase (MAAI) and plays a putative isomerase role in the catabolic pathway of phenylalanine and tyrosine in addition to the GSH-dependent transformation of α-halogenated acids. GSTZ1 is preferentially expressed in hepatocytes and renal proximal tubule cells where phenylalanine and tyrosine are catabolized.

GSTZ1-deficient mice have an elevated urinary excretion of FAA (Free amino acid) and were subject to renal injury following phenylalanine and tyrosine overload. Four families have been identified that lack GSTZ1 members that were died within the first year of life. Whereas the clinical data for GSTZ1 are insufficient to deduce a role for GSTZ1 in inherited genetic disease, it is plausible that a perturbation in GSTZ1-mediated tyrosine metabolism is contributory to the described pathology. Whether this tyrosine undergoes signal mechanism is not known.

Mu Class

Five GST isoforms belonging to the mu class (GSTM1–5) have been described. A gene cluster located on chromosome 1 encodes for GSTM1–5. The GSTM1 gene contains four different alleles allowing for several M1 class polymorphisms. GSTM1*A and M1*B are functionally identical and differ at K173N amino-acid substitution (McLellan et al. 1997). The presence of the GSTM1*A allele has been associated with a decreased risk of bladder cancer with the implication that detoxification of possible bladder-specific carcinogens may occur in these individuals. In addition McLellan et al. (1997) described a rapid enzyme activity phenotype in Saudi Arabian individuals attributed to a tandem M1 gene duplication resulting in two functional M1 genes. It was inferred that this rapid detoxification phenotype could have an increased protective effect against carcinogens.

Loss of GSTM enzyme function is ascribed to a homozygous deletion of this gene resulting in the GSTM1*0 allele. It has been suggested that the

mutation is a result of an unequal crossing-over of the M1 and M2 loci which are in close physical proximity and share 99% nucleotide sequence identity. The frequency of GTSM1*0 individuals is approximately 67% in Australians, 50% in Caucasians, and 22% in Nigerians. The GSTM null phenotype is associated with an increased risk of the lung, colon, and bladder cancer and has also been associated with response rates to some chemotherapy. Cytosolic prostaglandin E was purified from the human brain and characterized as GSTM2 (Beuckmann et al. 2000). The GSTM2*B allele has been shown to catalyze the conjugation of GSH to aminochrome, a redox cycling product of dopamine. Products formed during the redox cycling of catecholamines (such as dopamine) contribute to the processes involved in neurodegenerative diseases such as Parkinson's disease and schizophrenia; therefore, GSTM2*B may play a cytoprotective role in neurodegeneration.

The GSTM3 locus contains two alleles, A and B. The GSTM3*B allele has a three base pair deletion in intron 6 that introduces a recognition motif for the transcription factor YY1. GSTM3*A and *B are expressed in the brain; yet, there appears to be no direct relationship between GSTM3 expression and the incidence of astrocytomas. The GSTM3*AA genotype is associated with an increased risk for laryngeal squamous cell carcinoma, whereas GSTM3*BB was putatively protective. In addition, GSTM3*AA was shown to occur more frequently in patients with multiple cutaneous basal cell carcinoma than GSTM3*BB. The roles of GST M-4 and GST M-5 were not known.

Theta Class

The Theta class of GSTs consists of two different subfamilies: GSTT1 and GSTT2. Genes encoding both proteins are colocalized on chromosome 22 and are separated by 50 kb. Polymorphisms exist between these two and within both genes including a null phenotype (GSTT1*0) that exhibits decreased catalytic activity and has been associated with an increased risk of cancers of the head, neck, and oral cavity (Strange and Fryer 1999).

GST Pi

The GST Pi class is encoded by a single gene spanning approximately 3 kb and located on chromosome 11. In this group, four active, functionally different polymorphisms (GSTP1*A–D) have been identified. The GSTP1 genotype has been associated with differences in chemotherapeutic response and cancer susceptibility and is over expressed in a wide variety of tumors including ovarian, NSCLC, breast, colon, pancreas, and lymphoma (Tew 1994). Although it is well established that GSTπ is over expressed in a wide variety of solid tumors, prostate cancer is the only example in which the absence or reduced expression of GSTπ is associated with tumor incidence. GSTπ is widely expressed in normal prostate tissue; however, its presence is undetectable in malignant cells. Studies examining the absence of GSTπ in human prostate cancer show that hypermethylation of the GSTπ regulatory region is the most common somatic alteration identified. This alteration results in the loss of GSTπ expression and is proposed to occur during pathogenesis of the disease. Recently, a methyl-CpG binding domain protein has been identified that mediates hypermethylation of the GSTπ regulatory region. These findings provide a possible target for restoration of GSTπ activity. So the expression of GST proteins is independent on the concentration of each class GST protein in any tissue. GST expression (and/or activity) of specific isoforms is lost in some individuals with allelic variation.

Recently, Dang et al. (2005) examined the effect of GSTP1 on the survival and proliferation of human colon cancer cells. GSTP1 wild type and deficient colon cancer cells were grown under serum deprivation and low-density seeding conditions, and cellular apoptosis, oxidative stress, and kinase signaling were examined. Lack of GSTP1 expression resulted in an increase in cellular oxidative stress and resulted in apoptosis when cells were cultured under growth-limiting conditions. In addition, the presence of GSTP1 was essential for the mediation of MAPK kinase and ERK kinase signaling. GSTP1 plays a critical role in protection from cell cycle arrest and oxidative stress under growth-limiting conditions.

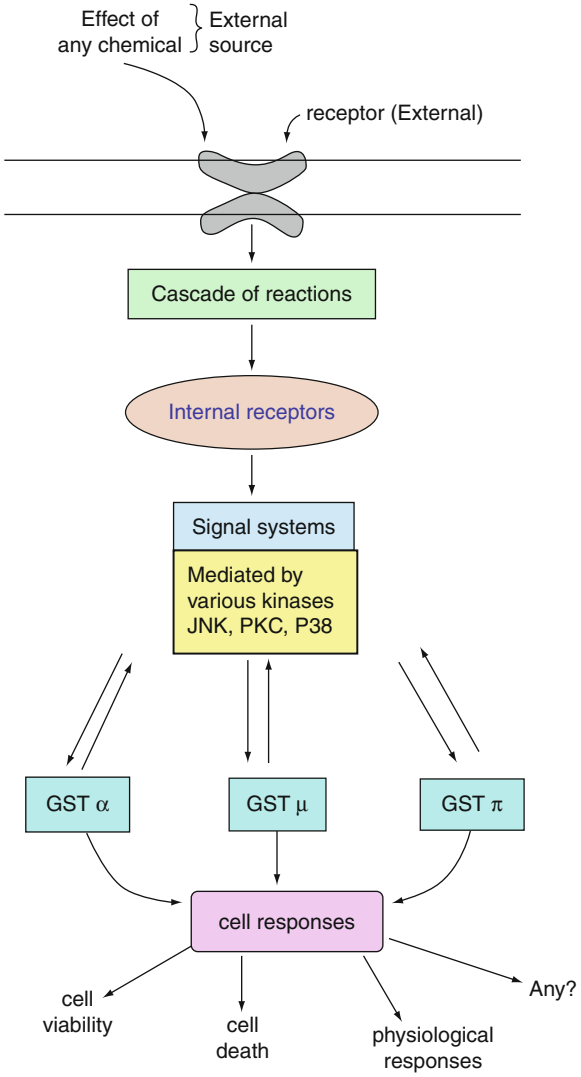
GSTs as Signaling Molecule

GSTs comprise a multigene family, of which GSTp is the most prevalent and ubiquitous non-hepatic isozyme. Recently, GSTs have also been shown to act as modulators of signal transduction pathways that

control cell proliferation and cell death. Because of their cytoprotective role and their involvement in the development of resistance to anticancer agents, GSTs have become attractive drug targets. Among cellular functions attributed to GSTs is ligand binding and xenobiotic detoxification (Tew 1994). Reduced glutathione (GSH) binds to the "G" site of GSTp (and other GST isozymes) and plays an important role in detoxification of reactive oxygen species (ROS) and the maintenance of the cellular redox state. Among factors implicated in regulating JNK activity are ROS and altered redox potential. ROS have also been associated with regulation of other signaling cascades, for example, certain isozymes of protein kinase C and mitogen-activated protein kinase. The addition of exogenous oxidants or antioxidants has been found to influence the activation of MAPK/JNK.

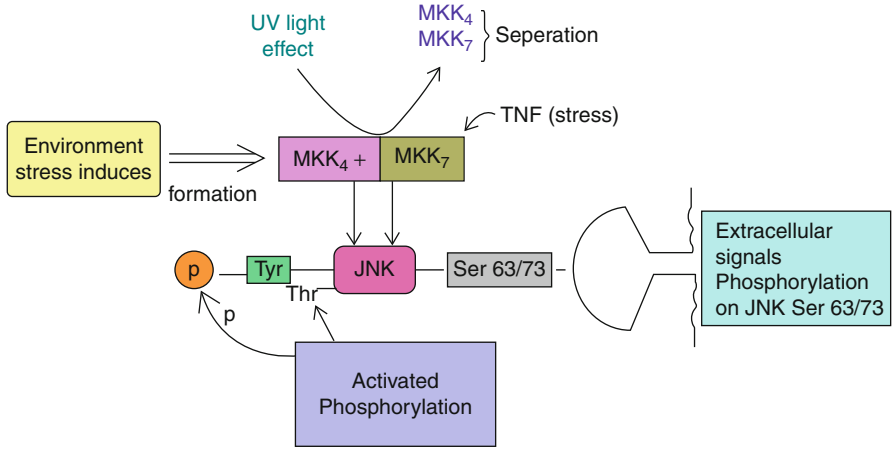
It has been speculated that the absence or decreased expression of GSTπ results in a reduced detoxification of possible carcinogens that may be causal to malignant transformation and disease progression. In addition, the GST-mediated conjugation of GSH to a number of anticancer drug substrates has long been linked to anticancer drug resistance in a variety of tumors. A disparity of this is that GSTπ has a weak affinity for the majority of anticancer drugs, although its increased expression is highly correlated with multidrug resistance. From this it can be inferred that the capacity of GSTs to regulate kinase-dependent proliferation pathways, especially in the case of GSTπ, may be of more consequence than its catalytic properties alone.

GSTs play a regulatory role in cellular signaling by forming protein–protein interactions with critical kinases involved in controlling stress response, apoptosis, and proliferation. The ligand-binding capacity of GST results in the negative regulation of signaling pathways through sequestration of signaling kinases. The first example of GST-mediated kinase regulation is the characterization of GSTπ as a Jun kinase (JNK) inhibitor (Adler et al. 1999). JNK has been implicated in proapoptotic signaling and may be required for the induced cytotoxicity of a variety of chemotherapy agents. Phosphorylation of c-Jun activates JNK resulting in subsequent activation of downstream effectors. In non-stressed cells, low JNK1 catalytic activity is orchestrated and maintained through its sequestration within the protein complex that includes at least GSTπ and JNK (Adler et al. 1999). However,



Glutathione-S-Transferases: As Signaling Molecules, Fig. 1 GSTs as signaling molecules model made from the work of Adler et al. 1999

under conditions of oxidative or chemical stress, a dissociation of the GSTπ–JNK complex occurs releasing GSTπ for oligomerization, and activation of released JNK allows for the subsequent induction of apoptosis (Fig. 1). The high levels of GSTπ in many tumors may be a consequence of an acquired dependence on the protein. Because of the proliferative nature of tumor cells, many kinase pathways are deregulated, and as a consequence, tumor cells may attempt to compensate by enhancing expression of GSTπ in an attempt to control kinase activity.



Glutathione-S-Transferases: As Signaling Molecules, Fig. 2 JNK phosphorylation based on the work of Dorion et al. 2002

Under non-stressed conditions, GSTπ inhibits JNK phosphorylation by sequestering JNK/c-Jun. Exposure to anticancer drugs or oxidative stress can alter the redox potential of the cell resulting in the oligomerization of GSTπ and the dissociation of the GSTπ–JNK complex. JNK can then become phosphorylated and subsequently activate downstream kinases and transcription factors. In some cases, transient or low exposure to stress can induce cell proliferation. During prolonged or high exposure, apoptosis can be induced.

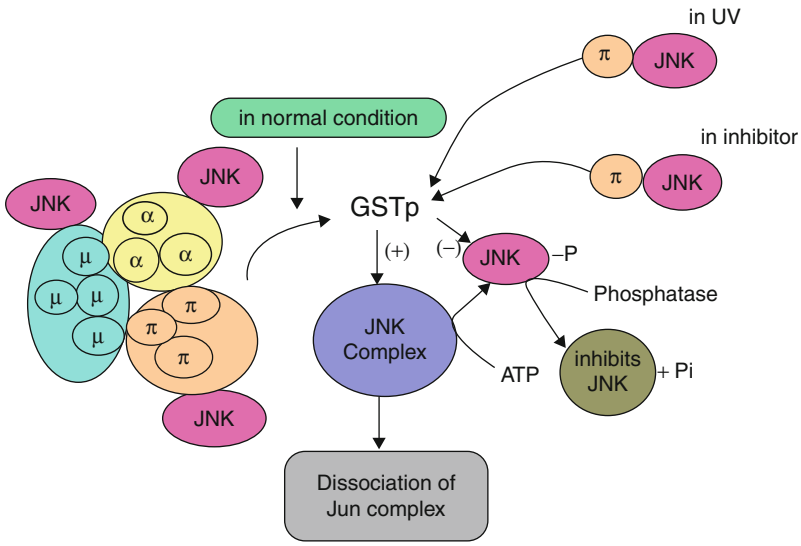
Another example of GST-mediated kinase regulation is evidence that GSTM1 binds to and inhibits the activity of ASK1 (apoptosis signal-regulating kinase). ASK1 is an MAP kinase that activates the JNK and p38 pathways leading to cytokine- and stress-induced apoptosis. ASK1 is activated in response to oxidative stress and heat shock. Under normal conditions, ASK1 exhibits low activity because of its sequestration via GSTM1. This protein–protein interaction forms a GSTM1–ASK1 complex, which is dissociated under stressful conditions leading to the release and activation of ASK1 (Fig. 2) (Dorion et al. 2002).

This mechanism is similar to the one proposed for GSTπ–JNK. In conditions, such as oxidative stress or heat shock, GSTM1 oligomerizes allowing for the release of ASK1 and subsequent induction of apoptosis (Dorion et al. 2002). GSTM1 plays a regulatory role in the heat shock–sensing pathway, while thioredoxin plays a regulatory role in the oxidative stress–sensing pathway that leads to p38 activation. Impaired clinical response to therapy in a variety of tumor types has been associated with an altered expression of GSTM1. Thus, any enzymatic influence GSTM1 plays in anticancer drug resistance is only further augmented by its role in kinase regulation.

GSTμ and thioredoxin (Trx) can act as inhibitors of ASK1. Stresses such as heat shock or reactive oxygen species can result in the release of ASK1 from the GSTμ–ASK1 or TRX–ASK1 complex (respectively). ASK1 oligomerizes and is activated through autophosphorylation, which in turn activates downstream kinases such as ▶ MKK4/MKK7, MKK3/MKK6, JNK, and p38. The fate of the cell (either proliferation or apoptosis) is dependent upon the time/concentration exposure to the stress.

Forced expression of GSTM1 blocked ASK1 oligomerization and repressed ASK1-dependent apoptotic cell death. GST-mediated regulation of the kinase pathways adds a new dimension to their known role in metabolism and cellular homeostasis.

In addition to acting as a negative regulator of kinase activation, GSTπ has also been shown to play a necessary role in the glutathionylation of 1-cys peroxiredoxin (1-cysPrx). Oxidation of the catalytic cysteine of 1-cysPrx has been associated with its loss of peroxidase activity. Recently, it was shown that heterodimerization of 1-cysPrx with GSTπ mediates the glutathionylation of the previously oxidized cysteine thus restoring its peroxidase activity. From this study, it was concluded that the glutathionylation and subsequent GSH-mediated reduction of 1-cysPrx requires heterodimerization with GSTπ. This provides the first example in which GSTπ functions in the glutathionylation of oxidized cysteine residues.



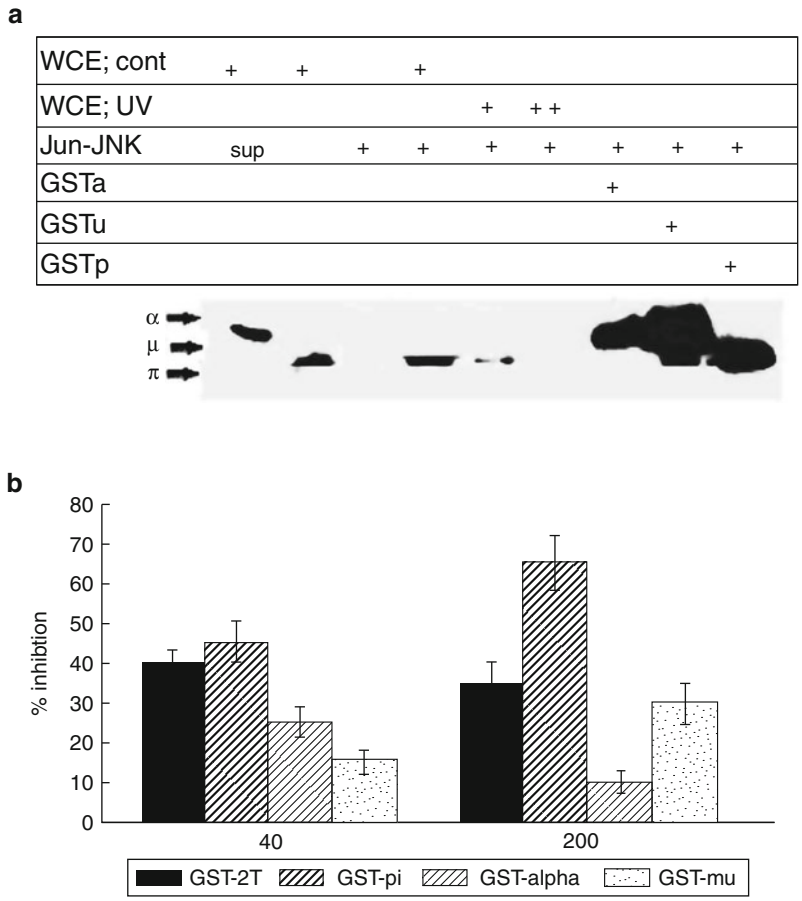
Glutathione-*S*-Transferases: As Signaling Molecules, Fig. 3 Mechanism of GSTp on JNK complex (+ effect; − no effect). (**a**) GSTp as a JNK inhibitor. GSTp was added at the indicated concentrations (micrograms) to the preformed Jun–JNK complex and the level of Jun phosphorylation was measured by means of autoradiography (Courtesy from Ishibashi group 1992). (**b**) To measure the effect of GSTp on JNK phosphorylation, JNK was immunoprecipitated from UV-treated cells and incubated with GSTp followed by Western blot with antibodies to phospho-JNK. (**c**) As a positive control, JNK from UV-treated cells was incubated with no protein, dual specificity phosphatase (Ishibashi et al. 1992), or GSTp before carrying out immunoblot analysis with antibodies to phospho-JNK. Quantification via densitometer scanning revealed 35% inhibition of JNK phosphorylation by PP, whereas GSTp did not elicit such inhibition. (**d**) The immunoblot study has revealed that the c-Jun phosphorylation level after incubation with the inhibitor for the indicated time periods (minutes) cannot be altered by the GSTp protein. So from a, b, c, and d it is known that GSTp has role but phosphorylation of JNK is mediated by other kinases

In addition, Townsend et al. (2003) have found that cells deficient in GSTP1-1 and/or GSTP2-2 have a reduced capacity to respond to oxidative or nitrosative stress by enacting glutathionylation of a select group of target proteins. These findings imply that GSTπ may play a direct role in control of post-translational glutathionylation reactions.

To determine whether GSTp affected the degree of JNK or Jun phosphorylation, increasing concentrations of GSTp (purified form) were added to preformed Jun–JNK complex, which contained the phosphorylated form of JNK, obtained from UV-treated cells. GSTp decreased JNK phosphorylation of c-Jun in a dose-dependent manner (within a range of 0.05–1 mg), but it did not decrease the number of phosphate groups on JNK, as revealed by immunoblots with phospho-JNK antibodies. Dual activity protein phosphatase (Ishibashi et al. 1992) was used as a positive control in these reactions.

To determine whether GSTp affects the number of phospho groups on c-Jun, cellular extracts from normally growing cells were incubated with preformed Jun–JNK complex for the indicated periods of time before or after the phosphorylation step with [g-32P] ATP. The extent of c-Jun phosphorylation was not altered when cell extracts were added after the phosphorylation reaction. This observation suggested that GSTp did not reduce the number of phospho groups on c-Jun (Fig. 3).

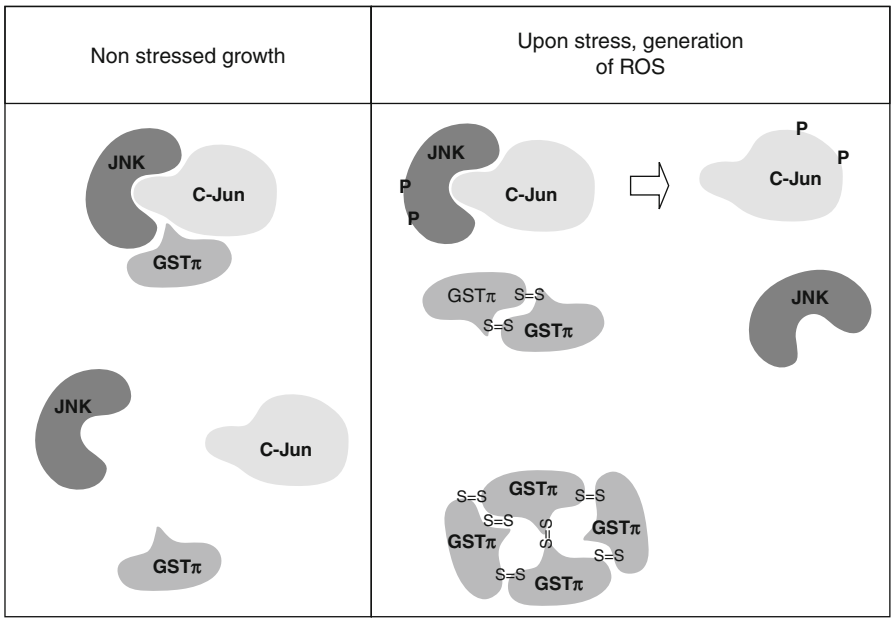
Later incubation of whole-cell extract prepared from unstressed mouse fibroblasts with the his Jun–JNK complex identified GSTp as the associated protein. A marked decrease in this association was found in proteins prepared after UV irradiation. In addition to GSTp, isozymes of the GSTα and GSTμ families were also capable of associating with the Jun–JNK complex in vitro. GSTp exhibited greater JNK inhibitory activity than did GSTμ, which was more potent than GSTα (Fig. 3b). Bacterially expressed GST (GST-2T) also mediated JNK inhibition. This excludes the possibility that the inhibitor activity was dependent on any putative GST-associated cellular component (Fig. 4a).



Glutathione-*S*-Transferases: As Signaling Molecules, Fig. 4 (**a**) GSTp associates with Jun–JNK in vitro. The preformed Jun–JNK complex was incubated with whole-cell extract (10 mg) prepared before (WCE cont) or after (WCE UV) UV irradiation or with purified forms of GST isozymes (Ciaccio et al. 1991), as indicated. Following extensive washes, complex-bound and non-bound (absorbed on Jun–JNK; sup) material was analyzed on immunoblots with polyclonal antibodies that recognize multiple forms of GST (Ramgamaltha and Tew 1991). *Arrows* point to the identified forms of GSTp. (**b**) Effect of different GST isozymes on JNK activity. Preformed his Jun–JNK was incubated with the indicated forms of GST (α, α; μ, μ; p, p; 2T, bacterially produced form of GST) purified as described in Materials and methods before the addition of [g-32P]ATP. Autoradiography demonstrates the degree of c-Jun phosphorylation in the presence of various GSTs

Ciaccio et al. study, as conducted above, has revealed that GSTp in different forms can associate with JNK proteins and in the presence of inhibitor α, μ and π GSTs are associated more as indicated by antibody interaction studies (Fig. 4a).

Changes in ROS affect GST oligomerization and its association with, and inhibition of, JNK were next assessed whether modulation of the cellular redox potential would affect JNK inhibition by GSTp. Further they monitored the GST–JNK complex in vivo by means of immunoprecipitation followed by immunoblot analysis. Exposure of mouse fibroblast cells to either UV or H_2O_2 reduced the amount of the JNK–GSTp complex and increased JNK activity, whereas pretreating cells with the free radical scavengers *N*-acetylcysteine (NAC) or the ethyl-ester of glutathione (eeGSH) prevented JNK dissociation from GSTp and maintained GSTp inhibitory activity. GST–JNK–Jun association is inversely correlated with JNK activity. Only the monomeric form of GSTp was capable of mediating efficient inhibition of JNK phosphorylation of c-Jun. GSTp inhibition of JNK is due primarily to their association, which is released upon the conversion of GSTp from a monomer to a dimer form.



Glutathione-*S*-Transferases: As Signaling Molecules,
Fig. 5 Modification of GST proteins on their association and dissociation upon binding with various proteins (Courtesy from Tew 1994)

Since JNK efficiently targets the ubiquitination of its nonphosphorylated associated proteins c-Jun, ATF2, and p53, we determined the possible effects of GSTp on ubiquitination of JNK substrates in this reaction. Under non-stress growth conditions, c-Jun exhibits a short half-life, which is prolonged upon phosphorylation by JNK (Musti et al. 1997). Transfection of GSTp cDNA into 3T3 mouse fibroblasts increased the level of c-Jun ubiquitination in vivo. Since the level of ubiquitinated Jun is inversely correlated with its degree of phosphorylation (Musti et al. 1997), the increase in ubiquitinated c-Jun is an expected result of the GSTp inhibition of basal JNK activity, which reduces the number of c-Jun molecules that undergo phosphorylation. The noticeable increase in ubiquitinated c-Jun molecules provides an example of the physiological significance of JNK inhibition under normal growth conditions.

Model of GST inhibition on JNK signaling, based on Tew's (1994) findings, is proposed as follows (Fig. 5): under non-stressed conditions, GSTp can be free or part of a complex with Jun–JNK. Upon stress, in which ROS are formed, GSTp forms dimers and larger aggregates which cannot accommodate Jun–JNK, thus enabling JNK phosphorylation of c-Jun, which as a result is a stable and active transcription factor.

The link between GSTs and the MAP kinase pathway provides a rationale as to why many of the selecting drugs are neither subject to conjugation with GSH, nor substrates for GSTs (Tew 1994). Many anticancer agents induce apoptosis via activation of the MAP kinase pathway, specifically via JNK and p38 (Davis 2000). Elevated levels of GST are associated with increased resistance to apoptosis initiated by a variety of stimuli (Cumming et al. 2001).

Aberrant cellular signaling is also a hallmark of the malignant phenotype, and thus high levels of GSTπ in many tumors may be either a cause or effect of the transformation process. The pathology of prostate cancer strongly supports these conclusions. Hypermethylation of the GSTπ regulatory region is the most common somatic alteration identified in human prostate cancer (Lin et al. 2001). This alteration results in the loss of GSTπ expression, and is proposed to occur during pathogenesis of the disease. Recently, a methyl-CpG-binding domain (MBD) protein that mediates hypermethylation of the GSTπ regulatory region has been identified. These findings provide a possible target for restoration of GSTπ activity. GST expression and/or activity of specific isoforms are lost in some individuals with allelic variation. Although it has been speculated that reduced detoxification of possible carcinogens may be causal to malignant transformation and disease progression, a more plausible link may be through an altered capacity to regulate kinase-dependent proliferation pathways.

As GST isozymes (in particular GSTπ) are frequently upregulated in many solid tumors and lymphomas, prodrugs activated by GST-mediated catalysis

have become a viable drug design concept. Additionally, the regulatory properties of GSTπ in kinase cascades have provided a translational opportunity to target GSTs in myeloproliferative pathways, with the consequent clinical testing of new agents in myelodysplastic syndrome.

Conclusion

The first GST described was originally identified as "ligandin" due to its ability to interact covalently and noncovalently with various compounds that are not substrates for enzymatic activity, including steroids, thyroid hormones, bile acid, bilirubin, and heme (Danielson and Mannervik 1985). While the ligand-binding function remains unclear, sequestering molecules may serve a regulatory role, preventing cytotoxic ligands from interacting with their targets. Supporting this conclusion, recent studies have demonstrated a regulatory role for the π and μ classes of GSTs in the mitogen-activated protein (MAP) kinase pathway that participates in cellular survival and death signaling.

Acknowledgment Our department is funded by DST FIST and UGC BSR, New Delhi Grants.

References

Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, et al. EMBO J. 1999;18:1321–34.

Beato M, Chavez S, Truss M. Transcriptional regulation by steroid hormones. Steroids. 1996;61(4):240–51.

Beuckmann CT, Fujimori K, Urade Y, Hayaishi O. Identification of mu-class glutathione transferases M2-2 and M3-3 as cytosolic prostaglandin E synthases in the human brain. Neurochem Res. 2000;25:733–8.

Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, et al. J Biol Chem. 2000;275:24798–806.

Ciaccio PJ, Tew KD, La Creta FP. Enzymatic conjugation of chlorambucil with glutathione by human glutathione *S*-transferases and inhibition by ethacrynic acid. Biochem Pharmacol. 1991;42:1504–7.

Coles B, Nowell SA, MacLeod SL, Sweeney C, Lang NP, Kadlubar FF. Mutat Res. 2001;482:3–10.

Cumming RC, Lightfoot J, Beard K, Youssoufian H, O'Brien PJ, Buchwald M. Nat Med. 2001;7:814–20.

Dang DT, Chen F, Kohli M, Rago C, Cummins JM, Dang LH. Cancer Res. 2005;65:9485–94.

Danielson UH, Mannervik B. Biochem J. 1985;231:263–7.

Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell. 2000;103:239–52.

Dorion S, Lambert H, Landry J. J Biol Chem. 2002;277:30792–7.

Hammes SR. The further redefining of steroid-mediated signaling. Proc Natl Acad Sci USA. 2003;100(5):21680–2170.

Han J, Lee J-D, Bibbs L, Ulevitch RJ. Science. 1994;265:808–11.

Hanna MH, Nowicki JJ, Fatone MA. Extracellular cyclic AMP (cAMP) during development of the cellular slime mold *Polysphondylium violaceum*: comparison of accumulation in the wild type and an aggregation-defective mutant. J Bacteriol. 1984;157(2):345–9.

Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu Rev Pharmacol Toxicol. 2005;45:51–88.

Hunter T. Cell. 1995;80:225–36.

Ip YT, Davis RJ. Curr Opin Cell Biol. 1998;10:205–19.

Ishibashi T, Bottaro DP, Chan A, Miki T, Aaronson SA. Expression cloning of a human dual-specificity phosphatase. Proc Natl Acad Sci USA. 1992;89:12170–4.

Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD. EMBO J. 1997;16:7054–66.

Kistler J, Stroud RM, et al. Structure and function of an acetylcholine receptor. Biophys J. 1982;37(1):371–83.

Latash ML. Neurophysiological basis of movement. Champaign: Human Kinetics; 2007. ISBN 0736063676.

Lawler S, Fleming Y, Goedert M, Cohen P. Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases *in vitro*. Curr Biol. 1998;8:1387–90.

Lechner C, Zahalka MA, Giot J-F, Moler NPH, Ullrich A. Proc Natl Acad Sci USA. 1996;93:4355–9.

Lin X, Tascilar M, Lee WH, Vles WJ, Lee BH, Veeraswamy R, et al. Am J Pathol. 2001;159:1815–26.

Mannervik B, Alin P, Guthenberg C, Jensson H, Tahir MK, Warholm M, et al. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc Natl Acad Sci USA. 1985;82:7202–6.

McLellan RA, Oscarson M, Alexandrie AK, Seidegard J, Evans DA, Rannug A, et al. Mol Pharmacol. 1997;52:958–65.

Musti AM, Treier M, Bohmann D. Reduced ubiquitindependent degradation of c-Jun after phosphorylation by MAP kinases. Science. 1997;275:400–2.

New L, Han J. Trends Cardiovasc Med. 1998;8:220–8.

Ramgamaltha S, Tew KD. Immunohistochemical localization of glutathione-*S*-transferases alpha, mu and pi in normal tissue and carcinomas from human colon. Carcinogenesis. 1991;12:2383–7.

Reece J, Campbell N. Biology. San Francisco: Benjamin Cummings; 2002. ISBN 0-8053-6624-5.

Rodbell M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature. 1980;284(5751):17–22.

Ruxana Begum SK, Kedam TR. Effect of acrylamide on chick embryo liver GSTs. Med J Nutr Met. 2010;3(1):31–3; Adv Cancer Res. 52:205–55.

Schroder K, et al. Interferon-γ an overview of signals, mechanisms and functions. J Leukoc Biol. 2004;75:163–89.

Silverthorn DU. Human physiology. 4th ed. San Francisco: Benjamin Cumming; 2006.

Sprague Jr GF. Signal transduction in yeast mating: receptors, transcription factors, and the kinase connection. Trends Genet. 1991;7(11–12):393–8.

Strange RC, Fryer AA. IARC Science. Lyon publishers, France, 1999. p. 231–49.

Sugden D, Davidson K, et al. Melatonin, melatonin receptors and melanophores: a moving story. Pigment Cell Res. 2004;17(5):454–60.
Tew KD. Glutathione-associated enzymes in anticancer drug resistance. Cancer Res. 1994;54:4313–20.
Tournier C, Dong C, Turner TK, Jones SN, Flavell RA, Davis RJ. MKK7 is an essential component of the JNK signal transduction pathway activated by pro-inflammatory cytokines. Genes Dev. 2001;15:1419–26.
Townsend D, Tew K. Am J Pharmacogenomics. 2003;3:157–72.
Wang L, Xu J, Ji C, Gu S, Lv Y, Li S, et al. Int J Mol Med. 2005;16:19–27.

Glycogen Synthase

▶ Glycogen Synthase Kinase-3

Glycogen Synthase Kinase-3

James Woodgett
Samuel Lunenfeld Research Institute, Mount Sinai Hospital & Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada

Synonyms

Adenomatous polyposis coli; APC; Casein kinase-I; CK-I; EMT; Epithelial to mesenchymal transition; GSK-3; Glycogen synthase; GS; Phosphoinositide 3-kinase; PI3K; PKB; Protein kinase B/Akt

Historical Background

Glycogen synthase kinase-3 (GSK-3) is a highly conserved protein-serine/threonine kinase that was first isolated from skeletal muscle in 1980 as one of five enzymes capable of phosphorylating glycogen synthase (Embi et al. 1980). In resting tissues, GSK-3 phosphorylation inhibits glycogen synthase, the rate-limiting enzyme of glycogen synthesis. In subsequent work, insulin was found to cause inactivation of GSK-3 (via induction of PKB/Akt) and this relieves the suppression of glycogen synthase, leading to enhanced glucose conversion into glycogen in response to insulin. In mammals GSK-3 is encoded by two genes that generate highly related proteins termed GSK-3α and GSK-3β that have molecular masses of 51 and 46 kDa, respectively. In brain, the *GSK-3β* gene is alternatively spliced to generate a version with a 13 amino acid insert termed GSK-3β2. In addition to its initial role in glycogen metabolism, GSK-3 has since been demonstrated to regulate many cellular processes by suppressing substrates and to be regulated itself by several upstream pathways. As exemplified by insulin, extracellular signal-dependent control of GSK-3 then relieves its blockade and allows targets to become active. Hence, this protein kinase acts as a regulatory switch that throttles the flux through numerous signaling pathways initiated by diverse stimuli (reviewed in: Forde and Dale 2007; Doble and Woodgett 2007). Deregulation of GSK-3 has been implicated in the development of cancer, diabetes, Alzheimer's disease, schizophrenia, and bipolar disorder. Indeed, GSK-3 is a validated target of lithium, which is frequently prescribed to patients with bipolar disorder (Klein and Melton 1996). Given its involvement in many pathophysiological processes, GSK-3 is a tempting therapeutic target and several pharmaceutical companies have active GSK-3 inhibitor discovery programs. However, its proclivity for involvement in multiple pathways also raises issues of selectivity. It is therefore imperative to assess the full spectrum of GSK-3 cell functions to predict clinical efficacy and avoid surprises.

Signaling Pathways Involving GSK-3

▶ *Phosphoinositide 3-kinase* (▶ *PI3K*): The first pathway biochemically shown to directly regulate GSK-3 activity was ▶ phosphoinositide 3-kinase (▶ PI3K). This pathway is induced by a wide variety of stimuli, including insulin and polypeptide mitogens. Activating mutations in ▶ PI3K are frequently observed in human cancers and inactivation of ▶ PTEN, the lipid phosphatase that reverses the action of ▶ PI3K, is also found, albeit less commonly, in tumors (and is responsible for the cancer predispositions of Cowden's disease). ▶ PI3K activation of PKB/Akt leads to phosphorylation of GSK-3α and β at serine 21 and 9, respectively, leading to their partial inactivation. Although rarely mutated in human cancers, PKB/Akt was first identified as an oncogene. Cancer-associated ▶ PI3K mutations have also been linked to activation

of serum and glucocorticoid-activated kinase 3 (SGK3), which also phosphorylates GSK-3. ▶ p38 mitogen-activated protein kinase (p38 MAPK) can also inactivate GSK-3β via phosphorylation of Ser389 and Thr390 and this mode of regulation has been observed in neurons and thymocytes (Thornton et al. 2008).

Cyclic AMP: This second messenger is induced primarily in response to seven-transmembrane pass receptors that signal through G proteins that couple to ▶ adenylyl cyclase, as exemplified by the β-adrenergic/epinephrine receptors. Generated cyclic AMP binds to the regulatory subunits of cyclic AMP-dependent protein (PKA), causing dissociation from and relief of inhibition of the catalytic subunits. These phosphorylate a number of substrates, including GSK-3α and β at the same sites as PKB/Akt (Li et al. 2000). Complicating matters further, certain other protein-serine kinases such as p70 S6 kinase (S6K) are also capable of phosphorylating GSK-3 at these same sites.

Wnt: The canonical Wnt pathway acts primarily by raising cytoplasmic levels of β-catenin that then translocates to the nucleus to regulate gene expression in concert with LEF/TCF DNA-binding proteins. In unstimulated cells, cytoplasmic β-catenin levels are kept very low via phosphorylation by GSK-3 at three residues that target the protein for ubiquitinylation by the E3 ligase, βTrCP and subsequent destruction by the 26S proteasome. A small fraction (<10%) of cellular GSK-3 (α and β) and a priming kinase (casein kinase 1; CK1) are specifically associated with a "destruction complex" that comprises the adenomatous polyposis coli (APC) tumor suppressor and the Axin scaffolding protein. This complex constantly recruits newly synthesized β-catenin and targets it for degradation. In the presence of a suitable Wnt ligand, the LRP5/6 co-receptors become phosphorylated by GSK-3 and CK1, which creates a high-affinity binding site for Axin (Zeng et al. 2005). Through a poorly understood mechanism, the destruction complex reorganizes as a consequence allowing β-catenin to escape phosphorylation and, hence, accumulate. One of the primary transcriptional targets of β-catenin is the gene for Axin2, the polypeptide product of which acts to restore the function of the destruction complex leading to only transient Wnt signaling. APC is inactivated in ~70% of colon cancers and a further 15% show mutations in the phosphorylation site region of β-catenin. Axin is also mutated in several cancers including hepatocellular carcinomas, as is β-catenin. In each of these tumors, β-catenin is no longer subject to negative control and its levels and transcriptional activity increase dramatically (20-fold or more). Inactivation of both alleles of both GSK-3 genes results in a similar effect but disruption of only 3 of the 4 GSK-3 alleles has little effect on β-catenin regulation highlighting the fact that only a small fraction of GSK-3 is associated with the destruction complex and is this relevant to Wnt signaling (Doble et al. 2007). This selective sequestration/secondment of small fractions of GSK-3 to specific compartments underlies the principle by which GSK-3 participates in several distinct signaling pathways while maintaining signal specificity.

Notch: Activation of ▶ Notch receptors via their interaction with specific Delta-like and Jagged ligands leads to ectodomain truncation of ▶ Notch by Adam-like metalloproteinases followed by juxtamembrane cleavage by a γ-secretase complex and formation of an intracellular domain fragment (▶ $Notch_{ICD}$). This signaling fragment binds to a DNA-binding protein termed CSL or RJBPκ, which transactivates genes including members of the Hes/Hey and Sonic Hedgehog (Shh) family. Activated ▶ Notch signaling in ES cells (such as induced by expression of ▶ $Notch_{ICD}$) can increase stem cell proliferation, drive neural cell fates and, in later stages of development, modulate differentiation of endothelial and epithelial cell structures as well as hematopoietic stem cell differentiation and maintenance of myogenic satellite cells (Androutsellis-Theotokis et al. 2006). Deregulated expression of ▶ Notch1 is also associated with T-cell acute lymphoblastic leukemia. Inhibition of GSK-3 leads to stabilization of ▶ $Notch_{ICD}$. Thus, GSK-3 likely acts to downregulate Notch signaling once it has been initiated.

Hedgehog: The mammalian Hedgehog pathway is initiated by three polypeptide ligands (Sonic, Desert, and Indian Hedgehog) that activate Smoothened (Smo) serpentine protein to regulate the Gli family of transcriptional activators (reviewed in Jiang and Hui 2008). Briefly, in resting cells, a transmembrane protein termed Patched inhibits Smo allowing several protein kinases including GSK-3 to phosphorylate Gli and target it for cleavage into a repressor form (Gli^R). When present, processed Hedgehog ligand binds to Patched and relieves inhibition of Smo. This consequently promotes the activation of Gli (Gli^A) through

a complex process that includes interference of Gli phosphorylation/cleavage and inactivation of a suppressor protein termed Sufu. Gli^A then induces expression of various target genes (including itself and snail). Ectopic activation of Hedgehog signaling can lead to basal cell carcinomas and medulloblastomas.

These pathways each play important, fleeting, and often temporally interdependent roles in normal development. All share the commonality of frequent deregulation in cancer via chronic activation and loss of feedback control. The fact that GSK-3 plays a fundamental role in suppressing signaling in each of these pathways is remarkable and, at the same time, rather puzzling – representing a significant potential vulnerability for loss of control without obvious rationale for burdening a single protein kinase with the responsibility of keeping multiple important pathways in check.

Regulatory Quirks of GSK-3

While the catalytic domains of GSK-3α and β products are virtually identical in their protein kinase domains (excluding the possibility of isoform-selective small molecule ATP-binding site inhibitors), they share only 36% identity in the last 76 C-terminal residues (Woodgett 1990). GSK-3α also has a glycine-rich N-terminal extension compared to the β-isoform. In addition to mammals, the genomes of many other species such as fish, amphibians, and lizards encode genes for both isoforms, although bird genomes only contain GSK-3β and appear to have selectively lost GSK-3α (Allon et al. 2011).

Although GSK-3 phosphorylates its many substrates on serine or threonine residues, it transiently expresses tyrosine kinase activity toward itself upon initial folding (Lochhead et al. 2006). The phosphorylated tyrosine lies within the "T loop" in kinase subdomain VIII (Tyr279 for GSK-3α and Tyr216 for GSK-3β). The role of this phosphotyrosine is to allow the kinase domain to adopt an active conformation for exogenous substrates. Analysis of the crystal structure of GSK-3β revealed that unphosphorylated Tyr216 is in a conformation that interferes with substrate access whereas the phosphorylated tyrosine moves out of the path. Hence, unlike serine phosphorylation of the N-terminal region of GSK-3, tyrosine phosphorylation promotes activity. This posttranslational modification appears quite stable and unregulated. Treatment of cells with GSK-3 inhibitors causes slow loss of the phosphotyrosine at a rate consistent with its half-life. Hence, in normal conditions, the residue is phosphorylated stoichiometrically and remains so for its natural life (Lochhead et al. 2006).

GSK-3 has a strong preference for substrates that are already phosphorylated at a proximal serine/threonine lying C-terminal to the GSK-3 target residue. The so-called priming site of the substrate slots into a pocket comprising of three basic amino acids – Lys205, Arg96, and Arg180. Binding of the priming phosphate into this pocket on GSK-3 induces a conformational change, aligning the substrate for subsequent phosphorylation. Most substrates of GSK-3 demonstrate an absolute requirement for prior phosphorylation by another kinase at the "priming" residue located C-terminal to the site of subsequent phosphorylation by GSK-3 (at least 500-fold preference for the phosphorylated form). GSK-3 phosphorylates these substrates at the fourth or fifth serine or threonine residue N-terminal to the primed site (*pS/TXXXpS/T*), where the first pS/T (Ser or Thr) is the target residue, X is any amino acid (often Pro), and the last pS/T is the site for priming phosphorylation. Several protein kinases act as priming enzymes for GSK-3 substrates, including CDK-5, PAR-1, casein kinase I, ▶ casein kinase II, PKA, and PKC. In several cases, the residue initially phosphorylated by GSK-3 acts as a priming phosphate to induce phosphorylation of a second residue N-terminal to it. This leads to a series of phosphorylated residues as observed in glycogen synthase (priming kinase: ▶ casein kinase II) and β-catenin (priming kinase: casein kinase I).

Many substrates have been identified for GSK-3 to various degrees of rigor. These fall into three general categories: metabolism and regulation; structural/cytoskeletal; transcriptional regulators (Table 1). It is important to note that individual signals that regulate GSK-3 do not change the phosphorylation state of all of its targets. Rather, subsets of substrates are impacted due to local sequestration and other mechanisms.

Genetic Analysis of GSK-3 Functions

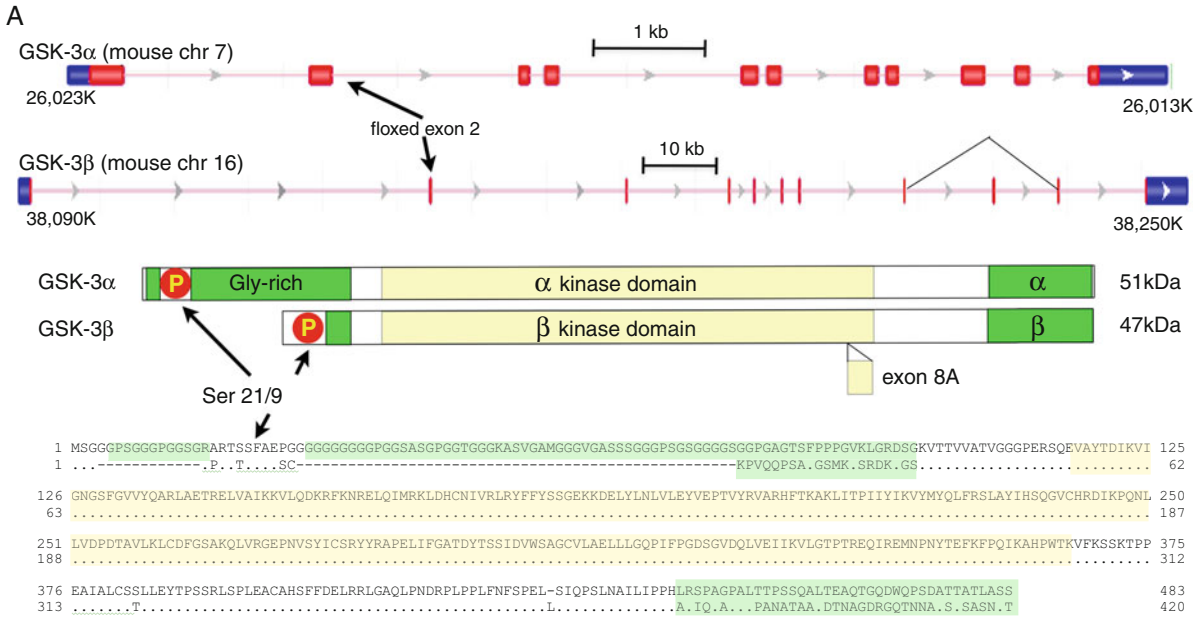
As mentioned above, mammals have two GSK-3 genes that encode two distinct proteins: GSK-33α

Glycogen Synthase Kinase-3, Table 1 GSK-3 substrates arranged into functional subgroups

Metabolism	
ATP-citrate lyase	Protein phosphatase 1
Glycogen synthase	Protein phosphatase 1 inhibitor-2
Pyruvate dehydrogenase	Acetyl CoA carboxylase
Signaling proteins	
Axin	Cyclic-AMP-dependent protein kinase – RII subunit
Adenomatous polyposis coli	Cyclin D1 and E
Cubitus interruptus/Gli	Eukaryotic initiation factor 2B
Per2	Cry2
Presenilin-1	Nucleoporin p62
p21 cdk inhibitor	Lipoprotein receptor-related protein 5/6
Insulin receptor substrate 1 and 2	Tuberous sclerosis 2
NGF receptor	Mcl-1
Structural proteins	
▶ Ncam	Collapsin response mediator proteins 2 and 4
Dynamin-like protein	Neurofilament heavy subunit
Kinesin light chain	Microtubule-associated protein 1B and 2
Ninein	Telokin (KRP) (kinase-related protein)
Tau	CLIP-associated protein 1 and 2
Paxillin	DF3/MUC1 (mucin-like glycoprotein)
Transcription factors	
β-catenin	Nuclear factor of activated T cells
Bcl3	CCAAT/enhancer-binding proteins α and β
Snail	Cyclic AMP response element-binding protein
GATA4	Microphthalmia-associated transcription factor
hypoxia-inducible factor 1	Glucocorticoid receptor
Heat shock factor-1	Bcl-2 interacting transcriptional repressor
c-▶ Myc and L-▶ Myc	SMAD1
c-Jun	▶ NF-κB (p65 and p105)
JunD	Notch
▶ P53	c-Myb

and GSK-3β (Woodgett 1990) (Fig. 1). GSK-3β is also differentially spliced in the brain to generate a minor (∼15% of total) isoform termed GSK-3β2, which contains a 13 amino acid insert within the kinase domain (exon 8A). GSK-3 orthologues have been found in all eukaryotes examined to date, including genetically tractable organisms. While structurally highly related, GSK-3α and GSK-3β are not functionally equivalent. This became obvious upon the generation of GSK-3β knockout mice (Hoeflich et al. 2000). Embryos carrying homozygous deletions of exon 2 of GSK-3β die between embryonic day 16.5 and birth due to liver degeneration caused by defects in NF-κB responses to maternal infection or (at birth) to patterning defects in the heart that preclude lung circulation of oxygenated blood. GSK-3α null animals, by contrast, are viable and demonstrate that GSK-3β can substitute for α but not vice versa (MacAulay et al. 2007). GSK-3α mutant mice do exhibit a variety of phenotypes including insulin sensitization and behavioral abnormalities. Conditional alleles of GSK-3α and β have also been generated. The first analysis of these animals involved rat insulin promoter-driven Cre that expresses the recombinase only in β-islet cells. These animals only lacked GSK-3β in the β-cells and had normal insulin responses. However, β-islet cell inactivation of GSK-3β rescued insulin resistance of insulin receptor substrate-2 mutant animals as well as insulin resistance caused by haploinsufficiency of the insulin receptor itself. Although the GSK-3α global knockout mice were insulin-sensitized, this effect was largely restricted to the liver, which exhibited significantly higher efficiency in glycogen deposition and was also dependent on strain background. Since GSK-3β null animals are inviable, floxed GSK-3β animals with either albumin-Cre or myosin light chain kinase-Cre have been generated to inactivate the gene in liver or skeletal muscle, respectively. These mice revealed inverse effects to the GSK-3α mutants, such that knocking out GSK-3β in the liver had no impact, whereas the animals lacking the enzyme in skeletal muscle were insulin-sensitized. These unanticipated results demonstrated that not only are the two isoforms of GSK-3 nonredundant, they also have tissue-specific roles.

An allelic series of embryonic stem cells have been engineered that are deficient in the various alleles of GSK-3α and GSK-3β. GSK-3 nullizygous ES cells proliferate normally but were highly refractive to differentiation cues and maintained expression of the pluripotency-associated transcription factors nanog and oct-4 even when grown as embryoid bodies in the absence of LIF (Doble et al. 2007). Likewise,



Glycogen Synthase Kinase-3, Fig. 1 *Structural architecture of GSK-3 genes and proteins*. Panel **A**. Chromosomal organization of mouse *GSK-3* genes. Coding exons are *red*, noncoding exons *blue*. The exon flanked by LoxP sites (exon2) is indicated. In the *middle* is a schematic topology of the two protein isoforms. *Pale yellow boxes* indicate the kinase domain, *green boxes* indicate the unconserved regions. The PKB/Akt phosphorylation site (Ser21/Ser9 in GSK-3α/β respectively) is shown by a *red-circled* "P." Below this are protein sequence alignments of mouse GSK-3α and GSK-3β, color-coded as above

treatment of mouse or human ES cells with GSK-3 inhibitors maintains pluripotency (Sato et al. 2004). GSK-3 therefore plays a critical role in supporting the capacity of embryonic stem cells to differentiate and, in its absence, these cells are effectively locked in a "ground state" (Ying et al. 2008).

Consequences of total inactivation of GSK-3: The block to differentiation in ES cells upon inactivation of GSK-3 is recapitulated upon genetic inactivation of the kinase in more differentiated or lineage-committed cells. For example, expression of Cre recombinase in presumptive neuronal precursor cells (via nestin-Cre) causes greatly enhanced neuronal precursor populations (as marked by Sox2) at the expense of differentiated neurons (Kim et al. 2009). These animals died at birth. Activation of the Wnt, ▶ Notch, Hedgehog, and PI-3K pathways downstream of GSK-3 was observed in the nullizygous neurons. Treatment of primary neuronal cultures with inhibitors (dominant-negative TCF4, γ-secretase inhibitor, cyclopamine and wortmannin, respectively) to these individual pathways revealed partial dependence on each for proliferation, with only a combination of the inhibitors achieving full suppression. This study also noted a loss of polarized cell division in dividing neurons in the subventricular zone, a process that is required for efficient stratification of the neuronal layers, suggesting an important role for GSK-3 in regulating spindle pole orientation. Similar suppression of differentiation to that observed in ES cells and neuronal precursors has been observed in other cell types and tissues in which all alleles of GSK-3 has been selectively genetically inactivated (Woodgett lab, Unpublished observations).

GSK-3 and Cancer Etiology

These animal models each share a similar property: differentiation is inhibited and there is expansion of precursor populations. These findings have implications for in vitro expansion of adult stem/progenitor cells in regenerative medicine but also provide insight into the early stages of tumor development. GSK-3 has been implicated in cancer at various levels, primarily as a tumor suppressor. Its inhibitory role in the major

signaling pathways described above that are most commonly activated in human cancers is well documented. However, the kinase has also been positively linked to pancreatic cancer and regulation of Snail, a transcription factor key to epithelial to mesenchymal transitions (EMT), a process associated with metastasis. A splice mutant of GSK-3β has recently been identified in a population of leukemic stem cells derived from blast crisis chronic myelogenous leukemia (CML) (Abrahamsson et al. 2009). Reintroduction of wild-type GSK-3β into the CML cells reduced engraftment efficiency suggesting a role in promoting stem cell expansion. By contrast, GSK-3 was found to play a tumor-promoting role in MLL-driven leukemias where its inhibition led to regression of the leukemic cells through destabilization of the CDK inhibitor, p27Kip1. In addition, inhibition of GSK-3 has been reported to induce glioma apoptosis through inhibition of ▶ NF-κB and to enhance sorafenib-stimulated apoptosis in melanoma cell lines. Hence, GSK-3's role in cancer progression remains to be properly elucidated.

Summary

GSK-3 is clearly an unusual protein kinase. It has an inhibitory influence in multiple signaling pathways that are critical for normal development as well as in metabolic control. Its high activity in "resting" tissue suggests its role is generally and suppressive. Signals must relieve the inhibitory action of GSK-3 and there are several means to achieve this including phosphorylation and sequestration. There are many inhibitors of GSK-3, a number of which are potent and relatively specific. Of note, these inhibitors do not discriminate between the isoforms or subcellular fractions. Chemical inhibition of GSK-3 is therefore a blunt tool for investigating the functions of the kinase. It is also recognized that the consequences of GSK-3 inhibition depend on the degree to which activity is suppressed. For example, mice lacking a single GSK-3 allele (equivalent to 25% inhibition) have clearly measurable behavioral phenotypes whereas almost complete inhibition by drugs is required to induce β-catenin stabilization. Lastly, research into this protein kinase has largely focused on the GSK-3β isoform. A search of PubMed identifies over 1300 articles with GSK-3β in the title but only 66 for GSK-3α. This is in spite of the fact that the two isoforms are largely, although not completely, redundant and no available small molecule inhibitors are selective to either isoform. Hence, much of the literature is overly focused on GSK-3β and many data are erroneously attributed to effects of GSK-3β without consideration of the role of GSK-3α. This "β bias" is both unnecessary and unfortunate in that much of the data pertain equally to the less appreciated isoform which likely pulls as much weight in cellular regulation as its sibling.

References

Abrahamsson AE, Geron I, Gotlib J, Dao KH, Barroga CF, Newton IG, Giles FJ, Durocher J, Creusot RS, Karimi M, Jones C, Zehnder JL, Keating A, Negrin RS, Weissman IL, Jamieson CH. Glycogen synthase kinase-3beta missplicing contributes to leukemia stem cell generation. Proc Natl Acad Sci USA. 2009;106:3925–9.

Allon LT, Pietrokovski S, Barkan S, Avrahami L, Kaidanovich-Beilin O, Woodgett JR, Barnea A, Eldar-Finkelman H. Selective loss of glycogen synthase kinase-3α in birds reveals distinct roles for GSK-3 isozymes in tau phosphorylation. FEBS Lett. 2011;585:1158–62.

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature. 2006;442:823–6.

Doble BW, Woodgett JR. Role of glycogen synthase kinase-3 in cell fate and epithelial-mesenchymal transitions. Cells Tissues Organs. 2007;185:73–84.

Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. Dev Cell. 2007;12:957–71.

Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP dependent protein kinase and phosphorylase kinase. Eur J Biochem. 1980;107:519–27.

Forde JE, Dale TC. Glycogen synthase kinase 3: a key regulator of cellular fate. Cell Mol Life Sci. 2007;64:1930–44.

Hoeflich KP, Luo J, Rubie EA, Tsao M-S, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature. 2000;406:86–90.

Jiang J, Hui CC. Hedgehog signaling in development and cancer. Dev Cell. 2008;15:801–12.

Kim WY, Wang X, Wu Y, Doble BW, Patel S, Woodgett JR, Snider WD. GSK-3 is a master regulator of neural progenitor homeostasis. Nat Neurosci. 2009;12:1390–7.

Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA. 1996;93:8455–9.

Li M, Wang X, Meintzer MK, Laessig T, Birnbaum MJ, Heidenreich KA. Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta. Mol Cell Biol. 2000;20:9356–63.

Lochhead PA, Kinstrie R, Sibbet G, Rawjee T, Morrice N, Cleghon V. A chaperone-dependent GSK3beta transitional

intermediate mediates activation-loop autophosphorylation. Mol Cell. 2006;24:627–33.
MacAulay K, Doble BW, Patel S, Hansotia T, Sinclair EM, Drucker DJ, Nagy A, Woodgett JR. Glycogen synthase kinase 3 alpha-specific regulation of murine hepatic glycogen metabolism. Cell Metab. 2007;6:329–37.
Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med. 2004;10:55–63.
Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL, Sabio G, Davis RJ, Matthews DE, Doble B, Rincon M. Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science. 2008;320:667–70.
Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/Factor A. EMBO J. 1990;9:2431–8.
Ying QL, Wray J, Nichols J, Batle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell renewal. Nature. 2008;453:519–23.
Zeng X, Tamai K, Doble B, Li S, Huang H, Habas R, Okamura H, Woodgett J, He X. A dual kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature. 2005;438:873–7.

Glycoprotein G

► Thrombospondin-1

Glycylpeptide N-Tetradecanoyltransferase

► NMT (N-Myristoyltransferase)

Gna12

► G Protein Alpha 12

GNAI

► G Protein α i/o/z

GNAO

► G Protein α i/o/z

GNAO /Gαo/Guanine Nucleotide Binding Protein/Alpha Activating Activity Polypeptide O (AGS10)

► Activators of G-Protein Signaling (AGS)

GNAO1

► G Protein α i/o/z

GNAT1

► G Protein Alpha Transducin

GNAT2

► G Protein Alpha Transducin

GNAZ

► G Protein α i/o/z

Gnb

► G Protein Beta/Gamma

Gng

► G Protein Beta/Gamma

GNRP

► RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor)

Go Alpha Subunit

▶ G Protein α i/o/z

gp96

▶ Grp94 (HSP90B1)

gpa-12 (*Caenorhabditis elegans* Homolog)

▶ G Protein Alpha 12

GPCR84

▶ GPR84

GPR100

▶ Relaxin Family Peptide Receptors (RXFP) 3 and 4

GPR105

▶ $P2Y_{14}$ Receptor

GPR84

Kimberly J. Perry and Jonathan J. Henry
Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Synonyms

GPCR84; G-protein-coupled receptor 84

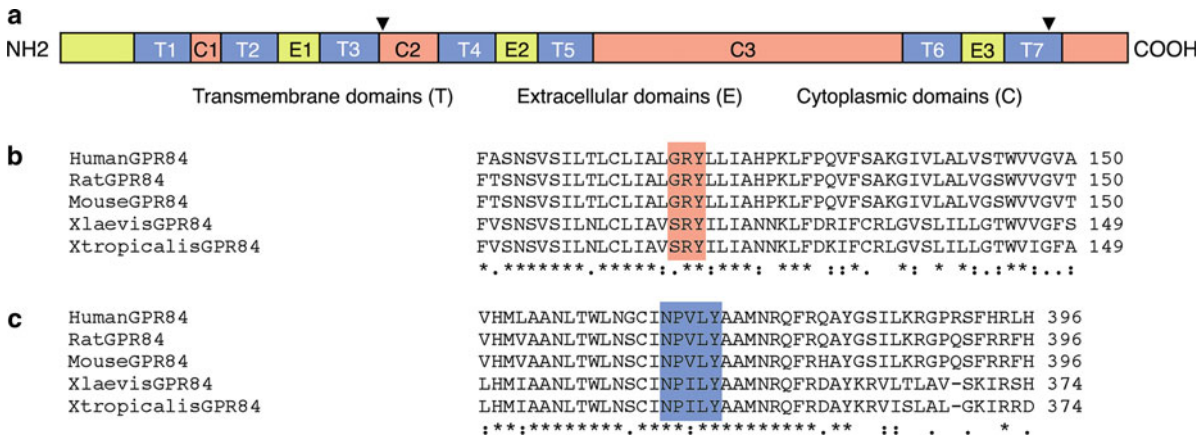
Historical Background

G-protein-coupled receptors constitute the largest and most diverse family of integral membrane proteins and are involved in a wide variety of physiological functions. Some of these functions include mediating responses to hormones, neurotransmitters, odorants and light, and regulation of the immune system and inflammation (Yousefi et al. 2001; Lattin et al. 2008; Weis and Kobilka 2008; Gloriam et al. 2009). GPR84 belongs to the rhodopsin subfamily of G- protein-coupled receptors and was first discovered during a comprehensive search of available expressed sequence tags (EST; Wittenberger et al. 2001). Since its discovery, *GPR84* has been found in many human tissues, in mouse spleen and liver, and in embryonic *Xenopus laevis* tissues (Wittenberger et al. 2001; Perry et al. 2010). This receptor was considered an orphan receptor until late 2006, when it was discovered that GPR84 functions as a receptor for medium-chain free fatty acids (FFAs, Wang et al. 2006).

Structure and Activation of GPR84

GPR84 is an integral membrane protein that consists of an extracellular amino terminus, seven transmembrane domains, and a cytoplasmic carboxy terminus (Fig. 1). The transmembrane domains contain the most sequence conservation among the rhodopsin family and are characterized by a stretch of 25–35 consecutive residues that are believed to represent seven alpha helices. Each alpha helix is arranged in the plasma membrane in a counterclockwise orientation and groups together to form a heptahelical bundle, which is considered the receptor (Fredriksson et al. 2003). Another signature element specific to GPR84 is the (D/G/S)RY motif in the second cytoplasmic domain. In human, mouse, and rat, this is represented as GRY, while in *Xenopus* it is represented as SRY (Fig. 1). The final characteristic element contained in the last transmembrane domain is the NPXXY motif, which is represented as NPVLY in human, mouse, and rat, and NPILY in *Xenopus* (Perry et al. 2010).

The arrangement of the transmembrane domains creates a ligand-binding pocket or receptor that is important for signaling events. Activation of this



GPR84, Fig. 1 Characteristic G-protein-coupled receptor (GPR) domains and conserved sequence motifs. (**a**) Schematic illustrating conserved features among known rhodopsin family members. N-terminal extracellular domain (NH2) is toward the *left*; C-terminal intracellular domain is toward the *right* (COOH). T1-T7, seven transmembrane domains; C1-C3, three cytoplasmic domains; E1-E3, three extracellular domains. *Black arrowhead* in C2 indicates the location of the typical (D/G/S)RY motif, further illustrated in (**b**). *Black arrowhead* within T7 indicates the location of the NPXXY motif, further illustrated in (**c**). (**b**) Protein comparison of known GPR84 sequences in reference to the (D/G/S)RY motif (*shaded*). (**c**) Protein comparison of known GPR84 sequences in reference to the NPXXY motif (*shaded*) (Source: Dev Dyn 2010;239:3024–7)

receptor occurs when the ligand binds, resulting in an interaction of the cytoplasmic part of the receptor with the heterotrimeric G protein (Weis and Kobilka 2008). The cellular response to the extracellular signal is mediated through second messenger cascades and this attenuation of GPCR signaling can be achieved by phosphorylation of the receptor. This results either in a conformational change affecting G protein binding or by reduced cell surface expression (Gainetdinov et al. 2004; Prossnitz 2004).

Identification of the ligands that activate GPCRs has been ongoing and many receptors still remain classified as orphan receptors. However, a library of biochemical intermediates was used to identify the ligands capable of activating GPR84 (Wang et al. 2006). This study identified medium-chain free fatty acids (FFAs) with carbon lengths of 9–14 as activators of GPR84. The most potent agonists of GPR84 were found to be capric acid, undecanoic acid, lauric acid, and the small molecule, diindolylmethane. Since diindolylmethane activates GPR84 with much greater potency, it has been used to investigate the effect of GPR84 activation on cytokine secretion (Wang et al. 2006). Interestingly, GPR84 is not activated by short-chain FFAs or by long-chain saturated and unsaturated FFAs.

Role in Immune Response

Several studies have emerged that have given some insight into the role of GPR84 during immune response in humans and mice. During a screen of human peripheral blood neutrophils, the *EX33* gene (later named GPR84) was found and identified as a novel G-protein-coupled receptor (Yousefi et al. 2001). Since movement of neutrophils into inflamed tissues is mediated by chemokines that regulate signaling through GPCRs (Adams and Lloyd 1997), it was of particular interest to determine whether EX33 was involved in this type of signaling mechanism. Additionally, identification of chemoattractants and their receptors has implications in the treatment of inflammatory diseases. Experiments were conducted to test *EX33* expression and revealed its presence in a variety of human cells and tissues (bone marrow, lung, colon, placenta). Abundant expression was noted in neutrophils and eosinophils in comparison with significantly lower levels in other cell types. This study was of particular interest because it presented the first evidence that EX33 was regulated by agonist-dependent mechanisms. In experiments where neutrophils were stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF; an inflammatory

cytokine), lower levels of EX33 proteins were detected, as compared to unstimulated neutrophils. In addition to its accepted involvement in neutrophil activation, the data suggests that GM-CSF might also be involved in the hindrance of neutrophils at the site of inflammation by downregulation of cell surface receptors (Yousefi et al. 2001).

A recent study to examine the biological function of GPR84 in mice utilized GPR84 knockout animals (Venkataraman and Kuo 2005). The proliferation rates of T and B cells were found to be similar in wild-type animals versus GPR84 knockout mice and though GPR84 is not necessary to modulate T and B cell proliferation, the absence of GPR84 in $CD4^+$ T cells results in the increased production of IL-4 by anti-CD3-activated cells. Additionally, when T cells were cultured in the appropriate stimuli, differentiation into Th1 or Th2 effector populations occurred. Those Th2 effector cells cultured from GPR84-deficient mice consistently secreted higher levels of IL-4, IL-5, and IL-13 in comparison to wild-type animals. Early cytokine signaling mechanisms are poorly understood, yet it can be suggested that hyper-Th2 cytokine production in GPR84-deficient mice can be correlated with their ability to synthesize more IL-4 protein during initial stimulation under Th2 differentiation conditions.

The inflammatory response of GPR84 was also noted in microglia (macrophage population of the brain) of mice analyzed after lipopolysaccharide (LPS)-induced endotoxemia (Bouchard et al. 2007). GPR84 is strongly expressed here and also in peripheral monocytic cells of the spleen. Additional evidence reveals that GPR84 is also expressed in clusters near blood vessels in microglia with induced experimental autoimmune encephalomyelitis (EAE) and the abundance of labeled clusters increases with the severity of the disease. Endogenous or exogenous inflammatory stimuli are capable of inducing *GPR84* gene transcription in the microglia. Soluble mediators such as TNF and IL-1 mediate the effect of LPS on GPR84 expression in the brain, although other mediators have yet to be discovered (Bouchard et al. 2007). The higher levels in microglia and populations of peripheral macrophages suggest that GPR84 may play a predominant role in populations of macrophages. Additional research has revealed that GPR84 is restricted to bone-marrow macrophage (BMM) and microglia in an unstimulated state (Lattin et al. 2008). However, upon stimulation with LPS, GPR84 is significantly upregulated in BMM and thioglycollate-elicited peritoneal macrophages (TEPMs), consistent with the previously mentioned study. These recent studies reveal a role for GPR84 in neuroinflammation and may present a useful tool in tracking macrophage populations.

Role in Fatty Acid Metabolism

In the presence of G protein G_{qi9}, both human and murine GPR84 are activated by medium-chain FFAs, suggesting that signaling occurs through the pertussis-sensitive $G_{i/o}$ pathway. GPR84 does not signal through the G_s-mediated pathway and is unlikely to signal through the G_q pathway.

Medium-chain FFAs were also shown to amplify LPS-stimulated production of IL-12 p40 directly through GPR84 (Wang et al. 2006). In the presence of naturally occurring medium-chain FFAs (capric acid, undecanoic acid, lauric acid) or in the presence of the small molecule agonist, diindolylmethane, an increase was observed in the secretion of IL-12 p40 subunit from LPS-stimulated RAW264.7 cells. IL-12 p40 is a proinflammatory cytokine responsible for the eradication of pathogens. This cytokine induces and maintains T helper 1 (Th1) responses and inhibits T helper 2 (Th2) responses (Scott 1993; Hsieh et al. 1993; Kopf et al. 1994). Consistent with these results is the increased production of Th2 cytokines in GPR84-deficient T cells (Venkataraman and Kuo 2005). Combined, these studies emphasize a role for GPR84 as a leukocyte-specific receptor for medium-chain FFAs.

Role in Development

Recent studies have focused on GPR84s role in immune response and so far little is known about its role during development. One study showed that GPR84 expression is also important for proper morphogenesis of the eye in the frog, *Xenopus laevis* (Perry et al. 2010). RT-PCR analysis of various embryonic stages of *Xenopus* shows that *GPR84* mRNA is present beginning at gastrulation through larval stages and more specifically in the retina, lens, and larval cornea epithelium. Previous mouse and human studies have not noted expression in the eye; however, these specific tissues do not appear to have been examined

(Yousefi et al. 2001; Venkataraman and Kuo 2005; Wang et al. 2006; Bouchard et al. 2007; Lattin et al. 2008).

GPR84 MO-mediated knockdown experiments revealed a role for GPR84 specifically in lens and retina morphogenesis (Perry et al. 2010). Significantly reduced lenses formed, but did not display secondary fiber cells and had defects in the lens epithelium. The retina was also significantly impacted, showing signs of improper differentiation of various neural retinal layers. Cell proliferation and apoptosis assays also show that GPR84 knockdown tissues not only display a higher number of proliferating cells, but also a higher number of apoptotic cells. Previous studies (Ohnuma et al. 2002; Casarosa et al. 2003) have demonstrated that the proper development of cells depends on the delicate balance of cell proliferation and fate determination. Impairing a cell's ability to exit the cell cycle can have significant implications in terms of the ultimate cell fate. It is possible that GPR84 could be a negative regulator of cell proliferation in the retina and lens, where suppressing proliferation at specific time points may be important for differentiation of the neural retina, lens, and cornea.

Summary

GPR84 is a transmembrane receptor originally identified through an EST search. Various medium-chain FFAs act as ligands to activate and induce a conformational change in the receptor. Interaction between the intracellular portion of the receptor and heterotrimeric G proteins results in activation of second messenger cascades and effector proteins. Mediation of cell signaling occurs through phosphorylation of GPR84 and results in lower binding efficiencies or reduced cell surface expression. Further analysis to determine the conformation of the active and inactive receptor will yield a better understanding of this signaling mechanism. *GPR84* expression has been noted to be higher in neutrophils and eosinophils. Inflammatory stimuli are able to induce GPR84 transcription in microglia and in clusters near blood vessels of microglia, and can be mediated by TNF and IL-1. GPR84-deficient mice result in $CD4^+$ T cells that produce higher levels of IL-4 and culturing those T cells produces Th2 effector cells that secrete higher levels of IL-4, IL-5, and IL-13. Increased secretion of IL-12 p40 occurs in LPS-stimulated RAW264.7 cells in the presence of medium-chain FFAs or diindolylmethane. *GPR84* expression is also important for proper eye development in *Xenopus*. Knockdown of *GPR84* expression results in defective lens formation, an improperly differentiated neural retina, higher proliferation rates, and an increase in cells undergoing apoptosis. Overall, GPR84 plays an important role in development, free fatty acid metabolism, and immune system regulation. Further understanding of the GPR84 signaling cascade may lead to promising therapies/drugs for the treatment of both metabolic and autoimmune diseases.

References

Adams DH, Lloyd AR. Chemokines: leukocyte recruitment and activation cytokines. Lancet. 1997;349:490–5.

Bouchard C, Pagé J, Bédard A, Tremblay P, Vallières L. G protein-coupled receptor 84, a microglia-associated protein expressed in neuroinflammatory conditions. Glia. 2007;55:790–800.

Casarosa S, Amato MA, Andeazzoli M, Gestri G, Barsacchi G, Cremisi F. *Xrx1* control proliferation and multipotency of retinal progenitors. Mol Cell Neurosci. 2003;22:25–36.

Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups and fingerprints. Mol Pharmacol. 2003;63:1256–72.

Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG. Desensitization of G protein-coupled receptors and neuronal functions. Annu Rev Nuerosci. 2004;27:107–44.

Gloriam DE, Foord SM, Blaney FE, Garland SL. Definition of the G protein-coupled receptor transmembrane bundle binding pocket and calculation of receptor similarities for drug design. J Med Chem. 2009;52:4429–42.

Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Pillars article: development of TH1 $CD4^+$ T cells through IL-12 produced by listeria-induced macrophages. Science. 1993;260:547–9.

Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H, Köhler G. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature. 1994;368:339–42.

Lattin JE, Schroder K, Su AI, Walker JR, Zhang J, Wiltshire T, Saijo K, Glass CK, Hume DA, Kellie S, Sweet MJ. Expression analysis of G protein-coupled receptors in mouse macrophages. Immunome Res. 2008;4:5.

Ohnuma S, Hopper S, Wang KC, Philpott A, Harris WA. Coordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the *Xenopus retina*. Development. 2002;129:2435–46.

Perry KJ, Johnson VR, Malloch EL, Fukui L, Wever J, Thomas AG, Hamilton PW, Henry JJ. The G-protein-coupled receptor, GPR84, is important for eye development in *Xenopus laevis*. Dev Dyn. 2010;239:3024–37.

Prossnitz ER. Novel roles for arrestins in the post-endocytic trafficking of G protein-coupled receptors. Life Sci. 2004;75:893–9.
Scott P. IL-12: initiation cytokine for cell-mediated immunity. Science. 1993;260:496–7.
Venkataraman C, Kuo F. The G-protein coupled receptor, GPR84 regulates IL-4 production by T lymphocytes in response to CD3 crosslinking. Immunol Lett. 2005;101:144–53.
Wang J, Wu X, Simonavicius N, Tian H, Ling L. Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. J Biol Chem. 2006;281:34457–64.
Weis WI, Kobilka BK. Structural insights into G-protein-coupled receptor activation. Curr Opin Struct Biol. 2008;18:734–40.
Wittenberger T, Schaller HC, Hellebrand S. An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. J Mol Biol. 2001;307:799–813.
Yousefi S, Cooper PR, Potter SL, Mueck B, Jarai G. Cloning and expression analysis of a novel G-protein-coupled receptor selectively expressed on granulocytes. J Leukoc Biol. 2001;69:1045–52.

G-Protein α_q (Gene Name: GNAQ)

Björn H. Falkenburger
Department of Neurology, RWTH University Medical Center, Aachen, Germany

Historical Background

Hormone receptors elicit cellular responses most often not directly, but through diffusible second messengers. In the early 1970s it was found that second messenger synthesis requires guanosine triphosphate (GTP). Yet, GTP binds neither to hormone receptors nor to enzyme effectors. Instead, GTP binds to GTP-sensitive transducers (see Gilman 1987). These G-proteins are heterotrimers of α, β, and γ subunits. The first Gα subunits to be identified were $G\alpha_s$ and transducin. $G\alpha_s$ triggers the formation of cAMP. Transducin mediates the effects of rhodopsin. Signaling by $G\alpha_s$ was found sensitive to pertussis toxin. Signaling by $G\alpha_i$, which inhibits cAMP formation, was found sensitive to cholera toxin. Formation of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC) was neither affected by pertussis toxin nor by cholera toxin. Therefore, a third class of Gα subunits was proposed. In 1990 Strathmann and Simon cloned murine Gα subunits that lack the sites modified by pertussis and cholera toxin. They termed them $G\alpha_q$ and suggested that they mediate signaling to PLC (Strathmann and Simon 1990).

Gene and Protein

The official gene symbol for $G\alpha_q$ in *Homo sapiens* and other mammals is GNAQ (guanine nucleotide-binding protein alpha q). GNAQ is the prototypical member of a G-protein family formed by GNAQ, GNA11, GNA14, and GNA15. As for the related $G\alpha_i$ family, the genomic organization is such that two $G\alpha_q$ genes are located in sequence, likely the result of tandem duplication (Wilkie et al. 1992). GNAQ is located on chromosome 9q21 together with GNA14. A very similar pseudogene is at 2q14.3–q21 (Dong et al. 1995).

The tertiary structure of $G\alpha_q$ has been solved (e.g., Nishimura et al. 2010). It is (reversibly) palmitoylated at *N*-terminal cysteins (C9, C10, Tsutsumi et al. 2009). The GTPase domain of $G\alpha_q$ is similar to the small, monomeric GTPase Ras. While not all combinations between Gβ and Gγ isoforms are possible, any of these Gβγ dimmers can in principle form a heterotrimer with $G\alpha_q$. The heterotrimeric G-protein $\alpha_q\beta\gamma$ is termed G_q after the α subunit.

Medical Relevance

Plasma membrane receptors signaling through G_q include the following G-protein coupled (GPCR) or seven-transmembrane domain (7TM) receptors: α_1 adrenoreceptor, M_1, M_3, M_5 muscarinic receptors, several P2Y purinergic (ATP, UTP) receptors, 5-HT_2 serotonin, B_2 bradykinin, H_1 histamine, GPR55 cannabinoid, PAR_2 trypsin, and group I metabotropic ► glutamate receptors. A close homologue of G_q mediates light perception in drosophila photoreceptors. Downstream responses include a rise in cytosolic calcium, which induces the secretion of hormones and neurotransmitters and the contraction of smooth muscles in vasculature and eye. Many of the GPCR activated by G_q are important drug targets. For instance, α_1 receptors mediate the actions of blood pressure–lowering drugs

and drugs treating benign prostatic hyperplasia, M_1 receptors mediate the effects of cholinergic drugs treating Alzheimer's disease, and H1 receptors are targeted by drugs treating allergies.

Mutations in GNAQ (and GNA11) have been associated with cancers, in particular uveal melanoma (Van Raamsdonk et al. 2009). $G\alpha_q$-deficient mice show cerebella ataxia and defective platelet activation (Offermanns 2001).

Overview of the G-Protein Cycle

In the classical view, $G\alpha_q$ has GDP bound at rest and forms a heterotrimer with $G\beta\gamma$ subunits. Activation of a G_q-coupled receptor (by ligand binding or other means) will lead to binding of G_q to the receptor and promote nucleotide exchange at $G\alpha_q$, i.e., the unbinding of GDP and the binding of GTP. $G\alpha_q$-GTP then dissociates from the receptor and from $G\beta\gamma$. This free $G\alpha_q$-GTP is the active form of $G\alpha_q$. It binds to effectors, most importantly isoforms of phospholipase C β (PLCβ). PLCβ hydrolyze the membrane phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP_2), forming the cytosolic second messenger inositol trisphosphate (IP_3) and the lipid diacylglycerol (DAG). In some cases, PLC activation depletes PIP_2. IP_3 releases calcium from intracellular stores; DAG activates protein kinase C (PKC); PIP_2 is an activator for many plasma membrane proteins. The endogenous GTPase activity of $G\alpha_q$ hydrolyzes GTP to GDP. This ends the activation of $G\alpha_q$. $G\alpha_q$-GDP then binds again to $G\beta\gamma$. If the receptor is still active, it can bind G_q again, catalyze nucleotide exchange, and induce another round of $G\alpha_q$ activity.

A More Detailed Description of the G-Protein Cycle

This section considers the individual steps of the G-protein cycle in more detail. Receptor activation evokes a conformational change in the receptor. This conformational changes occurs relatively fast (<100 ms in M_1R, Jensen et al. 2009). The affinity of the activated receptor for G_q is higher than the affinity of inactive receptor, leading to net binding of G-proteins to the receptor. For M_1R, G-protein binding takes about 200 ms. Because of the law of mass action, the speed of binding may depend on the local density of G-proteins in the vicinity of the receptor (Falkenburger et al. 2010).

Some investigators suggest, that heterotrimeric G_q is preassembled with G_q-coupled receptors. Whether – and to which extent – this is the case has remained controversial (discussed, e.g., in Hein and Bünemann 2009 and Falkenburger et al. 2010). Likely there is some finite affinity, and the fraction of receptors that have G_q bound at rest depends on the local density of G_q molecules in the vicinity of the receptor. A related question is whether an inactive receptor can catalyze nucleotide exchange, i.e., whether the receptor is "precoupled" to G_q. Some effects of the M_1R antagonist atropine can be interpreted in this way, but the extent of precoupling is certainly smaller than in adrenoreceptors. Finally, there is some recent evidence that different ligands may stabilize the receptor in different conformations, favoring binding of certain downstream partners over others and thus biasing signaling through one of several alternative pathways.

The active receptor acts as a GEF (guanine exchange factor) for $G\alpha_q$, speeding up the exchange of GDP to GTP. The rate-limiting step of nucleotide exchange is the unbinding of GDP from $G\alpha_q$. This step is what is accelerated when G_q is bound to activated receptor (Ross 2008). Without GAP (GTPase activating protein), the endogenous GTPase activity of $G\alpha_q$ is very slow (time constant of 30 s, Falkenburger et al. 2010), leaving $G\alpha_q$ (and $G\beta\gamma$) active for a fairly long time. Some effectors, including PLCβ, act as GAPs and accelerate the GTPase activity of $G\alpha_q$, speeding up recovery. Other GAPs for $G\alpha_q$ include RGS proteins (regulators of G-protein signaling) and the $G\alpha_q$ effector p115RhoGEFe (Ross and Wilkie 2000).

If PLCβ would only accelerate GTPase activity, it would turn off the molecule that activates it and PLCβ activation should be transient. However, this is not what was observed experimentally. PLCβ can be continually active for minutes. One possibility is that PLCβ also acts as GEF and accelerates nucleotide exchange (see Falkenburger et al. 2010). A different explanation is "kinetic scaffolding" (see Ross 2008), meaning that the GTPase activity of $G\alpha_q$ bound to PLCβ is so fast that GTP is hydrolyzed even before $G\alpha_q$-GTP has had a chance to dissociate from the receptor. The receptor (a GEF) then catalyzes again

nucleotide exchange and $G\alpha_q$ is kept at the receptor until the latter is no longer active. In both cases, fast GTPase activity is balanced by fast nucleotide exchange and $G\alpha_q$ bound to PLCβ is kept active by a rapid cycle of nucleotide exchange and GTP hydrolysis. The purpose of such seemingly futile GTP consumption is to allow rapid signaling. Without the GAP/GEF effect, responses would persist and blur into one another.

Spatial Aspects

The classical view holds that upon nucleotide exchange, G_q dissociates into $G\alpha_q$ and Gβγ. And indeed, some G-proteins do physically dissociate (Lambert 2008). Some Gβγ subunits even translocate to intracellular membranes upon activation while $G\alpha_q$ stays at the plasma membrane (Saini et al. 2009). However, for some G-proteins, activation results in a *decreased* distance between the Gα and Gβ subunits, which is inconsistent with subunit dissociation. Here, nucleotide exchange may merely result in a conformational rearrangement without physical dissociation (see Lohse et al. 2008). A related question is whether $G\alpha_q$ can stay bound to the receptor while binding and activating PLC. For $G\alpha_q$, an *increase* in the average distance to Gβ was observed upon activation along with a *decrease* in the distance to PLCβ (Jensen et al. 2009). This is consistent with the classical view. Nonetheless, effectors can be activated without dissociation of $G\alpha_q$ and Gβγ (Yuan et al. 2007). In addition to the mentioned activation-induced translocation of G-protein subunits, recent evidence suggests a continuous shuttling of G-protein heterotrimers between the plasma membrane and intracellular membrane compartments at rest (Saini et al. 2009).

Summary

$G\alpha_q$ mediates signaling from ubiquitous G_q-coupled plasma membrane receptors to PLCβ. Since its identification, considerable progress has been made in understanding kinetics and functioning of the GDP/GTP cycle. Further work is expected to decipher in more detail the spatial aspects of $G\alpha_q$ signaling, i.e., whether signaling molecules are preassembled in a large complex and information is transmitted mainly through conformational changes, or whether G-proteins indeed dissociate upon activation and translocate to associate with effectors.

Because many different receptors couple to G_q, a second challenge will be to better understand to what extent – and by which mechanisms – signaling from these different receptors can be kept separate within a single cell. For example, M_1 muscarinic receptors do elicit a calcium response in superior cervical ganglion cells, whereas bradykinin receptors do not (see Delmas et al. 2004).

Finally, it has become clear that $G\alpha_q$ can signal through other pathways than PLC. $G\alpha_q$ can activate Rho family GTPases through p115RhoGEFe, which activates RhoA by favoring nucleotide exchange there (Rojas et al. 2007). In addition, $G\alpha_q$ can bind to and activate Bruton's tyrosine kinase (Ma and Huang 1998), and possibly further kinases. Conversely, some Gβγ dimers can also activate isoforms of PLCβ, suggesting that not all effects of Gq are mediated by $G\alpha_q$. Therefore, even 20 years after cloning $G\alpha_q$ it can still be difficult to determine which molecules mediate a signaling event that is unaffected by pertussis and cholera toxins.

References

Delmas P, Crest M, Brown DA. Functional organization of PLC signaling microdomains in neurons. Trends Neurosci. 2004;27(1):41–7.

Dong Q, Shenker A, Way J, Haddad BR, Lin K, Hughes MR, et al. Molecular cloning of human $G\alpha_q$ cDNA and chromosomal localization of the $G\alpha_q$ gene (GNAQ) and a processed pseudogene. Genomics. 1995;30(3):470–5.

Falkenburger BH, Jensen JB, Hille B. Kinetics of M_1 muscarinic receptor and G protein signaling to phospholipase C in living cells. J Gen Physiol. 2010;135(2):81–97.

Gilman AG. G proteins: transducers of receptor-generated signals. Annu Rev Biochem. 1987;56:615–49.

Hein P, Bünemann M. Coupling mode of receptors and G proteins. Naunyn-Schmiedebergs Arch. Pharmacol.. 2009;379(5):435–43.

Jensen JB, Lyssand JS, Hague C, Hille B. Fluorescence changes reveal kinetic steps of muscarinic receptor-mediated modulation of phosphoinositides and Kv7.2/7.3 K^+ channels. J Gen Physiol. 2009;133(4):347–59.

Lambert NA. Dissociation of heterotrimeric G proteins in cells. Sci Signal. 2008;1(25):re5.

Lohse MJ, Hein P, Hoffmann C, Nikolaev VO, Vilardaga J-P, Bünemann M. Kinetics of G-protein-coupled receptor signals in intact cells. Br J Pharmacol. 2008;153(Suppl 1): S125–32.

Ma YC, Huang XY. Identification of the binding site for Gqα on its effector Bruton's tyrosine kinase. Proc Natl Acad Sci USA. 1998;95(21):12197–201.
Nishimura A, Kitano K, Takasaki J, Taniguchi M, Mizuno N, Tago K, et al. Structural basis for the specific inhibition of heterotrimeric G_q protein by a small molecule. Proc Natl Acad Sci USA. 2010;107(31):13666–71.
Offermanns S. In vivo functions of heterotrimeric G-proteins: studies in Gα-deficient mice. Oncogene. 2001;20(13):1635–42.
Rojas RJ, Yohe ME, Gershburg S, Kawano T, Kozasa T, Sondek J. $G\alpha_q$ directly activates p63RhoGEF and Trio via a conserved extension of the Dbl homology-associated pleckstrin homology domain. J Biol Chem. 2007;282(40):29201–10.
Ross EM. Coordinating speed and amplitude in G-protein signaling. Curr Biol. 2008;18(17):R777–83.
Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem. 2000;69:795–827.
Saini DK, Chisari M, Gautam N. Shuttling and translocation of heterotrimeric G proteins and Ras. Trends Pharmacol Sci. 2009;30(6):278–86.
Strathmann M, Simon MI. G protein diversity: a distinct class of α subunits is present in vertebrates and invertebrates. Proc Natl Acad Sci USA. 1990;87(23):9113–7.
Tsutsumi R, Fukata Y, Noritake J, Iwanaga T, Perez F, Fukata M. Identification of G protein α subunit-palmitoylating enzyme. Mol Cell Biol. 2009;29(2):435–47.
Van Raamsdonk CD, Bezrookove V, Green G, Bauer J, Gaugler L, O'Brien JM, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature. 2009;457(7229):599–602.
Wilkie TM, Gilbert DJ, Olsen AS, Chen XN, Amatruda TT, Korenberg JR, et al. Evolution of the mammalian G protein α subunit multigene family. Nat Genet. 1992;1(2):85–91.
Yuan C, Sato M, Lanier SM, Smrcka AV. Signaling by a non-dissociated complex of G protein βγ and α subunits stimulated by a receptor-independent activator of G protein signaling, AGS8. J Biol Chem. 2007;282(27):19938–47.

G-Protein-Coupled Receptor 84

▶ GPR84

G-Protein-Coupled Receptor Kinase 1

▶ G-Protein-Coupled Receptor Kinase 1 (GRK1)

G-Protein-Coupled Receptor Kinase 1 (GRK1)

Frank S. Chen and Ching-Kang (Jason) Chen
Department of Biochemistry and Molecular Biology, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA

Synonyms

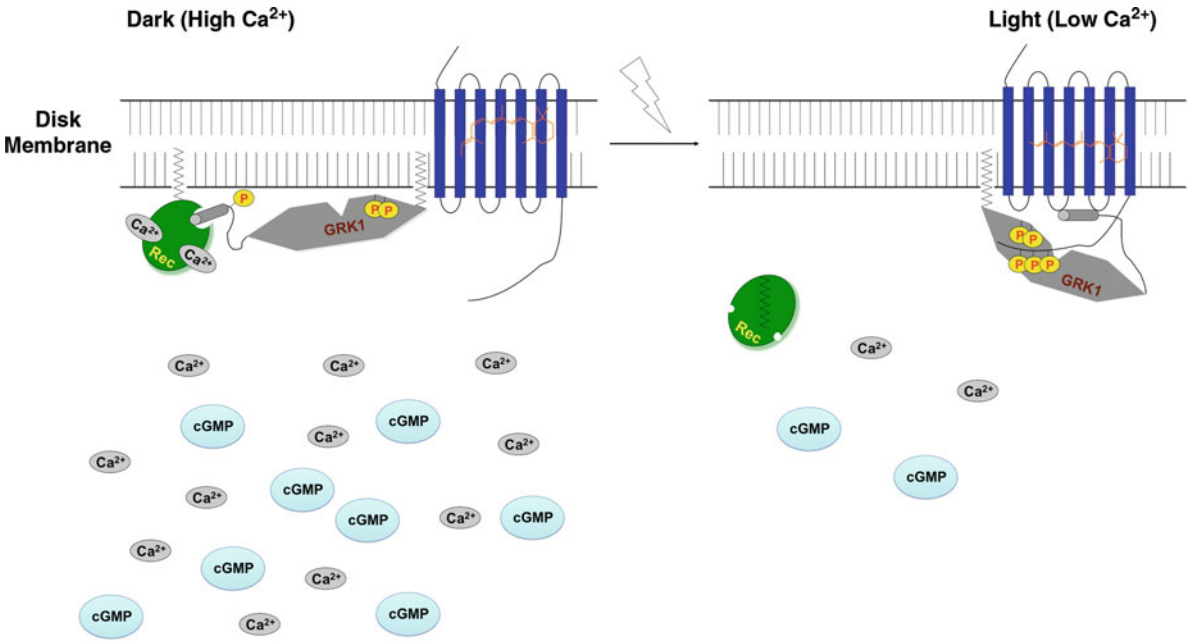
G-protein-coupled receptor kinase 1; GRK1; Rhodopsin kinase; RhoK; RK

Historical Background

The activity of G-protein-coupled receptor kinase 1 (GRK1) was first observed in the light-dependent phosphorylation of rhodopsin in rod outer segment (ROS). As GRK1 was unstable during biochemical manipulations, isolating it in sufficient quantity and quality was proven to be difficult over a period of 30 years (Maeda et al. 2003). The GRK1 gene was cloned in the early 1990s (Lorenz et al. 1991), and many findings regarding its roles in phototransduction recovery, light and dark adaptation, and stationary night blindness of human Oguchi disease took place in the following decade (Chen et al. 1999; Lyubarsky et al. 2000; Khani et al. 1998; Cideciyan et al. 1998). GRK1 is posttranslationally modified by isoprenylation and phosphorylation, and its catalytic activity can be regulated by other proteins such as recoverin (Chen et al. 1995) and protein kinase A (Horner et al. 2005). It is now firmly established that GRK1 is essential for deactivation of activated visual pigments in rod and cone photoresponses. GRK1 crystal structure has recently become available (Singh et al. 2008). The most recent discoveries and unsolved questions on GRK1 are summarized here.

Introduction

G-protein-coupled receptors (GPCR) with heptahelical transmembrane domains are the largest group of integral proteins enabling a cell to sense its outside environment by coupling to heterotrimeric G-proteins.



G-Protein-Coupled Receptor Kinase 1 (GRK1), Fig. 1 Current understanding of GRK1 function in photoreceptors

A wide range of physiological processes such as senses, hormonal actions, and neurotransmission employ GPCRs. The deactivation of GPCR signaling requires phosphorylation of C-terminal Serine and/or Threonine residues and this is catalyzed by G-protein-coupled receptor kinases (GRKs). Following GPCR phosphorylation, the interaction with G-protein is weakened and the affinity for arrestin is heightened. The binding of arrestin to phosphorylated receptors prevents coupling to G-proteins. In many cells, arrestin binding initiates receptor endocytosis to prolong and enhance desensitization (Dorn 2009). Keeping GPCR activity at bay by this simple but elegant deactivation mechanism is important, as loss-of-function mutations in GRKs have led to a variety of pathologic conditions. There are seven members in the GRK family that can be further subdivided into three groups: GRK1-like, GRK2-like, and GRK4-like. GRK1-like group consists of GRK1 and GRK7, which are expressed specifically in retinal photoreceptors. GRK2-like group contains GRK2 and GRK3, which are ubiquitously expressed. GRK4-like group is comprised of GRK4, GRK5, and GRK6. GRK4 expression is restricted to kidney, cerebellum, and testis, while GRK5 and GRK6 are expressed ubiquitously. All GRKs share a central kinase domain of approximately 270 amino acids, typical to that of other Ser/Thr kinases. The N-terminus of GRKs contains an RGS homology (RH) domain, which is found in regulators of G-protein signaling (RGS) proteins (Ribas et al. 2007; Dorn 2009). The RH domain of GRK1 and GRK2 has ~20–25% homology to that of the R7 subgroup of RGS proteins (data not shown). The physiological function of GRK1 RH domain remains unclear. The C-terminal regions of GRKs are most dissimilar with varying sizes and different protein motifs that contribute to the diversity of GRKs. GRK2 is critical in cardiac physiology, as its inactivation leads to cardiac abnormality and embryonic lethality in mice (Dorn 2009). GRK1 and GRK2 are the most extensively studied GRK members. GRK1 is required for visual pigment deactivation in rod and cone (Chen et al. 1999; Lyubarsky et al. 2000). Because of its unique expression pattern in retinal photoreceptors, knowledge stemming from studying GRK1 (Fig. 1) has contributed much to our understanding of GRK function in general.

In retinal rod, the phototransduction cascade is initiated when 11-cis-retinal, covalently linked to a Lysine residue in rhodopsin, is photoisomerized to all-trans-retinal. This induces a series of conformational changes in rhodopsin, leading to an active

conformation called Metarhodopsin II (R*), which is capable of catalyzing the exchange of GTP for GDP on the α subunit of heterotrimeric G-protein transducin ($G_{\alpha t}$). The GTP-bound transducin α subunit ($G_{\alpha t}$-GTP) activates phosphodiesterase 6 (Pde6) and results in rapid reduction of cGMP level and the closure of cGMP-gated (CNG) channels located on ROS plasma membranes. The reduction of cation influx leads to membrane hyperpolarization and a transient cessation of glutamate release at synaptic terminal. To reset a rod to detect additional photons, all active molecules during phototransduction, including R*, must be deactivated in a timely fashion. For R*, this is mediated by a two-step process, the first of which is phosphorylation of up to six Ser/Thr residues at the C-terminal tail by GRK1. Phosphorylated-R* is then recognized and bound by arrestin, preventing further receptor/G-protein coupling and allowing R* to decay (Chen 2005). More than a decade ago, a close relative of GRK1, called GRK7, was cloned from mammalian species (Weiss et al. 1998) and was subsequently suggested to be a cone opsin kinase. While GRK1 is expressed in both rod and cone, GRK7 is expressed only in cone and not in rod for human (Chen et al. 2001; Weiss et al. 2001). In species such as pigs and dogs, their cones only express GRK7. In rodents, GRK7 is absent and both rods and cones rely on GRK1 for receptor deactivation. Studying the differences between GRK1 and GRK7 has provided insights into the structure/function relationship of GRKs and the pathological mechanism of human Oguchi disease.

Role of GRK1 in Phototransduction, Oguchi Disease, and Light-Dependent Degeneration

Photoreceptors without GRK1 need longer time to dark adapt. In $GRK1^{-/-}$ photoreceptors, receptor deactivation is so slow that it becomes the slowest step that dominates the overall recovery phase of rod and cone phototransduction (Chen et al. 1999; Lyubarsky et al. 2000). Inactivating one copy of GRK1 also causes a noticeable delay in rod recovery, suggesting that GRK1 level may be critical in setting the duration of rod's photoresponses. However, while loss-of-function approach demonstrates the importance of GRK1 in rod recovery, it lacks the power to reveal whether R* phosphorylation dominate the recovery time course under normal conditions. Using a gain-of-function approach, Krispel et al. generated several transgenic mouse lines with varying levels (up to fourfold) of GRK1 and found in them that rods showed no statistically significant acceleration of recovery. Using a similar approach where the ternary GTPase accelerating protein (GAP) complex of R9AP/Gβ5-L/RGS9-1 is overexpressed, the recovery can be dramatically sped up. Together, this indicates that transducin deactivation is the overall rate-determining step in normal rod (Krispel et al. 2006). With transducin turn-off identified as the slowest step, is rhodopsin deactivation mediated by GRK1 the second slowest step in recovery? One strategy to identify the second rate-limiting step is to sufficiently speed up transducin GTP-hydrolysis until the second step becomes the slowest step. Another strategy is to use rods with much less GRK1 (Chen et al. 2010), whereas rod recovery was found to be faster than $GRK1^{-/-}$ but slower than that in normal rods. Under such condition the presence of background light was found to speed up rod recovery. Interestingly, the effect of background light depends on recoverin, a calcium-dependent GRK1 regulator (see below).

Photoreceptor degenerates in the presence of light delivered at high intensity or for a prolonged period (Hao et al. 2002). Genetic manipulation in mouse in recent years has identified several proteins that protect photoreceptors from light damage and GRK1 is one of them (Chen et al. 1999). Photoreceptor lacking arrestin also degenerates under intense light exposure. In double knockout ($GRK1^{-/-}$/$Arrestin^{-/-}$) mice, photoreceptors become extremely sensitive to light damage, which can occur as short as 1 min of light exposure (Choi et al. 2001; Hao et al. 2002; Krishnan et al. 2008). The susceptibility to light damage in $GRK1^{-/-}$, $Arrestin^{-/-}$, and double knockout photoreceptors presumably arises from the prolonged activation of rhodopsin. The degeneration in these animal models has been shown to activate both transducin-dependent and transducin-independent pathways to cause cell death (Hao et al. 2002; Fan et al. 2010). In the presence of bright light, photoreceptor without transducin still degenerates in a pathway that signals through activated rhodopsin. In contrast, the degeneration seen in $GRK1^{-/-}$ and $Arrestin^{-/-}$ photoreceptors under dim light condition is mediated by constant transducin activation. By mating these animals with $GNAT1^{-/-}$, the degeneration under dim light can be prevented (Hao et al. 2002). These evidences showcase the protective

properties of GRK1 and arrestin in rods against light damage by timely rhodopsin deactivation.

Since the loss of GRK1 leads to prolonged rhodopsin lifetime and cell death, can increasing GRK1 protect photoreceptor against degeneration? In a recent study by Whitcomb et al., BAC transgenic technique was used to overexpress GRK1 in rod photoreceptor to approximately threefold (GRK1+). Correspondingly, rhodopsin phosphorylation is also increased in GRK1 + rods. Retinal morphology in GRK1+ animals is similar to WT under regular illumination. However, when the animals are exposed to intense light (10,000 lux) for 12 h, GRK1+ photoreceptors display faster degeneration (Whitcomb et al. 2010). These data are contrary to what one may expect because GRK1 overexpression does not provide protection against light-induced degeneration, instead, hyperphosphorylation of rhodopsin may accelerate an unknown signaling event that leads to cell death. GRK1 concentration apparently needs to be maintained optimally within photoreceptors as an increase or loss of GRK1 can accelerate light-induced photoreceptor degeneration. While retinal degeneration has been described in many mouse strains in which they become useful models of human macular degeneration and retinitis pigmentosa, the cellular mechanisms that trigger apoptosis have been less understood. Future studies are needed to determine the signaling pathway(s) leading from rhodopsin light absorption to photoreceptor cell death to shed light into these dark and debilitating human conditions.

It is evident from animal studies that the loss of GRK1 results in severe defects in photoreceptors. Similarly, mutations in GRK1 and arrestin genes have been found in human patients with Oguichi disease, a rare form of stationary night blindness (Yamamoto et al. 1997). Color vision in Oguchi patients is typically normal, which indicates that cone function is unaffected. ERG analyses of Oguchi patients demonstrate severe delay in scotopic recovery and elevated rod thresholds, while their photopic responses are by and largely unaffected (Carr and Gouras 1965; Cideciyan et al. 1998; Zhang et al. 2005). Various GRK1 mutations have been identified and are listed in Table 1. Ectopically expressing GRK1 with Oguchi mutations (V380D, Ser536 (4-bp del), and exon 5 deletion) in COS7 cells has demonstrated a reduced ability to phosphorylate R* (Cideciyan et al. 1998; Khani et al. 1998). However, these mutations may also destabilize GRK1 in photoreceptors and this remains to be tested. As losing kinase activity or protein expression lead to defective rhodopsin phosphorylation and a prolonged dark adaptation time in Oguchi disease patients, one interesting discrepancy is worth noting here. Mouse without GRK1 has severe recovery defects in both cone and rod, while cones of most Oguchi disease patients appear spared. This is because of the expression of GRK7 (Maeda et al. 2003) in human cones. It is also interesting to note that cone ERG responses are diminished in patients with P391H GRK1 mutation, suggesting that this particular mutation may have a dominant effect in these cones affecting both GRK1 and GRK7 (Hayashi et al. 2007). The possibility that the photoreceptors with P391H mutation suffer some degree of degeneration leading to the cone defect remains, as shown in GRK1$^{-/-}$ mouse photoreceptors. The clinical presentation of Oguchi patients seems indistinguishable across the different mutations, although the molecular consequences of these mutations to GRK1 may be different.

Structure/Function of GRK1

Crystal structures of GRK1 have been solved to provide structure and function insights (Singh et al. 2008). Recent studies have further revealed the molecular interaction between GRK1 and recoverin, its calcium-dependent regulator. The NMR structure of the first 25 amino acid of GRK1 (RK25) in complex with Ca^{+2}-bound recoverin is solved. Residues 4–16 of RK25 form an amphipathic α-helix, where the hydrophobic surface interacts with a conserved groove lined with hydrophobic residues in recoverin. This N-terminal groove of recoverin, comprised of nine hydrophobic residues conserved across other neuronal calcium sensors, is implicated in target recognition (Ames et al. 2006). This N-terminal groove is exposed through the Ca^{2+}-myristoyl switch to allow recoverin binding to GRK1. The residues in GRK1 responsible for binding recoverin are V9, V10, A11, A14, and F15, which form a network of hydrophobic interactions to confer a K_d of 1.4 μM with recoverin. In addition, the N-terminal residues of GRK1 are required for rhodopsin binding (Higgins et al. 2006). Taken together, the binding of recoverin to GRK1 at the N-terminal domain prevents GRK1 from association to rhodopsin when Ca^{2+} level is high. As the calcium level drops in

G-Protein-Coupled Receptor Kinase 1 (GRK1), Table 1 GRK1 mutations in Oguchi disease reported to date

Patient	Mutation	Protein	Cone/rod function	Gender/age	Family history	References
#303-301	Exon 5 deletion	Frameshift mutation: premature stop codon	Normal/ delay	Male	Jewish ancestor Hungary/Ukraine	Yamamoto et al. (1997)
#303-302 (Case 3: NIH#03-37-33)	V380D, Ser536(4 bp-del)	Compound heterozygous (negative charge in catalytic domain/loss of last 22 residues involving isoprenylation)	Normal/ delay	Female, 16	Hungary, sister with #303-304	Carr and Gouras (1965), Yamamoto et al. (1997), Khani et al. (1998)
#303-303 (Case 1: NIH#05-49-42)	Exon 5 deletion	Frameshift mutation: premature stop codon	Normal/ delay	Female, 36	Jewish ancestor Hungary/Ukraine	Carr and Gouras (1965), Yamamoto et al. (1997)
#303-304	V380D, Ser536(4 bp-del)	Compound heterozygous (negative charge in catalytic domain/loss of last 22 residues involving isoprenylation)	Normal/ delay	Female, 13	Hungary, sister with #303-302	Carr and Gouras (1965), Yamamoto et al. (1997), Khani et al. (1998)
	Exon 5 deletion	Frameshift mutation: premature stop codon	Normal/ delay	Male, 6	Ashkenazi Jewish	Cideciyan et al. (1998)
Family 61029 (#11)	c.827 + 623_883del	Partial exon 3 deletion (1.1 kb)	Normal/ delay	Male, 19	Pakistan	Zhang et al. (2005)
Family 61029 (#13)	c.827 + 623_883del	Partial exon 3 deletion (1.1 kb)	Normal/ delay	Female, 16	Pakistan	
Family 61029 (#15)	c.827 + 623_883del	Partial exon 3 deletion (1.1 kb)	Normal/ delay	Female, 13	Pakistan	
JU#0008 (III-2)	P391H	Mutation in catalytic domain	Reduced/ delay	Male, 35	Japanese	Hayashi et al. (2007)
JU#0008 (III-3)	P391H	Mutation in catalytic domain	Reduced/ delay	Female, 31	Japanese	
Family RP19	c.614 C > A; p.S205X	Nonsense mutation: premature stop codon	Normal/ delay	8 affected individuals, age 3–66	Pakistan	Azam et al. (2009)
Yamamoto, Nobouski	Unknown	Unknown (shorter OS)	Normal/ delay	Male, 31	Japanese	Hashimoto and Kishi (2009)

photoreceptors upon illumination, the myristoyl group on recoverin is sequestered within the hydrophobic groove, resulting in dissociation of recoverin from GRK1 and allowing GRK1–rhodopsin interaction.

Several crystal structures of a truncated GRK1 were reported in various distinct conformations to demonstrate the key elements important for activity and interaction with R* (Singh et al. 2008). The kinase domain is similar to a typical Ser/Thr kinase (AGC Kinase), which contains a large (residues 181–268) and small (residues 269–454) kinase lobe and a C-terminal kinase extension. The active site is formed within a cleft between the large and small kinase lobe and complex with two Mg^{2+} and the nucleotide, ATP or ADP. At the active site, the surface of the large lobe is lined with basic residues to bind with rhodopsin's C-terminal peptides. The small lobe contains the phosphate-binding loop (P-loop) to interact with the triphosphate of ATP. However, the P-loop (Gly-rich B1-B2 turn) in GRK1 is observed to shift away from the nucleotide-binding site when compared to GRK2 and GRK6 and resembles more closely to the structure in Protein Kinase A without any bound nucleotide (Apo-PKA). In the apo-GRK1 structure, the kinase domain adopts a more flexible conformation as compared to nucleotide-bound GRK1. It is hypothesized

that the kinase domain adopts a rigid conformation upon nucleotide binding and become fully closed and active upon binding to R* at the second and third cytoplasmic loops.

Residues 455–511 form the C-terminal kinase extension domain in GRK1 and contain three regions: the C-terminal (large) lobe tether (residues 455–471), an active site tether (AST, residues 472–480), and an N-terminal (small) lobe tether (residues 498–511). The N-terminal and C-terminal lobe tethers have been previously described, while AST has not, due to its sequence variability among GRKs and AGC kinases. GRK1's AST can only be observed in the nucleotide-bound structure and becomes disorder in the apo-kinase, similar to the one in PKA. GRK1 AST includes the "tail loop" (Asp472-Tyr477), which packs closely to the active site, and is predicted to participate in substrate stabilization. The AST is connected to the N-terminal lobe tether through residues 481–489, which contains the autophosphorylation sites (Ser488/Thr489). The phosphorylation status of the two residues is unclear in the structure, although they are phosphorylated in mass spectrometric analysis in the presence of ATP (Singh et al. 2008). It is speculated that the autophosphorylation may participate in electrostatic interaction with Arg222 and Lys221 to stabilize the kinase domain.

The N-terminal structure of the truncated GRK1 is visible in one of the six crystals (form I, PDB 3C4W). The N-terminus (up to residue 32) forms extensive contacts with the RH domain and some with the kinase domain. Two novel phosphorylation sites at Ser5 and Thr8 are identified, but only Ser5 is phosphorylated in native bovine GRK1. Whether phosphorylation of these sites affects GRK1 activity during phototransduction remains to be determined. Interestingly, the N-terminal structure determined by crystallography is different from the RK25 amphipathic helix determined by NMR that complex with Ca^{2+}-recoverin. Recently, a more ordered GRK6 structure revealed the N-terminal region as a helix that interacted with the C-terminal kinase extension (C-tail) to stabilize the large and small kinase lobe and adopt a near-active conformation (Boguth et al. 2009). A set of residues from the small lobe (Arg190), N-terminal helix (Ala8, Asn9, Leu12, and Leu13), and C-tail (Ile472 and Lys475) forms a network of hydrophobic interactions and hydrogen bonds to stabilize the kinase. As these residues are highly conserved in GRK1, it is likely a similar intramolecular network is required for GRK1 to become activated. It is also possible that the amphipathic helix formation of GRK1 requires the presence of recoverin, the GPCR, or other cofactors. Further study is required to resolve the structure and interaction of N-terminus with other regions during GRK1 activation.

The RH domain of GRK1 is made up of nine α-helices that are typical of the RGS domain, with two additional GRK-specific helices. Other than serving as a GAP for transducin, the RH domain may have a role in GRK1 dimerization, but both speculations await experimental confirmation.

Regulations of GRK1 Activity

Several molecules have been identified to regulate GRK1 function in photoreceptors. As demonstrated through biochemical and structural analysis, recoverin interacts with GRK1 and negatively regulates GRK1 in a light-dependent manner (Fig. 1). In the dark, high level of cGMP opens the CNG channel on the plasma membrane and maintains a high Ca^{2+} concentration in the OS. The high Ca^{2+} level induces exposure of the myristoyl group of recoverin and allows it to translocate to OS. At the disk membrane, recoverin inhibits GRK1 activity through interactions of the N-terminal domains. As light stimulation leads to a drop in calcium concentration, the myristoyl group of recoverin becomes embedded within the protein and recoverin loses its ability to associate with the membrane and perhaps diffuses out of OS (Strissel et al. 2005). Without calcium, the inhibition of GRK1 is relieved to allow R* phosphorylation. While studies have shown that recoverin myristoylation is not required for its inhibition on GRK1, it does enhance the cooperative effect of recoverin on GRK1 (Chen et al. 1995). In mouse photoreceptors lacking recoverin, the recovery is faster compared to wild-type rods (Makino et al. 2004). To further test whether recoverin influences R* lifetime through regulating GRK1, Chen et al. underexpressed GRK1 to make rhodopsin deactivation the slowest step in rod recovery. Under such a condition, the presence of background light accelerates rod recovery but this effect disappears when recoverin is removed from photoreceptors. In the absence of recoverin, the inhibitory control on GRK1 is removed and rhodopsin phosphorylation occurs faster (Chen et al. 2010). Thus recoverin is indeed a negative regulator of GRK1 in photoreceptors.

In addition to recoverin regulation, PKA has also been found to phosphorylate and negatively regulate GRK1 (Horner et al. 2005). In vitro, GRK1 is phosphorylated at Ser21 when incubated with the catalytic subunit of PKA or PKA-holoenzyme stimulated with cAMP. Furthermore, urea-stripped ROS incubated with GRK1 and PKA has a 50% decrease in rhodopsin phosphorylation. The importance of Ser21 phosphorylation is seen with the phospho-mimetic mutation (S21E) of GRK1, which has decreased activity toward rhodopsin. In contrast, S21A GRK1 is resistant to PKA inhibition and constitutively phosphorylates rhodopsin. These evidences suggest a role of PKA in phosphorylating and regulating GRK1 in photoreceptor. Under high Ca^{2+} influx in the dark, calmodulin is activated and stimulates cAMP synthesis in photoreceptor. High cAMP level activates PKA to stimulate its phosphorylation on GRK1 at Ser21. Since the N-terminal residues of GRK1 are important in rhodopsin interaction, the phosphorylation at Ser21 introduces a charge residue and obstructs receptor-kinase recognition. Future studies are required to test PKA's action on GRK1 activity in vivo and its modulation on phototransduction.

One of the posttranslational modifications on GRK1 is isoprenylation, which is the covalent attachment of an isoprenoid group to a C-terminal cysteine residue. The modification allows the protein to associate with and function at the membrane. Isoprenylation occurs at the CaaX box motif (C, cysteine; a, any aliphatic residue; X, the residue that determines the type of isoprenoid group attached) (McTaggart 2006). The very C-terminus of GRK1 contains a CaaX box motif (C^{558}VLS) to promote isoprenylation (Inglese et al. 1992). The last three residues are proteolytic removed and a farnesyl group (15 carbon unit) is covalently linked to cysteine through a thioester bond. A methyl group is then added to complete the reaction events. In COS7 cells, GRK1 isoprenylation is abolished by the C558S mutation, and the protein loses its ability to associate with the plasma membrane. In addition, GRK1 without isoprenylation has reduced (~25%) R* phosphorylation activity, indicating that GRK1 isoprenylation is needed for full activity by anchoring the kinase to the disk membrane. It is unclear whether GRK1 isoprenylation and membrane association affects the intrinsic kinase activity, which can be examined by measuring the degree of autophosphorylation of the C558S GRK1 mutant. By changing the terminal serine to leucine at position 561 (Ser561Leu), the farnesylation of GRK1 can be changed to geranylgeranylation (20 carbon unit). When incubating geranylgeranylated GRK1 with urea-stripped ROS extract, the kinase is found to constitutively anchor to the disk membrane independent of light condition, whereas farnesylated GRK1 only localizes to membrane in the presence of light. Activated rhodopsin may serve as a docking site for farnesylated GRK1 to associate to the disk membrane, whereas geranylgeranylated GRK1 is self-sufficient to anchor to the membrane.

Similar to other kinases, GRK1 is found to be autophosphorylated at two residues, Ser488 and Thr489, at the C-terminus. Over the years, experiments studying GRK1 have used the autophosphorylation status as a method to assess the intrinsic kinase activity and compare it to the kinase's capability to phosphorylate R* (Horner et al. 2005; Higgins et al. 2006). To test the effect of autophosphorylation on GRK1, a synthetic peptide that corresponds to the autophosphorylation region is incubated with purified GRK1. The peptide did not affect GRK1's ability to phosphorylate rhodopsin as the kinase has low activity toward it. This suggests that the autophosphorylation domain in GRK1 does not serve as an autoinhibitory domain, which makes GRK1 unique among the protein kinases. Unphosphorylated GRK1 (Ser488 and Thr489 to alanines) have been shown to preferentially phosphorylate Ser338 on R*, whereas regular GRK1 phosphorylates Ser338 and Ser343. It is speculated that autophosphorylation influences the affinity and enhances the dissociation of GRK1 from phosphorylated rhodopsin to allow arrestin binding, but its exact physiological role remains to be elucidated.

Summary

As the kinase responsible for light-dependent rhodopsin phosphorylation, GRK1 is essential for timely R* deactivation during phototransduction recovery. The regulation of GRK1 through posttranslational modification (phosphorylation and isoprenylation) and other signaling molecules (PKA and recoverin) are important as slowing it down can change the rate-limiting step in rod recovery. Understanding the structure–function relationship of GRK1 is not complete and requires the crystal structure of a full length and active kinase preparation. As already demonstrated in several studies, changes in GRK1

level can have significant consequences in phototransduction recovery and may lead to sensitivity to light damage. More studies are needed to reveal the signaling events leading to photoreceptor degeneration in the absence of GRK1 and additional roles GRK1 may play in photoreceptors.

References

Ames JB, Levay K, et al. Structural basis for calcium-induced inhibition of rhodopsin kinase by recoverin. J Biol Chem. 2006;281(48):37237–45.

Azam M, Collin RW, et al. A novel mutation in GRK1 causes Oguchi disease in a consanguineous Pakistani family. Mol Vis. 2009;15:1788–93.

Boguth CA, Singh P, et al. Molecular basis for activation of G protein-coupled receptor kinases. EMBO J. 2009;29(19):3249–59.

Carr RE, Gouras P. Oguchi's disease. Arch Ophthalmol. 1965;73:646–56.

Chen CK. The vertebrate phototransduction cascade: amplification and termination mechanisms. Rev Physiol Biochem Pharmacol. 2005;154:101–21.

Chen CK, Inglese J, et al. Ca(2+)-dependent interaction of recoverin with rhodopsin kinase. J Biol Chem. 1995;270(30):18060–6.

Chen CK, Burns ME, et al. Abnormal photoresponses and light-induced apoptosis in rods lacking rhodopsin kinase. Proc Natl Acad Sci USA. 1999;96(7):3718–22.

Chen CK, Zhang K, et al. Characterization of human GRK7 as a potential cone opsin kinase. Mol Vis. 2001;7:305–13.

Chen CK, Woodruff ML, et al. Background light produces a recoverin-dependent modulation of activated-rhodopsin lifetime in mouse rods. J Neurosci. 2010;30(4):1213–20.

Choi S, Hao W, et al. Gene expression profiles of light-induced apoptosis in arrestin/rhodopsin kinase-deficient mouse retinas. Proc Natl Acad Sci USA. 2001;98(23):13096–101.

Cideciyan AV, Zhao X, et al. Null mutation in the rhodopsin kinase gene slows recovery kinetics of rod and cone phototransduction in man. Proc Natl Acad Sci USA. 1998;95(1):328–33.

Dorn 2nd GW. GRK mythology: G-protein receptor kinases in cardiovascular disease. J Mol Med. 2009;87(5):455–63.

Fan J, Sakurai K, et al. Deletion of GRK1 causes retina degeneration through a transducin-independent mechanism. J Neurosci. 2010;30(7):2496–503.

Hao W, Wenzel A, et al. Evidence for two apoptotic pathways in light-induced retinal degeneration. Nat Genet. 2002;32(2):254–60.

Hashimoto and Kishi. Shortening of the rod outer segment in Oguchi disease. Graefes Arch Clin Exp Ophthalmol. 2009 Nov;247(11):1561–3.

Hayashi T, Gekka T, et al. A novel homozygous GRK1 mutation (P391H) in 2 siblings with Oguchi disease with markedly reduced cone responses. Ophthalmology. 2007;114(1):134–41.

Higgins MK, Oprian DD, et al. Recoverin binds exclusively to an amphipathic peptide at the N terminus of rhodopsin kinase, inhibiting rhodopsin phosphorylation without affecting catalytic activity of the kinase. J Biol Chem. 2006;281(28):19426–32.

Horner TJ, Osawa S, et al. Phosphorylation of GRK1 and GRK7 by cAMP-dependent protein kinase attenuates their enzymatic activities. J Biol Chem. 2005;280(31):28241–50.

Inglese J, Glickman JF, et al. Isoprenylation of a protein kinase. Requirement of farnesylation/alpha-carboxyl methylation for full enzymatic activity of rhodopsin kinase. J Biol Chem. 1992;267(3):1422–5.

Khani SC, Nielsen L, et al. Biochemical evidence for pathogenicity of rhodopsin kinase mutations correlated with the oguchi form of congenital stationary night blindness. Proc Natl Acad Sci USA. 1998;95(6):2824–7.

Krishnan J, Lee G, et al. Characterization of phototransduction gene knockouts revealed important signaling networks in the light-induced retinal degeneration. J Biomed Biotechnol. 2008;2008:327468.

Krispel CM, Chen D, et al. RGS expression rate-limits recovery of rod photoresponses. Neuron. 2006;51(4):409–16.

Lorenz W, Inglese J, et al. The receptor kinase family: primary structure of rhodopsin kinase reveals similarities to the beta-adrenergic receptor kinase. Proc Natl Acad Sci USA. 1991;88(19):8715–9.

Lyubarsky AL, Chen C, et al. Mice lacking G-protein receptor kinase 1 have profoundly slowed recovery of cone-driven retinal responses. J Neurosci. 2000;20(6):2209–17.

Maeda T, Imanishi Y, et al. Rhodopsin phosphorylation: 30 years later. Prog Retin Eye Res. 2003;22(4):417–34.

Makino CL, Dodd RL, et al. Recoverin regulates light-dependent phosphodiesterase activity in retinal rods. J Gen Physiol. 2004;123(6):729–41.

McTaggart SJ. Isoprenylated proteins. Cell Mol Life Sci. 2006;63(3):255–67.

Ribas C, Penela P, et al. The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. Biochim Biophys Acta. 2007;1768(4):913–22.

Singh P, Wang B, et al. Structures of rhodopsin kinase in different ligand states reveal key elements involved in G protein-coupled receptor kinase activation. J Biol Chem. 2008;283(20):14053–62.

Strissel KJ, Lishko PV, et al. Recoverin undergoes light-dependent intracellular translocation in rod photoreceptors. J Biol Chem. 2005;280(32):29250–5.

Weiss ER, Raman D, et al. The cloning of GRK7, a candidate cone opsin kinase, from cone- and rod-dominant mammalian retinas. Mol Vis. 1998;4:27.

Weiss ER, Ducceschi MH, et al. Species-specific differences in expression of G-protein-coupled receptor kinase (GRK) 7 and GRK1 in mammalian cone photoreceptor cells: implications for cone cell phototransduction. J Neurosci. 2001;21(23):9175–84.

Whitcomb T, Sakurai K, et al. Effect of g protein-coupled receptor kinase 1 (Grk1) overexpression on rod photoreceptor cell viability. Invest Ophthalmol Vis Sci. 2010;51(3):1728–37.

Yamamoto S, Sippel KC, et al. Defects in the rhodopsin kinase gene in the Oguchi form of stationary night blindness. Nat Genet. 1997;15(2):175–8.

Zhang Q, Zulfiqar F, et al. A variant form of Oguchi disease mapped to 13q34 associated with partial deletion of GRK1 gene. Mol Vis. 2005;11:977–85.

GPSM1/G-Protein Signaling Modulator 1 (AGS3)

▶ Activators of G-Protein Signaling (AGS)

GPSM2/G-Protein Signaling Modulator 2/LGN/mPINS (AGS5)

▶ Activators of G-Protein Signaling (AGS)

GPSM3/G-Protein Signaling Modulator 3/G18/NG1 (AGS4)

▶ Activators of G-Protein Signaling (AGS)

GREAT

▶ Relaxin Family Peptide Receptors (RXFP) 1 and 2

GRF

▶ RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor)

GRIP1

▶ Steroid Receptor Coactivator Family

GRK1

▶ G-Protein-Coupled Receptor Kinase 1 (GRK1)

Grp94

▶ Grp94 (HSP90B1)

Grp94 (HSP90B1)

Luisa Gorza[1] and Maurizio Vitadello[2]
[1]Department of Biomedical Sciences, University of Padova, Padova, Italy
[2]CNR-Institute of Neuroscience, Padova section, Padova, Italy

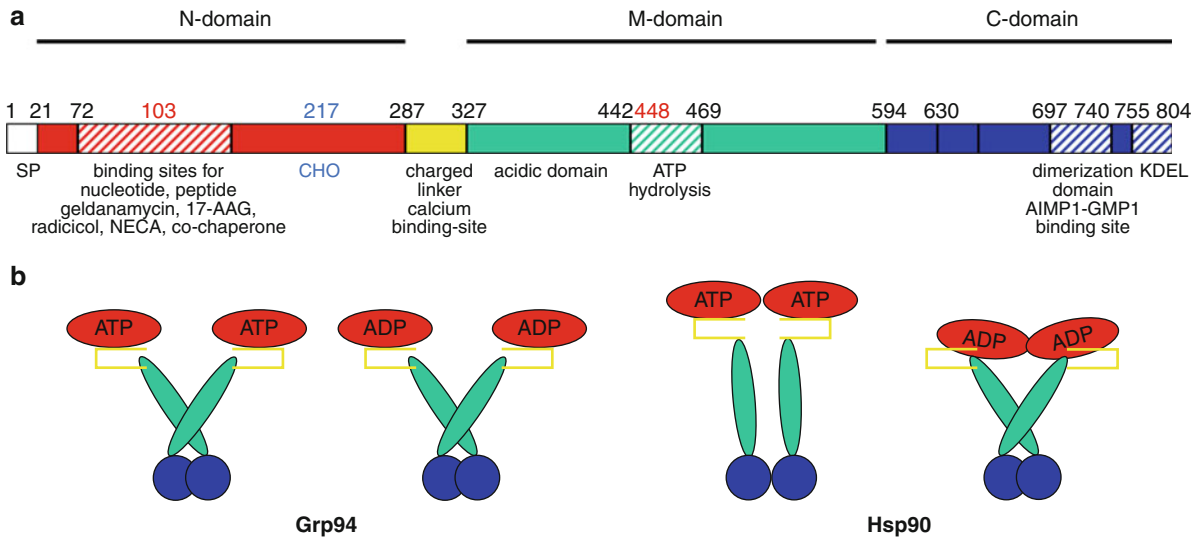
Synonyms

94 kDa glucose-regulated protein; Endoplasmin; Erp99; gp96; Grp94; Heat shock protein 90 kDa beta member 1; TRA1; Tumor rejection antigen gp96

Historical Background

Grp94 is the endoplasmic reticulum (ER) paralog of heat shock protein (Hsp) 90. This chaperone/stress protein was identified together with the other members of the family of glucose-regulated proteins because its levels significantly increased after treatment of mammalian cells with culture medium lacking glucose. Subsequent evidence showed that other ER stressors, such as calcium depletion, inhibition of glycosylation, and reducing agents, similarly affected Grp94 protein levels, making it one of the hallmarks of ER stress response (Lee 1987). Grp94 protein levels increase also after exposure to low-doses of endotoxin, hypoxia, and mild ischemia and in most tumors (Srivastava 2006; Glembotski 2008). The last feature, which is accomplished by Grp94 binding of tumor-specific peptides, explains the other widely used synonyms gp96 and TRA1 (Srivastava 2006).

The apparent Mr of Grp94 is around 94–100 kDa in most Vertebrate species and is the product of a single gene. Whereas Grp94 orthologs are detected in Metazoans, plants enclosed, no corresponding gene or protein has been so far identified in yeast and other monocellular eukaryotes, except for *Leishmania* (Eletto et al. 2010). The protein shows multiple Mr isoforms depending on the variable degree of glycosylation and on the presence of Ser/Thr- or Tyr-phosphorylation (Frasson et al. 2009 and references within). Whereas the functional significance of glycosylation remains still obscure, Ser/Thr-phosphorylation by Golgi casein kinase and Tyr-phosphorylation by Fyn Src-kinase are



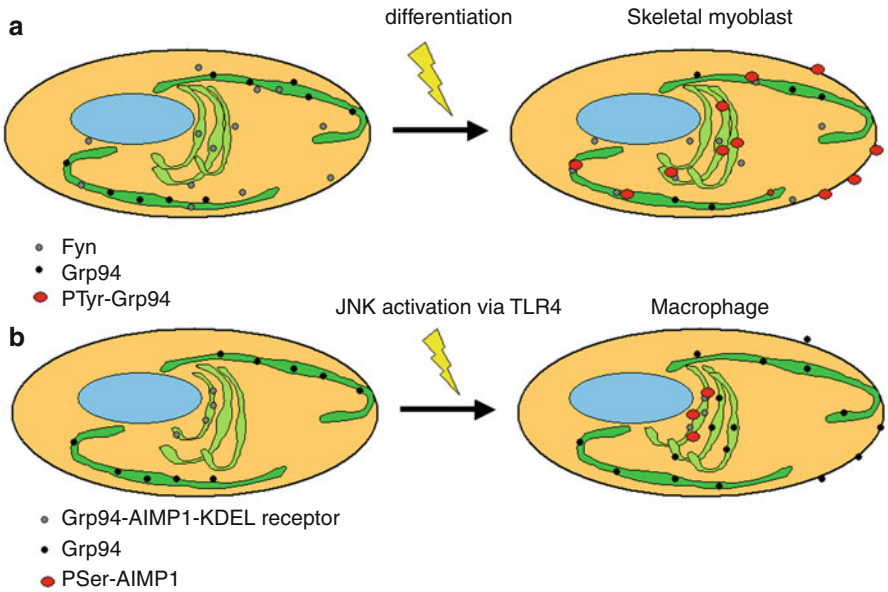
Grp94 (HSP90B1), Fig. 1 Diagrams illustrate Grp94 structure and configuration. (**a**) Primary structure of Grp94 and domain boundaries identification. Numbers identify amino acids. Numbers 103 and 448 identify amino acid residues involved in ATP hydrolysis; number 217 indicates an identified site of glycosylation. *Crosshatched* regions identify functions specified below and described in the text. SP: signal peptide; CHO: oligosaccharide. (**b**) Schematic representations of domain interactions of Grp94 and Hsp90 proteins observed after binding with ATP or ADP. At variance with Hsp90, whose conformation is changed to a catalytically active one by nucleotide binding, Grp94 ground-state conformation is apparently not affected

involved in Grp94 subcellular localization. Detailed analyses of Grp94 distribution in adult mammalian tissues and primary cells indicate relatively low expression levels of mRNA and protein in most tissues, except for lung bronchial epithelium, pancreatic islets, kidney tubular cells, and dendritic cells (Mao et al. 2010).

Structurally, Grp94 contains four domains (Eletto et al. 2010 and Fig. 1a). The N-terminal (N) domain contains a nucleotide binding site, a peptide binding site, unmapped sites responsible for interaction with dendritic cells during antigen presentation (Jockheck-Clark et al. 2010), the binding site for the co-chaperone Murine canopy 3 (CNPY3, Liu et al. 2010), and a putative transmembrane domain (Frasson et al. 2009 and references within). The second domain, as in Hsp90, is a charged linker between the N and middle (M) domains, but it is longer in Grp94 and functionally important for binding of both nucleotide and calcium. The M-domain contains residues involved in ATP hydrolysis after interaction with the N-terminal domain, and the C-terminal domain mediates the dimerization of Grp94 and ER localization via the C-terminal KDEL peptide. Grp94 displays a "twisted V" conformation as crystal structure (Fig. 1b), whereas in solution it has an extended, "chair-like" conformation, which shifts toward less extended, more closed conformations after nucleotide binding (Eletto et al. 2010).

Like other ER chaperones, such as Grp78 and calreticulin, Grp94 is a putative low affinity-high capacity Ca^{2+}-binding protein. Grp94 binds about 16–28 moles Ca^{2+} per mole protein with a few high affinity (Kd 1–5 μM) binding sites and the rest, lower affinity sites (Kd ~600 μM). Thus, Grp94 may bind about 4 moles of calcium/mole of protein in the presence of 1 mmol/L calcium, namely the range of free calcium concentration considered to be present within the ER (Eletto et al. 2010).

ER localization of Grp94 is regulated, like other ER chaperones, through the C-terminal KDEL sequence. The KDEL sequence is recognized by a specific receptor, which is mainly localized in the cis-Golgi. Dimers-oligomers of Grp94 interacting with the aminoacyl-tRNA synthetase-interacting multifunctional protein 1 (AIMP1; previously known as p43) bind to the receptor and lead to its oligomerization and rapid transport out of cis-Golgi (Kim et al. 2010). The KDEL receptor-Grp94 complex returns to the ER, where it dissociates, thus freeing the receptor for further cycling of transport. In addition to the ER, Grp94 is detected at the cell surface of several tumors and of immature cells



Grp94 (HSP90B1), Fig. 2 Signals responsible for Grp94 cell-surface localization. (**a**) Differentiation of skeletal myoblasts induced by low serum culture medium activates an ER-resident Fyn, which interacts with and Tyr-phosphorylates Grp94, promoting its migration to the Golgi compartment and the cell surface. (**b**) Exposure of circulating or spleen macrophages to bacterial lipopolysaccharide activates TLR4 and JNK-mediated AIMP1 phosphorylation, which dissociates Grp94-KDEL receptor complex and favor Grp94 export to the cell surface

(Srivastava 2006; Gorza and Vitadello 2000). Two signaling pathways responsible for Grp94 exit outside the ER have been so far identified (Fig. 2): (1) in differentiating myoblasts, the Tyr phosphorylation of Grp94 by Fyn Src-kinase is required for the chaperone translocation to Golgi and cell surface (Frasson et al. 2009) and (2) in LPS-stimulated monocytes and splenocytes, the Ser-phosphorylation of AIMP1 by c-Jun N-terminal kinase (JNK) releases the binding of the protein from Grp94 and the interaction with the KDEL receptor (Kim et al. 2010).

Another distinctive feature of Grp94 is the transmembrane configuration, which apparently involves a minor proportion of molecules and coexists in the ER compartment with the luminal configuration (Frasson et al. 2009 and references within). The precise mechanism of anchorage of Grp94 molecules to the cell surface is presently unknown; however, data suggest that it occurs through noncovalent, nonionic binding with other proteins (Srivastava 2006).

Physiological Functions

Protein Chaperone

Grp94 exists as a homodimer or higher-order oligomer. Although it has been shown to bind to some nascent ER proteins, Grp94 participates to the second major ER chaperone system, which operates, in addition and/or in alternative to the lectin system, on unfolded regions of proteins containing hydrophobic residues and is involved in folding and assembly of multimeric proteins, whose first and most studied example is the immunoglobulin heavy chain (Ma and Hendershot 2004). A large ER-localized multi-protein complex is initiated by the ER chaperone Grp78 that recognizes protein hydrophobic residues and recruits Grp94 and other molecular chaperones, among which are protein-disulfide isomerase, cyclophilin B, Grp170. As revealed by studies performed using chemical cross-linking, the complex forms also in the absence of protein synthesis (Ma and Hendershot 2004). The mechanism through which Grp94 exerts its chaperone function is still obscure, due to controversial evidence concerning ATPase activity, to the lack of co-chaperones and the paucity of client proteins. Conservation of amino acids involved in ATP binding and hydrolysis between Hsp90 and Grp94 would suggest a similar mechanism of nucleotide utilization. Grp94 binds adenosine nucleotides in vitro and mutations of either the ATP binding site, or the putative catalytic one, hamper chaperone activity in vivo (Randow and Seed 2001). However, structure analysis methods, including x-ray crystallography, mass spectrometry, and optical techniques showed that the ATP and ADP-bound forms of Grp94 are equivalent, that is,

they display identical twisted "V"-shape conformation with closed C domains and open N domains in opposing orientation (Eletto et al. 2010; Fig. 1b). Therefore, ATP binding is not sufficient to drive Grp94 into a hydrolytically productive conformation: the open N domain and the relaxed N–M orientation leave the catalytic residues too distant from the gamma-phosphate of the ATP, at variance with Hsp90 and other proteins of the GHKL ATPase/kinase superfamily, whose ATPase activity results from the contact of residues localized in both the N and M domains with the bound nucleotide. As argued by the structural analysis, co-chaperone(s) would be required by Grp94 to promote ATP hydrolysis by rotating the catalytic loop, and to regulate binding and release of client proteins. The ER resident protein CNPY3 has been recently identified as the first Grp94 co-chaperone, specifically involved in TLR folding (Liu et al. 2010). CNPY3 binds Grp94 close to the ATP pocket and in competition with nucleotides, suggesting that it regulates the folding of conformational intermediates without nucleotides, as shown for some Hsp90 co-chaperones (Eletto et al. 2010; Liu et al. 2010). Indeed, the same single point mutation at the highly conserved amino acid 103 (E103A) of Grp94, which was found to abolish ATPase activity and insulin-like growth factor (IGF) folding (Eletto et al. 2010), hampered the interaction with CNPY3 (Liu et al. 2010) and folding of Toll-like receptors (TLR) (Randow and Seed 2001), but it did not affect the folding of other client proteins, such as integrins (Randow and Seed 2001). At present, no recognition moiety has been identified for Grp94. Its small population of client proteins does not display common features other than the presence of disulfide bonds, and, in the case of integrins and TLR, shows selectivity even among family members (Table 1 and McLaughlin and Vandenbroeck 2011).

An unexpected role for Grp94 has been described in the ER quality control, that is, in targeting misfolded proteins to ER-associated degradation (ERAD). Misfolded and demannosylated protein substrates are bound by OS-9, a ER lumenal lectin, which associates with Grp94 in order to present substrates to the ubiquitination and dislocation apparatus. The role of Grp94 is presumably to facilitate or mediate substrate recognition, since degradation of mutated glycosylated proteins is impaired in Grp94-depleted cells (Eletto et al. 2010).

Grp94 (HSP90B1), Table 1 List of proteins requiring the Grp94 chaperone for folding, as shown by Grp94 knocking out or down experiments

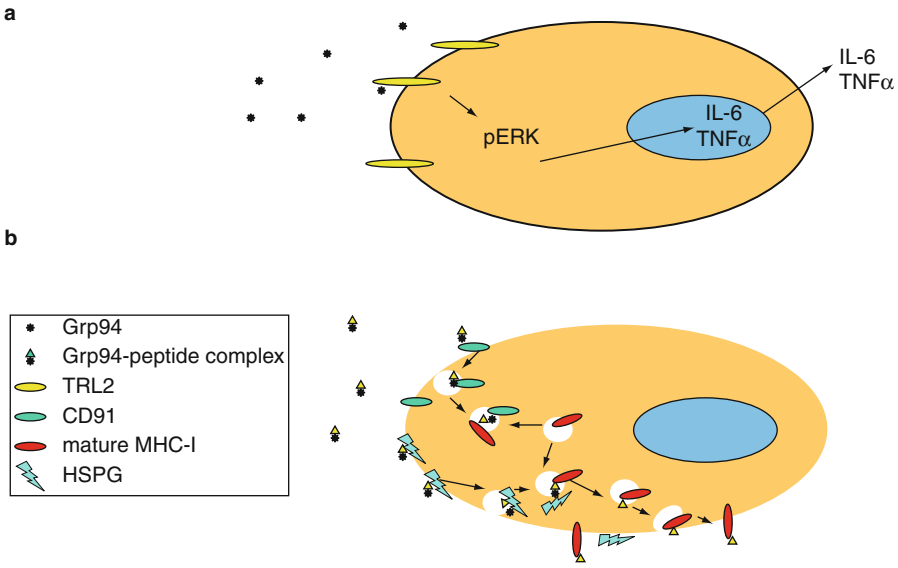
Proteins	References
Membrane receptors	
TLR (TLR1, TLR2, TLR4, TLR5, TRL6, TLR7, TLR9, TLR11, but not TLR3)	Randow and Seed (2001), Yang et al. (2007), Liu and Li (2008), Liu et al. (2010)
Integrins (α1, α2, α4, αD, αE, αL, αM, αV, αX, β2, β5, β6, β7, β8)	Randow and Seed (2001), Liu and Li (2008), Staron et al. (2010)
Secreted enzymes and molecules	
ADAMTS9 metalloprotease	Koo and Apte (2010)
Bile-salt-dependent lipase	Nganga et al. (2000)
IGFs (IGF-I, IGF-II)	Wanderling et al. (2007), Ostrovsky et al. (2009), Ostrovsky et al. (2010)

Immune Regulator

Grp94 is involved in both innate and adaptive immune responses.

In addition to play a key role in the folding of several TLR, Grp94 was proposed to activate professional antigen-presenting cells (pAPC) by interacting with TLR2 and ▶ TLR4. Contaminating endotoxin, either derived from recombinant Grp94 preparations or released even at very low levels from commensal flora, appears to have confounded the interpretation of these studies. However, after stringently controlling the level of endotoxin contamination and evaluating the inhibition of the Grp94 interaction by means of specific antibodies, a recent study demonstrated that this chaperone activates human macrophages primarily through TLR2 and induced both IL-6 and TNF-α release (Huang et al. 2009) (Fig. 3a). Some activation occurs through TLR4, concerning only IL-6 production. Besides, Grp94 appears to regulate the pathway activated by the TLR2 receptor downstream the mitogen extracellular kinase (▶ MEK)-ERK1/2. In kidney tubular cells, this chaperone binds and maintains in active conformation the serine-threonine protein phosphatase 5 (PP5), a negative modulator of ▶ Raf-1 (Mkaddem et al. 2009).

In addition to initiate the innate immune responses by interacting with TLR2 and TLR4, Grp94 contributes to the adaptive one. Several in vitro studies demonstrated that the N-terminal and charged linker domains of Grp94 comprise



Grp94 (HSP90B1), Fig. 3 Schematic representations of the pathways activated by exposure of pAPCs to Grp94 in innate and adaptive immune responses. (**a**) Interaction of soluble Grp94 with TLR2 induces IL-6 and TNF-α secretion in human macrophages through ERK1/2 pathway activation. (**b**) Peptides complexed to Grp94 are internalized by pAPCs after either receptor-mediated or fluid-phase endocytosis, and loaded onto mature MHC class I molecules in a post-ER compartment. HSPG: heparan sulfate proteoglycan

a peptide-binding domain, which is inhibited when small molecules such as radicicol, geldanamycin and NECA occupy the nucleotide pocket at the opposite site (Eletto et al. 2010). Although it is presently debated whether Grp94-peptide binding is physiologically relevant in vivo, it is fully recognized that Grp94 can direct the associated peptides into the Major Histocompatibility Complex (MHC) class I cross-presentation pathway of pAPCs (Srivastava 2006; Jockheck-Clark et al. 2010). Peptides bound to Grp94 are internalized by pAPCs by endocytosis and trafficked to a post-ER endosomal compartment, where they are processed and loaded onto mature MHC class I molecules (Fig. 3b). The mechanism of endocytic uptake of the Grp94-peptide complex remains controversial. A large body of evidence indicates the requirement of receptor-mediated internalization, via the low-density lipoprotein receptor-related protein 1 (▶ CD91) and/or the scavenger receptors, whereas recent results obtained in pAPC, engineered to knockdown or knockout CD91 expression, support a prominent role of the pathway of nonspecific fluid-phase uptake (Jockheck-Clark et al. 2010). In any case, the interaction of Grp94-peptide complex with pAPC elicits proinflammatory cytokine secretion and MHC class I/II up-regulation, leading to subsequent priming and activation of peptide-specific CD8+ T lymphocytes.

Calcium Binding Protein

Although each Grp94 molecule can bind between 16 and 28 Ca^{2+}, only one high affinity calcium-binding site has been mapped with certainty in the N-terminal portion of Grp94, in the charged linker domain (Eletto et al. 2010). In vitro studies showed that Ca^{2+} binding at this site stimulates Grp94 peptide binding. Due to its relative abundancy in the ER, Grp94 is expected to provide about 30 μM of Ca^{2+} storage capacity. This property, which is shared with other ER chaperones, like Grp78 and calreticulin, might imply the participation of Grp94 to the maintenance of calcium homeostasis. In addition to protein folding, ER is deputed to the storage and utilization of calcium. Release and uptake of calcium by the ER is involved in many signaling pathways and physiological responses, like protein secretion and muscle contraction. Depletion of calcium from the stores induces an ER stress response. Besides, alterations in ER free calcium concentration strongly influence cell survival. If the ER has less free calcium to release upon stress, then cytosolic calcium levels will not rise to the critical levels needed for triggering deleterious downstream effectors. Therefore, the increased expression of ER chaperones with calcium-binding properties should protect cells by decreasing the amount of releasable calcium. Such a hypothesis finds support from several studies showing that Grp94 overexpression counteracts the increase in intracellular Ca^{2+} evoked by exposure

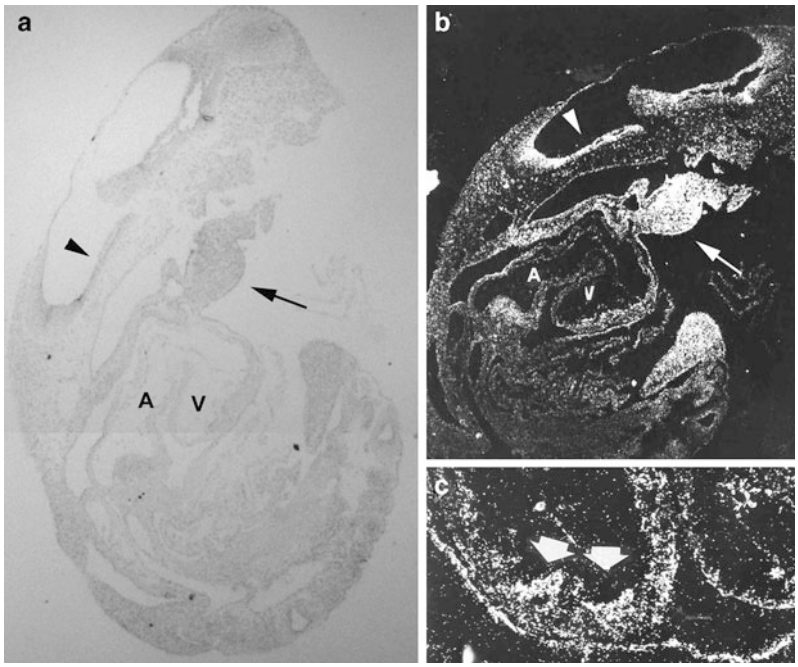
either to calcium ionophore or to oxidants, whereas cells engineered to decrease Grp94 expression appear unable to cope with it (Pizzo et al. 2010 and references within). Passive calcium release from the stores, induced in myogenic cells by means of sarcoendoplasmic calcium ATPase (SERCA) inhibition, showed that cellular levels of Grp94 inversely correlate to the rise in intracellular Ca^{2+} (Pizzo et al. 2010). This body of evidence points toward a role for Grp94 in preserving cell viability, when challenged by perturbations of Ca^{2+} homeostasis, suggesting that Grp94 may modify intracellular Ca^{2+} content, probably by interaction with calcium cycling proteins.

Physiological Function in Embryonic Development

Consistently with the observation that Grp94 is absent from most unicellular organisms, knockdown of Grp94 in different cell culture systems shows that low basal expression levels of the protein are sufficient to support cell growth and proliferation (Eletto et al. 2010). Similarly, two Grp94 gene mutations causing knocking out of the protein (the *Shepherd* mutation in *A. thaliana* and the frameshifts terminating Grp94 prematurely in the pre-B lymphocyte 70Z/3 cell line) are not cell-lethal, but rather affect selected processes. Although these data suggest that Grp94 does not appear to be essential for growth of individual mammalian cells in culture, survival of $grp94^{-/-}$ embryonic stem cells (ES) is strictly dependent from the presence of serum and IGFs. In addition, a parallel independent study showed that $grp94^{-/-}$ ES cells display an increased, compensatory, expression of ER chaperones, whereas in the presence of a 50% reduction of Grp94 levels, as it occurs in hemizygosis, there is no compensatory response (Mao et al. 2010). Furthermore, $grp94^{-/-}$ ES cells show a decreased ability to activate the XBP-1 pathway of the ER stress response. This observation reveals the involvement of Grp94 in the ER stress signaling, a function up to now mainly attributed to Grp78 (Glembotski 2008). In $grp94^{-/-}$ ES cells, the decreased expression of the ER stress-induced transcription factor XBP-1 might be due to the misfolding of the Grp94 client proteins TLR4 and TLR2, which in macrophages are known to activate the ER-stress sensor, inositol-requiring enzyme 1a (IRE 1), and its downstream target XBP-1 (Mao et al. 2010).

Conversely, Grp94 absence is responsible for the occurrence of lethal phenotypes in plants, nematodes, fruit flies, and mice (Eletto et al. 2010). In mammals, Grp94 transcripts are found in oocytes and, in 5–8.5-d embryos, protein expression is highest in the embryonic and extraembryonic ectoderm and lower in the visceral endoderm. Grp94 is also detected in mesoderm cells emerging from the primitive streak. At later stages during organogenesis (E9.5–13.5) Grp94 is expressed within the developing heart, neuroepithelium, and branchial arches (Fig. 4). Despite of the widespread and early expression of Grp94 during embryonic development, its requirement during differentiation and organogenesis differs depending on the pattern of expression and the essential function of the client proteins.

One relevant example is given by skeletal muscle progenitors, where Grp94 expression is required for differentiation and maturation (Eletto et al. 2010; Gorza and Vitadello 2000). The phenotype of $grp94^{-/-}$ mice is lethal at E7.5 and embryo bodies generated by $grp94^{-/-}$ ES cells show any type of tissue differentiation, except the formation of striated and smooth muscles. The loss of ability to give origin to muscle is due to the absolute requirement of Grp94 in folding of IGFs. When supplemented with IGF, $grp94^{-/-}$ ES cells are able to differentiate and express myogenin and contractile proteins; they can also fuse into myotube-like syncitia, although with far less efficiency of wild type ES cells. Since Grp94 depletion does not affect myoD levels, the chaperone is not required for the commitment to myogenesis. Conversely, Grp94 appears to be involved in myogenesis initiation. Few hours after switching the myogenic cell line C2C12 to differentiation medium, the protein translocates from the ER to the Golgi compartment and the cell surface, after interacting with and being Tyr-phosphorylated by the Src-kinase Fyn (Frasson et al. 2009 and Fig. 2a). It is presently unknown whether this re-localization is related to delivery of specific protein cargo; an identified relevant client is IGF-II, whose secretion and autocrine interaction with IGF-I and II receptors (IGF-R1) is required to proceed along the differentiation program. Eventually, Grp94 appears to be involved in myotube formation. Knocking down Grp94 protein levels to 40% of wild type C2C12 cells is permissive for myogenic differentiation, but not for myotube formation (Gorza and Vitadello 2000). Since IGF supplementation does not fully rescue the myotube phenotype, it is likely that



Grp94 (HSP90B1), Fig. 4 In situ hybridization analysis of Grp94 mRNA distribution in the E10 rabbit embryo. (**a**) and (**b**) show bright and dark field micrographs, respectively, of a sagittal section of the whole embryo. A and V indicate primordium of the atrial and ventricular myocardium, respectively. Strong hybridization signals are detectable at the level of branchial arches (*thin arrow*) and neuroepithelium (*arrowhead*). (**c**) The micrograph shows the myocardium of the ventricular primordium at higher magnification. *Large arrows* point to stronger hybridization signals at the level of ventricular trabeculae

other Grp94 client proteins play a relevant role at this later stage.

Another example of the tissue specificity of Grp94 involvement in tissue differentiation and maturation has been recently provided by the study of murine hematopoietic and lymphopoietic precursors after conditional knock out of Grp94 (Staron et al. 2010). Despite the essential role of the chaperone in the folding of most TLRs and integrins, only lymphopoiesis, that is, maturation of pro-B lymphocytes and development of thymocytes, is severely affected. Furthermore, this defect cannot be rescued by wild type precursors, indicating that the chaperone regulates B- and T-lymphopoiesis in a cell-intrinsic fashion.

Pathophysiological Functions

Cancer

Most neoplastic cells express high levels of chaperone/stress proteins. ER chaperones are upregulated probably because hypoxia and hypoglycemia are two characteristics of the tumor environment and inducers of the ER stress response. A direct consequence of this upregulation is the enhancement of neoplastic cell survival; an indirect one is the increase and the release of tumor-specific peptides bound to chaperones. Srivastava and coworkers first identified a series of structurally related, but distinct, Grp94 molecules associated with specific immunogenicity of chemically induced mouse sarcoma. Specific antitumor immunity, raised after purification of the Grp94-peptide complex from a highly metastatic variant of murine lung carcinoma, is mediated by $CD4^+$ and $CD8^+$ T lymphocytes and natural killer cells and suppresses tumor metastasis. Furthermore, vaccination with Grp94-peptide complexes isolated from different tumors elicits antitumor response against all the tumors (Srivastava 2006). These results, which have been confirmed experimentally by other laboratories, represent the starting point for the development of anticancer immunotherapy, which is presently tested in several clinical trials (Woods and Mulders 2009).

Grp94 is also involved in anticancer therapies aimed to disrupt Hsp90 chaperone function on several oncogenic proteins by means of molecules like geldanamycin and analogues, which inhibit ATPase activity (Fig. 1a). The effects of these molecules on Grp94 function in tumor cells are rarely investigated, although they might play a role in reducing active cell surface levels of transmembrane receptors implicated in cancer, such as the receptor for the epidermal growth factor (EGF receptor) and, most importantly, the release of IGFs (McLaughlin and Vandenbroeck 2011).

Autoimmune Diseases

Only immature cells and some neoplastic ones express Grp94 molecules at the cell surface. Spontaneous systemic lupus erythematosus-like autoimmune phenotype was experimentally obtained by inducing persistent expression of cell surface Grp94, via transgenic overexpression of an obligatory transmembrane form of Grp94 (Liu et al. 2006) or knock out of AIMP1, which allows the interaction of Grp94 with the KDEL receptor (Kim et al. 2010). A subsequent study excluded the direct role of the enforced cell surface expression of Grp94 demonstrating that disease phenotype occurs secondary to the concomitant increased expression of TLR4 and the enhanced sensitivity of the receptors to low levels of endotoxin released by the commensal flora (Liu et al. 2006). On the other hand, the inhibition of Grp94 localization to the surface of $AIMP^{-/-}$ cells, achieved by restoring KDEL receptor uptake, appeared efficient in ameliorating the disease phenotype (Kim et al. 2010). Despite these controversial findings, the participation of this chaperone in the pathogenesis of autoimmune diseases has been recently demonstrated for rheumatoid arthritis. Released Grp94 is increased in synovial fluid from the joints of human rheumatoid arthritis patients, where it interacts with the extracellular domains of both TLR2 and TLR4, promoting the self-perpetuating activation of synovial macrophages (Huang et al. 2009).

A major nonimmune role in the pathogenesis of autoimmune myositis is played by the ER stress response accompanying transgenic MHC class I upregulation in mouse skeletal muscle fibers and leading to NF-kB activation and increased expression of ER chaperones. Extensive analysis of patients' biopsies revealed that Grp94 overexpression was restricted to regenerating myofibers, which recapitulating muscle differentiation overexpressed also MHC class I molecules and other autoantigens (Vitadello et al. 2010). In this context, the immunomodulatory and pro-inflammatory effects of Grp94 upregulation appear to play a secondary role, bound to muscle regenerative events, which, however, may stimulate further the immune response.

Nevertheless, Grp94 involvement in the inflammatory and the adaptive immune response represents an interesting therapeutical target for autoimmune disorders. Grp94 chaperone activity on client proteins, such as TLRs and the p40 subunit of the interleukin 12 family of cytokines (IL-12), might be inhibited by radicicol and geldanamycin derivatives, which have been shown to reduce inflammatory markers and capillary leakage and modulate IL-12 family secretion levels (McLaughlin and Vandenbroeck 2011).

Ischemia-Reperfusion

Ischemia-reperfusion represents a strong inducer of the ER stress response. Several studies have been performed in the brain, where the activation of the ER stress response upregulates ER chaperone expression and is protective. Such an issue is far more relevant for mammalian cardiomyocytes, where control of intracellular calcium concentration $[Ca^{2+}]_i$ becomes critical during ischemia. Although evidence obtained in vitro and in vivo confirmed the activation of the ER stress response in ischemic-reperfused cardiomyocytes, it is less clear what function the ER stress response serves in the cardiac context. Several studies show that ER stress response contributes to ischemic damage, by inducing both apoptosis and autophagy-mediated cell death; conversely, others support the protective role of the ER stress response and of ER chaperones, among which is Grp94, in decreasing size of infarct or maintaining cardiomyocyte viability (Glembotski 2008). Such a discrepancy might depend upon the context of the study, in that protective aspects of the ER stress response predominate early after the ischemic injury, whereas pro-apoptotic features occur later. Although the mechanism through which Grp94 exerts cardioprotection remains to be determined, a body of evidence point to its role in the maintenance of calcium homeostasis. Grp94 overexpression improves myocyte survival after exposure to oxidative stress, a usual consequence of ischemia-reperfusion, because it reduces the degree of protein oxidation (Pizzo et al. 2010). Calcium dyshomeostasis is generated by and generate further oxidative stress. Grp94 overexpression, which can also be pharmacologically upregulated, appears to disrupt this pathway, by reducing the amount of releasable calcium from the stores.

Ischemia/reperfusion injury also induces an innate immune response, leading to an inflammatory reaction and tissue damage that have been attributed to engagement of TLR2 and 4. In the ischemic kidney, Grp94 contributes to the activation of ERK1/2 downstream pathway by dissociating from PP5, and thus

inactivating this negative modulator. Although multiple pathway activated by ischemia-reperfusion act upon the ERK1/2 cascade of renal tubular cells, the dissociation of Grp94 from PP5 is dependent only from TLR2 activation (Mkaddem et al. 2009).

Inhibitors and Expression Regulators

The small number of client proteins and the involvement in specific cellular events make this chaperone an interesting target to be inhibited or increased, depending of the desired outcome.

Known inhibitors of both ATP hydrolysis and peptide binding, such as radicicol, geldanamycin, and analogues, are already tested in clinical trials mostly as inhibitors of Hsp90 chaperone activity (McLaughlin and Vandenbroeck 2011). The nucleotide analog 5′-*N*-ethylcarboxamidoadenosine (NECA), a broad-spectrum adenosine A_2 receptor antagonist, which binds Grp94 and not Hsp90, is presently used only in vitro. Recently, the molecule S-methyl 2-(4,6-dimethoxypyrimidine-2-yloxy)-3-methylbutanoate (GPM1) has been shown to directly interact with hydrophobic residues of Grp94 within the dimerization domain (Fig. 1a), facilitating its oligomerization and retrograde transport to endoplasmic reticulum via the KDEL receptor. In vivo administration of this compound reduced maturation of pAPCs and activation of B and T cells, alleviating the symptoms associated to an experimental model of systemic lupus erythematosus in mice ($AIMP1^{-/-}$) (Han et al. 2010).

Although upregulation of Grp94 is achieved pharmacologically together with that one of the other ER chaperones – one example is the ER stress response induced by celecoxib, a nonsteroidal anti-inflammatory drug, and its derivatives (McLaughlin and Vandenbroeck 2011) – a more selective Grp94 involvement apparently follows the administration of curcumin (Pizzo et al. 2010). Similarly to celecoxib, also curcumin is a SERCA inhibitor and able to induce an ER stress response by depleting calcium stores. A single pulse of curcumin at low dosage selectively upregulates Grp94 expression and contains the effects of oxidative stress on protein carbonylation and cell survival, whereas the benefits of such a short curcumin treatment cannot be observed, when Grp94 is knocked down by antisense cDNA (Pizzo et al. 2010).

Summary

Grp94 is a chaperone/stress protein of the ER and is expressed only in multicellular organisms. At variance of the paralog Hsp90, Grp94 is involved in the folding of a limited population of client proteins and only one co-chaperone has been identified so far. Grp94 absolute requirement for embryonic development derives from the specific role of the client proteins played in critical contexts, like IGFs for differentiation of muscle tissues, and TLR and integrins for lymphopoiesis.

In addition to chaperone activity, Grp94 serves different functions. It binds calcium and participates, through still unknown mechanisms, to the control of intracellular calcium homeostasis. When increased, consequent to the ER stress-response, it exerts cytoprotection against different stressors, such as calcium overload and reactive oxygen species. Grp94 may change its subcellular localization, migrating to Golgi and the cell surface, depending on the cell type and the activation of signal transduction pathways involving Src-kinase or JNK. Cell surface and released Grp94 molecules act as immune regulator of both the innate and adaptive responses by allowing maturation of pAPCs. The protein interacts with TLR2 and initiates inflammation promoting IL-6 and TNF-α secretion from pAPCs. It participates to the adaptive immune response by binding peptides and being internalized in post-endoplasmic reticulum endosomal compartment of pAPCs, where the carried peptide is loaded onto mature MHC class I molecules and can recruit specific $CD4^+$ and $CD8^+$ T lymphocytes.

Although this last property suggested the use of peptide-bound Grp94 as anticancer vaccine, the other features identify this ER chaperone as an interesting target for pharmacological therapy. Hampering the chaperone activity of Grp94 or increasing the protein retention into the ER reduces the release of pro-inflammatory and immune modulatory signals. Future attempts to change selectively Grp94 protein levels would permit to vary cell resistance against lethal stresses.

References

Eletto D, Darsh D, Argon Y. GRP94 in ER quality control and stress responses. Sem Cell Dev Biol. 2010;21:479–85.

Frasson M, Vitadello M, Brunati AM, La Rocca N, Tibaldi E, Pinna LA, Gorza L, Donella-Deana A. Grp94 is Tyr-phosphorylated by Fyn in the lumen of the endoplasmic

reticulum and translocates to Golgi in differentiating myoblasts. Biochim Biophys Acta-Mol Cell Res. 2009; 1793:239–52.
Glembotski CC. The role of the unfolded protein response in the heart. J Mol Cell Cardiol. 2008;44:453–9.
Gorza L, Vitadello M. Reduced amount of the glucose-regulated protein GRP94 in skeletal myoblasts results in loss of fusion competence. FASEB J. 2000;14:461–75.
Han JM, Kwon NH, Lee JY, Jeong SJ, Jung HJ, Kim HR, Li Z, Kim S. Identification of gp96 as a novel target for treatment of autoimmune disease in mice. PLoS One. 2010;5(3):e9792. doi:10.1371/journal.pone.0009792.
Huang QQ, Sobkoviak R, Jockheck-Clark AR, Shi B, Mandelin AM, Tak PP, Haines GK, Nicchitta CV, Pope RM. Heat shock protein 96 is elevated in rheumatoid arthritis and activates macrophages primarily via TLR2 signaling. J Immunol. 2009;182:4965–73.
Jockheck-Clark AR, Bowers EV, Totonchy MB, Neubauer J, Pizzo SV, Nicchitta CV. Re-examination of CD91 function in GRP94 (glycoprotein 96) surface binding, uptake, and peptide cross-presentation. J Immunol. 2010;185: 6819–30.
Kim G, Han JM, Kim S. Toll-like receptor 4-mediated c-Jun N-terminal kinase activation induces gp96 cell surface expression via AIMP1 phosphorylation. Biochem Biophys Res Commun. 2010;397:100–5.
Koo BH, Apte SS. Cell-surface processing of the metalloprotease pro-ADAMTS9 is influenced by the chaperone GRP94/GP96. J Biol Chem. 2010;285:197–205.
Lee AS. Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. Trends Biochem Sci. 1987;12:20–3.
Liu B, Li Z. Heat shock protein HSP90b1 (grp94, gp96) optimizes B cell function via chaperoning integrins and Toll-like receptors but not immunoglobulins. Blood. 2008;112(4):1223–30.
Liu B, Yang Y, Dai J, Medzhitov R, Freudenberg MA, Zhang PL, Li Z. TLR4 up-regulation at protein or gene level is pathogenic for lupus-like autoimmune disease. J Immunol. 2006;177:6880–8.
Liu B, Yang Y, Qiu Z, Staron M, Hong F, Li Y, Wu S, Li Y, Hao B, Bona R, Han D, Li Z. Folding of Toll-like receptors by the HSP90 paralogue gp96 requires a substrate-specific cochaperone. Nat Commun. 2010;1:79. doi:10.1038/ncomms1070.
Ma Y, Hendershot LM. ER chaperone functions during normal and stress conditions. J Chem Neuroanat. 2004;28:51–65.
Mao C, Wang M, Luo B, Wey S, Dong D, Wesselschmidt R, Rawlings S, Lee AS. Targeted mutation of the mouse grp94 gene disrupts development and perturbs endoplasmic reticulum stress signaling. PLoS One. 2010;5:e10852. doi:10.1371/journal.pone.0010852.
McLaughlin M, Vandenbroeck K. The endoplasmic reticulum protein folding factory and its chaperones: new targets for drug discovery? Br J Pharmacol. 2011;162:328–45.
Mkaddem SB, Werts C, Goujon JM, Bens M, Pedruzzi E, Ogier-Denis E, Vandewalle A. Heat shock protein gp96 interacts with protein phosphatase 5 and controls toll-like receptor 2 (TLR2)-mediated activation of extracellular signal-regulated kinase (ERK) 1/2 in post-hypoxic kidney cells. J Biol Chem. 2009;284:12541–9.
Nganga A, Bruneau N, Sbarra V, Lombardo D, Le Petit-Thevenin J. Control of pancreatic bile-salt-dependent-lipase secretion by the glucose-regulated protein of 94 kDa (Grp94). Biochem J. 2000;352:865–74.
Ostrovsky O, Makarevich CA, Snapp EL, Argon Y. An essential role for ATP binding and hydrolysis in the chaperone activity of GRP94 in cells. Proc Nat Acad Sci USA. 2009;106:11600–5.
Ostrovsky O, Eletto D, Makarewich C, Barton ER, Argon Y. Glucose regulated protein 94 is required for muscle differentiation through its control of the autocrine production of insulin-like growth factors. Biochim Biophys Acta Mol Cell Res. 2010;1803:333–41.
Pizzo P, Scapin C, Vitadello M, Florean C, Gorza L. Grp94 acts as a mediator of curcumin-induced antioxidant defence in myogenic cells. J Cell Mol Med. 2010;14:970–81.
Randow F, Seed B. Endoplasmic reticulum chaperone gp96 is required for innate immunity but not cell viability. Nat Cell Biol. 2001;3:891–6.
Srivastava PK. Therapeutic cancer vaccines. Current Opin Immunol. 2006;18:201–5.
Staron M, Yang Y, Liu B, Li J, Shen Y, Zúñiga-Pflücker JC, Aguila HL, Goldschneider I, Li Z. gp96, an endoplasmic reticulum master chaperone for integrins and Toll-like receptors, selectively regulates early T and B lymphopoiesis. Blood. 2010;115:2380–90.
Vitadello M, Doria A, Tarricone E, Ghirardello A, Gorza L. Myofiber stress-response in myositis: parallel investigations on patients and experimental animal models of muscle regeneration and systemic inflammation. Arthritis Res Ther. 2010;12:R52. doi:10.1186/ar2963.
Wanderling S, Eletto D, Makarewich C, Barton ER, Argon Y. Grp94 is essential for mesoderm induction and muscle development because it regulates insulin-like growth factor secretion. Mol Biol Cell. 2007;18:3764–75.
Woods CG, Mulders P. Vitespen: a preclinical and clinical review. Future Oncol. 2009;5:763–74.
Yang Y, Liu B, Dai J, Srivastava PK, Zammit DJ, Lefrancois L, Li Z. Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. Immunity. 2007;26:215–26.

GS

► Glycogen Synthase Kinase-3

GSK-3

► Glycogen Synthase Kinase-3

Gsp1

► Ran

GST

► Glutathione-S-Transferases: As Signaling Molecules

Gsto 1

► Glutathione-S-Transferases: As Signaling Molecules

Gt

► G Protein Alpha Transducin

Gtrgeo5

► Tead

Guanine Nucleotide Binding Protein

► G Protein Beta/Gamma

Guanine Nucleotide Binding Protein (G Protein), Gamma

► G Protein Beta/Gamma

Guanine Nucleotide Binding Protein, Alpha Inhibiting

► G Protein α i/o/z

Guanine Nucleotide Binding Protein, Alpha o

► G Protein α i/o/z

Guanine Nucleotide Binding Protein, Alpha z Subunit

► G Protein α i/o/z

Guanine Nucleotide Binding Protein, Beta

► G Protein Beta/Gamma

Guanine Nucleotide Binding Protein, Beta Polypeptide

► G Protein Beta/Gamma

Guanine Nucleotide Binding Protein, Gamma

► G Protein Beta/Gamma

Guanine Nucleotide Binding Regulatory Protein, Alpha i

► G Protein α i/o/z

Guanine Nucleotide Exchange Factor

► Rap GEF Family

Guanine Nucleotide Exchange Factor, Calcium- and DAG-Regulated

► RasGRP1

Guanylate Cyclase

Karl-Wilhelm Koch
Department of Biology and Environmental Sciences, University of Oldenburg, Oldenburg, Germany

Synonyms

Guanylyl cyclase

Historical Background

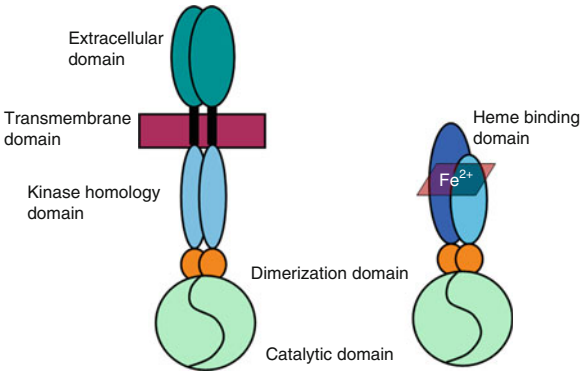
The concept of second messenger molecules in hormone signal transduction was developed in the 1950s by Earl W. Sutherland describing the cyclic nucleotide adenosine 3′, 5′-cyclic monophosphate (cAMP) as an intracellular messenger molecule. The main features of this concept are the binding of a hormone to the extracellular site of a transmembrane receptor protein which triggers an intracellular response mediated by a second messenger molecule (e.g., cAMP). Shortly after cAMP was discovered as second messenger in hormone signaling another cyclic nucleotide abbreviated cGMP (guanosine 3′, 5′-cyclic monophosphate) was first detected in rat urine (Ashman et al. 1963) and soon after was found in a variety of other tissues and biological samples. Every second messenger system requires the presence of synthesizing and degrading enzymes. For cyclic purine monophosphates these are adenylate and guanylate cyclases and specific forms of phosphodiesterases, respectively. Guanylate cyclases (GCs) catalyze the conversion of GTP to cGMP according to the reaction scheme: GTP → cGMP + pyrophosphate (PP_i). The development of suitable and effective enzymatic assays led in 1969 to several publications that report on the determination of GC activity. Consequent studies then revealed that GC activities cofractionated with soluble and particulate membrane fractions, but it was not before the mid to late 1980s that soluble and membrane-bound GCs were purified from mammalian sources to apparent homogeneity. Subsequent cloning studies in the late 1980s and early 1990s using tissue-specific cDNA libraries resulted in deduced primary structures of soluble and particulate GC isoforms. Studies on mammalian GCs were inspired by work on sea urchin sperm that is a rich source of a particulate GC and that is activated by certain peptides. In mammalians it was known already from reports in the 1970s that neuromediators like acetylcholine could regulate the cGMP level in perfused heart tissue, but the physiological pathways linking these steps remained unclear until the 1980s. Then several discoveries showed that cGMP is the second messenger of important physiological responses including smooth muscle relaxation, intestinal fluid and electrolyte homeostasis, and sensory physiology, in particular phototransduction. Several physiologically relevant molecules that regulate and/or trigger activation of GCs were identified and characterized in the last three decades. The most important so far are nitric oxide for the soluble isoforms and natriuretic peptides, paracrine intestinal hormones, and changes in cytoplasmic Ca^{2+}-levels in combination with Ca^{2+}sensor proteins for the different particulate isoforms. The identification of these regulatory molecules paved the way for integrating GC isoforms in signaling pathways or led to formulation of new signaling concepts (for historical aspects see Beavo and Brunton 2002; Kots et al. 2009; Sharma 2010).

Guanylate Cyclase Forms

Seven different forms of a membrane GC and two α- and β–subunits of the soluble form are expressed in a wide range of mammalian tissues (see under "Tissue Distribution"). They are classified on the basis of their amino acid sequences and according to their ligands or intracellular regulators (for each GC forms synonymous names are found in the literature and are given below):

Membrane-bound GCs	Soluble GCs
GC-A/ANF-RGC//NPR-A	sGC α1
GC-B/CNP-RGC/NPR-B	sGC α2
GC-C/STa-RGC	sGC β1
GC-D/ONE-GC	sGC β2
GC-E/ROS-GC1/retGC-1	
GC-F/ROS-GC2/retGC-2	
GC-G	

In addition GCs are also found in nonmammalian vertebrates like teleost fishes, in insects, nematodes, unicellular eukaryotic organisms, and bacteria (Baker and Kelly 2004; Ortiz et al. 2006; Rätscho et al. 2010).



Guanylate Cyclase, Fig. 1 Domain topography of membrane and soluble GCs

Some of these organisms express a larger variety of GCs than mammalians and are at least partially homologous to the GC forms found in mammalians.

Protein Structure and Topography

Membrane-bound and soluble GCs share some common structural features, but also differ in those protein domains that are important for their regulation by extracellular or intracellular factors (Lucas et al. 2000; Tamura et al. 2001). Figure 1 shows the main topographic features of these GC forms. Membrane (particulate) GCs are homodimers, whereas soluble GCs function as heterodimers. Both subtypes contain a cyclase catalytic domain with a high amino acid sequence homology among all GCs and significant homology to adenylate cyclases. Invariant amino acids in the catalytic domain define the substrate specificity (binding of GTP or ATP). The catalytic domain of the membrane-bound isoforms is extended by a C-terminal tail of varying length, which is not present in soluble GCs. Both subtypes also contain a dimerization domain that is N-terminal to the catalytic domain. This domain has the structural features of an amphipathic α–helix allowing forming a two-stranded α–helical coiled coil. The third large domain of the cytoplasmic part of membrane-bound GCs is the kinase homology domain that consists of approximately 250 amino acids. It shows partial amino acid sequence homology to protein tyrosine kinase receptors and harbors an ATP-binding motif that is identical or similar to the ATP-binding motif of the catalytic subdomains of protein kinases (the sequence GxGxxG that is rich in glycine is conserved in some GCs, but not in all). Particulate GCs are anchored in the membrane by a single transmembrane domain that has an amino acid sequence typical for an α-helical transmembrane region found in other membrane receptor proteins. While it is clear that this segment is important for membrane localization it is unresolved whether it also mediates transmembrane signaling. The different particulate subtypes might differ in this aspect. A short segment of amino acids called the juxtamembrane domain is sandwiched between the kinase homology and the transmembrane domain. Depending on the subtype of particulate GC this region consists of 25–100 amino acids. The more extended forms of the juxtamembrane region are found in the sensory GCs.

Instead of a kinase homology domain soluble GCs contain a heme-binding domain that is N-terminally located from the amphipathic dimerization domain (Fig. 1). The two heme-binding domains of one soluble GC heterodimer coordinate a heme prosthetic group (a porphyrin ring structure with a central ferrous ion (Fe^{2+})) that is required for the enzyme to become activated by nitric oxide (see below).

The least homology among all membrane-bound GCs is found in the extracellular domain. This large domain consists of approximately 500 amino acids and harbors the ligand-binding region, in which the ligand can be either a hormone or an odorant molecule depending on the GC subtype. Sensory GCs that are expressed in the photoreceptor cells of the vertebrate retina are an exception of this rule. No external ligand is known for the extracellular domain of these GCs and in the case of GCs expressed in rod photoreceptor cells the corresponding extracellular domain is located in the disk lumen.

Tissue Distribution

The natriuretic peptide receptor GCs GC-A and GC-B show the most diverse tissue distribution (Lucas et al. 2000; Tamura et al. 2001). GC-A is expressed in adrenal gland, pituitary gland, adipose tissue, cerebellum, aorta, heart, kidney, liver, spleen testis, colon, brain stem, retina, cochlea, ovary thymus, and others. Expression of GC-B largely overlaps with that of GC-A, but a more specific expression pattern is found for GC-C, the intestinal receptor for guanylin and uroguanylin. This GC is mainly present in the colon

and intestine, but is also found in the kidney, testis, and liver. A similar expression profile (lung, kidney, skeletal muscle, and intestine) is found for GC-G. Different from these receptor-type GCs are the sensory GCs that display a very restricted expression profile. For example, the odorant receptor GC-D is found in a subset of sensory neurons of the olfactory neuroepithelium (Sharma and Duda 2010; Zufall and Munger 2010) and the retina-specific GCs ROS-GC1 and ROS-GC2 (GC-E and GC-F) are predominantly expressed in photoreceptor cells of the vertebrate retina (Koch et al. 2010). In addition, ROS-GC1 is also found in the pineal gland.

Soluble GC forms are widely distributed and are mainly present in lung, heart, liver, kidney, cerebellum, skeletal muscle, and retina, whereby expression of the β2 isoform seems to be more restricted.

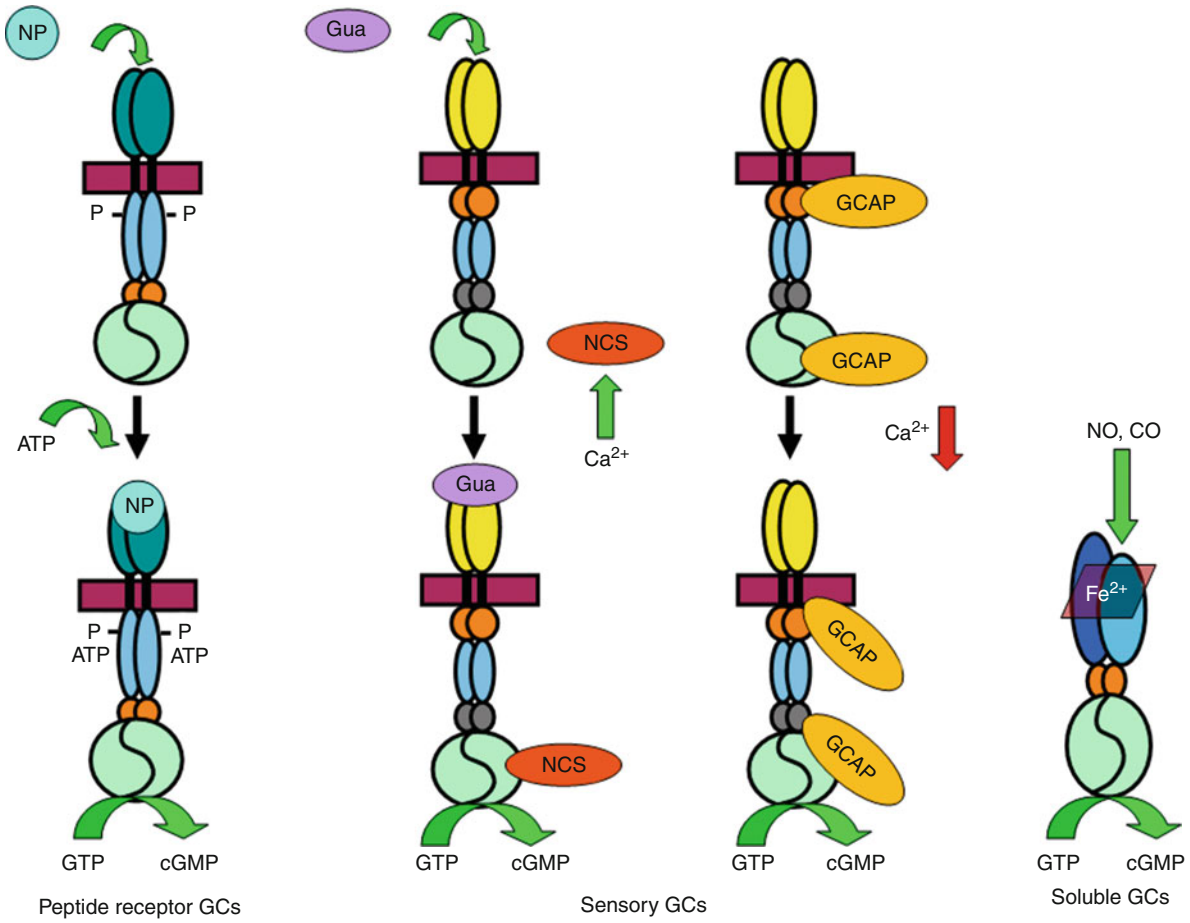
Mode of Activation and Regulation

Natriuretic peptides (NP) act on two types of membrane-bound GCs, GC-A and GC-B, and are a family of three factors (Potter et al. 2009; Martel et al. 2010). These factors are named ANP, BNP, and CNP. The active forms of these hormones/paracrine factors are between 22 and 53 amino acids long and circulate in the blood stream after posttranslational proteolytic cleavage from longer forms. ANP and BNP are secreted from cardiac myocytes and act on GC-A, CNP is secreted in the brain, chondrocytes, and epithelial cells and acts on GC-B. All peptides bind to the extracellular domain of the GC subgroup of natriuretic peptide receptors and thereby increase the guanylate cyclase activity of the receptor. The basal state of GC-A and GC-B exhibits very low GC activity, is highly phosphorylated (at serine and threonine residues), and forms homodimers. Binding of the peptide induces a conformational change enabling ATP to bind to the intracellular KHD, which is an allosteric control step for transducing the hormone signal to the cyclase catalytic domain (Fig. 2). Prolonged exposure of natriuretic peptides to the receptors leads to dephosphorylation and desensitization. Another class of peptides, guanylin and uroguanylin, are the endogenous activator of the intestinal GC-C. In addition, GC-C is specifically activated by bacterial heat-stable enterotoxins (STa) that cause severe secretory diarrhea via a cGMP-mediated signaling pathway (Lin et al. 2009). The odorant receptor GC-D is also activated by uroguanylin and guanylin as well as by natural urine stimuli and bicarbonate leading to an increase in cGMP synthesis (Zufall and Munger 2010). A further control mode is the regulation by so-called neuronal Ca^{2+}-sensor (NCS) proteins that regulate the GC activity from the cytoplasmic part of the receptor (Fig. 2). GC-D is related to the other sensory GCs (ROS-GC1 and ROS-GC2) that mainly operate in the outer segments of vertebrate photoreceptors and are regulated by intracellular NCS proteins named guanylate cyclase-activating proteins (GCAPs). Light triggers the decrease of the intracellular messenger cGMP and with a short delay the decrease of the cytoplasmic Ca^{2+}-concentration as well. Changing concentrations of Ca^{2+} are sensed by GCAPs that form a complex with the target GC and undergo a Ca^{2+}-induced conformational change. This in turn is thought to lead to an increase of catalytic activity at the cyclase catalytic domain (Fig. 2). No ligands are known for retina-specific GCs and it is unknown whether binding of a ligand to the extracellular (intradiskal) site is at all necessary. Binding of ATP to the intracellular KHD is known to enhance the GC activity (Koch et al. 2010).

Soluble GCs are activated by a fundamentally different mechanism. Gaseous molecules like NO and CO bind to the heme prosthetic group that is held between the two subunits forming the heterodimeric GC (Fig. 2). Binding of NO to the Fe^{2+} in the porphyrine ring of the heme group triggers the breakage of histidine coordinating bond and leads in consequence to a conformational change. By this mechanism the GC activity can be stimulated by about 5,000-fold (Garthwaite 2010).

Signaling Pathways and Physiological Responses Involving Guanylate Cyclases

The different GC forms are involved in complex physiological responses mirrored in the large variety of tissue. For example, the natriuretic peptide receptor GC-A is involved in regulation of blood pressure as this was demonstrated by transgenic mice lacking the receptor or the activating peptide ANP. Key steps in blood pressure regulation are vascular smooth muscle relaxation and contraction, which are under control of intracellular Ca^{2+}-spikes. In the kidney, ANP controls

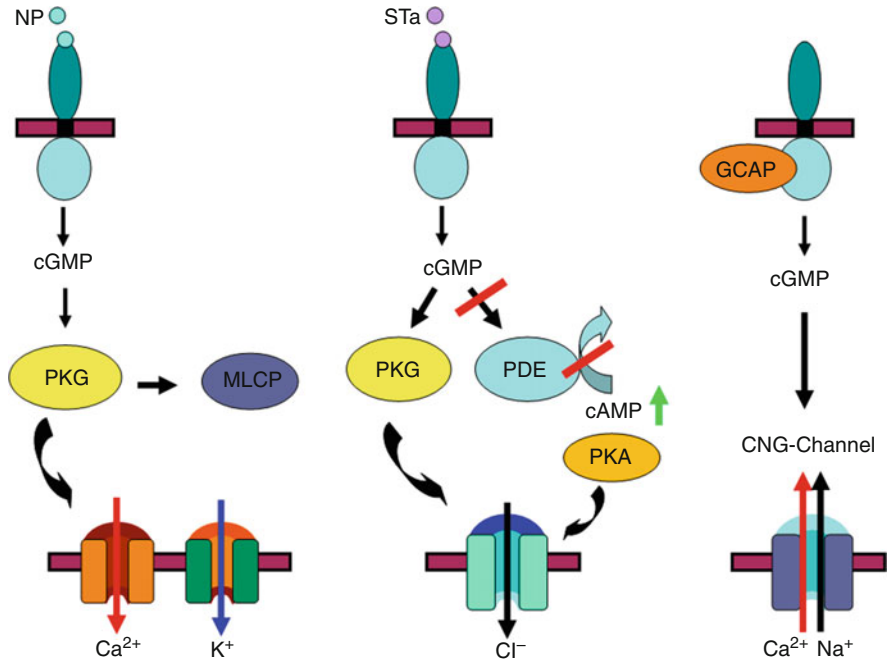


Guanylate Cyclase, Fig. 2 Modes of GC activation and regulation. Natriuretic peptides (NP) bind and thereby activate peptide receptor GCs that are phosphorylated (-P) in the basal state. The hormone signal is transferred to the catalytic domain under control of ATP. Sensory GCs are under control of Ca^{2+}-sensor proteins (NCS, GCAP) at the intracellular site. GC-D in olfactory sensory neurons is also activated by guanylin (Gua) and uroguanylin at the extracellular site, whereas no ligand appears necessary for the activation of photoreceptor GCs. GCAPs sense changes in intracellular Ca^{2+} and in turn activate photoreceptor GCs at low Ca^{2+}-concentrations. NO and CO can pass the cell membrane and stimulate soluble GCs

the fluid and electrolyte secretion by an increase of the glomerular filtration rate. These responses are triggered by an elevation of the intracellular cGMP level (Lucas et al. 2000; Tamura et al. 2001; Martel et al. 2010). Three main intracellular targets of cGMP are known so far including protein kinase G (PKG), a cAMP-hydrolyzing phosphodiesterase (PDE), and cyclic nucleotide-gated (CNG) channels. In particular PKG and PDE are involved in blood pressure regulation. PKG is known to activate or inhibit Ca^{2+}-transport systems in vascular smooth muscle cells by phosphorylation. These include Ca^{2+}-activated potassium channels, Ca^{2+}-ATPases in the plasma membrane and in the sarcoplasmic reticulum, voltage-dependent Ca^{2+}-channels, and the inositol 1,4,5-triphosphate receptor. In addition PKG controls enzymes like myosin light chain phosphatase (Fig. 3).

Additional effects of cGMP that are mediated via natriuretic peptide signaling are cardiac hypertrophy and fat metabolism. The factor CNP that acts on GC-B stimulates bone growth and is involved in vascular remodeling.

The paracrine hormones guanylin and uroguanylin regulate the fluid and electrolyte homeostasis in intestinal epithelial cells by binding to the extracellular site of GC-C. A similar ligand-receptor interaction is



Guanylate Cyclase, Fig. 3 Comparison of signaling pathways involving membrane-bound GCs and synthesis of cGMP. Targets of cGMP are PKG, cAMP-specific PDE, and CNG-channels. PKG can phosphorylate and thereby regulate many targets including Ca^{2+}-transport systems, Ca^{2+}-activated K^+-channels, and myosin light chain phosphatase (MLCP). Inhibition of PDE by cGMP that is synthesized by GC-C in intestine can lead to an increase of cAMP and activation of PKA, in particular during toxin (STa)-induced activation of GC-C. Both kinases (PKG and PKA) phosphorylate the CFTR and thereby increase its chloride-permeability. CNG-channels in photoreceptor cells bind directly to cGMP leading to an opening of the channel and an influx of Na^+ and Ca^{2+} into the cell

observed with bacterial enterotoxins leading also to an increase of intracellular cGMP. The latter is a major cause of secretory diarrheal disease. Elevated levels of cGMP activate PKG type II, but can also inhibit a cAMP-specific PDE leading to an increase in cAMP levels and in consequence to an activation of protein kinase A (PKA). PKGII and PKA then phosphorylate and thereby activate the cystic fibrosis transmembrane regulator (CFTR) a chloride-ion channel in the intestinal brush border membrane (Fig. 3).

Sensory GCs are predominantly expressed in sensory cell types and are part of signaling pathways that have CNG-channels instead of PKGs as main downstream targets of cGMP. Among them GC-D is a receptor GC that can be activated by the ligand peptides guanylin and uroguanylin (see above). GC-D is present in a subpopulation of olfactory sensory neurons and not part of the predominant cAMP-signaling pathway in most olfactory sensory neurons. Intracellular signaling of GC-D is linked to Ca^{2+}-signaling pathways as well, since Ca^{2+}-binding proteins of the NCS protein subfamily were shown to regulate GC activity by binding to intracellular regions of GC-D. These NCS proteins are GCAP1, neurocalcin-δ, frequenin, and ▶ hippocalcin. The biological role of GC-D has been discussed in the context of chemosensory detection of the metabolic status (Sharma and Duda 2010; Zufall and Munger 2010).

Vision in vertebrate photoreceptor cells involves membrane-bound GCs that are regulated by intracellular Ca^{2+}-binding proteins named GCAPs. No extracellular ligands are known so far. Mammalian rod and cone cells harbor two of these GCs named ROS-GC1 and ROS-GC2 or GC-E and GC-F, respectively. They synthesize the intracellular messenger of visual excitation, cGMP, thereby keeping the CNG-channels open to allow influx of Na^+ and Ca^{2+} from the extracellular medium in the dark adapted state of the cell. GCAPs are an important regulatory part of an intracellular feedback loop, since they activate ROS-GCs

at low Ca^{2+}-concentration and inhibit them at higher Ca^{2+}-concentration leading to resynthesis of cGMP after illumination, when the cytoplasmic Ca^{2+}-concentration has dropped (Fig. 3). ROS-GCs are key components of signal transduction in rod and cone cells by controlling the second messenger level. Thereby they are important for the cell's recovery to the dark state after illumination and its adaptational properties (Koch et al. 2010).

Soluble GCs are the target of the versatile messenger molecule NO that is synthesized by nitric oxide synthase isoforms. NO is, for example, mediating smooth muscle relaxation and is involved in the central and peripheral nervous system and in the immune response of macrophages. NO can act on other targets than soluble GCs, but increase of cGMP production by NO leads in general to activation of PKG and subsequent phosphorylation of PKG targets.

Summary

The second messenger cGMP and its synthesizing enzymes (membrane and soluble GCs) have become essential parts of different signaling pathways that mediate important physiological functions. These include, for example, blood pressure regulation, kidney and smooth muscle function, olfaction, and vision. Components of the corresponding signaling pathways have been characterized at the molecular level and the physiological impact of GCs has been investigated by a combination of biochemical, genetic, and physiological studies. For these systems a deeper mechanistic understanding at the structural level has also partially been achieved. Elucidating the structure-function relationships of GCs and their regulatory components will also guide future directions of pharmaceutical and therapeutical inventions, since several diseases correlate with dysfunctions of GC signaling systems. These include, for example, hypertension, different forms of retinal degeneration, and colorectal cancer. Another challenge is to dissect other signaling pathways, in which cGMP has been implicated, but information on a participating GC in these pathways is missing. Examples are other sensory cell systems (e.g., sensing of tastants, heat, and pain) or the Wnt-frizzled signaling pathway in early embryonic development (Wang and Malbon 2004). Finally, some nonmammalian organisms express a larger variety of GC forms than mammalians. It will be a promising task to investigate the properties and the operation principles of these GC forms in an environmental and evolutionary context.

References

Ashman DF, Lipton R, Melicow MM, Price TD. Isolation of adenosine 3′,5′-monophosphate and guanosine3′,5′-monophosphate from rat urine. Biochem Biophys Res Commun. 1963;11:330–4.

Baker DA, Kelly JM. Structure, function and evolution of microbial adenylyl and guanylyl cyclases. Mol Microbiol. 2004;52:1229–42.

Beavo JA, Brunton LL. Cyclic nucleotide research – still expanding after half a century. Nat Rev Mol Cell Biol. 2002;3:710–8.

Garthwaite J. New insight into the functioning of nitric oxide-receptive guanylyl cyclase: physiological and pharmacological implications. Mol Cell Biochem. 2010;334:221–32.

Koch KW, Duda T, Sharma RK. Ca^{2+}-modulated vision-linked ROS-GC guanylate cyclase transduction machinery. Mol Cell Biochem. 2010;334:105–15.

Kots AY, Martin E, Sharina IG, Murad F. A short history of cGMP, guanylyl cyclases, and cGMP-dependent protein kinases. In: Schmidt HHHW et al., editors. cGMP: generators, effectors and therapeutic implications, handbook of experimental pharmacology, vol. 191. Berlin/Heidelberg: Springer; 2009. p. 1–14.

Lin JE, Li P, Pitari GM, Schulz S, Waldman SA. Guanylyl cyclase C in colorectal cancer: susceptibility gene and potential therapeutic target. Future Oncol. 2009;5:509–22.

Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepnik KP, Waldman SA. Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev. 2000;52:375–413.

Martel G, Hamet P, Trembly J. Central role of guanylyl cyclase in natriuretic peptide signaling in hypertension and metabolic syndrome. Mol Cell Biochem. 2010;334:53–65.

Ortiz CO, Etchberger JF, Posy SL, Frokjaer-Jensen C, Lockery S, Honig B, Hobert O. Searching for neuronal left/right asymmetry: genomewide analysis of nematode receptor-type guanylyl cyclases. Genetics. 2006;173:131–49.

Potter LR, Yoder AR, Flora DR, Antos LK, Dickey DM. Natriuretic peptides: their structures, receptors, physiological functions and therapeutic applications. In: Schmidt HHHW et al., editors. cGMP: generators, effectors and therapeutic implications, handbook of experimental pharmacology, vol. 191. Berlin/Heidelberg: Springer; 2009. p. 341–66.

Rätscho N, Scholten A, Koch KW. Diversity of guanylate cyclases in teleost fishes. Mol Cell Biochem. 2010;334:207–14.

Sharma RK. Membrane guanylate cyclase is a beautiful signal transduction machine: overview. Mol Cell Biochem. 2010;334:3–36.

Sharma RK, Duda T. Odorant-linked ROS-GC subfamily membrane guanylate cyclase transduction system. Mol Cell Biochem. 2010;334:181–9.

Tamura N, Chrisman TD, Garbers DL. The regulation and physiological roles of the guanylyl cyclase receptors. Endocrine J. 2001;48:611–34.
Wang HY, Malbon CC. Wnt-frizzled signaling to G-protein-coupled effectors. Cell Mol Life Sci. 2004;61:69–75.
Zufall F, Munger SD. Receptor guanylyl cyclases in mammalian olfactory function. Mol Cell Biochem. 2010;334:191–7.

Guanylyl Cyclase

▶ Guanylate Cyclase

Guanylyl Cyclase C

Nirmalya Basu and Sandhya S. Visweswariah
Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka, India

Synonyms

GUC2C; GUCY2C; hSTAR; STAR

Historical Background

Guanylyl cyclase C (GC-C) is the target for a family of heat-stable enterotoxins (ST) produced by enterotoxigenic *Escherichia coli* (ETEC), *Vibrio cholera* non-01, and *Yersinia enterocolitica*. Stable toxin-mediated diarrheas are observed frequently in infants and contribute significantly to incidences of Travelers' Diarrhea. Early studies demonstrated that the effects of ST were mediated by increases in intracellular cGMP levels in intestinal cells, and the receptor for ST was almost exclusively expressed in the apical microvillar compartment of the intestinal brush-border epithelia (De Jonge 1975; Schulz et al. 1990). Subsequently, the receptor for ST peptides was identified as a member of the membrane-associated guanylyl cyclase family, and binding of ST to GC-C led to activation of the receptor, resulting in the production and accumulation of cGMP in enterocytes (Schulz et al. 1990). Effectors of cGMP in intestinal cells include protein kinase G (PKG), cyclic nucleotide gated ion channels (CNG), and the cystic fibrosis transmembrane conductance regulator (CFTR), which regulate net fluid and ion efflux into the intestinal lumen manifesting as watery diarrhea in humans and farm animals (Basu et al. 2010).

Peptide Ligands

ST peptides are low molecular weight peptides that differ slightly in their amino acid composition, but have a cysteine rich core with a conserved disulfide bonding pattern essential for the heat stability and biological activity of these peptides (Gariepy et al. 1987). Nearly a decade after the isolation of bacterial ST peptides, a peptide that increased cGMP levels in T84 human colon carcinoma cells was purified from the extracts of rat jejunum and named guanylin. This was soon followed by the isolation of a related peptide, uroguanylin. These endogenous ligands as well as ST peptides are synthesized as precursor proteins which have little or no intrinsic biological activity until cleaved by converting enzymes that release the active peptides for interaction with GC-C (Forte 2004). Guanylin and uroguanylin are less potent in stimulating chloride secretion in comparison with ST peptides, since the affinity of guanylin and uroguanylin for GC-C is lower than that of ST. Uroguanylin is effective in stimulating cGMP production in cells at a pH of ~5.5, whereas guanylin has a reduced efficacy at an acidic pH, and is active at an alkaline pH. This is because acidic pH markedly decreases the affinity of guanylin binding to GC-C but enhances that of uroguanylin. The presence of both guanylin and uroguanylin in the gastrointestinal tract allows for effective control of cGMP-mediated regulation of salt and water transport over a broad pH range that is encountered in the extracellular fluid of the intestinal tract (Hamra et al. 1997). Recently a novel peptide agonist has been developed for GC-C (Busby et al. 2010).

Domain Organization of GC-C

The mRNA of GC-C is translated to a polypeptide of 1,073 amino acids, which adopts the multidomain architecture similar to that seen in other receptor guanylyl cyclases. The N-terminal signal sequence of 23 amino acid residues undergoes proteolysis on transit to the endoplasmic reticulum, generating a mature polypeptide of 1,050 amino acids with a theoretical molecular mass of 120 kDa. From the N to the C terminus of GC-C, an extracellular ligand binding domain (ECD)

is followed by a single transmembrane helix, and an intracellular domain consisting of juxtamembrane, kinase-homology (KHD), linker, guanylyl cyclase (GCD), and the C-terminal tail (CTD) domains. The minimal catalytic unit required for the guanylyl cyclase activity of GC-C is a homodimer, although higher order oligomers have been reported and may be the physiologically relevant forms. As with all guanylyl cyclases, the active site is thought to lie at the interface of the two guanylyl cyclase domains of the homodimer. Other than the intestinal tract, GC-C is also expressed at lower levels in various extra-intestinal tissues such as the kidney, seminiferous tubules of the testis, perinatal liver, brain, and epididymis (Basu et al. 2010).

The ECD of GC-C shows low sequence similarity to the ECDs of other receptor guanylyl cyclases, reflecting the differences in the ligands that bind each individual receptor. GC-C binds ST peptides with a high affinity (Kd $\sim$ 0.1 nM) and guanylin (Kd $\sim$ 10 nM) and uroguanylin (Kd $\sim$ 1 nM) with lower affinity. The ECD of GC-C shows N-linked glycosylation resulting in 130 kDa and 145 kDa forms of the receptor. The 145 kDa form contains sialic acid and galactose residues and is present on the plasma membrane, whereas the 130 kDa form contains high mannose structures and is primarily resident in the endoplasmic reticulum. The 130 kDa is the precursor for the 145 kDa form, and though both differentially glycosylated forms bind ST with equal affinity, cGMP production in the presence of ligands is seen only with the 145 kDa form (Ghanekar et al. 2004).

GC-C harbors a domain of approximately 250 residues between the juxtamembrane and the guanylyl cyclase domains, which shares a significant homology to protein tyrosine kinases, and is called the KHD. The crucial position of the KHD in receptor guanylyl cyclases suggests that it transduces the signal of ligand binding from the ECD to the guanylyl cyclase domain. This is supported by the fact that complete and partial deletions of the KHD in GC-C led to inactivation of the receptor. A sequence alignment of the KHD of GC-C with tyrosine and serine-threonine kinases reveals that the highly conserved HRD motif present in active protein kinases is replaced with a HGR motif in the KHD of GC-C. The absence of the catalytic aspartate residue predicts that the KHD of GC-C is a pseudokinase (Rudner et al. 1995).

A $\sim$70 amino acid region connecting the KHD and the guanylyl cyclase domain is referred to as the linker region. A recent bioinformatic analysis has suggested that the linker region in receptor GCs serves as a signaling helix, which could regulate the activities of these receptors, and adopt a coiled-coil structure. Mutational analysis of the linker region has demonstrated its importance in repressing the guanylyl cyclase activity of GC-C in the absence of ligand and permitting ligand-mediated activation of the cyclase domain (Saha et al. 2009).

The primary structure of the guanylyl cyclase domain is highly conserved in both receptor and soluble GCs and is similar to the catalytic domain of adenylyl cyclases. Guanylyl cyclases belong to the Class III nucleotide cyclase family. Homodimeric receptor GCs could have two active sites, and the allostery seen in biochemical assays suggests that there are indeed two substrate binding sites per dimer of enzyme (Parkinson et al. 1994). Recent crystal structures of the guanylyl cyclases Cya2 from cyanobacterium *Synechocystis* PCC6803 and CYG12 from the green algae *Chlamydomonas reinhardtii* reveal the presence of a head-to-tail homodimer with the two monomers in a wreath-like arrangement.

GC-C and the retinal GCs (GC-E and GC-F) possess a short C-terminal domain (CTD) of approximately 60 residues. It has been suggested that the CTD may be involved in interaction of receptor GCs with the cytoskeleton. Deletion of the CTD in GC-C led to a loss of ligand-mediated activation of the receptor. The CTD contains a unique region of 11 highly conserved amino acids, which acts as an apical sorting signal (Hodson et al. 2006).

Regulation

One of the hallmarks of any signaling system is the intricate and diverse modes of regulation that it is subjected to, and GC-C is no exception.

Transcriptional regulation. GC-C mRNA is encoded by the gene GUCY2C present at the locus 12p12 in *Homo sapiens* and contains 27 exons. The regulatory region lies $\sim$ 2 kb upstream of the gene and has binding sites for caudal type homeobox gene-2 (Cdx2), hepatocyte nuclear factor 4 (HNF4), GATA-4, glucocorticoid receptor, and nuclear factor-IL6 (NF-IL6). HNF4, Cdx2, and GATA-4 are expressed in the intestine, and Cdx2 and HNF4 are responsible for the predominant expression of GC-C in the intestine as well as in cell-lines derived from colorectal carcinoma tissue such as Caco2 and T84. Protein

kinase C (PKC) has been shown to transcriptionally regulate expression of GC-C. Activation of PKC by phorbol esters such as phorbol 12-myristate 13-acetate (PMA) led to a down regulation of GC-C mRNA levels in T84 cells (Roy et al. 2001).

Desensitization. Refractoriness to prolonged exposure of cells to ligands of GC-C, in terms of cGMP production, has been observed. In Caco2 cells, selective removal of the 145 kDa form from the surface of the receptor is seen on extended treatment with ST (Ghanekar et al. 2003). Increased activity of the cGMP-binding, cGMP-specific phosphodiesterase, PDE5, was found to contribute to cellular refractoriness in T84 cells, reducing the levels of cGMP in the cell (Bakre et al. 2000). Exposure of HEK293 cells stably expressing GC-C to ST for prolonged periods did not result in desensitization, indicating that cell-specific mechanisms contribute to cellular refractoriness.

Inactivation of GC-C occurs in vitro on ST incubation prior to addition of MgGTP as the substrate for the guanylyl cyclase assays. Inactivation of GC-C was alleviated in the presence of ATP, indicating a role for ATP (and perhaps the KHD) in regulation of GC-C activity (Gazzano et al. 1991).

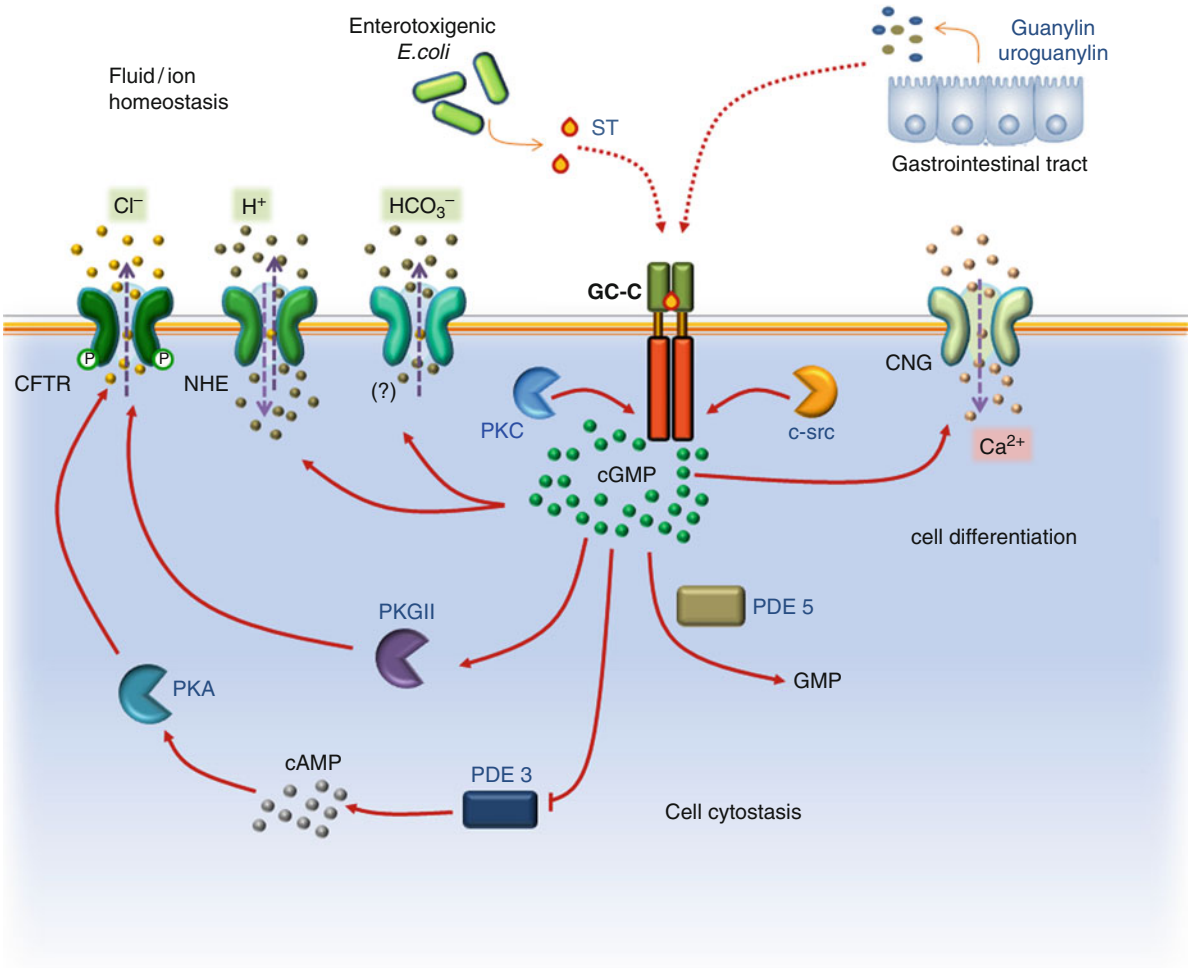
Phosphorylation. PKC phosphorylates GC-C on Ser1029 in the CTD of GC-C and enhances ST-mediated GC-C activation in terms of increased cGMP production (Wada et al. 1996). This is in contrast to the role of PKC in transcriptional down-regulation of GC-C. Phosphorylation at tyrosine residues has not been reported in any receptor GC except GC-C. The GC-C intracellular domain was tyrosine phosphorylated when expressed in *E. coli* cells harboring the tyrosine kinase Elk (EphB1). Recent data shows that GC-C is a substrate for inhibitory phosphorylation by c-src, resulting in reduced ligand-mediated cGMP production. The tyrosine at position 820 gets phosphorylated, and this generates a docking site for the c-src SH2 domain leading to interaction and further activation of ▶ c-src. Therefore, GC-C could be involved in cross-talk with tyrosine kinases especially in colon carcinoma cells where c-src kinase expression and activity is high (Basu et al. 2009).

Allosteric regulation. ATP is able to potentiate ligand-mediated activation of GC-C and consequent cGMP production in vitro, in the presence of Mg-GTP as substrate. In contrast, ATP inhibits the cyclase activity of GC-C when Mn-GTP is used as substrate. ATP mediates its effects by binding to the KHD of GC-C and ATP hydrolysis is not required for inhibition of the guanylyl cyclase activity. Lysine 516 in the KHD of GC-C corresponds to the conserved lysine in the VAIK motif present in tyrosine kinases that is involved in coordinating the α- and β-phosphates of ATP. Mutation of Lys516 to an Alanine abolished ATP binding and the subsequent regulation of GC-C activity by the adenine nucleotide (Jaleel et al. 2006). The KHD is also the site of action for some of the 2-substituted adenine nucleotides which were found to be potent allosteric inhibitors of GC-C (Parkinson et al. 1997). Tyrphostins, known to be inhibitors of tyrosine kinases, were found to noncompetitively inhibit the activity of GC-C, by binding to the catalytic domain of GC-C, and indeed, nucleotide cyclases in general (Jaleel et al. 2004). Till date, there are no specific inhibitors of any receptor guanylyl cyclase, but it is conceivable that inhibitors directed to the individual KHDs could be more specific, since this domain is unique to receptor guanylyl cyclases and is not found in soluble guanylyl cyclases.

Downstream Signaling and Disease Phenotypes

Cyclic GMP produced by GC-C executes its cellular functions by interacting with three types of target proteins: (a) cGMP-dependent protein kinases (PKG), (b) cyclic nucleotide-gated (CNG) channels, and (c) cGMP-regulated cyclic nucleotide phosphodiesterases (Fig. 1). The endogenous peptides, guanylin, and uroguanylin are involved in the regulation of salt and water transport across the intestinal epithelia. ST peptides serve as superagonists of the receptor resulting in ST-induced watery diarrhea. GC-C knockout (KO) mice are refractory to the actions of ST that are seen in WT mice (Fig. 1).

An increase in cGMP levels can regulate ion and fluid transport in the intestine in different ways. Sodium absorption in the intestine is partly governed by the Na^+/H^+ exchanger (NHE), which absorbs NaCl in combination with the chloride/anion exchanger. Cyclic GMP is known to inhibit the NHE, thereby decreasing sodium and chloride absorption. Intestinal chloride efflux is mediated by the cystic fibrosis transmembrane conductance regulator (CFTR), which is located in the apical membrane of Cl^- secreting epithelial cells. Increase in levels of intracellular cGMP results in the activation of cGMP-dependent protein



Guanylyl Cyclase C, Fig. 1 Overview of GC-C signaling: GC-C expressed on the surface of intestinal cells is the receptor for the endogenous ligands uroguanylin and guanylin, or ST produced by enterotoxigenic *E. coli*. Ligand-mediated activation of GC-C results in the production and accumulation of intracellular cGMP. Guanylyl cyclase activity of GC-C can be potentiated by phosphorylation by protein kinase C (PKC) and inhibited by phosphorylation by c-src. Cyclic GMP activates the sodium/hydrogen exchanger (NHE) causing hydrogen ion (H^+) efflux. Bicarbonate ion (HCO_3^-) is also secreted by unidentified channels activated by cGMP. Cyclic GMP–dependent Protein Kinase (PKGII) is activated by elevated levels of cGMP. Cyclic GMP inhibits the activity of the cAMP phosphodiesterase PDE3, thereby increasing levels of cAMP in the cell which in turn activates cAMP-dependent protein kinase (PKA). PKGII and PKA phosphorylate the cystic fibrosis transmembrane conductance regulator (CFTR) leading to chloride ion (Cl^-) efflux. These processes maintain fluid-ion homeostasis in the intestine. Cyclic GMP also directly activates cyclic nucleotide gated channels (CNG) leading to Ca^{2+} influx. Signaling downstream of Ca^{2+} leads to cell differentiation and migration. GC-C signaling is terminated by hydrolysis of cGMP to 5′ GMP by the cGMP binding, cGMP-specific phosphodiesterase, PDE5

kinase II and protein kinase A, which phosphorylate CFTR thus activating the chloride channel. The reduced chloride absorption by the NHE and increased chloride secretion by the CFTR in response to cGMP are thought to be the chief mechanisms by which ST mediates its effects (Basu et al. 2010).

The intestinal epithelium undergoes homeostatic cycles of proliferation, migration, differentiation, and apoptosis. Imbalance between cell proliferation and apoptosis leads to the formation of tumors within the intestinal tract. In colon cancer, the expression of mRNAs encoding uroguanylin and guanylin is

markedly suppressed, whereas mRNA expression of GC-C is comparable in colon cancer and normal colonic mucosa. Oral administration of uroguanylin suppressed the formation and apparent progression of polyps in the $APC^{Min/+}$ mouse model of colorectal cancer. Recent reports have suggested that ST or uroguanylin treatment of T84 and Caco2 cells inhibits proliferation by delaying progression through the cell cycle. The cytostatic effects of GC-C agonists could be mimicked by 8-Br-cGMP, a cell-permeable cGMP analog, but could not be prevented by inhibitors of the known downstream effectors of elevated cGMP, such as PKG, PKA or PDE3. However, L-*cis*-diltiazem, a CNG channel inhibitor, as well as removal of extracellular Ca^{2+}, prevented ST-mediated inhibition of proliferation. This suggested that the anti-proliferative action of GC-C agonists was mediated by a cGMP signaling mechanism, which regulates Ca^{2+} influx through CNG channels. Thus, Ca^{2+} serves as the third messenger in the signaling cascade linking GC-C at the cell surface to regulation of proliferation in the nucleus.

Over a million people suffer from colon cancer in developed countries, but its incidence is relatively low in underdeveloped and developing countries. A common epidemiological characteristic of these developing nations is the prevalence of ETEC. Periodic infections with ST producing bacteria in the intestine could be therapeutic for people living in the developing nations, since the action of the ST peptides induces cell cytostasis, thus providing resistance to intestinal neoplasia (Pitari et al. 2007).

Summary

The GC-C/cGMP signaling axis is important in maintaining fluid-ion homeostasis in the intestinal tract. In addition, evidence for its anti-neoplastic role in the development of colon carcinoma is rapidly accumulating. It would be interesting in the future to look at the molecular players responsible for the cytostatic effect exerted by the ligands for GC-C. The function of GC-C expressed in extra-intestinal tissue is largely unknown but some of the key components may be common to that operative in the intestine. To elucidate these mechanisms and to evaluate their importance for the pathophysiology of an organism are important topics for future research.

References

Bakre MM, Sopory S, Visweswariah SS. Expression and regulation of the cGMP-binding, cGMP-specific phosphodiesterase (PDE5) in human colonic epithelial cells: role in the induction of cellular refractoriness to the heat-stable enterotoxin peptide. J Cell Biochem. 2000;77:159–67.

Basu N, Bhandari R, Natarajan VT, et al. Cross talk between receptor guanylyl cyclase C and c-src tyrosine kinase regulates colon cancer cell cytostasis. Mol Cell Biol. 2009; 29:5277–89.

Basu N, Arshad N, Visweswariah SS. Receptor guanylyl cyclase C (GC-C): regulation and signal transduction. Mol Cell Biochem. 2010;334:67–80.

Busby RW, Bryant AP, Bartolini WP, et al. Linaclotide, through activation of guanylate cyclase C, acts locally in the gastrointestinal tract to elicit enhanced intestinal secretion and transit. Eur J Pharmacol. 2010;649:328–35.

De Jonge HR. The localization of guanylate cyclase in rat small intestinal epithelium. FEBS Lett. 1975;53:237–42.

Forte Jr LR. Uroguanylin and guanylin peptides: pharmacology and experimental therapeutics. Pharmacol Ther. 2004;104:137–62.

Gariepy J, Judd AK, Schoolnik GK. Importance of disulfide bridges in the structure and activity of *Escherichia coli* enterotoxin ST1b. Proc Natl Acad Sci USA. 1987;84:8907–11.

Gazzano H, Wu HI, Waldman SA. Activation of particulate guanylate cyclase by *Escherichia coli* heat-stable enterotoxin is regulated by adenine nucleotides. Infect Immun. 1991; 59:1552–7.

Ghanekar Y, Chandrashaker A, Visweswariah SS. Cellular refractoriness to the heat-stable enterotoxin peptide is associated with alterations in levels of the differentially glycosylated forms of guanylyl cyclase C. Eur J Biochem. 2003;270:3848–57.

Ghanekar Y, Chandrashaker A, Tatu U, et al. Glycosylation of the receptor guanylate cyclase C: role in ligand binding and catalytic activity. Biochem J. 2004;379:653–63.

Hamra FK, Eber SL, Chin DT, et al. Regulation of intestinal uroguanylin/guanylin receptor-mediated responses by mucosal acidity. Proc Natl Acad Sci USA. 1997;94:2705–10.

Hodson CA, Ambrogi IG, Scott RO, et al. Polarized apical sorting of guanylyl cyclase C is specified by a cytosolic signal. Traffic. 2006;7:456–64.

Jaleel M, Shenoy AR, Visweswariah SS. Tyrphostins are inhibitors of guanylyl and adenylyl cyclases. Biochemistry. 2004; 43:8247–55.

Jaleel M, Saha S, Shenoy AR, et al. The kinase homology domain of receptor guanylyl cyclase C: ATP binding and identification of an adenine nucleotide sensitive site. Biochemistry. 2006;45:1888–98.

Parkinson SJ, Carrithers SL, Waldman SA. Opposing adenine nucleotide-dependent pathways regulate guanylyl cyclase C in rat intestine. J Biol Chem. 1994;269:22683–90.

Parkinson SJ, Alekseev AE, Gomez LA, et al. Interruption of Escherichia coli heat-stable enterotoxin-induced guanylyl cyclase signaling and associated chloride current in human intestinal cells by 2-chloroadenosine. J Biol Chem. 1997;272:754–8.

Pitari GM, Li P, Lin JE, et al. The paracrine hormone hypothesis of colorectal cancer. Clin Pharmacol Ther. 2007;82:441–7.

Roy N, Guruprasad MR, Kondaiah P, et al. Protein kinase C regulates transcription of the human guanylate cyclase C gene. Eur J Biochem. 2001;268:2160–71.
Rudner XL, Mandal KK, de Sauvage FJ, et al. Regulation of cell signaling by the cytoplasmic domains of the heat-stable enterotoxin receptor: identification of autoinhibitory and activating motifs. Proc Natl Acad Sci USA. 1995;92:5169–73.
Saha S, Biswas KH, Kondapalli C, et al. The linker region in receptor guanylyl cyclases is a key regulatory module: mutational analysis of guanylyl cyclase C. J Biol Chem. 2009;284:27135–45.
Schulz S, Green CK, Yuen PS, et al. Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell. 1990;63:941–8.
Wada A, Hasegawa M, Matsumoto K, et al. The significance of Ser 1029 of the heat-stable enterotoxin receptor (STaR): relation of STa-mediated guanylyl cyclase activation and signaling by phorbol myristate acetate. FEBS Lett. 1996; 384:75–7.

Guanylyl Cyclase Receptors

Sandhya S. Visweswariah and Najla Arshad
Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka, India

Synonyms

GC-A; GC-B; GC-C; GC-D; GC-G; ret-GC; sGC

Historical Background

In 1971 when Sutherland received the Nobel Prize for the discovery of the second messenger cAMP, the functions of cGMP were still a mystery, even though cGMP had been isolated from rat urine in 1963, and an enzyme that cleaved the phosphodiester bond was discovered the following year. The levels of cGMP are regulated by synthesis by guanylyl cyclases, extrusion from the cell, and/or degradation by phosphodiesterases (Kots et al. 2009). Two classes of guanylyl cyclases, one that is cytosolic (soluble) and the other that is found associated with the particulate or membrane fraction of cells could be identified. The two forms of guanylyl cyclases are composed of polypeptides of different sizes, with different biochemical properties (Kimura and Murad 1974) (Receptor membrane cyclases Fig. 1).

Soluble Guanylyl Cyclases

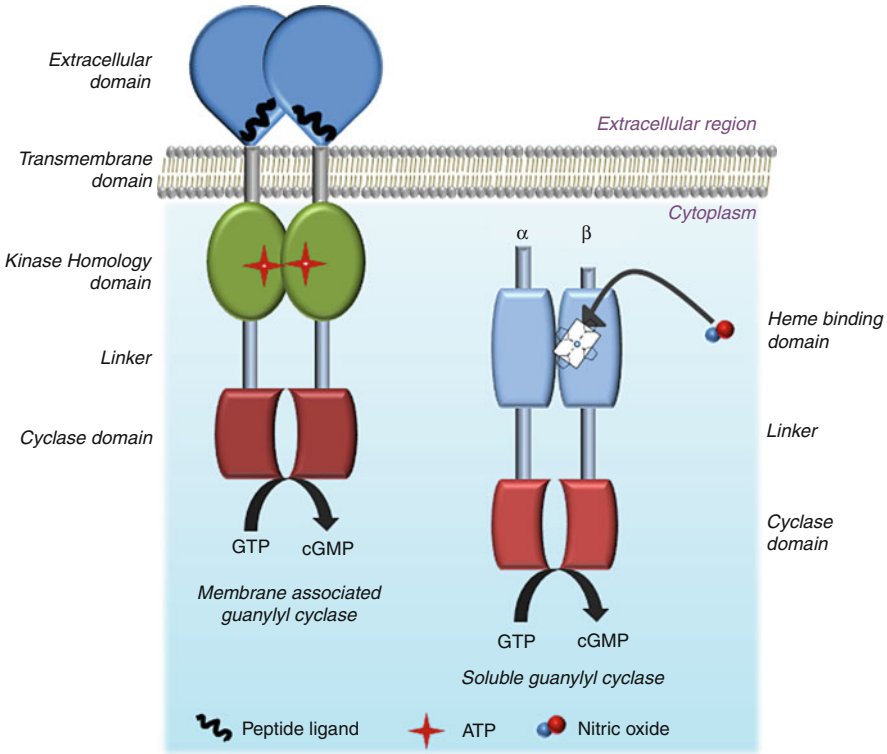
The soluble guanylyl cyclases (sGC) are heterodimers of two dissimilar subunits, alpha and beta. There are four types of alpha subunits, namely, α1, α2, α3, and $\alpha2_i$, and three types of beta subunits, namely, β1, β2, and β3. All the heterodimers are active, albeit to varying extents, except those containing $\alpha2_i$. The $\alpha2_i$ is a splice variant of α2 and provides a mechanism for regulating the activity of the α2/β1 form of sGC by acting as a dominant negative protein in cells. The crystal structure of the sGC from the green algae *Chlamydomonas reinhardtii* has been solved (Winger et al. 2008) and the structure of a human sGC has been deposited (pdb code: 2WZ1).There is also a report on the crystal structure of Cya2, a prokaryotic guanylyl cyclase from cyanobacterium *Synechocystis* PCC6803 (Rauch et al. 2008).

The sGCs are ubiquitously expressed and are responsible for smooth muscle relaxation, neurotransmission, and inhibition of platelet migration (Kots et al. 2009). Nitric oxide (NO) binds to the N-terminal heme-binding H-NOX domain. In the absence of NO, the iron atom of the heme moiety bonds with a histidine residue present in the protein, keeping the cyclase domain inactivate. NO binding to the heme moiety leads to the disruption of this interaction, altering the conformation of the enzyme, thereby resulting in its activation. Protoporphyrin IX can bind to and activate sGCs independent of heme or NO (Lucas et al. 2000). Carbon monoxide also regulates sGCs in a manner similar to that of NO, although its affinity for the enzyme is much lower than that of NO. ATP allosterically inhibits the activity of the soluble guanylyl cyclase (Derbyshire et al. 2009).

The C-terminal cyclase domains in each subunit are connected to the N-terminus by a linker region which contributes to the dimerization of sGC. Since the α-subunits of sGCs lack the critical residues required for catalytic activity, they heterodimerize with the β-subunits to give rise to a functional active site (Lucas et al. 2000; Perkins 2006).

Membrane-Associated Guanylyl Cyclases

Seven classes of membrane-associated guanylyl cyclases have been characterized. They are GC-A,



Guanylyl Cyclase Receptors, Fig. 1 Schematic of the domain organization of receptor guanylyl cyclases. Shown also is the topology of the enzymes discussed in this article either being present in the membrane of the cell or in the cytosol. Membrane-associated GCs are shown as dimers, though there is evidence that they can oligomerize in cells. Receptor GCs can exist as dimers even in the absence of their respective ligands

GC-B, ▶ GC-C, GC-D, and GC-G, and the retinal GCs, GC-E, and GC-F. All the receptor guanylyl cyclases share a common domain organization with a single-pass transmembrane region. They have an extra cellular domain (ECD), the amino acid sequence of which is poorly conserved across the different classes, thereby imparting ligand specificity. This domain is followed by a transmembrane domain and an intracellular domain (ICD). The ICD is comprised of a juxtamembrane domain followed by a regulatory kinase homology domain (KHD) which binds ATP. Following the KHD is a linker region which connects to the guanylyl cyclase domain. Ligand binding to the extracellular domains results in receptor activation and increased production of cGMP in the cell.

The Natriuretic Peptide Family Receptors

These comprise three receptors, namely, Natriuretic Peptide Receptors A, B, and C. The ligands for these receptors are natriuretic peptides – atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP).

Guanylyl Cyclase A/Natriuretic Peptide Receptor A (GC-A/NPR-A)

The mammalian cDNA encoding guanylyl cyclase A (NPR-A) was identified using the gene sequence of the sea urchin particulate guanylyl cyclase receptor as a probe to screen a cDNA library (Kots et al. 2009). NPR-A preferentially binds ANP and BNP. NPR-A is found in the heart, kidney, adrenal cortex, and vasculature.

Vasodilation and natriuresis are the most commonly studied aspects of signaling via NPR-A. GC-A prevents hypertrophy of the heart by relaxing the vascular musculature, thereby reducing the cardiac output. In kidneys, NPR-A assists in the secretion of sodium and reduction of aldosterone secretion by the adrenal cortex, thus reducing blood pressure (Martel et al. 2010).

Transcription of NPR-A is regulated by Sp1. NPR-A has been shown to downregulate its own expression by a feedback inhibition loop via a cGMP response element. Alternative splice variants of NPR-A can heterodimerize (Martel et al. 2010). The

juxtamembrane domain in NPR-A has been shown to modulate the proteolytic cleavage of the ECD, thereby regulating its activity (Huo et al. 1999). Posttranslational modifications which include glycosylation are implicated in the folding of NPR-A and its trafficking to the surface of the cells. When activated by its ligands, NPR-A is deposphorylated at serine and threonine residues in its KHD, which then attenuates the activation by the ligand, leading to desensitization and signal downregulation (Koller et al. 1993). Imbalance in circulating levels of natriuretic peptides and expression of NPR-A receptor is seen in hypertensive mice, making this receptor a potential target for gene therapy (Martel et al. 2010).

Guanylyl Cyclase B/Natriuretic Peptide Receptor B (GC-B/NPR-B)

Using the sequence of NPR-A, NPR-B was cloned from rat brain cDNA and human placenta. NPR-B is primarily located in the pituitary gland, adrenal gland, ovary, endothelium, vascular smooth muscle, and kidney (Schulz 2005). NPR-B binds CNP with highest affinity amongst the natriuretic peptides. Apart from the relaxation of the heart and lowering of blood pressure, NPR-B signaling also relaxes the smooth muscle of the oviduct, colon, and trachea. In the brain, CNP and NPR-B play a role in gonadotropin secretion. Expression of NPR-B in the ovary and testis may be responsible for development of mature ovarian follicles and regulation of sperm transport by relaxing the seminiferous tubules. NPR-B also promotes bone development and causes skeletal overgrowth on overexpression (Schulz 2005).

No cGMP response element has been identified in the NPR-B promoter (Martel et al. 2010). In NPR-B, glycosylation may play a role in ligand binding (Schulz 2005). The lack of an intact KHD in NPR-B causes stunted skeletal growth in humans, and splice variants of NPR-B lacking the KHD or intracellular domains are inactive and can act as dominant negatives, hindering bone development (Hachiya et al. 2007).

Only one other protein has been identified which binds all the natriuretic peptides, the NPR-C receptor. This receptor has no guanylyl cyclase activity as it lacks the intracellular domain. NPR-C is expressed in the heart, lung, vascular smooth muscle cells, kidney glomeruli, adrenal gland, brain cortex, and pituitary gland (Martel et al. 2010).

The primary function of the NPR-C receptor was thought to be sequestration and metabolic clearance of natriuretic peptides and it was therefore designated as the clearance receptor. The 37 amino acid long cytoplasmic tail of NPR-C has been shown to mediate inhibition of adenylyl cyclase activity on ligand binding to the receptor (Anand-Srivastava 2005).

Guanylyl Cyclase C (GC-C)

Guanylyl cyclase C (GC-C) is the third member of the family of receptor guanylyl cyclases that was identified. It was identified as the receptor for the bacterial heat-stable enterotoxin peptides produced by pathogenic strains of *Escherichia coli* (Schulz et al. 1990). GC-C is predominantly expressed in the intestinal tract.

Endogenous ligands for GC-C are guanylin and uroguanylin and have been purified from intestinal mucosa and urine. They are cysteine-rich peptides, similar to the stable toxin peptide, but, they have a 10–100-fold lower affinity for GC-C. It is believed that they regulate intestinal fluid and ion transport. Activation of GC-C, following binding of its ligands, results in increased synthesis cGMP. Accumulation of intracellular cGMP activates cyclic nucleotide-dependent protein kinases, PKA and PKG, leading to the phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) and increased chloride secretion through CFTR. Ion efflux leads to increased fluid secretion from the cell, resulting in a watery diarrhea. This manifests as traveler's diarrhea in adults which is self-limiting but may be fatal in infants (Basu et al. 2010).

GC-C prexists as an oligomer in the absence of ligand. GC-C is differentially glycosylated, but glycosylation may not be an essential requirement for ligand binding. GC-C is a substrate for inhibitory phosphorylation by c-src, resulting in reduced ligand-mediated cGMP production. Phosphorylation of a serine residue in the C-terminus by protein kinase C results in potentiation of ligand-stimulated activity in vivo and in vitro. Prolonged exposure of GC-C to its ligands leads to cell-specific desensitization of the receptor, by mechanisms that are not clearly understood at present. Intestine and kidney-enriched PDZ protein (IKEPP) and c-src are the only proteins to be identified that

interact with GC-C and regulate its activity (Basu et al. 2010). Ligands for GC-C have been shown to have a cytostatic effect on intestinal cells and thereby act as a barrier to tumorogenesis. A GC-C knock-out mouse has no obvious phenotype, but is resistant to stable toxin-mediated diarrhea. This raises the question of the evolutionary pressure that maintains a functional GC-C in a variety of species.

Guanylyl Cyclase D (GC-D)

The canonical olfactory signaling occurs when the cyclic nucleotide gated (CNG) ion channel are activated by cAMP in the olfactory neurons. When it was found that odorants triggered cGMP production which activated a cGMP gated ion channel, the olfactory guanylyl cyclase GC-D was identified. This gene is found to be well conserved in the rodents and canines, but is a pseudogene in a number of primate species, including the human (GC-E). A very small subset of the olfactory sensory neurons (OSN) of mice expresses GC-D, and these neurons terminate in necklace glomeruli (Zufall and Munger 2010). Non-primates detect more olfactory cues than primates, and may even detect pheromones via GC-D (Young et al. 2007). Recent reports suggest that uroguanylin and/or guanylin can activate GC-D.

GC-D can also be activated by bicarbonate ions which are produced by carbonic anhydrase specifically expressed in the GC-D subset of OSNs. The seemingly unrelated pathways appear to impinge at this point and reports have shown that rodents can be trained to avoid CO_2 concentrations as low at 0.5%. It is speculated that this is a means to detect other animals in the vicinity. There is also evidence that guanylyl cyclase activating protein (GCAP1) could also modulate a calcium-dependent activation of GC-D (Zufall and Munger 2010).

Retinal Guanylyl Cyclases: Guanylyl Cyclase E/F (GC-E/F)

In the mid-1990s, membrane-bound receptor guanylyl cyclases were identified in bovine photoreceptors. A gene encoding a particulate guanylyl cyclase was isolated from retinal cDNA based on its similarity to previously characterized GCs (Shyjan et al. 1992). This gene is referred to as retGC1 or retGC2 in humans or GC-E/GC-F in other mammals. RetGC2 is exclusively expressed in the retina while expression of retGC1 is seen in the pineal gland and retina. The mRNA of retGC1 has been detected in the olfactory bulb of fish, cochlear nerve and organ of Corti (Hunt et al. 2010).

GC-E and GC-F are important in retinal phototransduction and regulate the opening and closure of cGMP-gated ion channels. These channels are open in the dark and close on perception of light. The loss of either retGC results in compromised visual signal transduction suggesting that both retGCs are necessary for normal phototransduction.

The retGCs are orphan receptors as no ligands for the ECD have been discovered till date. These receptors are regulated by proteins which associate with their ICDs. Guanylate cyclase activating proteins (GCAPs) are always found associated with the retGCs. In resting depolarized cells, GCAPs are bound by Ca^{2+} ions which prevent activation of retGC. Upon light stimulus, the cGMP-specific phosphodiesterase, PDE6, found in photoreceptor cells is activated and degrades cGMP present in the cell, leading to closure of the CNG, thereby decreasing Ca^{2+} levels in the now hyperpolarized cell. The Ca^{2+} ions dissociate from GCAP, permitting them to activate retGC. Cyclic GMP levels are now elevated resulting in the reopening of the ion channels and reversion of the cell to a depolarized state. Despite the importance of both retGCs for visual signal transduction in mice, visual impairments studied so far in humans map only to the retGC1 locus. Recessive mutations in retGC1 are a major cause of Leber congenital amaurosis (LCA). Retinitis pigmentosa also is caused by a mutation in GC1. Other mutations in retGC1 are responsible for 35% of all rod-cone dystrophies (Hunt et al. 2010).

Guanylyl Cyclase G (GC-G)

The GC-G is an orphan receptor and transcripts have been found in mouse testis, kidney, and in the Grueneberg ganglion (Lin et al. 2008). This receptor may play a role in thermo-sensation, a process which has been shown to be via cGMP signaling in *C. elegans* (Zufall and Munger 2010).

Summary

Almost 50 years after the discovery of cGMP, its function in vision, olfaction, vasodilatation, and fluid ion homeostasis has been established. The various guanylyl cyclases have been identified and characterized but

questions still remain as to the ligands and exact functions of some of the receptors. The complex domain organization of these receptor guanylyl cyclases indicate that they may be regulated in multiple ways and be involved in cross talk with other signaling pathways, and studies along these lines will be of interest in future.

References

Anand-Srivastava MB. Natriuretic peptide receptor-C signaling and regulation. Peptides. 2005;26:1044–59.

Basu N, Arshad N, Visweswariah SS. Receptor guanylyl cyclase C (GC-C): regulation and signal transduction. Mol Cell Biochem. 2010;334:67–80.

Derbyshire ER, Fernhoff NB, Deng S, et al. Nucleotide regulation of soluble guanylate cyclase substrate specificity. Biochemistry. 2009;48:7519–24.

Hachiya R, Ohashi Y, Kamei Y, et al. Intact kinase homology domain of natriuretic peptide receptor-B is essential for skeletal development. J Clin Endocrinol Metab. 2007;92:4009–14.

Hunt DM, Buch P, Michaelides M. Guanylate cyclases and associated activator proteins in retinal disease. Mol Cell Biochem. 2010;334:157–68.

Huo X, Abe T, Misono KS. Ligand binding-dependent limited proteolysis of the atrial natriuretic peptide receptor: juxtamembrane hinge structure essential for transmembrane signal transduction. Biochemistry. 1999;38:16941–51.

Kimura H, Murad F. Evidence for two different forms of guanylate cyclase in rat heart. J Biol Chem. 1974;249:6910–6.

Koller KJ, Lipari MT, Goeddel DV. Proper glycosylation and phosphorylation of the type A natriuretic peptide receptor are required for hormone-stimulated guanylyl cyclase activity. J Biol Chem. 1993;268:5997–6003.

Kots AY, Martin E, Sharina IG, et al. A short history of cGMP, guanylyl cyclases, and cGMP-dependent protein kinases. Handb Exp Pharmacol. 2009;19:1–14.

Lin H, Cheng CF, Hou HH, et al. Disruption of guanylyl cyclase-G protects against acute renal injury. J Am Soc Nephrol. 2008;19:339–48.

Lucas KA, Pitari GM, Kazerounian S, et al. Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev. 2000;52:375–414.

Martel G, Hamet P, Tremblay J. Central role of guanylyl cyclase in natriuretic peptide signaling in hypertension and metabolic syndrome. Mol Cell Biochem. 2010;334:53–65.

Perkins WJ. Regulation of soluble guanylyl cyclase: looking beyond NO. Am J Physiol Lung Cell Mol Physiol. 2006;291:L334–6.

Rauch A, Leipelt M, Russwurm M, et al. Crystal structure of the guanylyl cyclase Cya2. Proc Natl Acad Sci USA. 2008;105:15720–5.

Schulz S. C-type natriuretic peptide and guanylyl cyclase B receptor. Peptides. 2005;26:1024–34.

Schulz S, Green CK, Yuen PS, et al. Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell. 1990;63:941–8.

Shyjan AW, de Sauvage FJ, Gillett NA, et al. Molecular cloning of a retina-specific membrane guanylyl cyclase. Neuron. 1992;9:727–37.

Winger JA, Derbyshire ER, Lamers MH, et al. The crystal structure of the catalytic domain of a eukaryotic guanylate cyclase. BMC Struct Biol. 2008;8:42.

Young JM, Waters H, Dong C, et al. Degeneration of the olfactory guanylyl cyclase D gene during primate evolution. PLoS ONE. 2007;2:e884.

Zufall F, Munger SD. Receptor guanylyl cyclases in mammalian olfactory function. Mol Cell Biochem. 2010;334:191–7.

Guanylyl Cyclase–Activating Protein

▶ GCAP (Guanylate Cyclase–Activating Protein)

GUC2C

▶ Guanylyl Cyclase C

GUCY2C

▶ Guanylyl Cyclase C

Gz Alpha Subunit

▶ G Protein α i/o/z

Gα GAPs

▶ RGS Protein Family

Gα12

▶ G Protein Alpha 12

Gαt

▶ G Protein Alpha Transducin

H

H411

▶ Fibulins

HACS1

▶ SAMSN1 (SAM Domain, SH3 Domain, and Nuclear Localization Signal)

Ha-Ras

▶ RAS (H-, K-, N-RAS)

HBEGF

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

HB-EGF (Heparin-Binding EGF-Like Growth Factor)

Ryo Iwamoto and Eisuke Mekada
Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

Synonyms

Diphtheria toxin receptor; DTR; DTS; DTSF; HBEGF; HEGFL; Heparin-binding epidermal growth factor-like growth factor

Historical Background

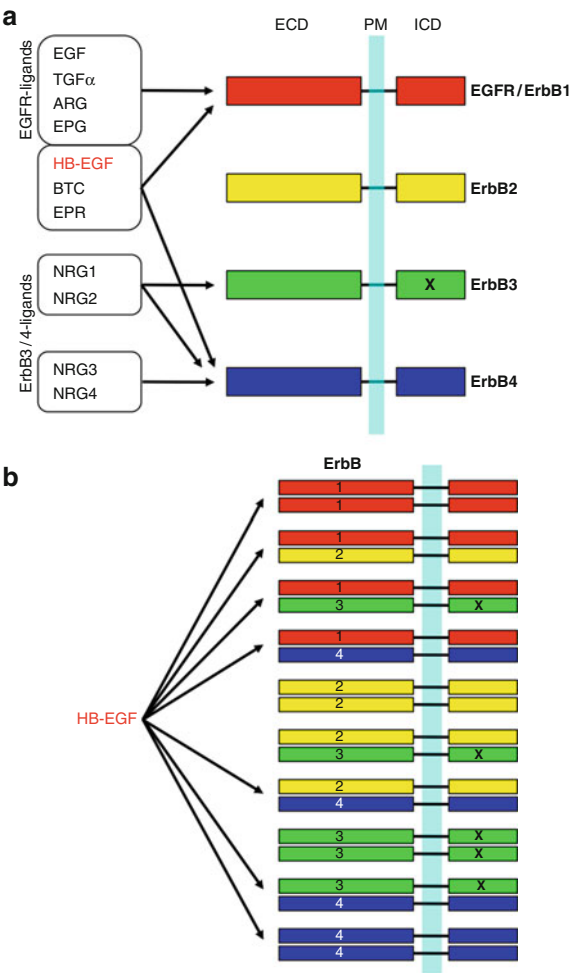
Since the discovery of epidermal growth factor (EGF) in 1962, a total of seven mammalian ligands that bind the EGF receptor (EGFR/ErbB1) have been identified, including transforming growth factor-α (TGFα), amphiregulin (ARG), betacellulin (BTC), epiregulin (ERG), epigen (EPG), and heparin-binding EGF-like growth factor (HB-EGF) (Harris et al. 2003). Historically, HB-EGF was the fourth growth factor to be identified among EGFR ligands. Major findings regarding HB-EGF are:

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

- Discovery of HB-EGF (Higashiyama et al. 1991)
- Identification of HB-EGF as the diphtheria toxin receptor (DTR) (Naglich et al. 1992; Iwamoto et al. 1994)
- Identification of HB-EGF's major role in EGFR transactivation (Prenzel et al. 1999)
- Establishment of HB-EGF-null mouse lines (Iwamoto et al. 2003; Jackson et al. 2003)
- Proof of the physiological significance of HB-EGF shedding control in vivo (Yamazaki et al. 2003)
- Discovery of the nuclear translocation of HB-EGF-CTF (Nanba et al. 2003)
- Identification of HB-EGF as a promising target for cancer therapy (Miyamoto et al. 2004)
- Proof of the physiological significance of the interaction between HB-EGF and HSPGs in vivo (Iwamoto et al. 2010)

HB-EGF and ErbB Family Receptor Tyrosine Kinases

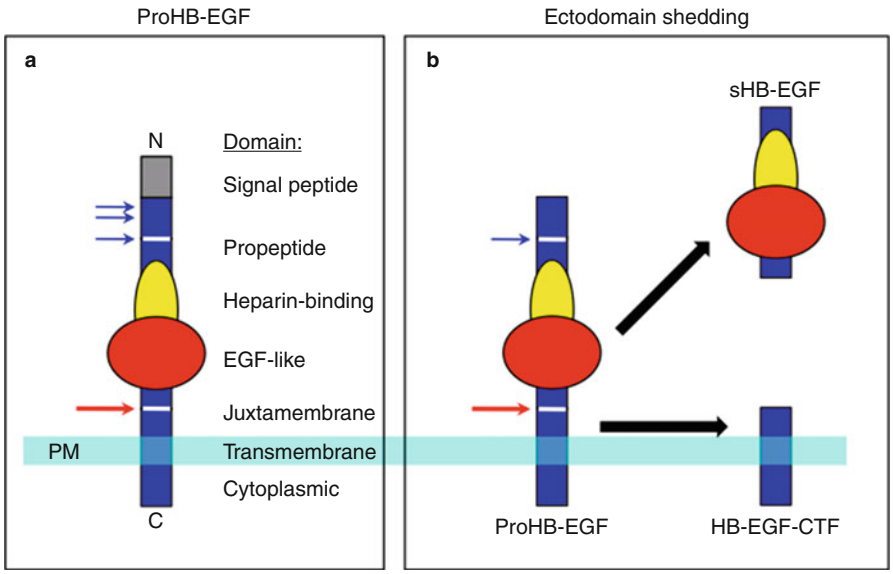
HB-EGF is a heparin-binding member of the EGFR ligands that was initially identified in the conditioned medium of human macrophage-like cells (Higashiyama et al. 1991). HB-EGF directly binds to and activates EGFR and ErbB4. The ErbB family of receptor tyrosine kinases (RTKs) consists of four receptors: EGFR/ErbB1, ErbB2, ErbB3, and ErbB4. The EGF family of growth factors binds to and activates ErbB RTKs, resulting in the formation of homo- and heterodimers, autophosphorylation of specific tyrosine residues within their cytoplasmic domains, and subsequent intracellular signaling. In vertebrates, EGF family members vary in their ability to activate distinct ErbB homo- and heterodimers, which may partly account for the differences in their bioactivities. In the case of HB-EGF, although it binds to and activates EGFR and ErbB4 directly, it can also activate ErbB2 and ErbB3 indirectly by heterodimerization (Harris et al. 2003; Mekada and Iwamoto 2008) (Fig. 1).



HB-EGF (Heparin-Binding EGF-Like Growth Factor), Fig. 1 *Binding specificity of members of the EGF ligand family to members of the ErbB receptor family*. (**a**) EGF family ligands are separated into four categories by their specificity of binding to members of the ErbB receptor family. ErbB2 has no ligand. ErbB3 is deficient in kinase activity (X). NRG, neuregulin; ECD, extracellular domain; ICD, intracellular domain; PM, plasma membrane. (**b**) ErbB homo- and heterodimier combinations activated by HB-EGF

Ectodomain Shedding

Like other EGF family members, HB-EGF is synthesized as a type I transmembrane protein (proHB-EGF), composed of a signal peptide, propeptide, heparin-binding, EGF-like, juxtamembrane, transmembrane, and cytoplasmic domains (Fig. 2). ProHB-EGF is biologically active as a juxtacrine growth factor that signals to neighboring cells in a nondiffusible manner; it also functions as the receptor for diphtheria toxin (DTR) (Naglich et al. 1992; Iwamoto et al. 1994; Iwamoto and Mekada 2000). ProHB-EGF is cleaved at its juxtamembrane domain by metalloproteinases, in a process called "ectodomain shedding." Ectodomain



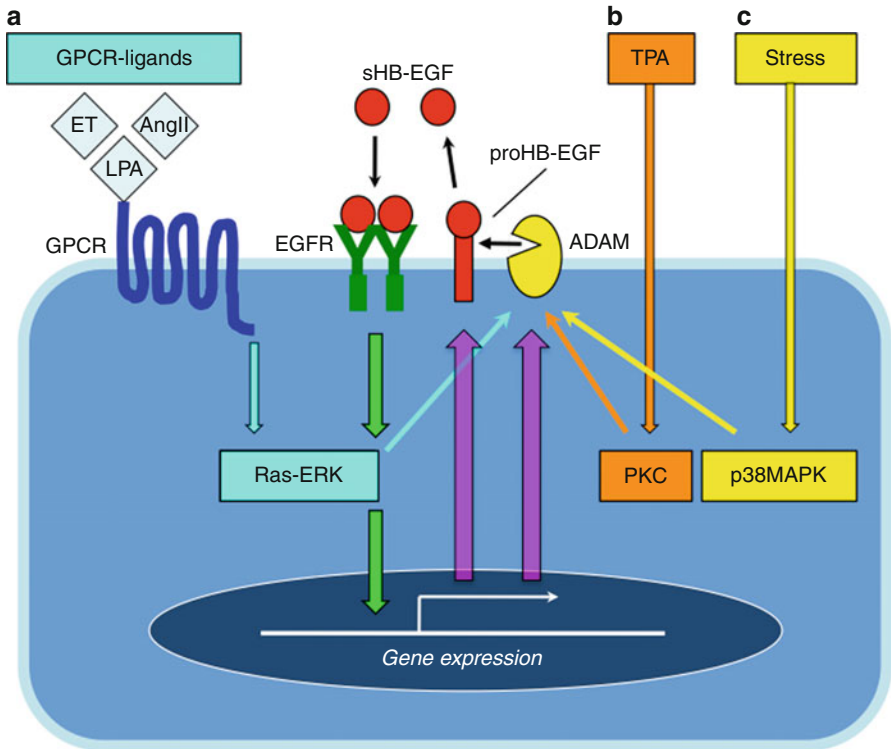
HB-EGF (Heparin-Binding EGF-Like Growth Factor), Fig. 2 *Ectodomain shedding of HB-EGF*. (**a**) Structure of proHB-EGF. The domain structure of the primary translation product of proHB-EGF is depicted. Ectodomain shedding cleaves off proHB-EGF in the juxtamembrane domain (a *large arrow*). Proteolytic cleavage also occurs at N-terminal sites (*small arrows*). MT1-MMP is involved in this cleavage. PM, plasma membrane. (**b**) Ectodomain shedding converts proHB-EGF to sHB-EGF and HB-EGF-CTF.

shedding of proHB-EGF yields a soluble ectodomain of HB-EGF (sHB-EGF) and a remnant carboxy (C)-terminal fragment (HB-EGF-CTF) (Fig. 2). sHB-EGF is a potent mitogen and chemoattractant for cells expressing its cognate ErbB receptors (Mekada and Iwamoto 2008). On the other hand, HB-EGF-CTF also functions as a signaling molecule. Subsequent to shedding, HB-EGF-CTF is phosphorylated and translocates into the nucleus, where it binds to and regulates several nuclear factors (Nanba et al. 2003; Higashiyama et al. 2008). Because proHB-EGF, sHB-EGF, and HB-EGF-CTF have distinct biological activities, ectodomain shedding is a critical process for HB-EGF proper function (Iwamoto and Mekada 2000; Higashiyama et al. 2008).

Shedding of proHB-EGF can be stimulated by various physiological and pharmacological stimuli, including G-protein-coupled receptor (GPCR) ligands and phorbol esters. Cellular stresses caused by inflammatory cytokines, reactive oxygen, and osmotic shock can also induce ectodomain shedding. These shedding stimuli activate several intracellular signaling molecules and result in the proteolytic cleavage of proHB-EGF by several metalloproteinases, including members of the ADAM family (Blobel 2005) (Fig. 3).

GPCR ligands, including lysophosphatidic acid (LPA), thrombin, angiotensin (ANG)-II, and endothelin, have been shown to stimulate EGFR tyrosine phosphorylation, in a process referred to as transactivation (Ohtsu et al. 2006). Transactivation of EGFR is a general function of GPCR signaling and is critical for the mitogenic activity of many GPCR ligands. Transactivation of EGFR is achieved by ectodomain shedding of EGFR ligands, and HB-EGF is often preferentially shed among the EGFR ligands (Prenzel et al. 1999). Ectodomain shedding of HB-EGF results in EGFR activation and subsequent Ras-ERK activation. In turn, activation of the EGFR-Ras-ERK pathway promotes further shedding of EGFR ligands. EGFR signals also induce the transcription of EGFR ligands, resulting in the repeated activation of ligand shedding and EGFR activation (Fig. 3). Thus, stimuli that induce HB-EGF shedding may trigger multiple positive feedback loops that ensure substantial EGFR activation, which potentially contributes to oncogenesis, cancer progression, and other diseases (Miyamoto et al. 2006; Mekada and Iwamoto 2008).

Dysregulation of HB-EGF shedding appears to be implicated in several diseases, including cardiovascular pathologies (cardiac hypertrophy, atherosclerosis, and pulmonary hypertension), cystic fibrosis, and various cancers. Analysis of knock-in mice, expressing transmembrane domain-truncated HB-EGF ($HB^{\Delta tm}$), revealed that dysregulated secretion of sHB-EGF induces hyperplastic tissue abnormalities, indicating that ectodomain shedding of proHB-EGF must be strictly controlled in vivo (Yamazaki et al. 2003; Mekada and Iwamoto 2008).



HB-EGF (Heparin-Binding EGF-Like Growth Factor), Fig. 3 *Molecular pathways regulating HB-EGF ectodomain shedding*. (**a**) GPCRs-Ras-ERK pathway. GPCR ligands including lysophosphatidic acid (LPA), angiotensin II (Ang II), and endothelin (ET), activate GPCR, resulting in activation of the downstream Ras-ERK pathway, which induces HB-EGF shedding. (**b**) TPA-PKC pathway. Phorbol esters, including TPA, activate protein kinase C (PKC), which induces HB-EGF shedding. (**c**) Stress-▶ p38MAPK pathway. Cellular stresses including inflammatory cytokines, reactive oxygen, and osmotic shock activate the p38MAPK pathway, which induces HB-EGF shedding. In all cases, sHB-EGF released by ectodomain shedding activates EGFR and the downstream Ras-ERK pathway, resulting in the formation of a positive feedback loop for promoting cell proliferation and migration

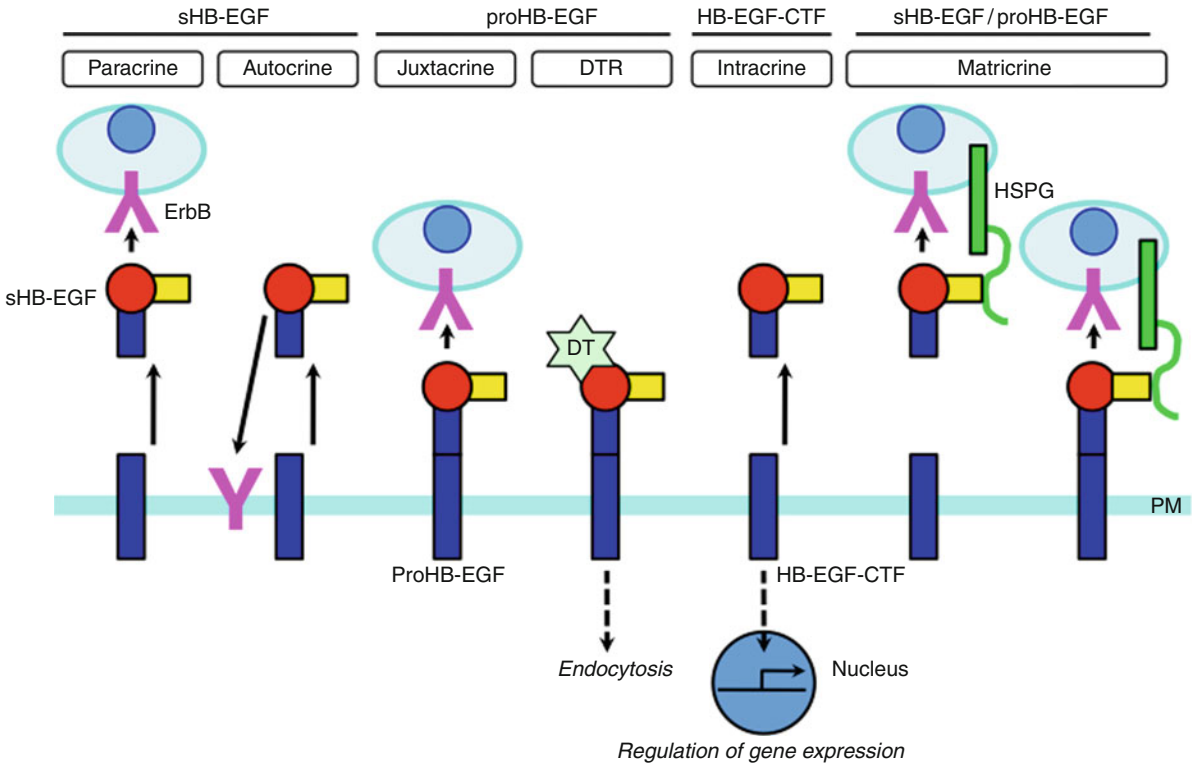
Modes of Action

Ectodomain shedding of proHB-EGF yields sHB-EGF and HB-EGF-CTF. The conversion of the molecular forms and the heparin-binding activity of HB-EGF suggest several modes of HB-EGF action (Fig. 4).

Paracrine and autocrine: Conventionally it is thought that sHB-EGF diffusively functions in a "paracrine" manner on cells expressing EGFR and/or ErbB4. Alternatively, sHB-EGF functions in an "autocrine" manner on its own cell, which also expresses EGFR and/or ErbB4. Studies of knock-in mice expressing an uncleavable mutant form (HB^{uc} mice) indicated that in vivo the major functions of HB-EGF are mediated by sHB-EGF (Yamazaki et al. 2003; Mekada and Iwamoto 2008).

Juxtacrine and DTR function: proHB-EGF functions in a "juxtacrine" manner by signaling to neighboring cells in a nondiffusible manner. Although sHB-EGF is known to be a potent mitogen, several lines of evidence from in vitro studies suggest that proHB-EGF has a distinct activity from sHB-EGF (Iwamoto and Mekada 2000). ProHB-EGF also functions as the cellular receptor for DT (DTR), mediating the entry of DT into the cytoplasm (Naglich et al. 1992; Iwamoto et al. 1994). Tetraspanin CD9 associates with proHB-EGF, which dramatically increases proHB-EGF activity as a DTR, by increasing the number of functional DTRs on the cell surface; however, the molecular mechanism underlying this process remains unclear (Iwamoto et al. 1994; Iwamoto and Mekada 2000).

Intracrine: HB-EGF-CTF has been found to be biologically active and functions in an "intracrine" manner (Nanba et al. 2003; Higashiyama et al. 2008). Subsequent to shedding, it is phosphorylated and



HB-EGF (Heparin-Binding EGF-Like Growth Factor), Fig. 4 *HB-EGF action modes.* Paracrine and autocrine by sHB-EGF: sHB-EGF acts diffusively on cells expressing EGFR and/or ErbB4 (paracrine). Alternatively, sHB-EGF acts on HB-EGF-expressing cells that also express EGFR and/or ErbB4 (autocrine). Juxtacrine and DTR function by proHB-EGF: ProHB-EGF acts by signaling to neighboring cells in a nondiffusible manner (juxtacrine). ProHB-EGF also functions as the cellular receptor for DT (DTR), mediating the entry of DT into the cytoplasm. Intracrine by HB-EGF-CTF: Subsequent to shedding, HB-EGF-CTF translocates into the nucleus, where it binds to several nuclear factors and regulates cell proliferation and apoptosis. Matricrine by sHB-EGF or proHB-EGF with HSPG: sHB-EGF interacts with HSPG as a co-receptor. ProHB-EGF also interacts with HSPGs, which up-regulates proHB-EGF activities

translocates into the nucleus, where it binds to several nuclear factors including PLZF and Bcl-6, and regulates cell proliferation and apoptosis.

Matricrine: HB-EGF has a high affinity for heparin and heparan sulfate (HS) and functions in a "matricrine" manner. Cell surface HS-proteoglycans (HSPGs) modulate various HB-EGF activities. Although the heparin-binding domain per se is not essential for HB-EGF activity, this domain suppresses the activity of the EGF-like domain and binding of heparin or HS to this domain inhibits this suppressive effect (Mekada and Iwamoto 2008). A recent study revealed that association of proHB-EGF with HSPGs promotes its localization to sites of cell–cell contact and prevents shedding (Prince et al. 2010). These studies suggested that HSPGs would be important regulators of HB-EGF activity, though the physiological significance was unclear. Recently, it has been reported that similarly to the HB-EGF-null and HB^{uc} mice, the knock-in mice that express a mutant HB-EGF, lacking heparin-binding activity ($HB^{\Delta hb}$ mice), show developmental abnormality in the cardiac valves, indicating that interaction between HB-EGF and HSPGs is essential for the physiological function of HB-EGF in this process (Iwamoto et al. 2010). Moreover, another recent study revealed that the interaction of HB-EGF with HSPGs is regulated by the processing of the N-terminal portion of HB-EGF by MT1-MMP, a membrane-bound metalloprotease (Koshikawa et al. 2010). Since the discovery of HB-EGF, it has been known that the processing of this portion of HB-EGF occurs in addition to ectodomain shedding (Fig. 2); however, its biological meaning remained unclear. N-terminal processing of HB-EGF

by MT1-MMP converts HB-EGF into a heparin-independent growth factor with enhanced mitogenic activity and thereby expression of both proteins co-stimulates tumor cell growth in vitro and in vivo. Thus, this processing might be a regulatory mechanism for the interaction of HB-EGF with HSPGs in HB-EGF-mediated processes.

Physiological Functions in Mice

Recent studies using HB-EGF-null and the mutant knock-in mice revealed that HB-EGF has critical roles in several physiological processes (summarized in Table 1) as described below (Mekada and Iwamoto 2008).

Heart muscle homeostasis: More than half of HB-EGF null mice died after birth and the survivors developed severe heart failure with grossly enlarged ventricular chambers and decreased contractility, symptoms resembling human dilated cardiomyopathy. This phenotype resembled that of mice conditionally lacking ErbB2. The data indicated that HB-EGF activation of ErbB2 and ErbB4 in cardiomyocytes is essential for normal heart function. The HB^{uc} mice showed an essentially similar phenotype, indicating that sHB-EGF functions by paracrine in this process.

Heart valve development: HB-EGF null mice also developed grossly enlarged cardiac valves. This enlargement is due to the increased proliferation of mesenchymal cells in the cardiac jelly during the later stage of cardiac valve development. This phenotype resembled that displayed by mice lacking EGFR and ADAM17, one of the sheddases for HB-EGF. The HB^{uc} mice also showed an essentially similar phenotype, indicating that sHB-EGF functions in this process. These studies proposed a model for valve development in which sHB-EGF secreted by ADAM17 from endocardial cells activates EGFR in mesenchymal cells, resulting in suppression of cell proliferation. As mentioned above, recently it has been revealed that HB-EGF must interact with HSPGs to properly function in this process (Iwamoto et al. 2010). Interestingly, although HB-EGF-null mice had abnormal heart chambers and lung alveoli, $HB^{\Delta hb/\Delta hb}$ mice did not exhibit these defects. Thus, the interaction with HSPGs is essential for HB-EGF function, especially in cardiac valve development.

Skin epidermis: Lack of HB-EGF resulted in a defect in eyelid closure in mouse embryos. Together with studies using HB^{uc}, waved-2 (a hypomorphic EGFR mutant strain), and TGFα null mice, the data indicate that sHB-EGF secreted from the tip of the leading edge of migrating epithelium activates the EGFR and ERK pathways, and that synergy with TGFα is required for the leading edge extension in epithelial sheet migration during eyelid closure. A similar mode of paracrine HB-EGF function in epithelial sheet migration underlies the skin wound healing process.

Lung development: HB-EGF is involved in distal lung development. In HB-EGF null newborns, abnormally thick saccular walls occurred accompanied by a significant increase in cell proliferation during the perinatal stage, indicating that HB-EGF suppresses distal lung cell proliferation. Together with studies using waved-2 and TGFα null mice, these findings indicate that HB-EGF has a suppressive function that contributes to decelerating distal lung cell proliferation synergistically with TGFα, through EGFR, during perinatal distal lung development.

Blastocyst implantation: HB-EGF has been suggested to be involved in the interaction between the blastocyst and the uterus during the implantation process. HB-EGF is expressed in the uterine luminal epithelium at the site of the blastocyst before the attachment reaction. Maternal HB-EGF is critical for implantation. Maternal deficiency of HB-EGF defers on-time implantation, leading to a compromised pregnancy outcome. ARG partially compensates for the loss of HB-EGF in this process.

Implications in Pathology

Recent clinical and basic studies have shed light on the relationship between the role of ectodomain shedding of the EGF family of growth factors and pathophysiology. Among EGF family, HB-EGF has been prominently studied regarding this issue (summarized in Table 2; Ohtsu et al. 2006; Miyamoto et al. 2006; Higashiyama et al. 2008).

Cardiac hypertrophy: HB-EGF shedding by a metalloproteinase ADAM12, followed by EGFR transactivation has been reported to be involved in cardiac hypertrophy. When cardiomyocytes are stimulated by GPCR-agonists, shedding of HB-EGF via

HB-EGF (Heparin-Binding EGF-Like Growth Factor), Table 1 Physiological functions and modes of action of HB-EGF

		Phenotypes							
Tissue	Process	KO	UC	ΔHB	HB-EGF expression	Effected region	Effects	Mode of action	References
Heart muscle	Homeostasis	DCM-like	DCM-like	Normal	Cardiomyocytes	Cardiomyocytes	Survival/ contraction	Paracrine	Iwamoto et al. (2003), Yamazaki et al. (2003), Iwamoto et al. (2010)
Heart valves	Development	Enlarged	Enlarged	Enlarged	Endocardial cells	Mesenchymal cells	Inhibition of proliferation	Para/ matricrine	Iwamoto et al. (2003), Yamazaki et al. (2003), Iwamoto et al. (2010)
Skin	Eyelid closure	Delay	Delay	n.d.	Leading edge of migrating epithelial sheet	Migrating epithelial sheet	Promotion of migration	Paracrine	Mine et al. (2005)
Skin	Wound healing	Delay	Delay	n.d.	Leading edge of migrating epithelial sheet	Migrating epithelial sheet	Promotion of migration	Paracrine	Shirakata et al. (2005)
Lung	Alveolization	Thickened alveolar wall	n.d.	Normal	Alveolar cells	Alveolar cells	Inhibition of proliferation	n.d.	Minami et al. (2008), Iwamoto et al. (2010)
Uterus	Implantation	Reduced	n.d.	n.d.	Endometrium	Blastocyst	Promotion of implantation	n.d.	Xie et al. 2007

KO HB-EGF-null mice, *UC* Knock-in mice expressing uncleavable mutant HB-EGF, *ΔHB* Knock-in mice expressing heparin-binding domain-truncated mutant HB-EGF, *DCM* Dilated cardiomyopathy, *n.d* not determined

HB-EGF (Heparin-Binding EGF-Like Growth Factor), Table 2 Effect of shedding dysregulation on HB-EGF-mediated processes and pathology

Shedding	Experimental model	Abnormality/ Pathology	Tissue	Inducer	Considered sheddase	References
Decreased	HB-EGF KO and UC mice	DCM-like	Heart			Iwamoto et al. (2003), Yamazaki et al. (2003)
		Valve hypertrophy	Heart		ADAM17	Iwamoto et al. (2003), Yamazaki et al. (2003)
		Eyelid closure defect	Skin		ADAM17	Mine et al. (2005)
		Defect in hyperplasia	Skin	tRA		Kimura et al. (2005)
	HB-EGF KO mice	Wound healing defect	Skin	Wounding		Shirakata et al. (2005)
	HB-EGF, ADAM17, and EGFR KO mice	Valve hypertrophy	Heart		ADAM17	Jackson et al. (2003)
Increased	Soluble HB-EGF mutant knock-in mice	Hypertrophy	Skin/ heart			Yamazaki et al. (2003)
	Heart hypertrophic mice	Hypertrophy	Heart	GPCR-agonists	ADAM12	Asakura et al. (2002)
	Cell culture	Atherosclerosis	Artery	GPCR-agonists	ADAM17	Ohts et al. (2006)
	Cell culture	Atherosclerosis	Artery	LRPs		Kawakami and Yoshida (2005)
	Hypertensive rats	Hypertension	Artery	GPCR-agonists	MMP7	Hao et al. (2004)
	Cell culture	Cystic fibrosis	Lung	*S. aureus* LTA	ADAM10	Lemjabbar and Basbaum (2002)
	Cell culture, xenografted nude mice	Ovarian cancer	Ovary	LPA		Miyamoto et al. (2004)

ADAM12 activation and subsequent transactivation of EGFR occur. In mice with cardiac hypertrophy, KB-R7785 (a metalloproteinase inhibitor) inhibits the shedding of HB-EGF and attenuates hypertrophic changes.

Atherosclerosis and pulmonary hypertension: Atherogenesis in the arterial wall is characterized by the formation of fibrous lesions and proliferation of neointimal smooth muscle cells (SMCs), and HB-EGF is a potent chemoattractant and mitogen for vascular SMCs. Large amounts of HB-EGF mRNA and protein are expressed in SMCs and macrophages in human atherosclerotic plaques. EGFR transactivation by sHB-EGF through ANG II-GPCR activation is required for the vascular SMC hypertrophy, and ADAM17 may be involved in this process. Remnant lipoproteins (RLPs) have also been reported as inducers of atherosclerosis, mediated by EGFR transactivation via HB-EGF shedding. Stimulation of GPCR with phenylephrine, which causes EGFR transactivation through MMP7 shedding of HB-EGF, has been suggested to be involved in the development and progression of hypertension. In spontaneously hypertensive rats (SHR), MMP levels are high at all time points. Administration of doxycycline, the only clinically approved MMP inhibitor, reduces systolic blood pressure and attenuates HB-EGF shedding in mesenteric arteries of SHR.

Cystic fibrosis: In the lungs of cystic fibrosis patients, it has been suggested that the pathway, bacterial lipoteichoic acid-GPCR-induced ADAM10 activation followed by HB-EGF shedding, and EGFR transactivation, may contribute to this pathology. Overproduction of mucus is a direct result of the activation of mucin gene expression by gram-positive bacteria. Bacterial lipoteichoic acid activates the platelet-activating factor receptor, which is a GPCR. This results in activation of ADAM10, cleavage of proHB-EGF and activation of EGFR.

Cancers: Emerging evidence has implicated dysregulation of ectodomain shedding of EGF family growth factors, especially HB-EGF, in the proliferative potential of tumor cells. It has been revealed that HB-EGF is a critical factor for ovarian cancer progression and thus suggested that HB-EGF could be a novel

targeting molecule for therapy of this cancer. Subsequently, CRM197 (a nontoxic mutant DT that neutralizes HB-EGF activity) is undergoing clinical development as an anticancer drug. Tumor progression involves the interaction of cancer cells with the cancer-surrounding stroma. A recent study, using CRM197 and humanized HB-EGF-expressing knock-in mice, has revealed that not only HB-EGF in cancer cells but also cancer stroma-derived HB-EGF contributes to tumor growth (Ichise et al. 2010).

Summary

HB-EGF is a member of the EGF family of growth factors, which binds to and activates EGFR and ErbB4. HB-EGF is synthesized first as a transmembrane proHB-EGF. Ectodomain shedding of proHB-EGF yields sHB-EGF and HB-EGF-CTF. Each form of HB-EGF has a distinct biological activity, and functions in several modes of action. Thus, ectodomain shedding is a critical process that must be strictly controlled for HB-EGF to function properly. Dysregulated shedding of HB-EGF causes severe developmental abnormalities in mice and has also been implicated in several pathologies. HSPGs are also important regulators of HB-EGF functions. Interaction with HSPGs is essential for HB-EGF function in mouse cardiac valve development. N-terminal processing of HB-EGF by MT1-MMP is a regulatory step for this interaction. HB-EGF has been implicated in several developmental, physiological, and pathological processes. Although HB-EGF has conventionally been thought of as a growth-promoting factor, especially in pathological processes including cancers, HB-EGF does not function as a growth-promoting factor, but rather as a growth-inhibitory or migration-promoting factor in developmental and physiological processes. Thus, future studies will clarify the mechanism regulating HB-EGF normal function in vivo (not as a growth-promoting factor), which will lead to novel therapeutic approaches for several HB-EGF-mediated pathologies.

References

Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. Nat Med. 2002;8:35–40. doi:10.1038/nm0102-35.

Blobel CP. ADAMs: key components in EGFR signalling and development. Nat Rev Mol Cell Biol. 2005;6:32–43. doi:10.1038/nrm1548.

Hao L, Du M, Lopez-Campistrous A, Fernandez-Patron C. Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor–receptor pathway. Circ Res. 2004;94:68–76. doi:10.1161/01.RES.0000109413.57726.91.

Harris RC, Chung E, Coffey RJ. EGF receptor ligands. Exp Cell Res. 2003;284:2–13. doi:10.1016/S0014-4827(02)00105-2.

Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science. 1991;251:936–9. doi:10.1126/science.1840698.

Higashiyama S, Iwabuki H, Morimoto C, Hieda M, Inoue H, Matsushita N. Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. Cancer Sci. 2008;99:214–20. doi:10.1111/j.1349-7006.2007.00676.x.

Ichise T, Adachi S, Ohishi M, Ikawa M, Okabe M, Iwamoto R, Mekada E. Humanized gene replacement in mice reveals the contribution of cancer stroma-derived HB-EGF to tumor growth. Cell Struct Funct. 2010;35:3–13. doi:csf.30.35/csf.09025.

Iwamoto R, Mekada E. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. Cytokine Growth Factor Rev. 2000;11:335–44. doi:10.1016/S1359-6101(00)00013-7.

Iwamoto R, Higashiyama S, Mitamura T, Taniguchi N, Klagsbrun M, Mekada E. Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. EMBO J. 1994;13:2322–30.

Iwamoto R, Yamazaki S, Asakura M, Takashima S, Hasuwa H, Miyado K, Adachi S, Kitakaze M, Hashimoto K, Raab G, Nanba D, Higashiyama S, Hori M, Klagsbrun M, Mekada E. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. Proc Natl Acad Sci USA. 2003;100:3221–6. doi:10.1073/pnas.0537588100.

Iwamoto R, Mine N, Kawaguchi T, Minami S, Saeki K, Mekada E. HB-EGF function in cardiac valve development requires interaction with heparan sulfate proteoglycans. Development. 2010;137:2205–14. doi:10.1242/dev.048926.

Jackson LF, Qiu TH, Sunnarborg SW, Chang A, Zhang C, Patterson C, Lee DC. Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. EMBO J. 2003;22:2704–16. doi:10.1093/emboj/cdg264.

Kawakami A, Yoshida M. Remnant lipprotein and atherogenesis. J Atheroscler Thromb. 2005;12:73–6.

Kimura R, Iwamoto R, Mekada E. Soluble form of heparin-binding EGF-like growth factor contributes to retinoic acid-induced epidermal hyperplasiav. Cell Struct Funct. 2005;30:35–42. doi:10.1247/csf.30.35.

Koshikawa N, Mizushima H, Minegishi T, Iwamoto R, Mekada E, Seiki M. Membrane type 1-matrix metalloproteinase cleaves off the NH2-terminal portion of heparin-binding

epidermal growth factor and converts it into a heparin-independent growth factor. Cancer Res. 2010;70:6093–103. doi:10.1158/0008-5472.CAN-10-0346.
Lemjabbar H, Basbaum C. Platelet-activating factor receptor and ADAM10 mediate responses to Staphylococcus aureus in epithelial cells. Nat Med. 2002;8:41–6. doi:10.1038/nm0102-41.
Mekada E, Iwamoto R. HB-EGF. UCSD-Nature Molecule Pages. 2008. doi:10.1038/mp.a002932.01.
Minami S, Iwamoto R, Mekada E. HB-EGF decelerates cell proliferation synergistically with TGFalpha in perinatal distal lung development. Dev Dyn. 2008;237:247–58. doi:10.1002/dvdy.21398.
Mine N, Iwamoto R, Mekada E. HB-EGF promotes epithelial cell migration in eyelid development. Development. 2005;132:4317–26. doi:10.1242/dev.02030.
Miyamoto S, Hirata M, Yamazaki A, Kageyama T, Hasuwa H, Mizushima H, Tanaka Y, Yagi H, Sonoda K, Kai M, Kanoh H, Nakano H, Mekada E. Heparin-binding EGF-like growth factor is a promising target for ovarian cancer therapy. Cancer Res. 2004;64:5720–7. doi:10.1158/0008-5472.CAN-04-0811.
Miyamoto S, Yagi H, Yotsumoto F, Kawarabayashi T, Mekada E. Heparin-binding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy. Cancer Sci. 2006;97:341–7. doi:10.1111/j.1349-7006.2006.00188.x.
Naglich JG, Metherall JE, Russell DW, Eidels L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. Cell. 1992;69:1051–61. doi:10.1016/0092-8674(92)90623-K.
Nanba D, Mammoto A, Hashimoto K, Higashiyama S. Proteolytic release of the carboxy-terminal fragment of proHB-EGF causes nuclear export of PLZF. J Cell Biol. 2003;163:489–502. doi:10.1083/jcb.200303017.
Ohtsu H, Dempsey PJ, Eguchi S. ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. Am J Physiol Cell Physiol. 2006;291:1–10. doi:10.1152/ajpcell.00620.2005.
Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature. 1999;402:884–8. doi:10.1038/47260.
Prince RN, Schreiter ER, Zou P, Wiley HS, Ting AY, Lee RT, Lauffenburger DA. The heparin-binding domain of HB-EGF mediates localization to sites of cell-cell contact and prevents HB-EGF proteolytic release. J Cell Sci. 2010;123:2308–18. doi:10.1242/jcs.058321.
Shirakata Y, Kimura R, Nanba D, Iwamoto R, Tokumaru S, Morimoto C, Yokota K, Nakamura M, Sayama K, Mekada E, Higashiyama S, Hashimoto K. Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. J Cell Sci. 2005;118:2363–70. doi:10.1242/jcs.02346.
Xie H, Wang H, Tranguch S, Iwamoto R, Mekada E, DeMayo FJ, Lydon JP, Das SK, Dey SK. Maternal heparin-binding-EGF deficiency limits pregnancy success in mice. Proc Natl Acad Sci USA. 2007;104:18315–20. doi:10.1073/pnas.0707909104.
Yamazaki S, Iwamoto R, Saeki K, Asakura M, Takashima S, Yamazaki A, Kimura R, Mizushima H, Moribe H, Higashiyama S, Endoh M, Kaneda Y, Takagi S, Itami S, Takeda N, Yamada G, Mekada E. Mice with defects in HB-EGF ectodomain shedding show severe developmental abnormalities. J Cell Biol. 2003;163:469–75. doi:10.1083/jcb.200307035.

hBRM

► SWI/SNF Chromatin Remodeling Complex

hD52 (Human)

► TPD52 (Tumor Protein D52)

Heat Shock Protein 90 kDa Beta Member 1

► Grp94 (HSP90B1)

HEGFL

► HB-EGF (Heparin-Binding EGF-Like Growth Factor)

Hek (Human)

► EphA3, Erythropoietin-Producing Hepatocellular Carcinoma Cell Receptor A3

Hematopoietic Cells Phosphatase (HCP)

► PTPN6

Hematopoietic Progenitor Kinase 1

► HPK1

Hemicentin

▶ Fibulins

Heparin-Binding Epidermal Growth Factor-Like Growth Factor

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

Heterogeneous Nuclear Ribonucleoprotein D

▶ hnRNP D (AUF1)

Hfz5

▶ FZD (Frizzled)

Hfz6

▶ FZD (Frizzled)

Him4

▶ Fibulins

HIPK2

Thomas G. Hofmann and Eva Krieghoff-Henning
Cellular Senescence (A210), German Cancer Research Center, Heidelberg, Germany

Synonyms

PKM (hamster); STANK

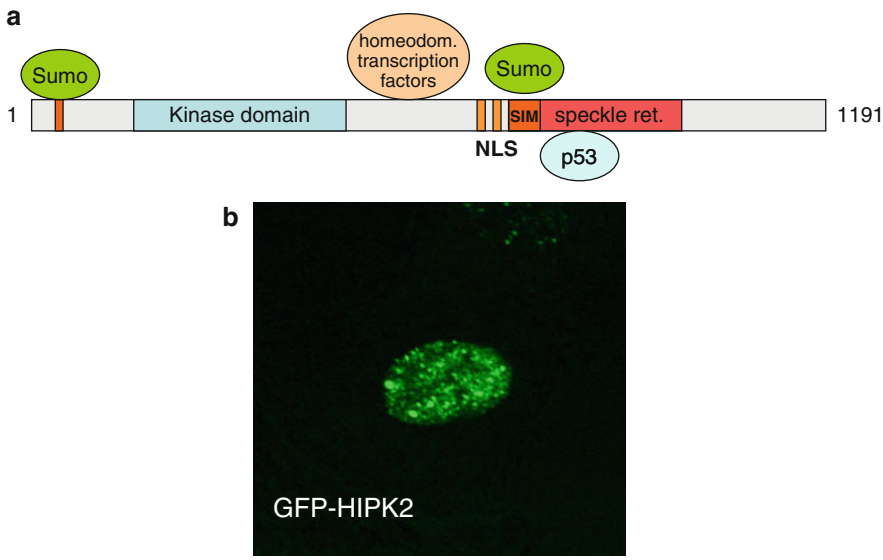
Historical Background

The homeodomain-interacting protein kinase 2 (HIPK2) was first described in 1998 as member of a novel protein kinase family (HIPK1-3) able to interact with homeodomain transcription factors of the NK-2 family and to enhance their repressor activity (Kim et al. 1998). Over the next years, it was shown that HIPK2 very likely is an autophosphorylating Ser/Thr kinase which localizes to nuclear speckles (see Fig. 1), and a number of interaction partners and putative targets such as the death receptor CD95, the corepressor Groucho, or a STAT3 peptide were identified. The HIPK2 genes were mapped to Chr. 7q32-42 in humans and to Chr. 6B in the mouse.

The findings that influenced the direction of HIPK2 research most profoundly for the next decade were published in 2002, when independent groups reported that HIPK2 is a DNA damage-responsive kinase that is partially co-recruited with the tumor suppressor ▶ p53 into PML bodies upon cytotoxic stress, most prominently by UV irradiation (D'Orazi et al. 2002; Hofmann et al. 2002). HIPK2 was shown to phosphorylate p53 on Serine 46, thereby enhancing the proapoptotic activity of the transcription factor p53 and programmed cell death. Moreover, it was shown in 2003 that HIPK2 is also able to promote p53-independent cell death by targeting the corepressor C-terminal binding protein (CtBP) for degradation, thus increasing the transcription of proapoptotic CtBP target genes. Since then, the important regulatory role of HIPK2 in determining cell fates upon genotoxic stress, but also in various developmental processes, has been elucidated in great detail.

The HIPK2 Protein

HIPK2 is a 130 kDa protein of 1191 amino acids in humans that is well conserved from flies to humans. It harbors an N-terminal kinase domain that was published to be very homologous to DYRK family kinases. It was shown that HIPK2 most probably autophosphorylates at multiple Serine/Threonine residues. Mutation of the conserved residue K221 within the ATP-binding site of the kinase domain results in an inactive kinase. HIPK2 preferentially targets Serine or Threonine residues that are followed by a Proline residue (SP/TP sites) in its substrates. Correlating with its



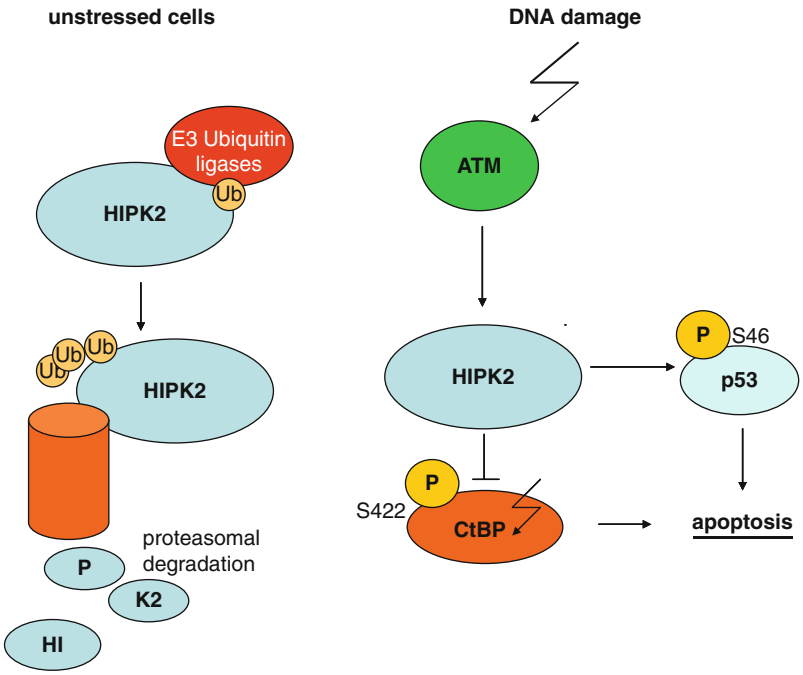
HIPK2, Fig. 1 The HIPK2 protein. (**a**) Schematic drawing of important domains and interactions of the HIPK2 protein. NLS, nuclear localization signal; SIM, SUMO interaction motif. (**b**) Nuclear speckle localization of exogenously expressed GFP-HIPK2 in U2OS cells

observed localization in nuclear substructures, it contains several C-terminal NLS sequences and a speckle retention signal. HIPK2 was also shown to be covalently SUMOylated at several sites, the principal site being K25, and to have two adjacent SUMO interaction motifs (SIMs) within the C-terminus that influence its ability to be recruited to promyelocytic leukemia (PML) nuclear bodies (de la Vega et al. 2010 and references cited therein). Recently, it has been shown that HIPK2 is an unstable protein in unstressed cells which is kept low in cells via proteasomal degradation, and the region that is responsible for this degradation has also been mapped to the C-terminus (Calzado et al. 2009a, b; Sombroek and Hofmann 2009; Winter et al. 2008). Moreover, it has been speculated that the HIPK2 C-terminus may form an auto-inhibitory loop that might block HIPK2 activity in the absence of cofactors like Han11 (Ritterhoff et al. 2010) or Axin which was reported to bind to the HIPK2 C-terminus and to enhance the kinase activity of HIPK2 towards p53.

HIPK2 and the DNA Damage Response

The role of HIPK2 in cells suffering from genotoxic stress is the best studied aspect of the functions of HIPK2 on a molecular level. As mentioned previously, HIPK2 can promote apoptosis in irreparably damaged cells by p53-dependent or p53-independent means (Fig. 2). HIPK2 protein levels are very low in unstressed cells due to efficient protein degradation via the ubiquitin/proteasome system. Several E3 ubiquitin ligases have been identified that earmark HIPK2 for degradation, namely, seven in absentia-homolog (Siah)-1 and -2, WD40 repeat/SOCS box protein 1 (WSB-1), and the p53 E3 ligase mouse double minute (MDM)2/HDM2 (reviewed in Calzado et al. 2009b and Sombroek et al. 2009).

The region within HIPK2 that is required for destabilization of HIPK2 by these proteins is located in its C-terminus, between amino acids 600 and 800 for Siah-1 and starting from amino acid 838 for MDM2. Upon DNA damage inflicted by various genotoxic stimuli like UV and ionizing irradiation or treatment with adriamycin or cisplatin, however, HIPK2 degradation is blocked, HIPK2 protein accumulates and phosphorylates its target genes. The process by which HIPK2 degradation is inhibited is best characterized for Siah-1, which is phosphorylated by the damage-activated checkpoint kinases ATM and/or ATR at Ser19, leading to the loosening of the HIPK2/Siah-1 interaction and therefore to HIPK2 stabilization. When this occurs in p53-proficient cells, HIPK2 phosphorylates p53 on Serine residue 46 (or 58 in mice) in a PML- and Sp100-dependent manner. Ser46 phosphorylation very likely causes enhanced acetylation of p53 on K382 by the HAT protein CREB-binding protein (CBP), another HIPK2 interactor, and these modifications lead to an altered promoter specificity



HIPK2, Fig. 2 HIPK2 within the DNA damage response. In unstressed cells, HIPK2 is almost completely degraded via the proteasomal pathway, whereas genotoxic stress causes HIPK2 stabilization and greatly enhanced ability of HIPK2 to promote apoptosis by phosphorylating its target proteins p53 and/or CtBP, thereby altering the promoter specificity of the p53 protein and promoting degradation of the antiapoptotic CtBP protein

of the p53 transcription factor and to the increased transcription of proapoptotic p53 target genes such as *Puma* and *Noxa*. Alternatively, HIPK2 can lead to derepression of proapoptotic genes by phosphorylating the transcriptional repressor CtBP, inducing its degradation via the proteasomal pathway. This results in upregulation of CtBP targets such as *Bax*, *Noxa*, and ▶ PTEN which then drive apoptosis in the damaged cells (for reviews, see Bitomsky and Hofmann 2009 and Puca et al. 2010). HIPK2-mediated apoptosis has also been linked to the presence of the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2) (Bracaglia et al. 2009), but the underlying mechanism has not been determined. Accordingly, it has been observed that HIPK2 can act as haploinsufficient tumor suppressor in a two-stage model of skin carcinogenesis in mice. Involvement of HIPK2 in tumor suppression, however, will be discussed in more detail in a separate chapter. Likewise, the involvement of HIPK2-mediated apoptosis in normal development will be discussed later. One interesting aspect of HIPK2 regulation upon genotoxic stress is the transient stabilization that occurs after sublethal damage but does not result in Ser46 phosphorylation. The transient nature of this stabilization is probably brought about by negative feedback loops involving the Siah-1 and MDM2 ubiquitin ligases, both of which are p53 target genes which accumulate upon DNA damage. However, the functions of the transiently increased HIPK2 levels after mild stress have not been elucidated so far. Another interesting aspect of HIPK2 regulation is the question whether HIPK2 is activated in addition to its accumulation, and if so, which modification and/or interactions might be required for that. One described mechanism activating HIPK2 after damage in addition to stabilization is caspase cleavage. It was shown that HIPK2 can be cleaved after D977 and D916 in a process involving caspases 3 and 6, and that the cleaved products are more active with respect to p53 phosphorylation than corresponding uncleavable HIPK2 constructs (reviewed in Sombroek et al. 2009). Nevertheless, the issue of HIPK2 activation by other means remains subject to intense investigation.

HIPK2 in Development

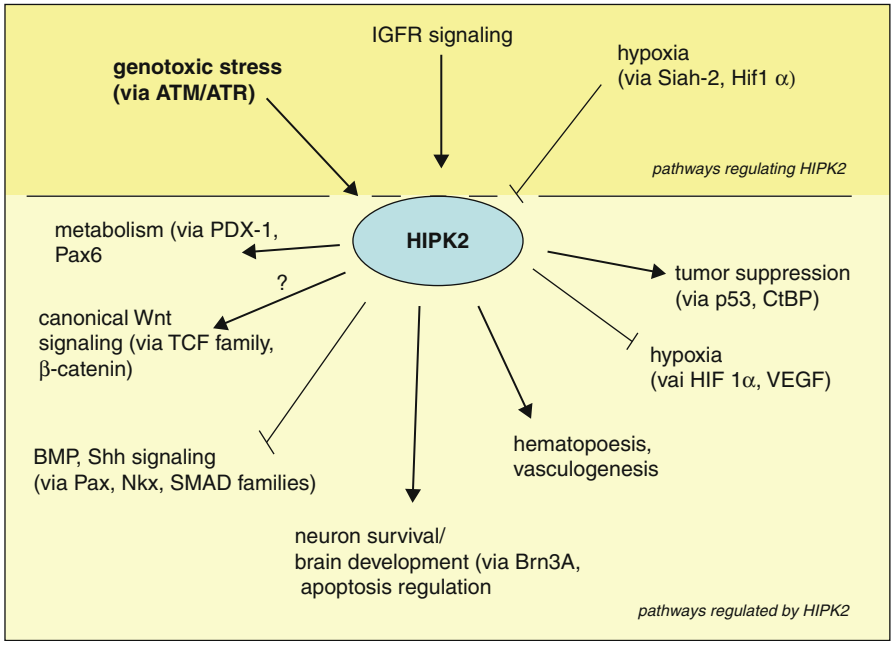
Apoptosis is an important process shaping many tissues and organs. For example, in brain development, neurons are produced in very large numbers and the excess is then eliminated by apoptosis. Indeed, it was shown that in sensory and sympathetic neurons for instance in trigeminal ganglia, HIPK2 overexpression induces massive apoptosis and thus seems to be

important for the removal of excess neurons by programmed cell death, likely by regulating the pro-survival factor Brn3a. Somewhat paradoxically, another study suggested that HIPK2 knockout actually increases apoptosis in trigeminal neurons, which would suggest an as-yet-unrecognized pro-survival function of HIPK2 (reviewed in Rinaldo et al. 2007). The phenotypes of knockout mice generated independently in different laboratories (Isono et al. 2006; Trapasso et al. 2009; Hattangadi et al. 2010; Inoue et al. 2010) suggest that there is some degree of redundancy between HIPK2 and HIPK1. Analysis of double HIPK1/2 knockout mice (Isono et al. 2006) revealed disturbed hematopoiesis, vasculogenesis, and angiogenesis in one study, and embryonic death from embryonic day 9 onward and exencephaly, eye phenotypes, and fusion of dorsal root ganglia in another study. Moreover, the latter also revealed developmental defects like the formation of ectopic ribs, indicative of disturbed Hox gene regulation, and an influence of HIPKs on the ▶ Sonic hedgehog (Shh) pathway via Pax1 and -3. MEFs from double knockout were also reported to be more resistant to UV-induced apoptosis. HIPK2 single knockouts from independent groups showed a "clasping of hindlimbs" phenotype when suspended by the tail, albeit to greatly varying percentages. One study also reported shuffling gait and other motoric abnormalities resembling a Parkinson's-like disease, and significantly enhanced apoptosis in neurons at E17.5 and P0. Another independent study (Trapasso et al. 2009) reported increased stem and progenitor cell compartments in mouse skin when HIPK2 was knocked out, suggesting that HIPK2 may restrict the proliferation of the stem and progenitor cells in the skin, maybe by interfering with the Wnt signaling pathway. Interestingly, the study reporting only 15% of "clasped hindlimbs" in their HIPK2 knockout observed a growth reduction of HIPK2-deficient animals in comparison to their littermates that was already observable at birth and persisted to adulthood, again indicating a role for HIPK2 in cell proliferation rather than cell death. However, the underlying mechanisms for a putative growth-stimulating role of HIPK2 have not been identified so far.

In *Drosophila*, knockout of the only existing HIPK gene results in defective eye formation and frequent death at the pupal or embryonal stages. *Drosophila* HIPK phosphorylates Groucho at multiple residues, resulting in upregulation of the Notch pathway (Lee et al. 2009). This connection has not (yet) been described for vertebrates. In addition, it could be shown that HIPK is required for collective programmed cell death of epithelial cells in the developing wing, similar to previously determined apoptotic factors like Dronc or Dark (Link et al. 2007).

Analyses in cell culture have yielded a set of interactors for HIPK2 that point to possible involvement of HIPK2 in several signal transduction pathways important for important developmental processes (reviewed in Rinaldo et al. 2007; Puca et al. 2010) (Fig. 3). Most prominently, the Wnt pathway has been shown to be connected to HIPK2 activity at several points. For instance, Wnt-1 induces the HIPK2- and NLK-dependent degradation of the transcription factor ▶ c-myb. Moreover, both human and *Drosophila* HIPK have been shown to interact with and phosphorylate both ▶ β-catenin and transcription factors of the Lef/TCF-family (Hikasa et al. 2010), thereby directly modulating the canonical Wnt signaling pathway, which is important for axis specification, neural tube patterning where it seems to counteract Shh signaling, and regulation of stem cell compartments. However, data concerning the interactions and effects of HIPK2 within the canonical Wnt signaling pathway appear partially contradictory and await further investigation. HIPK2 also seems to interfere with the Shh pathway at the level of its downstream homeobox transcription factors; as mentioned previously, HIPK2 likely interacts with Pax transcription factors on the one hand and the Nkx family on the other hand. Thus, HIPK2 might influence important morphogenetic processes such as motoneuron formation. HIPK2 interaction with Pax6 may also be responsible for the observed disturbances in eye formation in mice which frequently have abnormally small eyes without a lens and with incorrect lamination and cell arrangement in the retina. Furthermore, HIPK2 was recently shown to interact with the transcription factor cAMP-responsive element-binding protein (▶ CREB) (Sakamoto et al. 2010) and to increase its association with CBP, which also plays a role in neuron development and survival.

It has also been reported that HIPK2 can interact with the corepressor c-ski and with SMAD1 and thereby counteract bone morphogenetic protein (BMP) signaling, which is implicated in embryo polarity, heart and CNS development as well as bone formation.



HIPK2, Fig. 3 The HIPK2 network. The most important signals and pathways that alter HIPK2 (*upper part*) or are modulated by HIPK2 (*lower part*) that are currently known are depicted schematically

HIPK2 and HIPK1 depletion also impacts very strongly on fetal liver hematopoiesis (Hattangadi et al. 2010), preventing upregulation of many erythropoietic and heme-biosynthesis-associated genes, but the exact target(s) of the HIPK2 in this system has not been identified to date.

HIPK2 and Cancer

Given the observations that HIPK2 can induce apoptosis in both p53-proficient and p53-deficient cells upon genotoxic stress, it is highly likely that HIPK2 can act as a tumor suppressor in many tissues (Krieghoff-Henning and Hofmann 2008; Sombroek and Hofmann 2009 and references cited therein). This question has been addressed directly in mice in a skin carcinogenesis model, where deletion of only one HIPK2 allele already renders the mice more sensitive to skin cancer formation, with an even stronger phenotype in full knockouts. Thus, HIPK2 acts as haploinsufficient tumor suppressor in mouse skin. Moreover, there are a number of human tumors, such as thyroid and breast carcinoma, that show a high incidence of HIPK2 downregulation on the mRNA level. In one case each of myelodysplastic syndrome and AML, point mutations in the HIPK2 sequence were found: R868W and N958I, and these point mutants were found to localize aberrantly, affecting their ability to transactivate p53 target genes. Moreover, there is evidence for a tumor-promoting effect of the HIPK2 interactor HMGA1, which is highly overexpressed in many breast tumors and seems to recruit HIPK2 to the cytoplasm, thereby preventing its association with PML-NBs and hence p53 Ser46 phosphorylation. Interestingly, HIPK2 protein is degraded in a possibly HIF1- and Siah-2-dependent manner under hypoxic conditions, which occur very frequently in larger tumors. HIPK2 also co-localizes with the tumor suppressor PML in PML nuclear bodies, and while PML may actually stabilize HIPK2, HIPK2 was shown to phosphorylate PML IV at several residues, increasing PML SUMOylation and its ability to induce apoptosis. Finally, injection of colorectal cancer RKO cells into mice causes tumors that grow much faster if the cells are HIPK2-depleted. Moreover, depletion of HIPK2 in RKO cells leads to integrin subunit β4 upregulation (Bon et al. 2009), which is strongly associated with increased migration and metastatic potential. In breast cancer, HIPK2 nuclear positivity was reported to be inversely correlated with β4 expression. These observations all suggest that in many tumors HIPK2 is indeed inactivated, strengthening the hypothesis that HIPK2 is a tumor suppressor protein. Reports on pilocytic astrocytoma, however, indicate that HIPK2 may also be upregulated by

amplification in a subset of human tumors, although the driving factor of cancer formation in these samples may be BRAF which is located in the same region (Cin et al. 2011). In cervical cancer, HIPK2 nuclear positivity was positively correlated with tumor stage and tumor dedifferentiation, suggesting that HIPK2 may also have a growth-promoting role. However, the molecular basis of this potential role has not been determined so far.

HIPK2, Aging and Metabolism

HIPK2 may not be regulated solely by DNA damage, but also by the metabolic state of the cell. Vice versa, HIPK2 activity also appears to contribute to metabolic regulation in the presence or absence of genotoxic stress. Interestingly, it is becoming more and more obvious that metabolism, genotoxic stress, and cellular aging in particular are closely interlinked. Caloric restriction is probably extending the organismal lifespan, as shown in *C. elegans* and mice, most likely by limiting the amount of DNA-damaging reactive oxygen species (ROS).

As mentioned previously, HIPK2 plays an important regulatory role in pancreas development and function. One important HIPK2 target in this context is the Insulin Promoter Factor/Pancreatic Duodenal Homeobox 1 (IPF-1/PDX-1)-dependent upregulation of the *insulin* gene, *glut2*, *glucokinase*, etc., in the developing pancreatic epithelium and in β-cells (Boucher et al. 2009). HIPK2 seems to regulate the nucleocytoplasmic distribution of IPF-1 (An et al. 2010). HIPK2 can also regulate the *proglucagon*, *somatostatin*, and *insulin* promoters via Pax6, which also impacts on pancreas development and function. HIPK2 may also play a role in ROS metabolism, by virtue of its ability to suppress transcription of *Nox1*, encoding the catalytic subunit of a NADPH oxidase which can generate ROS (Puca et al. 2010).

Conversely, it was shown that HIPK2 is upregulated in aging neurons, which can be counteracted by caloric restriction, arguing that the presence of ROS induces HIPK2, and placing HIPK2 in the aging-associated insulin-like growth factor (IGF1)-R signaling cascade (Li et al. 2009).

A very general way in which HIPK2 regulates metabolic and other processes within the cell is its ability to recruit and/or interact with the HAT enzyme p300 and its paralog CBP, which are important co-transcription factors for a variety of transcription factors, including the CREB protein, which itself was shown to be a HIPK2 target (Sakamoto et al. 2010), and which has also been linked to lifespan extension in *C. elegans*.

Summary

Although discovered relatively recently, HIPK2 has already been shown to regulate several important processes within eukaryotic cells. The best studied function of HIPK2 is to induce programmed cell death in response to genotoxic stress, either by p53 phosphorylation at Serine residue 46 or by degrading the antiapoptotic transcriptional repressor CtBP. Furthermore, HIPK2 has been shown to act as transcriptional regulator in various transcription complexes, most prominently those formed by homeobox transcription factors. Especially with respect to the developmental functions of HIPK2, a certain degree of redundancy with the related kinase HIPK1 seems to occur, so that developmental defects are in some cases only clearly detectable when both proteins are depleted. Single and double knockout animals suggest roles for HIPK2 in brain development, hematopoiesis, pancreas development, and body patterning, in part by regulating apoptosis, but also by modulating the activity of several important signaling pathways such as canonical Wnt, TGFβ, or BMP. In part, the detailed consequences of the described interactions and phosphomodifications of the HIPK2 targets identified so far still remain to be elucidated on the organismal level.

Moreover, the prominent role of HIPK2 in the DNA damage response and first hints obtained by analyzing human tumors as well as a two-step carcinogenesis model in mice suggest that HIPK2 may act as a tumor suppressor in many tissues, and indicate that cancer patients might benefit from specific activation of HIPK2 in tumors.

References

An R, da Silva Xavier G, Semplici F, Vakhshouri S, Hao HX, Rutter J, et al. Pancreatic and duodenal homeobox 1 (PDX1) phosphorylation at serine-269 is HIPK2-dependent and

affects PDX1 subnuclear localization. Biochem Biophys Res Commun. 2010;399(2):155–61.
Bitomsky N, Hofmann TG. Apoptosis and autophagy: regulation of apoptosis by DNA damage signalling - roles of p53, p73 and HIPK2. FEBS J. 2009;276(21):6074–83.
Bon G, Di Carlo SE, Folgiero V, Avetrani P, Lazzari C, D'Orazi G, et al. Negative regulation of beta4 integrin transcription by homeodomain-interacting protein kinase 2 and p53 impairs tumor progression. Cancer Res. 2009;69(14):5978–86.
Boucher MJ, Simoneau M, Edlund H. The homeodomain-interacting protein kinase 2 regulates insulin promoter factor-1/pancreatic duodenal homeobox-1 transcriptional activity. Endocrinology. 2009;150(1):87–97.
Bracaglia G, Conca B, Bergo A, Rusconi L, Zhou Z, Greenberg ME, et al. Methyl-CpG-binding protein 2 is phosphorylated by homeodomain-interacting protein kinase 2 and contributes to apoptosis. EMBO Rep. 2009; 10(12):1327–33.
Calzado MA, de la Vega L, Moller A, Bowtell DD, Schmitz ML. An inducible autoregulatory loop between HIPK2 and Siah2 at the apex of the hypoxic response. Nat Cell Biol. 2009a;11(1):85–91.
Calzado MA, De La Vega L, Munoz E, Schmitz ML. From top to bottom: the two faces of HIPK2 for regulation of the hypoxic response. Cell Cycle. 2009b;8(11):1659–64.
Cin H, Meyer C, Herr R, Janzarik WG, Lambert S, Jones DT, et al. Oncogenic FAM131B-BRAF fusion resulting from 7q34 deletion comprises an alternative mechanism of MAPK pathway activation in pilocytic astrocytoma. Acta Neuropathol. 2011;121(6):763–74.
D'Orazi G, Cecchinelli B, Bruno T, Manni I, Higashimoto Y, Saito S, et al. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat Cell Biol. 2002;4(1):11–9.
de la Vega L, Frobius K, Moreno R, Calzado MA, Geng H, Schmitz ML. Control of nuclear HIPK2 localization and function by a SUMO interaction motif. Biochim Biophys Acta. 2010;1813(2):283–97.
Hattangadi SM, Burke KA, Lodish HF. Homeodomain-interacting protein kinase 2 plays an important role in normal terminal erythroid differentiation. Blood. 2010;115(23): 4853–61.
Hikasa H, Ezan J, Itoh K, Li X, Klymkowsky MW, Sokol SY. Regulation of TCF3 by Wnt-dependent phosphorylation during vertebrate axis specification. Dev Cell. 2010;19(4): 521–32.
Hofmann TG, Möller A, Sirma H, Zentgraf H, Taya Y, Dröge W, Will H, Schmitz ML. Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat Cell Biol. 2002;4(1):1–10.
Inoue T, Kagawa T, Inoue-Mochita M, Isono K, Ohtsu N, Nobuhisa I, et al. Involvement of the Hipk family in regulation of eyeball size, lens formation and retinal morphogenesis. FEBS Lett. 2010;584(14):3233–8.
Isono K, Nemoto K, Li Y, Takada Y, Suzuki R, Katsuki M, et al. Overlapping roles for homeodomain-interacting protein kinases hipk1 and hipk2 in the mediation of cell growth in response to morphogenetic and genotoxic signals. Mol Cell Biol. 2006;26(7):2758–71.
Kim YH, Choi CY, Lee SJ, Conti MA, Kim Y. Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. J Biol Chem. 1998;273(40):25875–9.
Krieghoff-Henning E, Hofmann TG. HIPK2 and cancer cell resistance to therapy. Future Oncol. 2008;4(6):751–4.
Lee W, Andrews BC, Faust M, Walldorf U, Verheyen EM. Hipk is an essential protein that promotes notch signal transduction in the drosophila eye by inhibition of the global co-repressor Groucho. Dev Biol. 2009;325(1):263–72.
Li H, Costantini C, Scrable H, Weindruch R, Puglielli L. Egr-1 and Hipk2 are required for the TrkA to p75(NTR) switch that occurs downstream of IGF1-R. Neurobiol Aging. 2009;30(12):2010–20.
Link N, Chen P, Lu WJ, Pogue K, Chuong A, Mata M, et al. A collective form of cell death requires homeodomain interacting protein kinase. J Cell Biol. 2007;178(4):567–74.
Puca R, Nardinocchi L, Givol D, D'Orazi G. Regulation of p53 activity by HIPK2: molecular mechanisms and therapeutical implications in human cancer cells. Oncogene. 2010;29(31):4378–87.
Rinaldo C, Prodosmo A, Siepi F, Soddu S. HIPK2: a multitalented partner for transcription factors in DNA damage response and development. Biochem Cell Biol. 2007;85(4):411–8.
Ritterhoff S, Farah CM, Grabitzki J, Lochnit G, Skurat AV, Schmitz ML. The WD40-repeat protein Han11 functions as a scaffold protein to control HIPK2 and MEKK1 kinase functions. EMBO J. 2010;29(22):3750–61.
Sakamoto K, Huang BW, Iwasaki K, Hailemariam K, Ninomiya-Tsuji J, Tsuji Y. Regulation of genotoxic stress response by homeodomain-interacting protein kinase 2 through phosphorylation of cyclic AMP response element-binding protein at serine 271. Mol Biol Cell. 2010; 21(16):2966–74.
Sombroek D, Hofmann TG. How cells switch HIPK2 on and off. Cell Death Differ. 2009;16(2):187–94.
Trapasso F, Aqeilan RI, Iuliano R, Visone R, Gaudio E, Ciuffini L, et al. Targeted disruption of the murine homeodomain-interacting protein kinase-2 causes growth deficiency in vivo and cell cycle arrest in vitro. DNA Cell Biol. 2009;28(4): 161–7.
Winter M, Sombroek D, Dauth I, Moehlenbrink J, Scheuermann K, Crone J, et al. Control of HIPK2 stability by ubiquitin ligase Siah-1 and checkpoint kinases ATM and ATR. Nat Cell Biol. 2008;10(7):812–24.

Hippocalcin

Masaaki Kobayashi and Ken Takamatsu
Department of Physiology, Toho University School of Medicine, Ohta-ku, Toyko, Japan

Synonyms

BDR

Historical Background

Hippocalcin was originally cloned from a rat brain cDNA library based on the partial amino acid sequences from a calcium-binding protein of molecular mass 23,000 (P23k), which was purified during a survey of recoverin-like immunoreactivity in the brain (Kobayashi et al. 1992). During the 1990s, a number of calcium-binding proteins structurally related to hippocalcin were identified in the nervous system and named NCS (neuronal calcium sensor) proteins. There are 14 protein coding genes in the NCS family in the human genome (Burgoyne 2007; Braunewell and Klein-Szanto 2009). Hippocalcin is a highly conserved protein having an identical amino acid sequence in rat, mouse, bovine, and human genes (Kobayashi and Takamatsu 2009).

Protein Chemical Characteristics

Hippocalcin, a 23 kDa protein of 193 amino acid residues, has three functional EF-hand calcium-binding domains and an N-terminal glycine residue covalently linked to myristic acid (Kobayashi et al. 1993). Calcium binding to the EF-hands of hippocalcin induces a conformational change that results in exposure of the myristoyl group and association of hippocalcin with the cellular membranes. This process is referred to as a calcium myristoyl switch and is a prominent characteristic of some members of the NCS family including recoverin, VILIP1, VILIP2, and VILIP3 (Burgoyne 2007; Braunewell and Klein-Szanto 2009). Hippocalcin undergoes a calcium myristoyl switch in HeLa cells transfected with hippocalcin-EYFP (enhanced yellow fluorescent protein) and the maximal rate of translocation to the membrane is approximately 1 sec. Half maximal translocation occurred at approximately 300 nM free calcium with a dynamic range of calcium-sensitivity of 200–800 nM free calcium. In hippocampal neurons, spontaneous and action potential-dependent translocation of hippocalcin-YFP fluorescence were observed in different parts of neuronal processes, reaching peak translocation within 1–5 sec (Markova et al. 2008). These results suggest that hippocalcin would be able to affect interact with target proteins on membranes when free calcium levels are only slightly elevated above resting levels. N-myristoylated hippocalcin as well as an N-myristoylated hippocalcin peptide (1-14) were shown to interact with liposomes containing phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) with high affinity (Kd = 50 nM) in a Ca^{2+}-dependent manner (O'Callaghan et al. 2005). Expression of hippocalcin(1-14)-ECFP (enhanced cyan fluorescent protein) partially displaced the pleckstrin homology (PH) domain of phospholipase δ1, a PtdIns(4,5)P_2-specific binding partner, from the plasma membrane in living cells. These results suggest that the interaction with PtdIns(4,5)P_2 is likely to be a physiologically significant event contributing to the targeting of hippocalcin.

Hippocalcin has been shown to interact with ▶ MLK (mixed-lineage kinase) 2, neuronal apoptosis inhibitory protein (NAIP), and the β2-adaptin subunit of the AP2 adaptor complex (Nagata et al. 1998; Lindholm et al. 2002; Palmer et al. 2005). Hippocalcin also interacts with the microsomal cytochrome b5, calmodulin (CaM)-dependent cyclic nucleotide 3′,5′-phosphodiesterase (PDE), and CAPS1, a calcium-dependent activator protein involved in secretion. However, the functional consequences of these interactions have not yet been determined (Kobayashi and Takamatsu 2009).

Distribution

Hippocalcin is expressed in various regions of the rat hippocampus, as well as other regions of the brain (Kobayashi et al. 1992; Kobayashi and Takamatsu 2009). In the hippocampus, hippocalcin mRNA and immunoreactivity are detected at high levels in the pyramidal cells of Ammon's horn and at moderate levels in the granule cells of the dentate gyrus. Hippocalcin is also found at significant levels in other regions of the brain, including the pyramidal cells of cerebral cortex layers II–VI, the caudate-putamen, taenia tecti, claustrum, olfactory tubercle, anterior olfactory nucleus and olfactory bulb, as well as in the ganglion cell layer and amacrine cell layer in the retina, and in the apical layer in the olfactory epithelium. No hippocalcin protein expression has been detected outside of the central nervous system, although expressed sequence tag (EST) sequences have been found in a few peripheral tissues (Braunewell and Klein-Szanto 2009).

In hippocampal pyramidal cells, hippocalcin is located in the cytoplasm and associated with the

plasma membrane of the cell body, dendrites and axon. The hippocalcin concentration in hippocampal pyramidal cells is estimated to be greater than 30 μM. Hippocalcin reversibly translocates from the cytosol to the plasma membrane and the trans-Golgi network of the perinuclear region in transfected HeLa cells and in hippocampal cells via the calcium myristoyl switch mechanism (O'Callaghan et al. 2003).

Physiological Functions

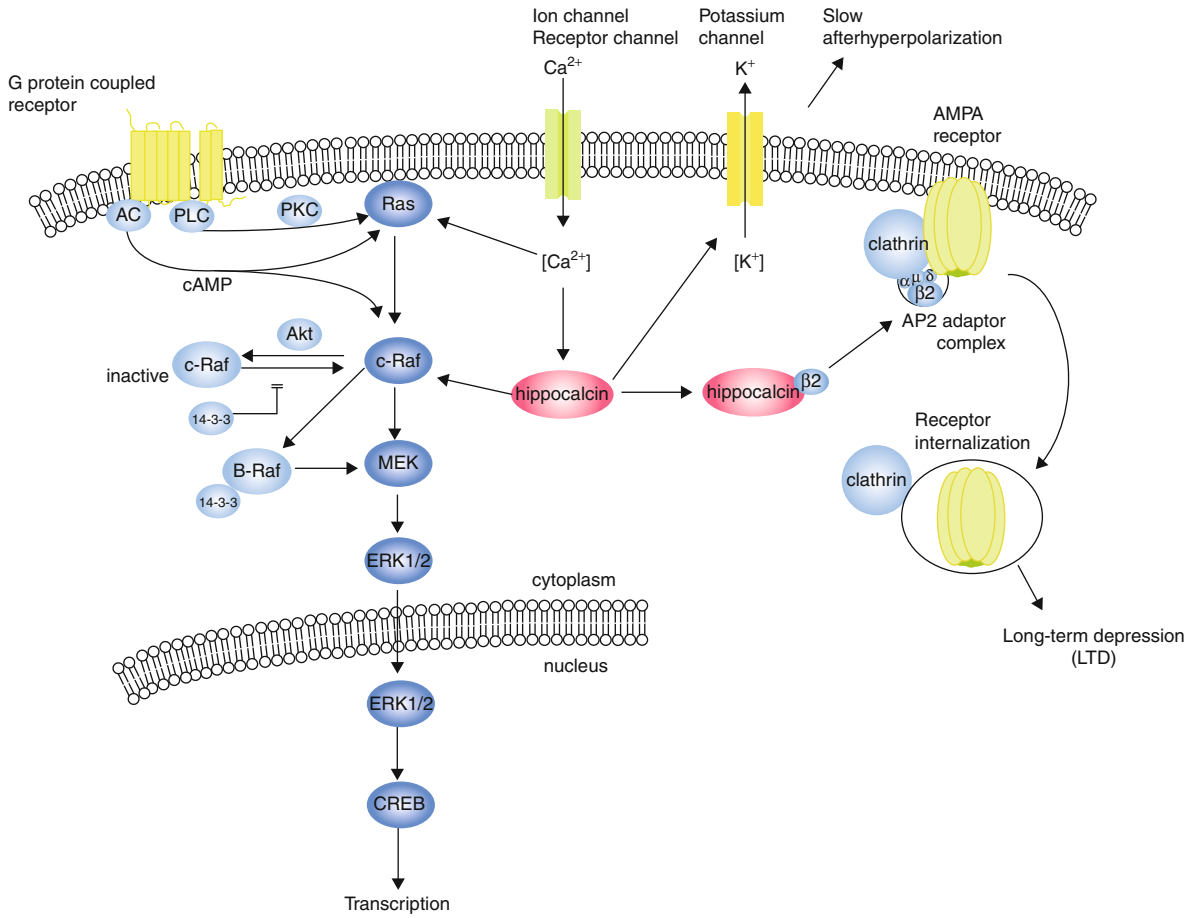
Roles in Neural Plasticity

The hippocampus is an important cortical region for associative learning and memory, and is especially important in the performance of spatial positioning discrimination. Hippocalcin may be involved in these functions. Hippocalcin deficient (hippocalcin$^{-/-}$) mice displayed deficits in spatial memory and working (trial-dependent) associative memory (Kobayashi et al. 2005). Hippocalcin may be involved in memory formation via regulation of extracellular signal-regulated kinase (ERK) cascade. In hippocalcin$^{-/-}$ mice, NMDA stimulation- and depolarization-induced phosphorylation of the cAMP response element binding protein (▶ CREB) was attenuated in hippocampal neurons (Kobayashi et al. 2005). Impaired CREB activation may be caused by a malfunctioning ERK cascade, since an ERK cascade inhibitor blocked stimulation-dependent CREB phosphorylation in control hippocampal slices but not in hippocalcin$^{-/-}$ mice. When the Ca^{2+}-dependent Ras/Raf/▶ MEK/ERK signaling cascade was further examined, no direct effect of hippocalcin on Raf-1 kinase or MEK kinase was observed (Noguchi et al. 2007). Hippocalcin also had no effect on the activation of Ras. However, hippocalcin$^{-/-}$ mice do display a defect in NMDA- and depolarization-induced activation of Raf-1 kinase and ERK. Therefore, hippocalcin may act on an alternative Raf-1 kinase activation pathway, such as protein kinase B (▶ PKB)/Akt or 14-3-3 protein activation of Raf-1. Notably, hippocalcin, in conjunction with the small GTPase Cdc42, led to an increase in calcium-dependent ▶ phospholipase D activation. In NIH3T3 cells, phospholipase D activation induced by overexpression of hippocalcin was dependent on upregulation of phospholipase D expression via activation of the ERK cascade (Oh et al. 2006). These results indicate that hippocalcin affects activity-dependent gene expression via regulation of ▶ MAP kinase signaling (see Fig. 1).

Moreover, hippocalcin has been implicated in hippocampal NMDA receptor-dependent long-term depression (LTD) (Palmer et al. 2005; Fig. 1). Hippocalcin binds directly to the β2-adaptin subunit of the AP2 adaptor complex, which couples clathrin to the cytosolic domains of membrane-bound proteins destined to be internalized. In hippocampal neurons, this hippocalcin-AP2 complex bound only to the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type ▶ glutamate receptor. The calcium-dependent endocytosis of the AMPA receptor is a key event in N-methyl-D-aspartate (NMDA) receptor-dependent LTD. Expression of a mutated hippocalcin lacking all EF-hand structures in CA1 pyramidal neurons blocked synaptically evoked LTD without affecting basal AMPA receptor-mediated transmission or long-term potentiation (LTP). In addition, hippocalcin is the key intermediate between calcium influx in response to a train of action potentials and potassium channel activation, which mediates the slow after-hyperpolarization current (IsAHP) in hippocampal neurons (Tzingounis et al. 2007; Fig. 1). Brief depolarizations, which are sufficient to activate IsAHP in wild-type mice, did not elicit IsAHP in hippocalcin$^{-/-}$ mice. Introduction of hippocalcin into cultured rat hippocampal neurons led to a pronounced IsAHP, while neurons expressing a mutant hippocalcin lacking N-terminal myristoylation exhibited a small IsAHP similar to that recorded in uninfected neurons, confirming that hippocalcin gates the potassium channel that mediates IsAHP. These results demonstrate the involvement of hippocalcin in activity-dependent plasticity and memory formation.

Protection Against Neuronal Damage

Hippocalcin has been shown to have neuroprotective effects in several studies (see Fig. 2). Hippocalcin interacts with the neuronal apoptosis inhibitory protein (NAIP). Overexpression of hippocalcin alone did not substantially enhance cell survival, but co-expression of both hippocalcin and NAIP synergistically facilitates neuronal survival against calcium-induced cell death stimuli, such as ionomycin and thapsigargin. The interaction between hippocalcin and NAIP rescues neuroblastoma cells from calcium-induced cell death, but no significant effect on neuronal death induced by nerve

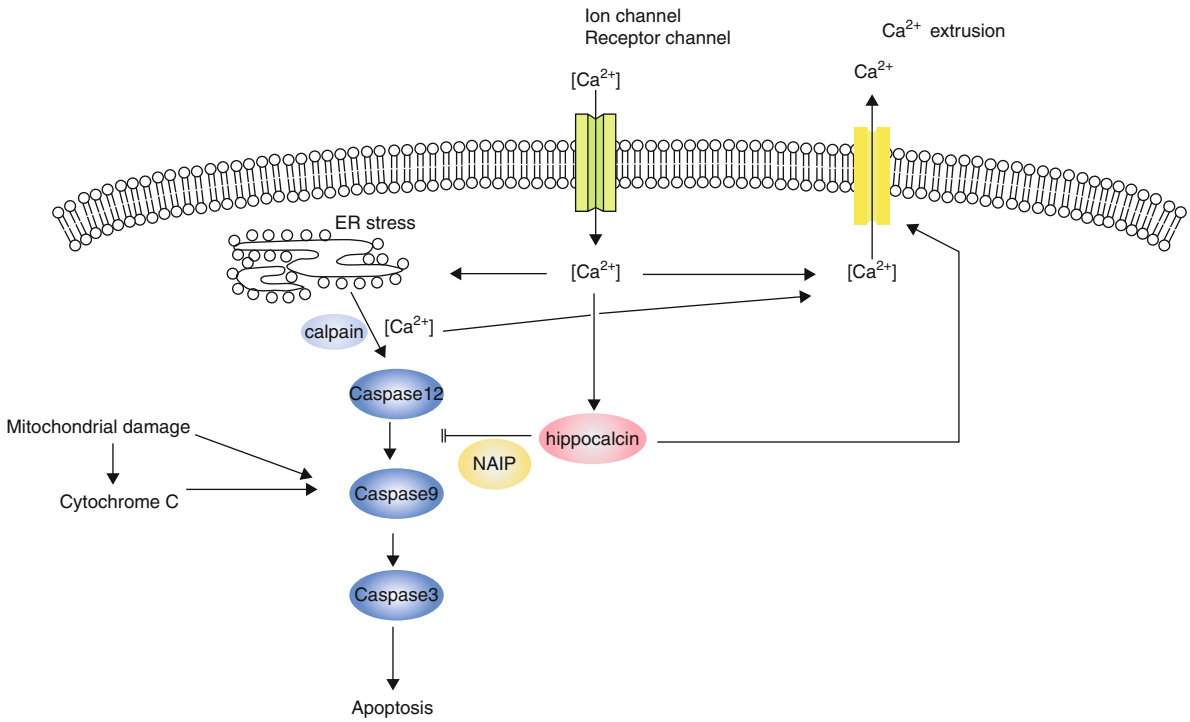


Hippocalcin, Fig. 1 Involvement of hippocalcin in neuronal excitability. *AC* = adenylyl Cyclase; *PLC* = phospholipase C; *PKC* = protein kinase C; *G protein* = guanine nucleotide-binding protein; *β2* = β2-adaptin subunit of the AP2 adaptor complex; *ERK* = extracellular signal-regulated kinase; *CREB* = cAMP response element binding protein

growth factor (NGF) withdrawal has been observed in sympathetic neurons (Lindholm et al. 2002). In hippocalcin$^{-/-}$ mice, systemic injection of kainic acid results in an increase in seizure-induced neuronal cell death in the CA3 field of Ammon's horn accompanied by increased caspase-3 activation (Korhonen et al. 2005). Injection of quinolinic acid, an NMDA receptor agonist, into the hippocampal CA1 region of hippocalcin$^{-/-}$ mice caused high levels of cell death in CA1 neurons (Masuo et al. 2007). Cultured hippocalcin$^{-/-}$ hippocampal neurons exhibited reduced levels of survival under basal culture conditions accompanied by an increase in caspase 12 activation, which can be caused by increased endoplasmic reticulum (ER) stress (Korhonen et al. 2005). A decrease in the survival rate of hippocalcin$^{-/-}$ hippocampal neurons was also observed independently, in a study in which the measurement of intracellular calcium in single cells revealed that calcium extrusion from hippocalcin$^{-/-}$ neurons was slower than that from wild-type neurons (Masuo et al. 2007). The involvement of hippocalcin in the upregulation of calcium extrusion was confirmed using hippocalcin-expressing COS7 cells. Thus, hippocalcin protects hippocampal neurons against various types of calcium-induced cell damage by interacting with NAIP, diminishing ER stress, and upregulating calcium extrusion (Fig. 2).

Regulation of Cyclic Nucleotide Signaling

Hippocalcin has been shown to modulate ▶ adenylyl cyclase and ▶ guanylyl cyclase activities in the olfactory epithelium where cilia of mature olfactory receptor neurons reside (see Fig. 3). In the olfactory cilia, odorant-induced activation of adenylyl cyclase and

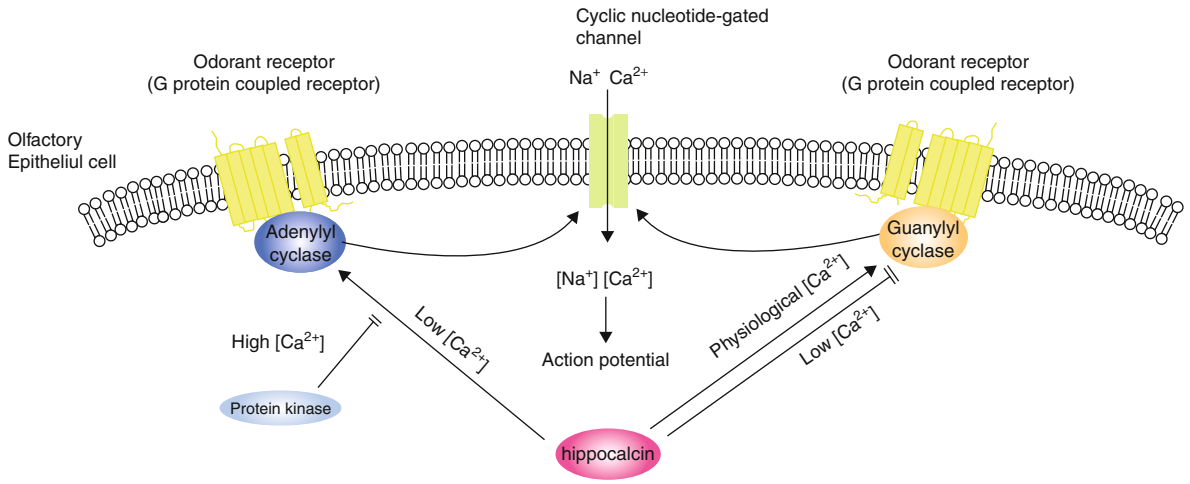


Hippocalcin, Fig. 2 Involvement of hippocalcin in the neuronal apoptosis induction pathway. *NAIP* = neuronal apoptosis inhibitory protein; *ER* = endoplasmic reticulum

guanylyl cyclase results in generation of cAMP and cGMP, respectively. As the levels of cyclic nucleotides increase, olfactory cyclic nucleotide-gated channels open to allow an influx of sodium and calcium ions, leading to the generation of an action potential. Hippocalcin increased adenylyl cyclase activity in olfactory cilia at low calcium levels and decreased adenylyl cyclase activity at high calcium levels in reconstitution system (Mammen et al. 2004). At low calcium levels, protein kinase treatment of cilia inhibited the effect of hippocalcin on adenylyl cyclase activity, whereas untreated cilia or protein phosphatase-treated cilia showed increases in hippocalcin-mediated adenylyl cyclase activity. At high calcium levels, only protein phosphatase-treated cilia showed significant increases in hippocalcin-mediated adenylyl cyclase activity. Calcium influx by odorant stimulation induces phosphorylation of adenylyl cyclase, which may reduce adenylyl cyclase activity and be involved in the mechanisms of odorant adaptation. In contrast to the effect on adenylyl cyclase, hippocalcin significantly inhibited the activity of particulate guanylyl cyclase activity within a limited range of free calcium concentrations (1–10 nM) (Mammen et al. 2004). The inhibitory effect of hippocalcin on guanylyl cyclase activity decreased as calcium levels increased. At physiological levels of calcium, hippocalcin increased guanylyl cyclase activity with an EC_{50} of 0.5 μM calcium (Krishnan et al. 2009). Studies using hippocalcin$^{-/-}$ mice demonstrated the involvement of hippocalcin in the total guanylyl cyclase-mediated signal transduction pathways to be approximately 30% (Krishnan et al. 2009). Thus, hippocalcin may be involved in the fine-tuning of stimulus detection and odor adaptation mediated by cyclic nucleotide signaling in the olfactory epithelium.

Involvement in Other MAP Kinase Cascade Signaling Pathway

Hippocalcin is involved in the mitogen-activated protein kinase (MAP kinase) pathway, including downstream gene expression (Braunewell and Klein-Szanto 2009; Kobayashi and Takamatsu 2009). In a yeast two-hybrid screen, hippocalcin was identified as a possible interacting partner of ▶ mixed-lineage kinase (MLK) 2 and MLK3 (Nagata et al. 1998; Kobayashi et al.



Hippocalcin, Fig. 3 Involvement of hippocalcin in cyclic nucleotide signaling pathways in the olfactory epithelium. *G protein* = guanine nucleotide-binding protein

unpublished data). MLKs are closely related to the MAP kinase kinase kinase (MAPKKK) family. MLK2 and MLK3 interact with the small GTPases Rac and Cdc42. MLK2 and MLK3 also interact with motor proteins of the kinesin superfamily and co-localize with the microtubule cytoskeleton, suggesting that they are involved in the regulation of cytoskeletal dynamics. MLK2 and MLK3 activate c-Jun N-terminal kinase (JNK), ERK, and p38 MAPK. The interaction of hippocalcin with MLKs is not calcium-dependent and has no influence on the kinase activity of MLKs in resting hippocalcin-transfected cells (Nagata et al. 1998). A recent report shows that hippocalcin and MLK2 are colocalized in the halo surrounding Lewy bodies in patients with Parkinson's disease (PD) (Nagao and Hayashi 2009). The fact suggests the association of both proteins with pathogenesis in PD; however, the molecular basis has not yet been determined.

Summary

Hippocalcin, a member of the NCS (neuronal calcium sensor) protein family, which is predominantly expressed in the hippocampus, is a highly conserved protein that has an identical amino acid sequence in rat, mouse, bovine, and human. Hippocalcin has three functional EF-hand calcium-binding domains and its N-terminal glycine residue is myristoylated. Hippocalcin is regulated by a calcium myristoyl switch mechanism within cells, allowing it to translocate from the cytosol to intracellular membranes of the trans-Golgi network and to the plasma membrane in response to an increase in free calcium. Hippocalcin is involved in the activity-dependent activation of the MAP kinase pathway via of ERK signaling and activation of CREB, key gene expression events mediating the long lasting synaptic plasticity underlying learning and memory. Spatial and associative learning abilities in hippocalcin-null mutant (hippocalcin$^{-/-}$) mice are impaired, as determined by the probe test, Morris water maze, and visual discrimination learning tasks. Hippocalcin is implicated in hippocampal NMDA receptor-dependent LTD via binding the β2-adaptin subunit of the AP2 adaptor complex, which mediates internalization of the GluR1 subunit of AMPA receptor. The slow after-hyperpolarization current (IsAHP), which is activated by brief depolarizations, was not elicited in hippocalcin$^{-/-}$ hippocampal neurons. This observation indicates that hippocalcin mediates potassium channel activation in response to calcium influx. Hippocalcin exhibits a protective effect against calcium-induced cell death by interacting with NAIP, diminishing ER stress, and upregulating calcium extrusion. Hippocalcin is also expressed in olfactory receptor neurons and has been found to regulate the activities of ciliary adenylyl cyclase and particulate guanylyl cyclase in a calcium-dependent manner. Thus, hippocalcin may act as a multifunctional modulator in calcium signaling pathways, such as those underlying neuronal plasticity, neuronal excitability, neuronal cell death, and olfaction.

References

Braunewell KH, Klein-Szanto AJ. Visinin-like proteins (VSNLs): interaction partners and emerging functions in signal transduction of a subfamily of neuronal Ca^{2+} -sensor proteins. Cell Tissue Res. 2009;335:301–16. doi:10.1007/s00441-008-0716-3.

Burgoyne RD. Neuronal calcium sensor proteins: generating diversity in neuronal Ca^{2+} signalling. Nat Rev Neurosci. 2007;8:182–93. doi:10.1038/nrn2093.

Kobayashi M, Hippocalcin TK. UCSD-Nature molecule pages. 2009. doi:10.1038/mp.a004097.01.

Kobayashi M, Takamatsu K, Saitoh S, Miura M, Noguchi T. Molecular cloning of hippocalcin, a novel calcium-binding protein of the recoverin family exclusively expressed in hippocampus. Biochem Biophys Res Commun. 1992;189:511–7. doi:10.1016/0006-291X(92)91587-G.

Kobayashi M, Takamatsu K, Saitoh S, Noguchi T. Myristoylation of hippocalcin is linked to its calcium-dependent membrane association properties. J Biol Chem. 1993;268:18898–904.

Kobayashi M, Masaki T, Hori K, Masuo Y, Miyamoto M, Tsubokawa H, et al. Hippocalcin-deficient mice display a defect in cAMP response element-binding protein activation associated with impaired spatial and associative memory. Neuroscience. 2005;133:471–84. doi:10.1016/j.neuroscience.2005.02.034.

Korhonen L, Hansson I, Kukkonen JP, Brännvall K, Kobayashi M, Takamatsu K, et al. Hippocalcin protects against caspase-12-induced and age-dependent neuronal degeneration. Mol Cell Neurosci. 2005;28:85–95. doi:10.1016/j.mcn.2004.08.015.

Krishnan A, Duda T, Pertzev A, Kobayashi M, Takamatsu K, Sharma RK. Hippocalcin, new Ca(2+) sensor of a ROS-GC subfamily member, ONE-GC, membrane guanylate cyclase transduction system. Mol Cell Biochem. 2009;325:1–14. doi:10.1007/s11010-008-0015-z.

Lindholm D, Mercer EA, Yu LY, Chen Y, Kukkonen J, Korhonen L, et al. Neuronal apoptosis inhibitory protein: Structural requirements for hippocalcin binding and effects on survival of NGF-dependent sympathetic neurons. Biochim Biophys Acta. 2002;1600:138–47. doi:10.1016/S1570-9639(02)00454-5.

Mammen A, Simpson PJ, Mamman A, Simpson JP, Nighorn A, Imanishi Y, et al. Hippocalcin in the olfactory epithelium: a mediator of second messenger signaling. Biochem Biophys Res Commun. 2004;322:1131–9. doi:10.1016/j.bbrc.2004.07.123.

Markova O, Fitzgerald D, Stepanyuk A, Dovgan A, Cherkas V, Tepikin A, et al. Hippocalcin signaling via site-specific translocation in hippocampal neurons. Neurosci Lett. 2008;442:152–7. doi:10.1016/j.neulet.2008.06.089.

Masuo Y, Ogura A, Kobayashi M, Masaki T, Furuta Y, Ono T, et al. Hippocalcin protects hippocampal neurons against excitotoxin damage by enhancing calcium extrusion. Neuroscience. 2007;145:495–504. doi:10.1016/j.neuroscience.2006.12.011.

Nagao M, Hayashi H. Mixed lineage kinase 2 and hippocalcin are colocalized in Lewy bodies of Parkinson's disease. J Neurol Sci. 2009;281:51–4.

Nagata K, Puls A, Futter C, Aspenstrom P, Schaefer E, Nakata T, et al. The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3. EMBO J. 1998;17:149–58. doi:10.1093/emboj/17.1.149.

Noguchi H, Kobayashi M, Miwa N, Takamatsu K. Lack of hippocalcin causes impairment in Ras/extracellular signal-regulated kinase cascade via a Raf-mediated activation process. J Neurosci Res. 2007;85:837–44. doi:10.1002/jnr.21180.

O'Callaghan DW, Tepkin AV, Burgoyne RD. Dynamics and calcium sensitivity of the Ca^{2+}/myristoyl switch protein hippocalcin in living cells. J Cell Biol. 2003;163:715–21. doi:10.1083/jcb.200306042.

O'Callaghan DW, Haynes LP, Burgoyne RD. High-affinity interaction of the N-terminal myristoylation motif of the neuronal calcium sensor protein hippocalcin with phosphatidylinositol 4,5-bisphosphate. Biochem J. 2005;391:231–8. doi:10.1042/BJ20051001.

Oh DY, Yon C, Oh KJ, Lee KS, Han JS. Hippocalcin increases phospholipase D2 expression through extracellular signal-regulated kinase activation and lysophosphatidic acid potentiates the hippocalcin-induced phospholipase D2 expression. J Cell Biochem. 2006;97:1052–65. doi:10.1002/jcb.20665.

Palmer CL, Lim W, Hastie PG, Toward M, Korolchuk VI, Burbidge SA, et al. Hippocalcin functions as a calcium sensor in hippocampal LTD. Neuron. 2005;47:487–94. doi:10.1016/j.neuron.2005.06.014.

Tzingounis AV, Kobayashi M, Takamatsu K, Nicoll RA. Hippocalcin gates the calcium activation of the slow afterhyperpolarization in hippocampal pyramidal cells. Neuron. 2007;53:487–93. doi:10.1016/j.neuron.2007.01.011.

HM145

► Chemokine Receptor CCR1

HM63

► FPR2/ALX

HMAT1

► Pea15

hnRNP D

► hnRNP D (AUF1)

hnRNP D (AUF1)

Jennifer Defren and Gary Brewer
Department of Molecular Genetics, Microbiology and Immunology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ, USA

Synonyms

AUF1; hnRNP D; Heterogeneous nuclear ribonucleoprotein D

Historical Background

AUF1, or hnRNP D (heterogeneous nuclear ribonucleoprotein D), was one of the first *trans*-acting factors identified that binds AU-rich elements (AREs) within the 3′ UTR (UTR – untranslated region) of many labile mRNAs (Brewer 1991). The importance of AREs in promoting rapid RNA turnover has been well characterized (Guhaniyogi and Brewer 2001). A mechanism was still under investigation when AUF1 was discovered. AUF1 as an ARE-binding protein (AUBP) was first identified in specific fractions of cytosol separated by sucrose gradient fractionation (Brewer 1991). Electrophoretic mobility shift assays (EMSAs) of these fractions revealed 37 and 40 kDa (kD) polypeptides that specifically bound ARE-containing *c-*►*myc* mRNA. Cell-free decay assays confirmed *c-myc* mRNA degradation occurred within the same fractions that included the ARE-binding activity. The aforementioned polypeptides, now referred to as p37 and p40, were reproducibly purified from the post-ribosomal, 130,000 × g supernatant, S130, by poly-U agarose chromatography. These polypeptides could cross-link in specific fashion to radiolabeled ARE-containing RNA substrates, including *c-fos* and GM-CSF 3′UTRs, with the same efficiency seen in the EMSAs (Brewer 1991). Cross-linking could be abolished by mutating the ARE sequence.

An antibody was generated from the poly-U agarose eluate and affinity-purified (Zhang et al. 1993). It detected p37 and p40, as well as a 45-kD polypeptide. Determination of their subcellular localization by cell fractionation and Western blot showed that p37 and p40 were present in both the nucleus and cytoplasm, while p45 was present exclusively in nuclear fractions, thereby explaining why it wasn't detected initially in the cytosolic S130 fractions.

Screening of a HeLa cDNA expression library with the affinity-purified p40 antibody allowed molecular cloning of p37, which was named AUF1 (ARE/poly (U)-binding/degradation factor 1) and later designated hnRNP D (Zhang et al. 1993; Wagner et al. 1998). Sequence analysis indicated that p37 was an RNA-binding protein distinct from hnRNP A, B, or C. Additional cDNA cloning and sequencing of genomic clones revealed that alternative splicing of the AUF1 pre-mRNA accounted for p37, p40, and p45, as well as a fourth isoform, p42 (Wagner et al. 1998).

Binding of purified AUF1 to AREs in vitro correlated with rapid decay of the transcript (DeMaria and Brewer 1996). Many short-lived cytokine mRNAs contain one or more AREs in their 3′UTR, generally consisting of repeats of overlapping AUUUA pentamers embedded within U-rich sequence (Guhaniyogi and Brewer 2001). EMSAs with cytoplasmic lysates of monocytes revealed that AUF1 bound to the ARE-containing GROα and IL-1β chemokine/cytokine mRNAs as part of a multi-subunit complex (Sirenko et al. 1997). In human monocytes, these mRNAs remain very labile until the receipt of various extracellular stimuli, whereupon the bound AUF1 and associated protein complex changes in composition, followed by transcript stabilization.

The above studies, as well as others not included in this discussion for purposes of brevity, indicate an important role for AUF1 in regulating immune responses, as many ARE-containing cytokine mRNAs undergo rapid decay until extracellular signaling pathways indicate a need for their transient stabilization. During this window of short-term stability, cytokines are produced in much greater quantities until they are down-regulated again by rapid mRNA decay. Knockdown of AUF1 in mice adversely affects survival, as the mice are unable to suppress overproduction of proinflammatory cytokines (Lu et al. 2006a). The levels of cytokine transcripts, including TNFα and IL-1β, remain constitutively high; as such, the mice die of severe endotoxic shock due to the runaway effects of cytokine production.

Characterization of AUF1 Protein Structure and Function

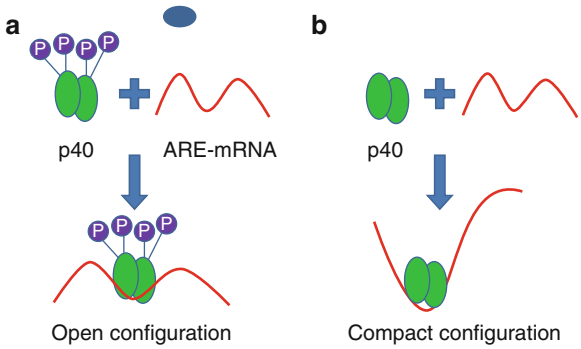
AUF1 Genomic Organization

The hnRNP D/AUF1 gene maps to human chromosome locus 4q21 and encodes four different isoforms by differential splicing of the pre-mRNA; a pseudogene lies on the X chromosome (Wagner et al. 1998; Dempsey et al. 1998). The isoforms differ in their molecular weights due to the inclusion or exclusion of specific exons in their respective mRNAs. All isoforms include a pair of RNA recognition motifs (RRMs) and a glutamine-rich region required for RNA binding and protein–protein interactions, respectively. A peptide sequence required for AUF1 dimerization in the absence of RNA is encoded by exon 1, present in all isoforms. The p40 and p45 isoforms contain an additional 19-amino acid insert encoded by exon 2 that in p40 can be phosphorylated in vitro by glycogen synthase kinase-3β and protein kinase A (Wilson et al. 2003a). The p42 and p45 isoforms have an additional 49-amino acid insert encoded by exon 7 toward the C-terminal end of the proteins, which in p45, confers protection from ubiquitination and subsequent degradation (Laroia and Schneider 2002).

Properties of AUF1 Isoforms

Differential splicing of exons 2 and 7 confers selective subcellular localization and ARE-binding affinities on the AUF1 isoforms (Zhang et al. 1993; Wagner et al. 1998). P37 and p40 are primarily found in the cytoplasm, while p42 and p45 are primarily nuclear. In terms of RNA-binding, the p37 isoform has the highest affinity for AREs, while p40 has the lowest. P42 has an affinity only slightly lower than p37, while the ARE-affinity of p45, though much lower than p37, is still much higher than p40. Altogether, the four isoforms exhibit an approximate 35-fold range of ARE-binding affinities (Wagner et al. 1998). The two isoforms with the highest binding affinity do not contain the exon 2-encoded amino acids.

The N-terminal exon 2 insert contains a pair of serines that can be reversibly phosphorylated on p40 – Ser 83 and Ser 87 (Wilson et al. 2003a,b). In vivo, the phosphates on the two serines are lost upon activation of various cell signaling pathways, correlating with an increase in ARE-mRNA stability, paralleled by changes in mRNA-protein structure (Fig. 1). In vitro studies indicate that p40 can bind to an ARE substrate as both a dimer and a tetramer (Wilson et al. 2003a). When p40 is phosphorylated, the bound RNA is held in an extended conformation versus a more compact configuration generated by the binding of unphosphorylated p40. In vivo, the extended conformation may allow easier access to the ARE by components of the cellular degradation machinery, subsequently accelerating mRNA decay.



hnRNP D (AUF1), Fig. 1 Phosphorylation of p40 induces changes in AUF1-mRNA conformation and accelerates ARE-mRNA decay. For simplicity, p40 is depicted as a dimer in the absence of other RNA-binding proteins. (**a**) When Ser 83 and Ser 87 are phosphorylated, the mRNA is held in an open conformation relative to the (**b**) more compact configuration generated by unphosphorylated bound AUF1. Note: AUF1 can also bind as tetramers to AREs in vitro. Phosphorylated p40 and an open mRNP conformation correlate with increased ARE-mRNA decay

AUF1 Can Compete with PABP in Binding to Polyadenylated Sequences

AUF1 cannot only promote mRNA decay via ARE binding, but may also function in dissociating PABP (poly-A binding protein) from transcript tails, depriving the mRNA of its 3′-end protection, and allowing digestion by deadenylases and exosomal proteins. Like PABP, AUF1 can also bind polyadenylated sequences. Affinity chromatography of Hela cytoplasmic lysates using poly(A) resin showed that the AUF1 isoforms could specifically bind the poly(A) sequence and not the control poly(C) resin (Sagliocco et al. 2006). This binding was not due to mRNA tethering, nor the amount of PABP present. Further analysis indicated endogenous AUF1 bound the poly(A) resin independently and non-consecutively of PABP, since PABP knockdown had no effect on the amount of AUF1 bound to the resin (Sagliocco et al. 2006).

In vitro, all recombinant AUF1 isoforms can associate cooperatively and sequentially in oligomeric fashion on an RNA substrate containing a poly(A) sequence 100 nucleotides long (Sagliocco et al. 2006). The presence or absence of an ARE in the substrate sequence does not affect the poly(A) binding affinity of AUF1. However, the presence of AUF1 can displace PABP on the poly (A) substrate via competitive binding in these EMSAs. Increasing AUF1 concentration reduced PABP-poly(A) complexes on the RNA substrate in a dose-dependent manner. Simultaneously, AUF1-poly(A) complexes increased. The above data indicating AUF1-PABP competition for the poly(A) tail is an additional step in regulating mRNA decay/stability.

ARE-mRNA Decay Mediated by AUF1 Is Linked to Translation Initiation Factors

AUF1 not only binds proteins at the 3′ end of the transcript like PABP, but also components of the translation initiation complex located at the 5′ end, hinting that translation also plays a role in ARE-mediated decay. Specifically, eIF4G can bind all AUF1 isoforms strongly in vitro, in the presence or absence of an ARE (Lu et al. 2006b), with p37 having the strongest affinity. Further experiments performed with truncation mutants of p37 (His-tagged) and a GST-tagged fragment of eIF4G in GST pulldown assays identified a 45-amino acid domain within the C-terminus of AUF1 necessary for eIF4G binding.

Besides binding to poly(A) sequences (see above), AUF1 can also directly bind to PABP itself in vitro (Lu et al. 2006b). The interaction can be abrogated by adding an RNA consisting of the TNFα ARE to the reaction, and to a lesser extent, by adding Hsp70, whose induction in vivo can stabilize mRNAs. In fact, AUF1 cannot interact with the ARE and PABP simultaneously in an in vitro assay. Interestingly, p37 and PABP can bind concurrently to eIF4G in the absence of an ARE. If an ARE is present, p37 detaches from PABP, leaving behind a stronger binding interaction between PABP and eIF4G. The dynamic AUF1-eIF4G-PABP complex implicates AUF1 in linking rapid mRNA decay to translation.

AUF1 Can Promote Translation of ARE-Containing mRNAs

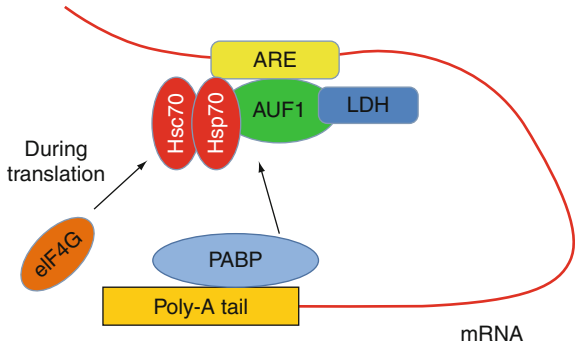
Posttranscriptional regulation of gene expression by AUF1 is not limited to accelerating mRNA decay. It can alternatively promote translation of ARE-mRNAs. For example, knocking down AUF1 expression does not affect *MYC* mRNA levels but rather reduces ▶ MYC protein levels at least threefold (Liao et al. 2007). By contrast, overexpression of the individual AUF1 isoforms, or all in combination, increases MYC protein levels by threefold or greater. As well, the presence of *MYC* mRNA on polyribosomes increases significantly upon overexpression of AUF1. Further analysis showed that the increase in *MYC* translation is dependent on the ARE. Together, these observations indicated roles for AUF1 in translational control.

The mechanism by which AUF1 promotes translation likely involves competitive binding with translational repressor/s. For example, TIAR knockdown increases *MYC* protein levels as well as the amount of AUF1 binding to the mRNA. Likewise, AUF1 knockdown increases the amount of TIAR protein bound to the transcript, resulting in reduced *MYC* translation. This reciprocal competition is ARE-dependent. However, the increase in MYC protein levels by TIAR knockdown is reversed by knocking down AUF1 simultaneously, with no effect on mRNA levels, suggesting that AUF1 can promote *MYC* translation directly and not solely by blocking TIAR binding.

AUF1 Binds to AREs as Part of a Multi-subunit Complex

AUF1 binds to ARE-containing mRNAs as part of a multi-subunit complex. As heat shock stabilizes ARE-mRNAs, it should not be surprising that AUF1 forms complexes with heat shock proteins Hsc70, Hsp70, and Hsp27 on polysomal mRNA (Laroia et al. 1999; Sinsimer et al. 2008). Hsp70 binding to AUF1 is concomitant with accumulation of stabilized ARE-mRNAs. Also, since heat shock downregulates the ubiquitin-proteosome pathway, which is necessary for AUF1-mediated mRNA decay, it is likely that ubiquitination and proteolysis of AUF1 itself is required for rapid mRNA degradation.

Other subunits of the ARE-bound protein complex include eIF4G and PABP (poly-A binding protein), which function in translation (Laroia et al. 1999). More recently, lactate dehydrogenase was identified as a protein that directly interacts with AUF1, though the significance is unclear (Pioli et al. 2002). The translation factor eIF4G is largely dissociated from the complex until heat-shock reduces its dissociation; this is accompanied by ARE-mRNA stabilization. The cross talk among subunits of the AUF1-containing



hnRNP D (AUF1), Fig. 2 Model for the regulation of ARE-mRNA decay by AUF1 and several known associating proteins. Hsp70/Hsc70, lactate dehydrogenase (LDH), PABP, and eIF4G are complexed with AUF1 to regulate ARE-mRNA decay. Some subunits may remain dissociated from the complex until needed for increased translation. eIF4G and PABP may promote a circular mRNP complex necessary for translational activation

complex of proteins and between proteins of various signaling pathways are still being elucidated, but it is apparent that ARE-mRNA decay is regulated by a complex process that is often AUF1-dependent and is affected by the rearrangements and posttranslational modifications of the ARE-bound protein complex.

Summary

AUF1 is a *trans*-acting factor that binds AU-rich elements within the 3′ UTRs of labile mRNAs. There are four isoforms generated by alternative pre-mRNA splicing: p37, p40, p42, and p45. They differ in their subcellular localizations and RNA-binding affinities, suggesting that each isoform has its own subset of target transcripts. AUF1 is needed to promote decay of many ARE-containing transcripts, not just encoding cytokines, but those encoding oncoproteins and anti-apoptotic factors as well (Lapucci et al. 2002). It does so as part of a multi-subunit, ARE-bound complex (Fig. 2). Its binding partners include Hsp70, Hsc70, Hsp27, eIF4G, PABP, lactate dehydrogenase, and other unidentified proteins. The AUF1-containing complex can also compete with RNA-binding proteins such as TIAR to control ARE-mRNA translation (Liao et al. 2007). AUF1-mediated mRNA decay is a complex process that is affected by heat shock, the ubiquitin-proteosome pathway, and posttranslational modification and/or subunit rearrangement of AUF1 and its associated proteins.

References

Brewer G. An A + U-rich element RNA-binding factor regulates c-*myc* mRNA stability in vitro. Mol Cell Biol. 1991;11:2460–6.

DeMaria C, Brewer G. AUF1 binding affinity to A + U-rich elements correlates with rapid mRNA degradation. J Biol Chem. 1996;271:12179–84.

Dempsey L, Li M, DePace A, Bray-Ward P, Maizels N. The human *HNRPD* locus to 4q21 and encodes a highly conserved protein. Genomics. 1998;49:378–84.

Guhaniyogi J, Brewer G. Regulation of mRNA stability in mammalian cells. Gene. 2001;265:11–23.

Lapucci A, Donnini M, Papucci L, Witort E, Tempestini A, Bevilacqua A, et al. AUF1 is a *bcl-2* A + U-rich element-binding protein involved in *bcl-2* mRNA destabilization during apoptosis. J Biol Chem. 2002;277:16139–46.

Laroia G, Schneider R. Alternate exon insertion controls selective ubiquitination and degradation of different AUF1 protein isoforms. Nucleic Acids Res. 2002;30:3052–8.

Laroia G, Cuesta R, Brewer G, Schneider R. Control of mRNA decay by heat-shock-ubiquitin-proteosome pathway. Science. 1999;284:499–502.

Liao B, Hu Y, Brewer G. Competitive binding of AUF1 and TIAR to *MYC* mRNA controls its translation. Genes Dev. 2007;20:3174–84.

Lu J, Sadri N, Schneider R. Endotoxic shock in AUF1 knockout mice mediated by failure to degrade proinflammatory cytokine mRNAs. Genes Dev. 2006a;20:3174–84.

Lu J, Bergman N, Sadri N, Schneider R. Assembly of AUF1 with eIF4G-poly(A) binding protein complex suggests a translation function in AU-rich mRNA decay. RNA. 2006b;12:883–93.

Pioli P, Hamilton BJ, Connolly J, Brewer G, Rigby W. Lactate dehydrogenase is an AU-rich element-binding protein that directly interacts with AUF1. J Biol Chem. 2002;277:35738–45.

Sagliocco F, Laloo B, Cosson B, Laborde L, Castroviejo M, Rosenbaum J, et al. The ARE-associated factor AUF1 binds poly(A) *in vitro* in competition with PABP. Biochem J. 2006;400:337–47.

Sinsimer K, Gratacós F, Knapinska A, Lu J, Krause C, Wierzbowski A, et al. Chaperone Hsp27, a novel subunit of AUF1 protein complexes, functions in AU-Rich element-Mediated mRNA decay. MCB. 2008;28:5233–7.

Sirenko O, Lofquist A, DeMaria C, Morris J, Brewer G, Haskill JS. Adhesion-dependent regulation of an A + U-rich element-binding activity associated with AUF1. Mol Cell Biol. 1997;17:3898–906.

Wagner B, DeMaria C, Sun Y, Wilson G, Brewer G. Structure and genomic organization of the human AUF1 gene: alternative pre-mRNA splicing generates four protein isoforms. Genomics. 1998;48:195–202.

Wilson G, Lu J, Sutphen K, Suarez Y, Sinha S, Brewer B, et al. Phosphorylation of $p40^{AUF1}$ regulates binding to A + U-rich mRNA destabilizing elements and protein-induced changes in ribonucleoprotein structure. J Biol Chem. 2003a; 278:33039–48.

Wilson G, Lu J, Sutphen K, Sun Y, Huynh Y, Brewer G. Regulation of A + U-rich element-directed mRNA turnover

involving reversible phosphorylation of AUF1. J Biol Chem. 2003b;278:33029–38.
Zhang W, Wagner B, Ehrenman K, Schaeffer A, DeMaria C, Crater D, et al. Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol Cell Biol. 1993;13:7652–65.

Homer

Simon Kaja[1], Andrew J. Payne[1], Stephanie L. Burroughs[1] and Peter Koulen[2]
[1]Department of Ophthalmology, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA
[2]Department of Ophthalmology and Department of Basic Medical Science, University of Missouri - Kansas City School of Medicine, Kansas City, MO, USA

Synonyms

Cupidin; Vesl

Historical Background

Vesl/Homer proteins are a family of scaffolding molecules, encoded by three genes (Homer1, Homer2, and Homer3) that are abundantly expressed in a variety of tissues, including the brain, retina, cardiac muscle, skeletal muscle, smooth muscle, liver, kidneys, spleen, testis, thymus, placenta, and intestine. Their primary function is to cluster proteins and modulate their activity. Despite their system-wide expression, Homer proteins are best characterized in the brain and the central nervous system, where their primary role is to cluster and modulate the function of synaptic proteins.

Molecular Determinants of Vesl/Homer Proteins

In mammals, Vesl/Homer proteins are encoded by three genes, Homer1, Homer2, and Homer3 that give rise to at least 22 splice variants. Homer1a and Ania-3 are short isoforms encoded by the Homer1 gene (Sgambato-Faure et al. 2006; Duncan et al. 2005). Both splice variants were originally identified after induction of their expression levels following excitatory synaptic activity, including induced convulsive seizures, long-term potentiation, and ischemic injury. Interestingly, compared to other immediate early genes (IEGs) that typically encode transcription factors, Homer1a and Ania-3 are synaptic proteins that can directly modulate protein–protein interactions (Sgambato-Faure et al. 2006; Duncan et al. 2005).

Other, typically long Homer isoforms are constitutively expressed and likely fulfill different functions compared with the short IEG isoforms of Homer1 (Duncan et al. 2005).

Homer proteins contain various conserved protein/protein interaction sites. Isoforms both short and long possess an Ena/vasodilator-stimulated phosphoprotein homology-1 (EVH1) domain within the first 110 N-terminal amino acids. The EVH1 domain is evolutionarily conserved and mediates the binding of Homer to its interaction partner. Through the EVH1 domain, Homer binds receptors and channel proteins such as the metabotropic ▶ glutamate receptor (mGluR), the inositol triphosphate (IP_3R), the ▶ ryanodine receptors (RyR), and transient receptor potential canonical channels (TRPCs). Other targets of Homer include GTPases, anchoring proteins such as shank, as well as transcription factors, including nuclear factor of activated T cells (NFAT). Furthermore, the Homer EVH1 domain shares the RX_5GLGF domain found in PDZ proteins (PSD/Disks/ZO-1 [epithelial tight junction protein]), which is thought to ensure correct targeting of ion channels.

All long Homer isoforms also share a C-terminal coiled-coil (CC) domain containing two leucine zipper motifs, which mediate the multimerization between Homer monomers. It is thought that long Homer isoforms form tetrameric structures with their CC domains aligned in parallel, exposing four EVH1 domains for ligand binding (Hayashi et al. 2006).

Expression and Localization of Homer Isoforms

Homer proteins are present in many different tissues, both excitable and non-excitable. In the brain, Homer isoforms are expressed abundantly and at similar levels in cortex, hippocampus, and cerebellum. All isoforms are particularly highly expressed in cerebellar Purkinje cells. Similarly, all Homer proteins have been detected

in skeletal and smooth muscle as well as heart. In non-excitable tissues, Homer protein expression has been reported for kidney, intestine, lung, spleen, liver, thymus, ovary, and testis. The expression of Homer isoforms has been reviewed in detail by Duncan et al. (2005).

Localization studies have shown that all Homer isoforms co-localize with their binding partner Group I mGluRs in the postsynaptic density of excitatory synapses in the brain (as reviewed in Duncan et al. (2005)). Specifically, Homer proteins are predominantly localized to the soma and apical dendrites. Subcellular fractionation studies have detected all Homer isoforms in the crude nuclear pellet, synaptosomal pellet, microsomal pellet, and PSD fraction. The additional presence of Homer2a/b in the soluble and synaptic vesicle fractions suggest a role for Homer 2 in receptor trafficking.

Following ischemic injury to the retina, Homer-1c was identified as an early marker for subtle changes prior to more severe neurodegenerative events (Kaja et al. 2003).

Binding Partners of Homer Proteins

Homer proteins interact with a variety of synaptic proteins. Particularly, the interaction of Homer proteins with calcium channels and neurotransmitter receptors both at the plasma membrane and at the endoplasmic reticulum (ER) has recently attracted interest due to the possible involvement of these interactions in age-related diseases of the nervous system.

Inositol-1,4,5-trisphosphate receptors (IP_3Rs) are intracellular ER channels of which activation results in calcium release from the ER lumen to the cytosol. Homer 1 proteins directly interact with the C-terminus of the IP_3R and decrease calcium flux through the channel (Duncan et al. 2005). Interaction with Homer-1c potentiates IP_3R activation (Tanaka et al. 2006), resulting in a decrease in dendritic branching (Duncan et al. 2005; Tanaka et al. 2006). The short isoform of Homer-1a has been shown to antagonize this direct interaction, resulting in reduced IP_3R activity and a concomitant increase in dendritic branching (Duncan et al. 2005; Tanaka et al. 2006).

Similarly to IP_3Rs, ▶ ryanodine receptors (RyRs) are large endoplasmic channels that extrude calcium from the ER lumen into the cytosol. Sequence analysis of RyRs has shown a number of EVH1 binding domains and a direct interaction between Homer and RyR has been experimentally verified (reviewed in Pouliquin and Dulhunty (2009)). Binding of Homer proteins to the RyR provides for a physical tethering of the RyR to the cytoskeleton, and for RyR crosstalk with plasma membrane proteins (Duncan et al. 2005; Pouliquin and Dulhunty 2009). However, the direct effects of Homer proteins on calcium release through the RyR are subject to controversy and speculation (Duncan et al. 2005; Pouliquin and Dulhunty 2009; Westhoff et al. 2003; Hwang et al. 2003).

Group 1 metabotropic glutamate receptors (mGluRs) are plasma membrane neurotransmitter receptors, which when activated initiate a pathway resulting in calcium release through the IP_3R. Homer proteins have been found to traffic Group 1 mGluRs to the plasma membrane of neurons (Duncan et al. 2005). mGluR binding of long Homer isoforms (specifically Homer-1b and Homer-1c) creates a ready pool of mGluRs on the ER membrane. Upregulation of Homer-1a displaces the long Homer isoforms and facilitates the trafficking of mGluR to the plasma membrane (Duncan et al. 2005; Kammermeier 2008). Competition between long and short Homer isoforms hence serves as a cellular mechanism of regulating neuronal excitability in response to stress and/or injury. Recent data furthermore indicates that Homer proteins facilitate the cross talk between mGluRs and *N*-methyl-D-aspartic acid NMDA receptors (Bertaso et al. 2010), corroborating the modulatory role of Homer in excitatory glutamate signaling.

Transient receptor potential canonical (TRPC) channels are plasma membrane, nonspecific cation channels. Interestingly, TRPC channels have the requisite EVH1 sites at both the N- and C-termini to facilitate the cooperative binding of Homer proteins. Binding of the long Homer isoform inhibits the TRPC channel, where the short isoform has the opposite effect (Duncan et al. 2005). An upregulation of Homer-1a in response to depletion of ER calcium stores thus provides a mechanism for activation TRPC channels and subsequent store refilling (Duncan et al. 2005; Rychkov and Barritt 2007). Binding of Homer to polycystin-1 has implications for regulation of intracellular calcium signaling in a number of cell types and organs and has been reported for the central nervous system (Stokely et al. 2006).

As discussed previously, Homer proteins interact and link plasma membrane receptors/channels to

intracellular ER channels at neuronal synapses. In addition, Homer proteins also interact with a variety of other proteins that facilitate and/or modulate synaptic function.

The *Dynamins* form part of a superfamily of proteins that participate in membrane trafficking events following localization to cytoplasmic and membrane compartments (Reems et al. 2008). Homer-1 and Homer-2 proteins bind to Dynamin III through their EVH1 domain, whereas the physical link between Dynamin III and Homer has been reported to be involved with positioning the endocytic zone to near the PSD (Duncan et al. 2005; Lu et al. 2007). As Dynamin III also interacts with mGluR5, Homer proteins have been suggested to play a role in recruiting Dynamin III to mGluR5 at the PSD (Duncan et al. 2005).

Shank is a scaffolding protein to which Homer can bind through the EVH1 domain and C-terminal leucine zipper motifs (Duncan et al. 2005). Crystallographic analysis of the postsynaptic density revealed that Homer together with Shank binds to form a mesh-like matrix structure, where the Homer–Shank complex may provide structural support to neuronal dendritic spines and provide an assembly stage for other PSD proteins (Hayashi et al. 2009). Overexpression of Homer-1c in hippocampal neurons resulted in the synaptic localization of Shank being reduced and actin being increased (Duncan et al. 2005). Additionally, depolymerization of actin reduced synaptic localization of both Homer-1c and Shank, suggesting Homer-1c to be involved in the accumulation of synaptic F-actin (Duncan et al. 2005).

Cupidin, or Homer-2, interacts with actin cytoskeletal regulators, Cdc42 and Drebrin, in dendritic spines (Shiraishi-Yamaguchi et al. 2009). The interaction between Cupidin and activated Cdc42 has been suggested to possess a possible role in the formation of mushroom-type spines in hippocampal neurons (Shiraishi-Yamaguchi et al. 2009). Drebrin is a dendritic spine F-actin binding protein and interacts with Cupidin via the N-terminal EVH1 domain (Shiraishi-Yamaguchi et al. 2009). These interactions suggest that Cupidin may play an important role in spine morphology by scaffolding multiple dendritic spine actin regulators (Shiraishi-Yamaguchi et al. 2009).

Huntington interacting protein (Hip1) and *Hip1 protein interactor (Hippi)* form complexes and activate caspcase-8 which leads to mammalian cell death during Huntington's Disease (Sakamoto et al. 2007). Homer-1c has been shown to protect striatal neurons from hippi-hip1 induced cell death. A mutant form of Homer-1c lacking the C-terminal region failed to protect the neurons from cell death or bind to Hippi, suggesting that a direct interaction between the Homer-1c C-terminal region and Hippi is required to induce protection and concluding that a Homer-1c/Hippi complex may be an important regulator for neuronal death during Huntigton's Disease (Sakamoto et al. 2007).

Soluble N-ethyl-maleimide-sensitive attachment protein receptor (SNARE) syntaxin 13 can bind with Homer-1c, which may participate in endosomal trafficking as co-expression in COS-7 cells results in the co-localization of Homer-1c and syntaxin 13 in intracellular vesicular structures (Duncan et al. 2005).

General Mechanism

Synaptic functions of Homer proteins are to a large extent regulated by the interplay of the short Homer-1a isoform and the various long Homer proteins. Homer-1a regulates long isoforms in a dominant-negative fashion by competing with the long isoforms for Homer binding sites on Homer ligands, demonstrating the potential involvement of the inducible short Homer isoforms in the regulation of intracellular signaling and trafficking processes controlled by ubiquitously expressed long Homer isoforms. Furthermore, the general mechanism of short and long Homer proteins suggests a general stimulus-dependent mechanism using the molecular determinants of Vesl/Homer proteins to regulate intracellular signaling pathways (Duncan et al. 2005).

Summary

Homer proteins are a family of scaffolding molecules, encoded by three genes: Homer1, Homer2, and Homer3. Two short Homer1 isoforms are known (Homer1a and Ania-3) that act as IEGs and are upregulated in response to seizures, LTP, and ischemic insult; in contrast, all long Homer isoforms are constitutively expressed. The long Homer isoforms are abundantly expressed in brain, muscle, and various non-excitable tissues. Homer proteins possess an

EVH1 domain for ligand binding and interaction, and a CC domain providing for tetramerization of multiple Homer proteins into scaffolding complexes. Homer proteins physically link plasma membrane proteins (such as group I mGluRs) with intracelluar Ca^{2+} channels (such as the ▶ IP_3Rs and RyRs), and hence are critically modulating synaptic activity. The short IEG Homer1 transcripts lack a CC domain and are thought to competitively disturb this physical interaction in a response to increased synaptic activity as experienced during LTP or following seizure activity or ischemic insult (Sgambato-Faure et al. 2006; Duncan et al. 2005).

Homer proteins are important modulators of synaptic activity. However, their role in disease and in non-excitable tissues requires further investigation and could critically contribute to protective mechanisms (Duncan et al. 2010).

Acknowledgments This study was supported in part by grants EY014227 from NIH/NEI, RR022570 from NIH/NCRR and AG010485, AG022550 and AG027956 from NIH/NIA, by the Felix and Carmen Sabates Missouri Endowed Chair in Vision Research (PK). We thank Margaret, Richard, and Sara Koulen for generous support and encouragement.

References

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F. Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. PLoS ONE. 2010;5:e9755.

Duncan RS, Hwang SY, Koulen P. Effects of Vesl/Homer proteins on intracellular signaling. Exp Biol Med Maywood. 2005;230:527–35.

Duncan RS, Goad DL, Grillo MA, Kaja S, Payne AJ, Koulen P. Control of intracellular calcium signaling as a neuroprotective strategy. Molecules. 2010;15(3):1168–95.

Hayashi MK, Ames HM, Hayashi Y. Tetrameric hub structure of postsynaptic scaffolding protein homer. J Neurosci. 2006;26:8492–501.

Hayashi MK, Tang C, Verpelli C, Narayanan R, Stearns MH, Xu RM, et al. The postsynaptic density proteins Homer and Shank form a polymeric network structure. Cell. 2009;137:159–71.

Hwang SY, Wei J, Westhoff JH, Duncan RS, Ozawa F, Volpe P, et al. Differential functional interaction of two Vesl/Homer protein isoforms with ryanodine receptor type 1: a novel mechanism for control of intracellular calcium signaling. Cell Calcium. 2003;34:177–84.

Kaja S, Yang SH, Wei J, Fujitani K, Liu R, Brun-Zinkernagel AM, et al. Estrogen protects the inner retina from apoptosis and ischemia-induced loss of Vesl-1 L/Homer 1c immunoreactive synaptic connections. Invest Ophthalmol Vis Sci. 2003;44:3155–62.

Kammermeier PJ. Endogenous homer proteins regulate metabotropic glutamate receptor signaling in neurons. J Neurosci. 2008;28:8560–7.

Lu J, Helton TD, Blanpied TA, Racz B, Newpher TM, Weinberg RJ, et al. Postsynaptic positioning of endocytic zones and AMPA receptor cycling by physical coupling of dynamin-3 to Homer. Neuron. 2007;55:874–89.

Pouliquin P, Dulhunty AF. Homer and the ryanodine receptor. Eur Biophys J. 2009;39:91–102.

Reems J-A, Wang W, Tsubata K, Abdurrahman N, Sundell B, Tijssen MR, et al. Dynamin 3 participates in the growth and development of megakaryocytes. Exp Hematol. 2008;36:1714–27.

Rychkov G, Barritt GJ. TRPC1 Ca(2+)-permeable channels in animal cells. Handb Exp Pharmacol. 2007;179:23–52.

Sakamoto K, Yoshida S, Ikegami K, Minakami R, Kato A, Udo H, et al. Homer1c interacts with Hippi and protects striatal neurons from apoptosis. Biochem Biophys Res Commun. 2007;352:1–5.

Sgambato-Faure V, Xiong Y, Berke JD, Hyman SE, Strehler EE. The Homer-1 protein Ania-3 interacts with the plasma membrane calcium pump. Biochem Biophys Res Commun. 2006;343:630–7.

Shiraishi-Yamaguchi Y, Sato Y, Sakai R, Mizutani A, Knopfel T, Mori N, et al. Interaction of Cupidin/Homer2 with two actin cytoskeletal regulators, Cdc42 small GTPase and drebrin, in dendritic spines. BMC Neurosci. 2009;10:25.

Stokely M, Hwang S-Y, Hwang J-Y, Fan B, King M, Inokuchi K, Koulen P. Polycystin-1 can interact with Homer 1/Vesl-1 in postnatal hippocampal neurons. J Neurosci Res. 2006;84(8):1727–37.

Tanaka M, Duncan RS, McClung N, Yannazzo JA, Hwang SY, Marunouchi T, et al. Homer proteins control neuronal differentiation through IP(3) receptor signaling. FEBS Lett. 2006;580:6145–50.

Westhoff JH, Hwang SY, Duncan RS, Ozawa F, Volpe P, Inokuchi K, et al. Vesl/Homer proteins regulate ryanodine receptor type 2 function and intracellular calcium signaling. Cell Calcium. 2003;34:261–9.

HPK1

Sebastian Königsberger and Friedemann Kiefer
Department Vascular Cell Biology, Max Planck Institute for Molecular Biomedicine, Mammalian Cell Signaling Laboratory, Münster, Germany

Synonyms

Hematopoietic progenitor kinase 1; Map4k1; Mitogen-activated protein kinase kinase kinase kinase 1

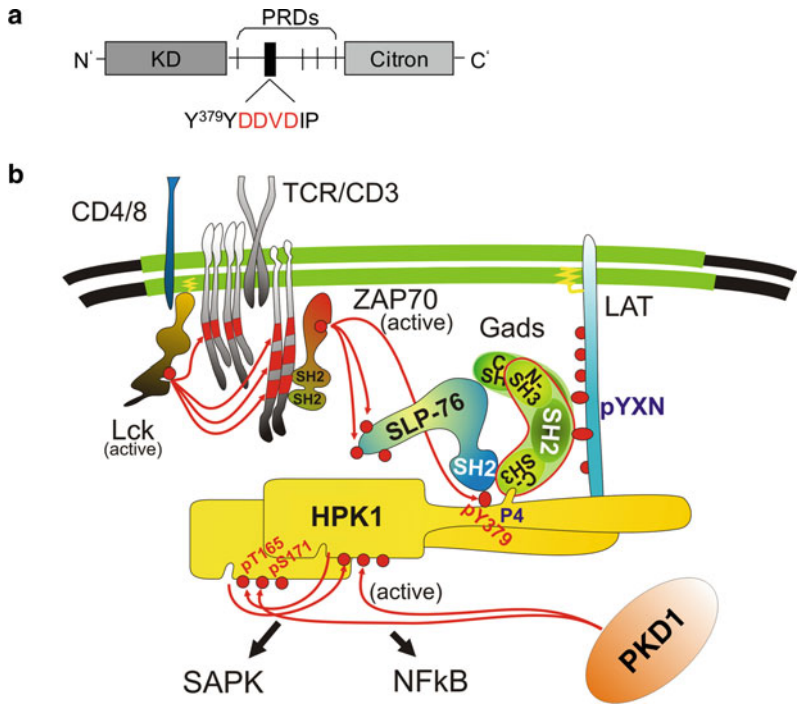
Historical Background

Since 1996, when hematopoietic progenitor kinase 1 (HPK1) was first described as an activator of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway (Kiefer et al. 1996), a range of information concerning activation, subcellular localization, putative interaction partners, and the regulatory function of HPK1 in immune cells was gathered. The initial studies showed that while HPK1 mRNA is ubiquitously detectable in embryonic tissues, expression in the adult is restricted to hematopoietic organs like thymus, bone marrow, and spleen. Protein database sequence comparison classified HPK1 as a member of the Ste20-related protein kinase family and is most closely related to the germinal center kinase (GCK), a MAP4 kinase. The structure of HPK1 comprises an N-terminal kinase domain, a central region containing proline-rich (SH3-binding) motifs and at least one tyrosine residue that serves as phosphorylation target, and a C-terminal citron homology domain of yet undefined function (Ling et al. 1999). In addition, a central caspase cleavage site alters effector properties during apoptosis induction (Arnold et al. 2001) as will be discussed in more detail in the following sections.

HPK1 Activation and Molecular Interactions

HPK1 was shown to be activated following genotoxic stress (Ito et al. 2001); growth factor stimulation via TGFβ (Zhou et al. 1999), EGF, PDGF (Ling et al. 1999), and erythropoietin (Nagata et al. 1999); prostaglanding E_2 stimulation (Sawasdikosol et al. 2003, 2007); and antigen receptor triggering (Liou et al. 2000; Liu et al. 2000; Sauer et al. 2001; Tsuji et al. 2001). In the case of BCR/TCR stimulation, HPK1 phosphorylation at Tyr^{379} in mice and Tyr^{381} in humans seems the prerequisite for further activational steps (Sauer et al. 2001; Tsuji et al. 2001). The upstream factors involved are Lck, ▶ Zap-70, ▶ LAT, and ▶ SLP-76 in T cells and Lyn, Syk, and SLP-65 in B cells (Liou et al. 2000). In addition, a detailed study by Arnold et al. revealed that Thr^{165}, Ser^{171}, and Thr^{175} located in the activation loop of the kinase are of critical importance for the optimal activation of HPK1 upon TCR stimulation (Fig. 1b). Accordingly, Lck and SLP-76 relocate HPK1 from the cytoplasm to the plasma membrane, where it follows transphosphorylation by protein kinase D1 and subsequent autophosphorylation reaches full activity. Interestingly, this study also demonstrated the recruitment of HPK1 to the interface of T-/B-cell conjugates, indicating a function at the level of immune synapse associated signaling.

To ensure proper activation, subcellular localization, and functionality, HPK1 constitutively or inducibly interacts with a range of adaptors and signaling molecules. Table 1 provides a list of reported interaction partners, the cell types in which the interaction was observed and, if determined, the HPK1-binding region responsible for the interaction. Grb2 and Grap together with Gads belong to the Grb2 family of adaptor proteins containing a SH2 domain flanked by two SH3 domains. The constitutive association of HPK1 with Gads (Grap2) was shown to be required for the synergistic activation of c-Jun and IL-2 transcription in COS-7/Jurkat T cells, whereas the binding to Grb2 in Cos1 cells was reported to link HPK1 to the EGF receptor. The finding that Grb2 family members locate HPK1 to the transmembrane adaptor LAT, led early on to the speculation that HPK1 might antagonize the recruitment of SLAP-130/▶ ADAP/Fyb, a positive regulator of IL-2 and NFAT activity. The constitutive association of HPK1 with the upstream adaptors Crk/CrkL in Jurkat T cells mediated by the proline-rich (PR) domain 2,4 – SH3 interaction was suggested to ensure synergistic activation of JNK and optimal IL-2 transcription (Ling et al. 1999; Oehrl et al. 1998). Crk family adaptor proteins are involved in a range of cell signaling pathways, including antigen receptor stimulation, integrin signaling, cell migration, and focal adhesion signal transduction, thus suggesting that HPK1 could be involved in the spatial bridging of antigen receptor signaling and integrin regulation. As already stated above, $Tyr^{379/381}$ phosphorylation of HPK1 is prerequisite for the binding to the central B- and T-cell adaptors SLP-76/65. Upon phosphorylation by Syk family protein tyrosine kinases (Syk/Zap-70), SLP76 family adaptors act as membrane proximal scaffolds enabling the coordinated activation of downstream pathways. As abrogated binding of HPK1 to mutated SLP-adaptors does not completely abolish its negative regulatory role on Erk, AP-1, and NFAT (Sauer et al. 2001), the synchronous recruitment to other adaptor molecules like Crk, Grb2, and also Nck family members might generate a complex pattern



HPK1, Fig. 1 Schematic HPK1 protein structure and inducible recruitment to the T-cell antigen receptor membrane-signaling scaffold. (**a**) The modular build of HPK1 contains an N-terminal kinase domain, a central region with proline-rich motifs involved in SH3 domain binding and several interspersed tyrosine residues, as well as a C-terminal citron homology domain. Phosphorylation of tyrosine 379 (Y^{379}) in mice is a mandatory initial step for the recruitment of HPK1 to SLP-76/65 adaptor molecules in T- and B cells; DDVD indicates the target sequence for caspase-mediated cleavage. (**b**) Upon T-cell receptor triggering, Zap-70-mediated phosphorylation of Y^{379} parallels HPK1 translocation to the membrane, auto (T165)- and transphosphorylation (S171) by PKD1, complex formation with SLP-76, LAT, and Gads, and the initiation of downstream signaling; *Gads* (Grap2), Grb2-related adaptor protein 2, *KD* kinase domain, *LAT* linker for activation of T cells, *Lck* lymphocyte-specific protein tyrosine kinase, *NFκB* nuclear factor "kappa light chain enhancer" of activated B cells; *PKD1* protein kinase D1, *PRDs* proline-rich domains, *SH3* Src-homology 3, *SLP-76/65* SH2 domain containing leukocyte protein of 76/65 kDa, *TCR* T-cell receptor, *Zap-70* zeta-chain-associated protein kinase 70 kDa

of regulation that still awaits complete resolution. As a matter of fact, HPK1 binds to the SLP-76 family adaptor Clnk with significantly higher affinity as compared to SLP-76 itself (Yu et al. 2001). Clnk can substitute for SLP-76 in immunoreceptor signaling and together with HPK1 acts synergistically on IL-2 promoter activity (Yu et al. 2001).

Abp1 (SH3P7, HIP55), a broadly expressed adaptor protein in human and mouse tissues binds HPK1 at the PR2 motif (Ensenat et al. 1999). As a member of the drebrin/Abp1 family of actin-binding proteins, Abp1 was shown to be recruited to the IS in activated T cells, where it promotes TCR downmodulation and negatively affects NFAT activation (Le Bras et al. 2004). Bam32 (DAPP1, PHISH) is an additional adaptor molecule binding to HPK1 in B cells, possibly via its N-terminal SH2 domain. It was found to be required for Rac1 activation, proper Ca^{2+} mobilization, and optimal PLCγ2 activation. Accordingly, $Bam32^{-/-}$ B cells exhibit defects in ERK, JNK, and HPK1 activity. Bam32 was also shown to colocalize with the BCR complex and clathrin to regulate BCR uptake and actin dynamics. Despite being a target for Bam32-mediated JNK activation, the relevance of HPK1 in the regulation of antigen receptor internalization and actin dynamics was not investigated so far. An interesting observation in this context was the finding by Nagata et al. that HPK1 constitutively binds to HS1, a well-established actin-regulatory protein (Uruno et al. 2003), in FD-EPO and SKT6 cells, although the functional significance of this interaction in vivo awaits further investigation. In addition to the binding of the above-mentioned adaptor molecules, HPK1 activates IKKß (Hu et al. 1999; Arnold et al. 2001) and,

HPK1, Table 1 List of adaptors and signaling molecules reported to interact with HPK1

Adaptor proteins binding HPK1 (reviewed in Boomer and Tan 2005)

Adaptor	Binding	Cell type	HPK1 domain
Grb2	Constitutive	Jurkat, Cos-1	PR1,2,4
Grap	Constitutive	Jurkat	n.d.
Gads	Inducible	Jurkat, Cos-7	PR2,4
Nckα	Inducible	Jurkat	All PR motifs
CrkI	Inducible	Jurkat, Cos-1	PR2,4
CrkII	Inducible	Jurkat, Cos-1	PR2,4
CrkL	Constitutive	Jurkat, Cos-1 Cos-7, HEK293	PR2,4
SLP-76	Inducible	Jurkat, J14	$pY^{379(m)/381(h)}$
SLP-65	Inducible	Wehi231.5	$pY^{379(m)/381(h)}$
Clnk	Inducible	Cos-1, $B6SutA_1$	n.d.
Bam32	Constitutive	BJAB	n.d.
Hip-55	Constitutive	HEK293	PR2
LAT	Inducible	Jurkat	Indirect

Additional binding partners of HPK1

Protein	Binding	Cell type	HPK1 domain	References
c-Abl	Inducible	Jurkat, HEK293	PR?	Ito et al. (2001)
HS1	Constitutive	FD-EPO, SKT6	n.d.	Nagata et al. (1999)
IKKβ	Constitutive	Cos1, BJAB, Jurkat, primary T cells	N′-terminal fr.	Brenner et al. (2005)
CARMA1	Constitutive	Jurkat, primary T cells	C′-terminal region	Brenner et al. (2009)
SKAP-HOM	Constitutive	Wehi 231 cells	n.d., indirect?	Königsberger et al. (2010)

n.d. not determined, *PR* proline rich

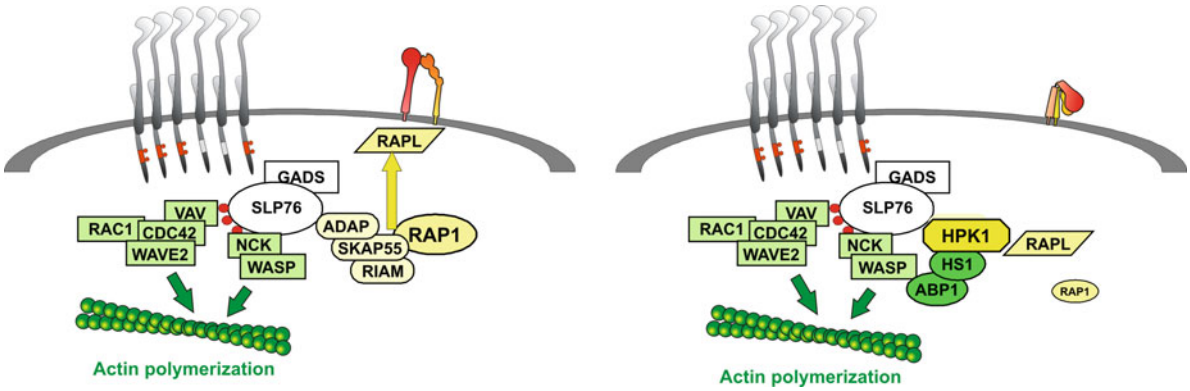
as recognized recently, phosphorylates CARMA1 (Brenner et al. 2009), which further establishes the function of the protein in NFκB regulation and apoptosis induction.

Functions in Cellular Activation and Apoptosis Induction

Already in early studies, HPK1 was shown to selectively activate the SAPK/JNK cascade via MEKK1 (Hu et al. 1996)/MLK-3 (Kiefer et al. 1996) phosphorylation, a process independent of Rac1 or Cdc42 GTPase function, which were known to feed into the SAPK/JNK pathway via PAK activation (Coso et al. 1995). Epistasis analysis established that HPK1 acts on MAP3 kinases (MEKK1, MLK-3) which in turn signal through MAP2 kinases (e.g., MKK4/7, MKK3/6) to the c-Jun N-terminal kinase (Tibbles et al. 1996; Ensenat et al. 1999; Liou et al. 2000). Besides this well-accepted function in triggering the SAPK/JNK pathway upon environmental stress, a growing list of publications shows that HPK1 is fundamentally involved in antigen receptor signaling processes.

An elegant study from di Bartolo et al. in 2007 focusing on negative regulatory components of proximal TCR signaling revealed a novel function of HPK1 in limiting/fine-tuning antigen receptor–mediated cellular activation. By phosphorylating SLP-76 at Ser^{376}, HPK1 enables the recruitment of 14-3-3 ε and ζ proteins, which limits PLCγ1 phosphorylation and IL-2 promoter activity. HPK1 was also shown to control proximal TCR signaling by limiting Vav, Lat, and PLCγ1 activation and promoting the interaction of SLP-76 with the 14-3-3 protein family member 14-3-3τ. The finding that $HPK1^{-/-}$ BMDCs show higher levels of activation markers and proinflammatory cytokines upon LPS stimulation makes them more potent in eliciting cytotoxic T-cell-mediated antitumor responses (Alzabin et al. 2010) and adds to the general view of HPK1 as a negative regulator of immunity and inflammation.

NFκB is a pleiotropic transcription factor regulating a range of genes relevant in immune and inflammatory responses. NFκB transcription factors are kept inactive in the cytoplasm by interaction with inhibitory IκB proteins. Antigen receptor stimulation triggers the activation of an IκB kinase (IKK) complex comprised of



HPK1, Fig. 2 Competition of HPK1 and ADAP for SLP-76 binding in lymphocyte inside-out integrin signaling. *Left*: Pro-adhesive interaction of the ADAP/SKAP55/RIAM module with SLP-76 leads to affinity (depicted)/avidity upregulation of β2 integrins via Rap1 recruitment and binding of the Rap1 effector RapL to the α integrin cytoplasmic tail. *Right*: HPK1 binding to SLP-76 limits integrin activation by sequestering the ADAP trimolecular complex and downstream signaling mediators; concomitant regulation of actin polymerization through the WAVE2 complex and WASP is indicated; *Cdc42* cell division cycle 42, GTP-binding protein, *Nck* noncatalytic region of tyrosine kinase adaptor protein 1, *Rac1* Ras-related C3 botulinum toxin substrate 1, *WASP* The Wiskott-Aldrich Syndrome protein, *Wave2* verprolin homology domain-containing protein 2

IKKα, β, and γ. Phosphorylation of IκB results in the cytoplasmic sequestration of IκB proteins and targeting for proteasomal degradation, while NFkB transcription factors are liberated to enter the nucleus and activate transcription. Full-length HPK1 facilitates NFκB activation in hematopoietic cell lines and primary murine $CD4^+$ T cells, while caspase-mediated cleavage at Asp^{385} within the DDVD cleavage site converts the C-terminal fragment of HPK1 into an NFκB inhibitor (Arnold et al. 2001; Schulze-Luehrmann et al. 2002). The N-terminal HPK-1 fragment, which is largely comprised of the kinase domain retains the ability to activate JNK, but is unable to bind adaptor proteins, which altogether enhances peripheral T-cell sensitivity to activation-induced cell death (AICD) (Brenner et al. 2005). This was further corroborated by a study of Brenner et al. in 2007 addressing AICD sensitivity in HPK1-C transgenic mice. In this model system, primary T- and B cells, constitutively expressing HPK1-C, were prone to CD95L-independent AICD, intimately caused by caspase-3/9 activity. In addition, HPK1 interacts with and phosphorylates the IKK-activating adaptor protein CARMA1 (caspase-recruitment domain [CARD]-containing signaling adaptor protein) at Ser^{551} upon TCR stimulation, which is a prerequisite for HPK1-dependent NFκB activation and IL-2 production (Brenner et al. 2009). Of note, HPK1 is also a target of caspase-3 during monocytic differentiation. In this context, the HPK1-N fragment confers IL-3 independent survival of mouse myeloid FDC-P1 progenitor cells and induces differentiation toward the monocytic lineage (Arnold et al. 2007). This, in contrast to the situation in lymphocytes, results in constitutive cytokine-independent signaling and prolonged cellular survival.

HPK1 as a Negative Regulator of Inside-Out Integrin Regulation

Integrins are heterodimeric cell-surface receptors mediating cell-cell and cell-extracellular matrix interactions (ECMs). Being composed of one α- and one β-subunit, integrins build up a family of at least 24 members in mammals, within which LFA-1 (lymphocyte function-associated antigen 1; $\alpha_L\beta_2$), VLA-4 (very late antigen 4; $\alpha_4\beta_1$), and $\alpha_4\beta_7$-integrins are particularly important for leukocyte function by binding cellular adhesion molecules such as ICAM-1/2, VCAM-1, MADCAM1, and the ECM component fibronectin. In the resting, low-affinity conformation, integrins are bent at the stalk region which sterically masks the ligand-binding headpiece. Upon inside-out activation through chemokines/antigen receptors and enhanced by outside-in triggering, integrins undergo a process of avidity regulation, including intracellular and extracellular conformational changes toward an open structure as well as valency changes facilitated by oligomerization.

Two recent studies by (Patzak et al. 2010) and Königsberger et al. indicated that HPK1 has a negative regulatory role in inside-out integrin activation. In addition to the hyperproliferative nature of HPK1$^{-/-}$ lymphocytes upon antigen receptor–mediated stimulation, these studies show that B and T cells lacking HPK1 exhibit a stronger basal adhesion to ICAM-1, most likely explained by an increase in the amount of the signaling active GTP-bound small GTPase Rap1 (Ras-related protein 1). Upon recruitment to SLP-76, a trimolecular complex containing ADAP (adhesion- and degranulation-promoting adaptor protein)/SKAP55 (Src kinase−associated phosphoprotein of 55 kDa)/RIAM (Rap1-GTP interacting adaptor molecule) binds the small GTPase Rap1 and thus facilitates integrin activation in T cells (Kliche et al. 2006; Menasche et al. 2007). Interestingly, HPK1 competes with ADAP for SLP-76 binding as HPK1$^{-/-}$ T cells show more SLP-76 pre-associated ADAP (Fig. 2) and this lack of ADAP localization leads to an increase in T-cell adhesion and spreading on ICAM-1. This finding adds another interesting feature to the portfolio of HPK1 functions in immune cells and opens a new field of investigation on the proteins' relevance in pathological settings of altered leukocyte adhesiveness like, for example, the involvement in the development of atherosclerosis.

Summary

Hematopoietic progenitor kinase 1 (HPK1) is a mammalian Ste20 homologue predominantly expressed in hematopoietic tissues of the adult. The protein is a specific activator of the SAPK/JNK pathway acting at the level of a MAP4 kinase and is therefore also referred to as MAP4K1. In leukocytes, HPK1 is activated by the canonical immunoreceptor signaling complexes. HPK1 interacts constitutively with SH3-containing adaptor proteins and is recruited to activated immunoreceptors upon tyrosine phosphorylation and interaction with SH2-containing adaptor proteins. In addition to the activation of the SAPK/JNK pathway, full-length HPK1 mediates T-cell receptor-proximal NF-κB activation by phosphorylating the adaptor protein CARMA1. Upon lymphocyte activation and during differentiation of monocytes, HPK1 is proteolytically processed toward a catalytic N-terminal fragment and an inhibitory C-terminal fragment (HPK1-C). This processing changes effector functions and converts HPK1 into a suppressor of NF-κB activation, which leads to sensitization of lymphocytes toward cell death. More recent insights suggest a negative regulatory function of HPK1 in lymphocyte integrin activation and hence make the kinase an interesting candidate to study in leukocyte adhesive processes.

References

Alzabin S, Pyarajan S, Yee H, Kiefer F, Suzuki A, Burakoff S, Sawasdikosol S. Hematopoietic progenitor kinase 1 is a critical component of prostaglandin E2-mediated suppression of the anti-tumor immune response. Cancer Immunol Immunother. 2010;59(3):419–29.

Arnold R, Liou J, Drexler HC, Weiss A, Kiefer F. Caspase-mediated cleavage of hematopoietic progenitor kinase 1 (HPK1) converts an activator of NFkappaB into an inhibitor of NFkappaB. J Biol Chem. 2001;276(18):14675–84.

Arnold R, Frey CR, Muller W, Brenner D, Krammer PH, Kiefer F. Sustained JNK signaling by proteolytically processed HPK1 mediates IL-3 independent survival during monocytic differentiation. Cell Death Differ. 2007;14(3):568–75.

Boomer JS, Tan TH. Functional interactions of HPK1 with adaptor proteins. J Cell Biochem. 2005;95:34–44.

Brenner D, Golks A, Kiefer F, Krammer PH, Arnold R. Activation or suppression of NFkappaB by HPK1 determines sensitivity to activation-induced cell death. EMBO J. 2005;24(24):4279–90.

Brenner D, Golks A, Becker M, Muller W, Frey CR, Novak R, et al. Caspase-cleaved HPK1 induces CD95L-independent activation-induced cell death in T and B lymphocytes. Blood. 2007;110(12):3968–77.

Brenner D, Brechmann M, Rohling S, Tapernoux M, Mock T, Winter D, et al. Phosphorylation of CARMA1 by HPK1 is critical for NF-kappaB activation in T cells. Proc Natl Acad Sci U S A. 2009;106(34):14508–13.

Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, et al. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell. 1995;81(7):1137–46.

Di Bartolo V, Montagne B, Salek M, Jungwirth B, Carrette F, Fourtane J, et al. A novel pathway down-modulating T cell activation involves HPK-1-dependent recruitment of 14-3-3 proteins on SLP-76. J Exp Med. 2007;204(3):681–91.

Ensenat D, Yao Z, Wang XS, Kori R, Zhou G, Lee SC, et al. A novel src homology 3 domain-containing adaptor protein, HIP-55, that interacts with hematopoietic progenitor kinase 1. J Biol Chem. 1999;274(48):33945–50 [published erratum appears in J Biol Chem 2000 May 5;275(18):14004].

Hu MC, Qiu WR, Wang X, Meyer CF, Tan TH. Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes Dev. 1996;10(18):2251–64.

Hu MC, Wang Y, Qiu WR, Mikhail A, Meyer CF, Tan TH. Hematopoietic progenitor kinase-1 (HPK1) stress response signaling pathway activates IkappaB kinases (IKK-alpha/beta) and IKK-beta is a developmentally regulated protein kinase. Oncogene. 1999;18(40):5514–24.
Ito Y, Pandey P, Sathyanarayana P, Ling P, Rana A, Weichselbaum R, et al. Interaction of hematopoietic progenitor kinase 1 and c-Abl tyrosine kinase in response to genotoxic stress. J Biol Chem. 2001;276(21):18130–8.
Kiefer F, Tibbles LA, Anafi M, Janssen A, Zanke BW, Lassam N, et al. HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. EMBO J. 1996;15(24):7013–25.
Kliche S, Breitling D, Togni M, Pusch R, Heuer K, Wang X, et al. The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. Mol Cell Biol. 2006;26(19):7130–44.
Königsberger S, Peckl-Schmid D, Zaborsky N, Patzak I, Kiefer F, Achatz G. HPK1 associates with SKAP-HOM to negatively regulate Rap1-mediated B-lymphocyte adhesion. PLoS One. 2010;5(9):e12468.
Le Bras S, Foucault I, Foussat A, Brignone C, Acuto O, Deckert M. Recruitment of the actin-binding protein HIP-55 to the immunological synapse regulates T cell receptor signaling and endocytosis. J Biol Chem. 2004;279(15):15550–60.
Ling P, Yao Z, Meyer CF, Wang XS, Oehrl W, Feller SM, et al. Interaction of hematopoietic progenitor kinase 1 with adapter proteins Crk and CrkL leads to synergistic activation of c-Jun N-terminal kinase. Mol Cell Biol. 1999;19(2):1359–68.
Liou J, Kiefer F, Dang A, Hashimoto A, Cobb MH, Kurosaki T, et al. HPK1 is activated by lymphocyte antigen receptors and negatively regulates AP-1. Immunity. 2000;12(4):399–408.
Liu SK, Smith CA, Arnold R, Kiefer F, McGlade CJ. The adaptor protein Gads (Grb2-related adaptor downstream of Shc) is implicated in coupling hemopoietic progenitor kinase-1 to the activated TCR. J Immunol. 2000;165(3):1417–26.
Menasche G, Kliche S, Chen EJ, Stradal TE, Schraven B, Koretzky G. RIAM links the ADAP/SKAP-55 signaling module to Rap1, facilitating T-cell-receptor-mediated integrin activation. Mol Cell Biol. 2007;27(11):4070–81.
Nagata Y, Kiefer F, Watanabe T, Todokoro K. Activation of hematopoietic progenitor kinase-1 by erythropoietin. Blood. 1999;93(10):3347–54.
Oehrl W, Kardinal C, Ruf S, Adermann K, Groffen J, Feng GS, et al. The germinal center kinase (GCK)-related protein kinases HPK1 and KHS are candidates for highly selective signal transducers of Crk family adapter proteins. Oncogene. 1998;17(15):1893–901.
Patzak IM, Königsberger S, Suzuki A, Mak TW, Kiefer F. HPK1 competes with ADAP for SLP-76 binding and via Rap1 negatively affects T-cell adhesion. Eur J Immunol. 2010;40(11):3220–5.
Sauer K, Liou J, Singh SB, Yablonski D, Weiss A, Perlmutter RM. Hematopoietic progenitor kinase 1 associates physically and functionally with the adaptor proteins B cell linker protein and SLP-76 in lymphocytes. J Biol Chem. 2001;276(48):45207–16.
Sawasdikosol S, Russo KM, Burakoff SJ. Hematopoietic progenitor kinase 1 (HPK1) negatively regulates prostaglandin E2-induced fos gene transcription. Blood. 2003;101(9):3687–9.
Sawasdikosol S, Pyarajan S, Alzabin S, Matejovic G, Burakoff SJ. Prostaglandin E2 activates HPK1 kinase activity via a PKA-dependent pathway. J Biol Chem. 2007;282:34693–9.
Schulze-Luehrmann J, Santner-Nanan B, Jha MK, Schimpl A, Avots A, Serfling E. Hematopoietic progenitor kinase 1 supports apoptosis of T lymphocytes. Blood. 2002;100(3):954–60.
Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR, et al. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. EMBO J. 1996;15(24):7026–35.
Tsuji S, Okamoto M, Yamada K, Okamoto N, Goitsuka R, Arnold R, et al. B cell adaptor containing src homology 2 domain (BASH) links B cell receptor signaling to the activation of hematopoietic progenitor kinase 1. J Exp Med. 2001;194(4):529–39.
Uruno T, Zhang P, Liu J, Hao JJ, Zhan X. Haematopoietic lineage cell-specific protein 1 (HS1) promotes actin-related protein (Arp) 2/3 complex-mediated actin polymerization. Biochem J. 2003;371(Pt 2):485–93.
Yu J, Riou C, Davidson D, Minhas R, Robson JD, Julius M, et al. Synergistic regulation of immunoreceptor signaling by SLP-76-related adaptor Clnk and serine/threonine protein kinase HPK-1. Mol Cell Biol. 2001;21(18):6102–12.
Zhou G, Lee SC, Yao Z, Tan TH. Hematopoietic progenitor kinase 1 is a component of transforming growth factor beta-induced c-Jun N-terminal kinase signaling cascade. J. Biol. Chem. 1999;274:13133–8.

HRK (Harakiri BCL2 Interacting Protein), DP5 (Neuronal Death Protein-5)

► BCL-2 Family

Hsk1 (Homologue of Cdc7 [seven] Kinase, *S. pombe*)

► Cdc7

Hsp 27 (Heat Shock Protein 27)

► p38 MAPK Family of Signal Transduction Proteins

Hsp25-Kinase

► Mapkap Kinase 2/3 (MK2/3)

Hsp27-Kinase

► Mapkap Kinase 2/3 (MK2/3)

Hspb1-Kinase

► Mapkap Kinase 2/3 (MK2/3)

hSTAR

► Guanylyl Cyclase C

HSTE14

► Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

hToll

► TLR4, Toll-Like Receptor 4

Human Mucosal Lymphocyte Antigen 1

► Alpha E Integrin

HUMMAT1H

► Pea15

Hyl

► CSK-Homologous Kinase

Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

Kathryn M. Appleton[1], Ian Cushman[2] and Yuri K. Peterson[1]
[1]Department of Pharmaceutical and Biomedical Sciences, The Medical University of South Carolina, Charleston, SC, USA
[2]Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, USA

Synonyms

HSTE14; MGC39955; MST098; MSTP098; PCCMT; PCMT; PPMT

Historical Background

Isoprenylcysteine carboxyl methyltransferase (Icmt) is the only known prenylcysteine protein-dependant methyltransferase and plays a critical role in the post-translational modification of prenylated proteins (Bergo et al. 2000). The Icmt ortholog from *Saccharomyces cerevisiae*, STE14p, was the first prenylcysteine carboxyl methyltransferase to be cloned and sequenced, and it is considered the founding member of this eukaryote protein methyltransferase family (Anderson et al. 2005). STE14p was originally recognized in a screen of mutant yeast, deficient of STE14, which rendered the yeast sterile due to their failure to methylate the mating pheromone a-factor, a protein crucial for fertility. STE14p is a 26 kDa integral membrane protein with multiple transmembrane (TM) spanning domains and is localized to the endoplasmic reticulum (ER) (Romano and Michaelis 2001). Human Icmt cDNA possess notable homology to STE14, and upon its expression in STE14-deficient yeast, the sterile phenotype is reversed (Svensson et al. 2006). Icmt orthologs such as *Schizosaccharomyces pombe* (mam4p), and *Xenopus laevis* (Xmam4p), also exhibit considerable amino acid homology with STE14p, and behave as functionally complimentary prenylcysteine carboxyl methyltransferases (Wright et al. 2009). STE14p and its orthologs lack significant amino acid conservation to other nucleotide, DNA, or protein methyltransferases, including the absence of the commonly observed consensus S-adenosylmethionine (SAM or AdoMet) binding motifs (Wright et al. 2009). The novelty and specificity of its biologic role and its unique evolutionary conservation, along with the Icmt knockout mouse being embryonic lethal, suggest that the function of Icmt is critical (Bergo et al. 2001).

CaaX/CXC Processing

Posttranslational modification of eukaryotic polypeptides is critical for proper function and localization of mature proteins. Many of these proteins are modified by a three step enzymatic process which is dictated by the succession of amino acids at the C-terminus. These proteins, termed CaaX proteins, contain a specialized carboxyl-terminal amino acid sequence that orchestrates modification of nascent proteins. Icmt is responsible for the final and only potentially reversible enzymatic step in CaaX protein processing, which involves methylation of the carboxylic acid of the

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

protein terminal isoprenylated cysteine residue. Ras, and other critical signaling proteins, including Rho GTPases, undergo this posttranslational modification via the CaaX motif (Anderson et al. 2005).

The CaaX sequence is represented by a cysteine, followed by two residues that are typically aliphatic (aa), and finally an amino acid which dictates which of two prenyl groups will be covalently attached to the cysteine in the motif by the enzymes ▶ protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase-I). Note there is a second class of prenylated proteins, the Rab CXC proteins, which include a CXC or CC sequence motif. CXC proteins are prenylated by protein geranylgeranyltransferase type II (GGTase-II). Icmt is capable of identifying substrates from both classes of proteins (Anderson et al. 2005). Despite the substrate prenylation specificity of the two CaaX prenyl transferases, it has been documented that alternate utilization of protein substrates does occur (Wright et al. 2009).

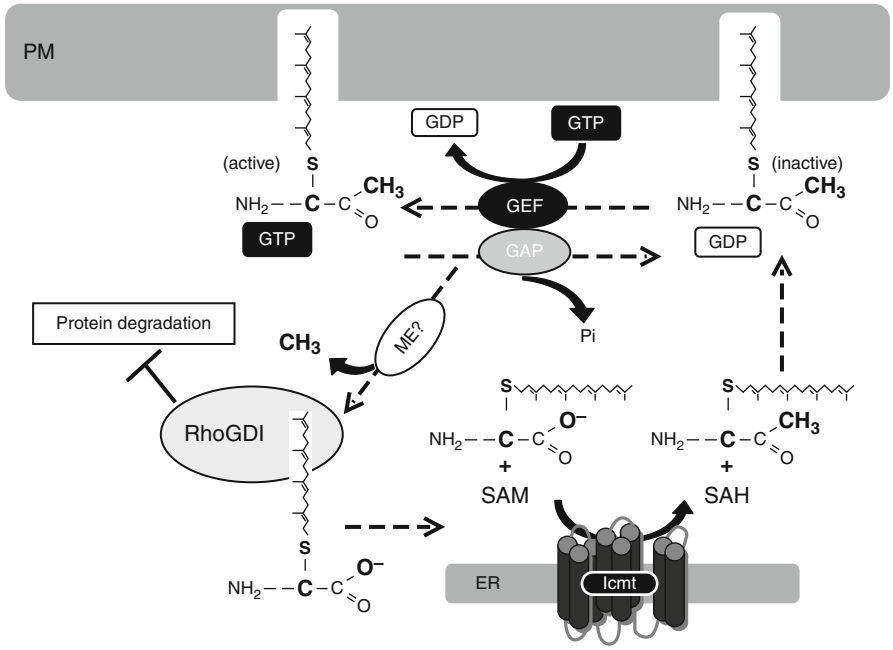
Following prenylation, the second step of CaaX protein modification involves Ras-converting enzyme I (RceI). This ER integral membrane protease cleaves the terminal three -aaX amino acid residues of the CaaX motif rendering the prenylated cysteine residue the new carboxy-terminus. Upon cleavage by RceI, CaaX proteins are specifically methylated on the alpha carboxyl group of the prenylated cysteine by Icmt. Methylation removes the negative charge from the carboxylic acid of the prenylated cysteine, and is therefore the final crucial step to modify proteins in order to increase their hydrophobicity, by leaving the protein with a terminal isoprenylcysteine methyl ester residue. The major observed effect of the isoprene lipid and methylation of the CaaX protein is a high degree of association with cell membranes.

CaaX modification is not only critical in participating in protein-membrane association, but also in protein–protein interactions as seen with Rho GDP-dissociation inhibitor (RhoGDI). RhoGDI regulates isoprenylated Rho GTPase activity and localization by its ability to avert Rho protein–membrane association via seizure of the hydrophobic carboxy-terminus portion of Rho GTPases (Cushman and Casey 2010). RhoGDI acts as an escort protein shuttling these Rho proteins in a guanine diphosphate (GDP) bound inactive state by as well as eliminating interaction with guanine exchange factors (GEFs) (Fig. 1). Rho GEFs stimulate nucleotide exchange and have decreased affinity to unmethylated Rho proteins (Papaharalambus et al. 2005). Icmt is the only potentially reversible step in the CaaX pathway, and therefore may play an important regulatory role for Rho family GTPases, such as Rac1 (Papaharalambus et al. 2005; Huizinga et al. 2008). Studies show an increase in binding of RhoGDI to the Rho GTPases, RhoA and Rac1, when Icmt is inhibited (Harrington et al. 2004; Michaelson et al. 2005). Recent evidence has provided a potential role for RhoGDI in seclusion of Rho GTPases from protein degradation pathways (Boulter et al. 2010).

Structure, Interactions, and Regulation

Icmt differs from other methyltransferases due to its unique ability to selectively bind and methylate prenylated and proteolysed CaaX proteins. Icmt also appears to be the sole enzyme responsible for methylesterification of the Rab CXC proteins (Bergo et al. 2000). Icmt is restricted to the ER, and hydropathy based topology predictions from sequence studies show STE14p likely navigates through the ER membrane with six TM segments (Wright et al. 2009). Functional evidence suggests STE14p is active as a homodimer and may even be able to further oligomerize, and the enzyme contains a GXXGXXG motif that is responsible for dimerization of several other TM proteins (Griggs et al. 2010). The human Icmt protein contains an additional N-terminal domain predicted to contain two additional TM segments for a total of eight TM segments (Wright et al. 2009). The evolution of this additional sequence grants consideration that Icmt has acquired a unique regulatory role in mammals (Wright et al. 2009).

Based on the well-characterized substrates of Icmt (farnesyl-, and geranylgeranylcysteine), synthetic prenylcysteine analogs were designed to act as efficient Icmt substrates and as classical competitive inhibitors. Establishment of an ordered sequential kinetic mechanism determined that Icmt binds first to the methyl donor SAM (Km $\sim$2μM) followed by the binding of a CaaX protein, thereby allowing methylation to occur (Baron and Casey 2004). The Icmt-catalyzed reaction leaves a newly methylated protein and S-adenosylhomocysteine (SAH or AdoHcy).



Icmt (Isoprenylcysteine Carboxyl Methyltransferase), Fig. 1 Proposed regulation of Rho GTPases by Icmt-catalyzed methylation and nucleotide dependent interactions. Depicted are the potential modified states of GTPases due to methyl, nucleotide, and RhoGDI cycling. RhoGDI functions as an escort protein by extracting unmethylated inactive GDP bound GTPases from the plasma membrane and sequestering GTPases in the cytosol away from GEFs and the protein degradation pathways. Guanine Exchange Factor (GEF): GTPase Activating Protein (GAP): Plasma Membrane (PM): Endoplasmic Reticulum (ER): Methyl Esterase (ME): Inorganic phosphate (Pi)

Currently, Icmt activity regulation appears to be mostly substrate availability dependent. The active guanine triphosphate (GTP) bound state of guanine nucleotide binding CaaX proteins, compared to the inactive GDP bound state, displays a characteristic increase in methylation proficiency (Kowluru et al. 1996). Despite apparent constitutive expression and activation in most cell types, there is evidence indicating Icmt is responsible for increased methylation of CaaX proteins as a consequence of particular ligands in specific cell types and that its expression can be regulated. Icmt is responsible for the methylation of a large number of Ras family GTPases and a variety of non-Ras signaling proteins such as the nuclear lamins, heterotrimeric G-protein gamma subunits, the prostacyclin and prostaglandin E2 receptors, and some phosphatases and kinases (Reid et al. 2004). Certain disease states such as Parkinson's disease, or physiologic conditions resulting from pharmacological treatment as seen with antifolate drugs like the dihydrofolate reductase inhibitor methotrexate, can elevate the Icmt reaction end product SAH (Winter-Vann et al. 2003). Like with many enzymes and their end products, SAH inhibits Icmt leading to cellular apoptosis and provides a partial mechanism for the therapeutic effects of drugs like methotrexate in cancer, arthritis, and psoriasis (Winter-Vann et al. 2003). Evidence supports a mechanistic role of Icmt in neuroblastoma differentiation due to a decrease in the methylation of key proteins induced by retinoic acid (Van Dessel et al. 2002). Detectable increase in methylation of CaaX and CXC proteins is observed in such cases as Cdc42 upon glucose treatment and Rap1 upon potassium treatment in beta cells (Kowluru et al. 1996).

Expression and Phenotype

Icmt is ubiquitously expressed but displays enhanced expression in testes, liver, and brain tissues, with expression of up to three splice variants being possible (Bergo et al. 2001). An alteration of Icmt expression has only been observed during mouse development, where detectable mRNA levels are observed by

postnatal day 11 and increase to reach a maximum expression by week four. The inactivation of one Icmt allele produces no apparent phenotype, whereas the deletion of Icmt locus in mice by homologous recombination results in embryonic lethality (Bergo et al. 2001). Icmt-deficient mice embryos die from anemia and cellular apoptosis at mid-gestation by day 11.5 with the primary afflicted tissue being testes, brain, and liver, with additional disruption of skeletal muscle development (Bergo et al. 2001).

Therapeutic Target

Mutations of the Ras proto-oncogene are frequent aberrancies observed in human cancer, and consequently, Ras proteins are the most studied CaaX protein. (Split the difference?) Icmt is critical for the proper plasma membrane targeting of H-, N-, and K-Ras, and localization is essential for their correct function. Icmt-deficient cells exhibit mislocation and cytoplasmic accumulation of Ras proteins (Michaelson et al. 2005). The demonstration of a reduction in K-Ras methylation being linked to slowed cell growth and attenuation of oncogenic transformation has lead to the consideration of Icmt inhibition as an attractive therapeutic target. Given the important role of oncogenic Ras protein in human tumorigenesis, it has been hypothesized that the inhibition of Icmt could be an efficacious strategy to block Ras-induced oncogenic transformation. A large effort has been undertaken in targeting FTase in CaaX processing, leading to the development of highly selective and potent FTase inhibitors, but these compounds were found to lack clinical efficacy. Icmt has been the favored alternate target due to the in-ability of Ras to promote transformation if it is unmethylated, irrespective if prenylation and proteolysis occurs (Svensson et al. 2006). Attempts to prevent Ras-induced cellular transformation by inhibiting Icmt with substrate analogs displayed limitations, due to collateral inhibition of other isoprenylated proteins and/or other methyltransferases. The necessity for a specific pharmacologic agent against Icmt led to the identification of cysmethynil, an Icmt specific inhibitor that has no observable effects on Icmt-deficient cells (Winter-Vann et al. 2005; Svensson et al. 2006). Icmt inhibition by cysmethynil results in decreased cell proliferation, and a reduction in Ras-induced oncogenic transformation (Svensson et al. 2006). Studies show the treatment of cysmethynil on human colon cancer cells results in the mislocation of Ras, a decline in anchorage-independent growth, and a reduction in the response to treatment with epidermal growth factor (EGF) (Winter-Vann et al. 2005; Svensson et al. 2006). The effects of cysmethynil are reversible upon rescue by over-expression of Icmt. Recently, evidence suggests a role for Icmt in cellular migration by impacting the function of Rho GTPases through methylation (Fig. 1). Many Rho GTPases are involved in cell migration by synchronizing changes in actin cytoskeleton from external stimuli, and therefore have implications in metastasis in oncogenic states. Cysmethynil inhibits migration in a highly metastatic breast cancer cell line, MDA-MB-231, with RhoA and Rac1 activity significantly diminished (Cushman and Casey 2009). This decrease in activity is attributed to the aforementioned observed increase in RhoGDI binding affinity to Rac and Rho; however, over-expression of Rho GTPases rescues cell migration repressed by inhibition of Icmt. The culmination of these data supports Icmt as a promising target in cancers commonly afflicted with aberrancies associated with prenylated proteins.

Summary

Icmt is a posttranslational modifying enzyme that specifically methylates prenylated proteins. Further evaluation of its structure and oligomerization state is critical for more in depth analyses of its mechanistic properties and function. Additional domain within human Icmt is still not understood despite suggestions it may play a regulatory role. The unique biologic function of methylation raises the question about the existence and regulation of a potential prenylcysteine dependant methylesterase. Addition of dynamic methylation/demethylation into RhoGDI cycling model will further characterize the impact of Icmt induced methylation in cell signaling and protein degradation. Regardless of the future outcome of Icmt inhibition as an effective cancer treatment, investigation of Icmt-catalyzed methylation of prenylated proteins is pivotal for a complete understanding of the consequences it plays in protein interactions.

References

Anderson JL, Frase H, Michaelis S, Hrycyna CA. Purification, functional reconstitution, and characterization of the *Saccharomyces cerevisiae* isoprenylcysteine carboxylmethyltransferase Ste14p. J Biol Chem. 2005;280(8):7336–45.

Baron RA, Casey PJ. Analysis of the kinetic mechanism of recombinant human isoprenylcysteine carboxylmethyltransferase (Icmt). BMC Biochem. 2004;5:19.

Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Young SG. Targeted inactivation of the isoprenylcysteine carboxyl methyltransferase gene causes mislocalization of K-Ras in mammalian cells. J Biol Chem. 2000;275(23):17605–10.

Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Gomes AQ, et al. Isoprenylcysteine carboxyl methyltransferase deficiency in mice. J Biol Chem. 2001;276(8):5841–5.

Boulter E, Garcia-Mata R, Guilluy C, Dubash A, Rossi G, Brennwald PJ, et al. Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. Nat Cell Biol. 2010;12(5):477–83.

Cushman I, Casey PJ. Role of isoprenylcysteine carboxylmethyltransferase-catalyzed methylation in Rho function and migration. J Biol Chem. 2009;284(41):27964–73.

Cushman I, Casey PJ. RHO methylation matters: a role for isoprenylcysteine carboxylmethyltransferase in cell migration and adhesion. Cell Adh Migr. 2010;5(1):11–15.

Griggs AM, Hahne K, Hrycyna CA. Functional oligomerization of the *Saccharomyces cerevisiae* isoprenylcysteine carboxyl methyltransferase, Ste14p. J Biol Chem. 2010;285(18):13380–7.

Harrington EO, Newton J, Morin N, Rounds S. Barrier dysfunction and RhoA activation are blunted by homocysteine and adenosine in pulmonary endothelium. Am J Physiol Lung Cell Mol Physiol. 2004;287(6):L1091–7.

Huizinga DH, Omosegbon O, Omery B, Crowell DN. Isoprenylcysteine methylation and demethylation regulate abscisic acid signaling in Arabidopsis. Plant Cell. 2008;20(10):2714–28.

Kowluru A, Seavey SE, Li G, Sorenson RL, Weinhaus AJ, Nesher R, et al. Glucose- and GTP-dependent stimulation of the carboxyl methylation of CDC42 in rodent and human pancreatic islets and pure beta cells. Evidence for an essential role of GTP-binding proteins in nutrient-induced insulin secretion. J Clin Invest. 1996;98(2):540–55.

Michaelson D, Ali W, Chiu VK, Bergo M, Silletti J, Wright L, et al. Postprenylation CAAX processing is required for proper localization of Ras but not Rho GTPases. Mol Biol Cell. 2005;16(4):1606–16.

Papaharalambus C, Sajjad W, Syed A, Zhang C, Bergo MO, Alexander RW, et al. Tumor necrosis factor alpha stimulation of Rac1 activity. Role of isoprenylcysteine carboxylmethyltransferase. J Biol Chem. 2005;280(19):18790–6.

Reid TS, Terry KL, Casey PJ, Beese LS. Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. J Mol Biol. 2004;343(2):417–33.

Romano JD, Michaelis S. Topological and mutational analysis of Saccharomyces cerevisiae Ste14p, founding member of the isoprenylcysteine carboxyl methyltransferase family. Mol Biol Cell. 2001;12(7):1957–71.

Svensson AW, Casey PJ, Young SG, Bergo MO. Genetic and pharmacologic analyses of the role of Icmt in Ras membrane association and function. Meth Enzymol. 2006;407:144–59.

Van Dessel GA, De Busser HM, Lagrou AR. Prenylcysteine carboxymethyltransferase type III activity is decreased in retinoic acid-treated SH-SY5Y neuroblastoma cells. Int J Biochem Cell Biol. 2002;34(5):477–86.

Winter-Vann AM, Kamen BA, Bergo MO, Young SG, Melnyk S, James SJ, et al. Targeting Ras signaling through inhibition of carboxyl methylation: an unexpected property of methotrexate. Proc Natl Acad Sci USA. 2003;100(11):6529–34.

Winter-Vann AM, Baron RA, Wong W, dela Cruz J, York JD, Gooden DM, et al. A small-molecule inhibitor of isoprenylcysteine carboxyl methyltransferase with antitumor activity in cancer cells. Proc Natl Acad Sci USA. 2005;102(12):4336–41.

Wright LP, Court H, Mor A, Ahearn IM, Casey PJ, Philips MR. Topology of mammalian isoprenylcysteine carboxyl methyltransferase determined in live cells with a fluorescent probe. Mol Cell Biol. 2009;29(7):1826–33.

ID4

► Inhibitor of DNA Binding 4 (ID4)

IDB4

► Inhibitor of DNA Binding 4 (ID4)

IFI10

► CXCL10

IFNG

► Interferon-Gamma

IFN-Gamma

► Interferon-Gamma

IFN-β2

▶ IL6

IFN-γ

▶ Interferon-Gamma

IkappaBzeta

▶ IkBz

IkBz

Balachandran Manavalan, Shaherin Basith and Sangdun Choi
Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Synonyms

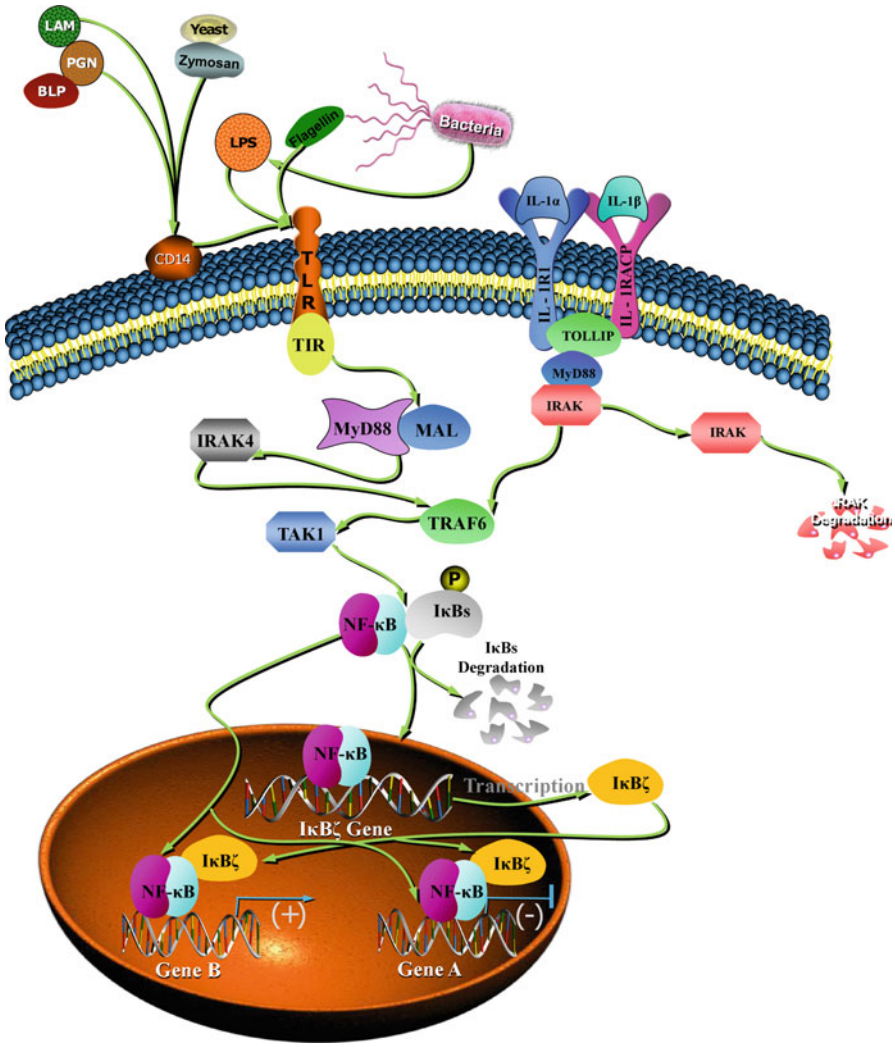
IkappaBzeta; IkB-zeta; IL-1 inducible nuclear ankyrin-repeat protein (INAP); Inap; Mail; Molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL); Molecule possessing ankyrin-repeats induced by lipopolysaccharide; NF-kappa-B inhibitor zeta; NFKBIZ; Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta

Historical Background

IκBζ, which is also known as molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL) or interleukin (IL)-1 inducible nuclear ankyrin-repeat protein (INAP), was discovered independently in three laboratories during 2000 and 2001 as a protein containing ankyrin repeats. The gene encodes a protein with an amino-terminal region nearly 450 amino acids in length of unknown structure that contains a nuclear localization signal (NLS) and a transactivation domain (TAD) followed by the seven recognizable ankyrin repeats at its carboxyl terminal. Treatment of cells with lipopolysaccharides (LPS) or the cytokine interleukin (IL)-1 (Haruta et al. 2001; Kitamura et al. 2000; Yamazaki et al. 2001) induced the expression of IκBζ, whereas tumor necrosis factor (TNF)-α (Totzke et al. 2006) treated cells did not show any IκBζ expression. The induced IκBζ is localized in the nucleus, where it interacts with the nuclear factor (NF)-κB subunit and other nuclear proteins via their ankyrin repeat domain (ARD), leading to a positive or negative regulation of its transcriptional activity depending on genes (Muta 2006). Thus, the innate immune system utilizes NF-κB as a major transcription factor and modulates its activity in a gene-specific manner via the regulatory factor IκBζ, which is specifically induced upon stimulation of the innate immune system. This multistep regulation of the transcriptions would be fundamental in the selective expression of genes upon cell activation. In this chapter, we summarize recent findings in nuclear IκBζ with an emphasis on its immunological aspects.

Induction of IκBζ and Its Functions

Many cellular responses are mediated by orchestrated gene expression. When cells are exposed to diverse inflammatory stimuli, such as microbial components, a large number of genes are induced to elicit inflammatory responses. These genes include cytokine/chemokine, antimicrobial peptides, and cell adhesion molecules, many of which are known to be induced through activation of transcription factor NF-κB (Akira and Takeda 2004; Hayden and Ghosh 2008; Hoffmann and Baltimore 2006). In resting cells, typical cytoplasmic IκB proteins (IκB-α, -β and -ε) mask the NLS of NF-κB, thereby preventing its translocation into the nucleus. The activation of cells with appropriate stimuli, particularly toll-like receptor (TLR) ligands or various host immune mediators such as proinflammatory cytokines and IL-1 superfamily proteins, induces activation of IκB kinase complex, which leads to the degradation of the cytoplasmic IκBs by the ubiquitin-proteosomal pathway. The NF-κB liberated from the IκBs is then translocated to the nucleus, where it binds to the promoter/enhancer region of the target genes, resulting in the regulation



IkBz, Fig. 1 Roles of IκBζ in inflammatory response. Activation of the TIR-containing receptors by TLR ligands elicits phosphorylation and ubiquitination-induced degradation of the cytosolic IκB proteins, which allows nuclear translocation of NF-κB. In the nucleus, NF-κB activates transcription of a subset of genes A, which includes IκBζ. The expression of IκBζ also requires a specific mRNA stabilization signal that comes from the TIR-containing receptor as well as activation of NF-κB. The expressed IκBζ associates with NF-κB, and the complex engages transcription of another subset of genes B. Simultaneously, IκBζ inhibits transcription of the subset of genes A

of transcription via recruitment of several co-activators and co-repressors. This transcriptional activation leads to the expression of primary/early response gene A, depicted in Fig. 1, which includes three atypical members, IκBζ, Bcl-3, and IκBNS. The induced IκBζ associates with NF-κB, and this complex then activates another subset of inflammatory gene B (Fig. 1). Simultaneously, IκBζ inhibits the transcriptional regulation of gene A.

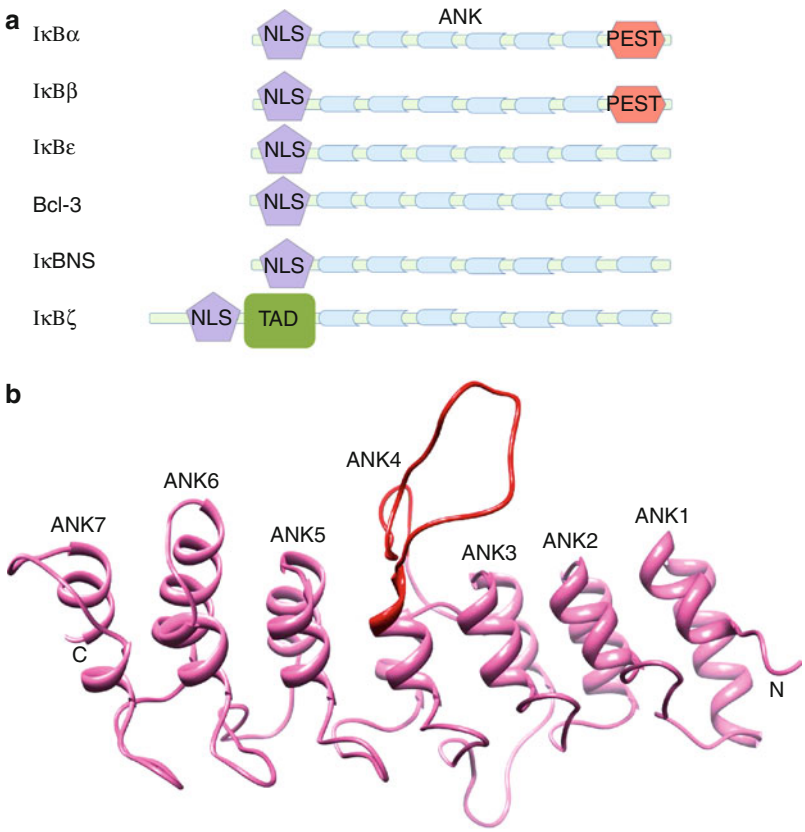
The predominantly expressed cytoplasmic IκB proteins, IκB-α, -β, and -ε, act exclusively as NF-κB inhibitors, whereas the nuclear IκB proteins, Bcl-3 and IκBζ, can both act as either a positive or negative regulator of NF-κB target genes. Bcl-3 can act as a positive regulator of NF-κB either by removing transcriptionally inactive p50 and p52 dimers from the IκB sites, thus allowing transcriptionally active heterodimers to take their places or by forming a ternary complex with DNA-binding p50 and p52 homodimers, thereby facilitating gene expression (Yamazaki et al. 2001; Totzke et al. 2006; Dechend et al. 1999; Fujita et al. 1993; Yamamoto et al. 2004). Unlike other classical IκB proteins, IκBζ is strongly expressed in response to treatment with different proinflammatory stimuli. IκBζ inhibits the transcriptional activity of NF-κB by associating with the p50/p65 heterodimer. More importantly and in contrast to other classical IκB proteins, IκBζ can also induce the expression of genes such as IL-6 and IL-12p40 by binding with the p50 homodimer.

IκBζ is encoded by a primary responsive gene, *Nfkbiz*, and its induction depends on NF-κB activation, suggesting that IκBζ regulated genes are induced via two-step machinery (Motoyama et al. 2005; Yamazaki and Takeshige 2008). Very little IκBζ is detected in unstimulated cells, and it is induced by various microbial substances that stimulate TLRs and IL-1β receptors, but not by TNF-α. Subsequent studies have shown that TNF-α induced transcription of *Nfkbiz* gene, but did not stabilize IκBζ mRNA, indicating that the stimulus-specific expression of IκBζ is determined post-transcriptionally. All TLRs possess ectodomains that recognize ligands from microbial substances and endodomains that facilitate the downstream signaling, which has been shown to be associated with the ectodomain through a transmembrane segment. With the exception of TLR3, which mediates downstream signaling through TRIF/TRAM (which is ▶ Myd88 independent), the other TLRs share common signaling pathways initiated by an adaptor protein, Myd88 (Myd88 dependent), which is necessary for the IκBζ induction. In macrophage cells, LPS stimulation leads to three variants (L, S, and D) that are generated by alternative splicing of IκBζ. The longer form, IκBζ (L, 1–728), is predominantly expressed upon LPS stimulation, while the shorter form IκBζ (S) has been observed at mRNA and protein levels in minor species. Although IκBζ (D) mRNA has been detected in macrophages, its corresponding protein level has not been found (Yamazaki et al. 2005). Overall, IκBζ (L and S) are functionally active when expressed in the cells.

IκBζ (1–728 amino acids) can be divided into N-terminal (1–450) and C-terminal (451–728) portions. The N-terminal region contains NLS and TAD, whereas the C-terminal region contains the ANK repeat domain, which plays an important role in interaction with NF-κB subunits, thereby regulating its functions (Fig. 2a). Truncated mutation studies of IκBζ (especially its N-terminal region) have shown $-K_{163}-R_{164}-X_{12}-K_{177}-R_{178}-$ to be indispensable for NLS, and the mutation of this portion has revealed that IκBζ localized in the cytosol and effectively inhibited NF-κB, which is similar to the cytosolic IκB proteins. IκBζ was initially characterized as a negative regulator of NF-κB, but subsequent studies demonstrated that it could also act as a positive regulator of NF-κB. Analysis using GAL4-fusion protein of IκBζ revealed that its N-terminal region (329–403) exhibits transcriptional activity after association with NF-κB (p50/p50) subunit (Yamazaki et al. 2005). To date, numerous functions of IκBζ have been reported, when bound to other nuclear proteins. (1) Overexpression of IκBζ augmented IL-6 production in response to LPS by interacting with p50 homodimer, whereas TNF-α production is inhibited through interaction with p50/p65 heterodimer, indicating specific target gene activity (Motoyama et al. 2005). (2) IκBζ physically and functionally interacts with STAT3, which is a member of the NF-κB signaling pathway, thereby inhibiting the transcriptional activation of STAT3 (Wu et al. 2009). (3) Human IκBζ expressed in response to TNF-α binds to the DNA-binding region of p50/p65 heterodimer, consequently leading to the inhibition of the TNF-α response (Totzke et al. 2006). (4) IκBζ mediates pre-initiation complex assembly and histone H3K4 methylation, leading to the activation of secondary response genes, thereby suggesting a role of IκBζ in the nucleosome remodeling (Kayama et al. 2008). (5) IκBζ is expressed in IL-17-producing helper T (T_H17) cells that play an important role in resistance to experimental autoimmune encephalomyelitis (EAE) (Okamoto et al. 2010).

Physiological Roles of IκBζ

Studies of IκBζ-deficient mice have demonstrated that IκBζ plays a role as a positive and negative regulator of NF-κB-mediated transcription (Yamamoto et al. 2004). Microarray studies have shown that IκBζ is an indispensable component of the LPS-induced transcription of genes represented by IL-6 and of the genes listed in Table 1. It should also be noted that IL-6 plays an important role in many inflammatory diseases including sepsis, heart attacks, and stroke, as well as in many human cancers including hepatocarcinoma, multiple carcinoma, and ovarian cancer. However, the transcription complex on IL-6 appears to differ depending on the stimuli. The role of IκBζ as a negative regulator was not evident in the isolated cells, probably because of redundant negative regulators of NF-κB. The inhibitory roles of IκBζ in NF-κB-mediated transcription are critical in fine-tuning to balance inflammatory reactions to maintain homeostasis in vivo. IκBζ knockout animals have atopic-like dermatitis and eye inflammation that supports a role of IκBζ in innate host defense. However, the inflammation caused by IκBζ deficiency is not so



IkBz, Fig. 2 IκB family members. (**a**) All IκB proteins harbor ankyrin repeats at their carboxy terminal region. The NH_2 terminal regulatory region of IκB-α, -β, and -ε contains specific sequences that are phosphorylated and ubiquitinated. The NH_2 terminal region of IκBζ contains the nuclear localization and transactivation domains that are necessary for the transcriptional process. (**b**) The homology models of the IκBζ ARD domain are shown in *bright pink*. The 28 amino acid residues occurring within the ANK4 are shown in *red*

IkBz, Table 1 Genes that require IκBζ for LPS-mediated induction

Category	Subset of genes
Cytokines	IL-6, IL-12 p40 subunit, IL-18, granulocyte macrophage stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), growth-differentiation factor (GDF) 15, Epstein-Barr-virus-induced gene (EBI) 3
Chemokines	CXC chemokine ligand (CXCL)5, CXCL13, chemokine ligand (CCL)7, CCL17
Enzymes	Histidine decarboxylase, caspase11, inositol polyphosphate-5-phosphatase B, deltex 2B, glutathione reductase, guanylate nucleotide-binding protein (GDP) 1
Receptors	Formyl peptide receptor 1, macrophage receptor with collagenous structure (MARCO)
Biological active peptides	Endothelin 1, ghrelin
Transcription factors	Basic leucine zipper transcription factor (BATF), CCAAT/enhancer-binding protein (C/EBP)-δ
Antimicrobial substances	Lipocalin 2/neutrophil gelatinase-associated lipocalin (NGAL)
Others	Tax-1 binding protein, extracellular proteinase inhibitor, solute carrier family 11 member 2 (S1c11a2), Src-like adaptor protein (SLAP), immunoglobulin heavy chain, immunoglobulin light chain, membrane spanning 4-domains (MS4A1), thrombospondin 1, immediate early response 3 (IER3/IEX1), disabled-2

clear. Accordingly, it will be important to test whether the skin and eye inflammation in IκBζ knockout mice is due to the lack of host defense molecules such as lipocalin, or lack of essential cytokines such as IL-6. However, the other function of IκBζ, suppression of NF-κB activity, cannot be excluded. Atopic dermatitis and ocular inflammation may also occur due to the over expression of cytokines that IκBζ might inhibit via the C-terminal ankyrin repeat.

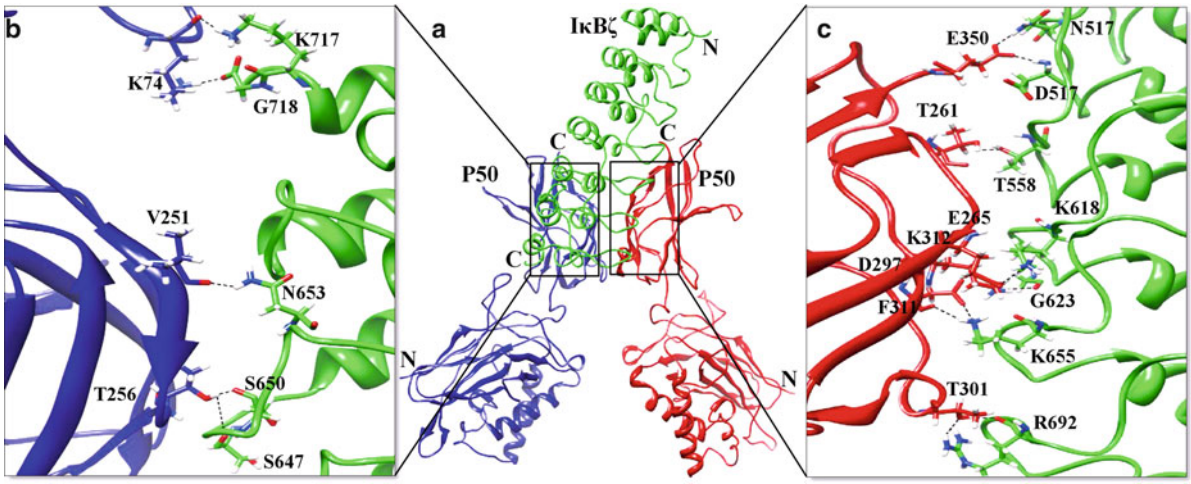
The factors that distinguish genes that are activated or inhibited by IκBζ appear to be present in the promoter of each gene. Biochemical studies have indicated that in addition to NF-κB binding sites, IκBζ is also required for transcriptional activation. Since the transcriptional activation activity of IκBζ on the GAL4-reporter system is much weaker than that of the NF-κB p65 subunit, other transcription factors are necessary for efficient IκBζ-mediated transcription (Yamazaki et al. 2008). IκBζ acts as a negative regulator of the promoter harboring canonical NF-κB-binding sequences alone. Moreover, all the nuclear IκB proteins are homologous to each other; therefore, these nuclear proteins may act as competitors for IκBζ or vice versa. In fact, IκBNS has been reported to inhibit LPS-mediated IL-6 production (Kuwata et al. 2006).

Structure-Based Activation and Inhibition Mechanism of Nuclear IκBζ

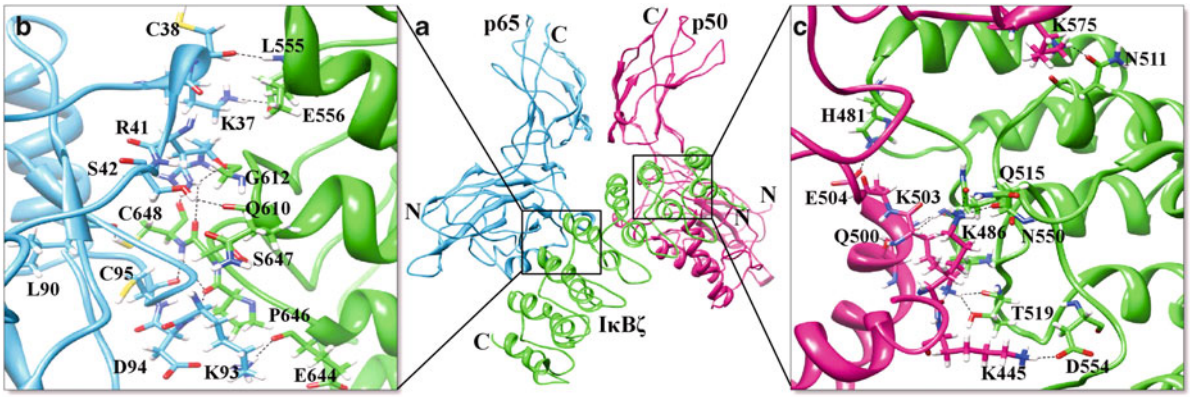
The primary sequence of human and mouse IκBζ share about 70% homology with the N-terminal region (1–450) and 97% sequence identity with the C-terminal ARD. Although there is sequence variation at the N-terminal region, the NLS and TAD are conserved. There has been speculation about the differences in the N-terminal region of human and mouse IκBζ, which differ in their ability to bind with NF-κB proteins. Mouse IκBζ binds only to p50/p50 homodimer, whereas human IκBζ binds to both the p65 and p50 subunits. Sequence analysis of the N-terminal region has not revealed any significant homology to proteins in the NCBI database. In addition, secondary structure prediction showed that the N-terminal has no ANK repeat followed by the C-terminal ARD. This type of architecture has also been reported in proteins such as the yeast ribosomal binding protein yar-1 (Lycan et al. 1996). No crystal structure is yet available for IκBζ ARD, but recent modeling studies have shown the IκBζ three-dimensional structure, which was built based upon the Bcl-3 crystal structure (Michel et al. 2001) (Fig. 2b). Each ANK repeat of the IκBζ models depicted two antiparallel α-helices, followed by a loop of variable length at a right angle. Each repeat began and ended with short β-hairpin turns that protruded away from the α-helix. This non-globular fold was stabilized through intra- and inter-repeat hydrophobic interactions. The represented structural motifs stack upon one another in a linear fashion to form a curved architecture. These motifs are known to facilitate protein–protein interaction, but have no known enzymatic activity (Mosavi et al. 2004). The presence of ankyrin repeats and their role in inflammatory signaling immediately suggested structural and functional homology with the ARD-containing classical IκB proteins. Despite the similarities, IκBζ differs structurally from the classical IκB inhibitors in its unique amino-terminal region, the number of ankyrin repeats, the presence of a unique 28 amino acids insertion within the helices of the α1 and α2 of ANK4 (similar to one that has been observed in IκBNS), and the complete absence of a PEST-like region within the IκBζ carboxy-terminus (Fig. 2b).

Recent studies have indicated that human TNF-α-induced IκBζ is associated with both the p50/p65 subunits of NF-κB in the nucleus, and inhibits the transcriptional activity of anti-apoptotic protein (Totzke et al. 2006). The structural basis of this inhibitory and activation mechanism was explained by docking studies (Manavalan et al. 2011), which have shown that IκBζ ARD associates with the DNA-binding domain of p50/p65 subunits of NF-κB and contains important residues that interact with the bases as well as sugar phosphate backbone present in the p50 and p65 subunit (Fig. 3). Hence, there will be no more p50/p65 subunits available to the promoter region, which ultimately results in inhibition of the transcription mediated by p50/p65 subunit. Generally, p65 subunits contain the TAD at the C-terminal end, which is important for its transcriptional activity (O'Shea and Perkins 2008). IκBζ inhibits p65 transactivation activity through its binding with the N-terminal DNA-binding domain.

Furthermore, IκBζ-p50/p50 complex revealed that ANK3-7 interact with the dimerization domain of the p50 subunit (Fig. 4). The binding orientation of IκBζ



IkBz, Fig. 3 Docking studies predict that the cofactor IκBζ ARD binds at the side of the p50/p65 heterodimer interface. (**a**) The p50/p65 heterodimers, represented as a ribbon diagram, are shown in magenta and cyan, respectively. Docked IκBζ is *green* in the ribbon diagram. (**b**) p65-IκBζ binding interface. Side chains of the amino acid contributing to the hydrogen bonding formation (marked as *black*) are represented by a stick model with the residue name and numbers shown next to them. (**c**) The p50-IκBζ binding interface is shown in a similar fashion as in (**b**)



IkBz, Fig. 4 IκBζ ARD-p50 homodimer interface. (**a**) The p50/p50 dimers are *blue* and *red* in the ribbon diagram. Docked IκBζ is *green* in the ribbon diagram. (**b**) The p50 (chain A)-IκBζ binding interface. Side chains of the amino acid contributing to the hydrogen bonding formation (marked as *black*) are represented by a stick model with the residue name and numbers shown next to them. (**c**) The p50 (chain B)-IκBζ binding interface is shown in a similar fashion as in (**b**)

with this homodimer is similar to that of the classical IκBα-p50/p65 heterodimer. Although the binding orientation is the same, there might be some differences in the regulation of NF-κB-dependent gene expression by IκBα and IκBζ. Activation of p65-containing NF-κB heterodimer by LPS or IL-1 leads to the expression of NF-κB-dependent genes, including IκB proteins IκBα and IκBζ. Following translocation, IκBα enters the nucleus, where it targets NF-κB p50/p65 dimers and removes them from DNA through the acidic PEST motif of IκBα and the basic DNA containing surfaces of the NF-κB p65 subunit that likely disrupt protein/DNA binding. In contrast, IκBζ enters into the nucleus and targets the p50/p50 homodimer, which is already bound to the promoter region, thereby blocking the transcription due to the unavailability of the TAD. Overexpression experiments have suggested that IκBζ exhibited transactivation potential (Motoyama et al. 2005); hence IκBζ mediates transcriptional activity by binding with DNA-bound p50/p50 homodimer,

thereby providing a transactivation domain to the NF-κB complex. Such IκBζ mediated transcription is important for the production of IL-6, antimicrobial peptides, lipocalin, hDB-2, and the genes listed in Table 1. Finally, it should be noted that, when compared with other IκB proteins, IκBζ possesses numerous functions that occur via binding with different nuclear proteins. Recent studies of MD (molecular dynamics) simulation of IκB have revealed that IκBζ possesses more thermodynamically flexible residues than other IκB members. These findings demonstrate that structural flexibility is the major factor that enables IκBζ to interact with different sets of nuclear proteins (Manavalan et al. 2010).

Summary

Studies of IκBζ have provided evidence for multistep regulation of inflammatory responses in TLR signaling. Upon cell activation by appropriate stimuli, primary responses are induced by rapid activation of the major transcription factor (NF-κB), which is activated through posttranslational modifications such as phosphorylation without *de novo* protein synthesis. During this period, transcriptional regulators such as IκBζ are induced via stimuli-specific mechanisms. Secondary response genes are activated and primary responses are gradually diminished via the combinations of major transcription factors and inducible regulators. Since the genes that are activated via secondary responses also include other transcription factors, stimulus-specific transcriptional activation would proceed in a multistep fashion with time after the stimulation. In vitro studies have shown that these nuclear IκB proteins interact with the p50 or p52 subunits of NF-κB. Only p50/p52 double knockout mice, but not single knockout mice, exhibit severely defective immune disorders such as osteopetrosis. However, some immunological phenotypes occur in mice lacking only one nuclear IκB protein. This condition may be compensated by utilizing other IκB proteins. Further studies are required to clarify and discover new and detailed physiological aspects of the nuclear IκB proteins in the future by using mice devoid of two or all three nuclear IκB proteins. In conclusion, it can be seen that nuclear IκBζ not only contributes to NF-κB mediated transcription, but also plays an important role in innate immune responses by modulating the expression of proinflammatory cytokines.

Acknowledgments This work was supported by the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (2012016803).

References

Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499–511.

Dechend R, Hirano F, Lehmann K, Heissmeyer V, Ansieau S, Wulczyn FG, et al. The Bcl-3 oncoprotein acts as a bridging factor between NF-kappaB/Rel and nuclear co-regulators. Oncogene. 1999;18(22):3316–23.

Fujita T, Nolan GP, Liou HC, Scott ML, Baltimore D. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev. 1993;7(7B):1354–63.

Haruta H, Kato A, Todokoro K. Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. J Biol Chem. 2001;276(16):12485–8.

Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell. 2008;132(3):344–62.

Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. Immunol Rev. 2006;210:171–86.

Kayama H, Ramirez-Carrozzi VR, Yamamoto M, Mizutani T, Kuwata H, Iba H, et al. Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IkappaBzeta. J Biol Chem. 2008;283(18):12468–77.

Kitamura H, Kanehira K, Okita K, Morimatsu M, Saito M. MAIL, a novel nuclear I kappa B protein that potentiates LPS-induced IL-6 production. FEBS Lett. 2000;485(1):53–6.

Kuwata H, Matsumoto M, Atarashi K, Morishita H, Hirotani T, Koga R, et al. IkappaBNS inhibits induction of a subset of toll-like receptor-dependent genes and limits inflammation. Immunity. 2006;24(1):41–51.

Lycan DE, Stafford KA, Bollinger W, Breeden LL. A new Saccharomyces cerevisiae ankyrin repeat-encoding gene required for a normal rate of cell proliferation. Gene. 1996;171(1):33–40.

Manavalan B, Basith S, Choi YM, Lee G, Choi S. Structure-function relationship of cytoplasmic and nuclear IkappaB proteins: an in silico analysis. PLoS One. 2010;5(12):e15782.

Manavalan B, Govindaraj R, Lee G, Choi S. Molecular modeling-based evaluation of dual function of IkappaBzeta ankyrin repeat domain in toll-like receptor signaling. J Mol Recognit. 2011;24(4):597–607.

Michel F, Soler-Lopez M, Petosa C, Cramer P, Siebenlist U, Muller CW. Crystal structure of the ankyrin repeat domain of Bcl-3: a unique member of the IkappaB protein family. EMBO J. 2001;20(22):6180–90.

Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY. The ankyrin repeat as molecular architecture for protein recognition. Protein Sci. 2004;13(6):1435–48.

Motoyama M, Yamazaki S, Eto-Kimura A, Takeshige K, Muta T. Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. J Biol Chem. 2005;280(9):7444–51.
Muta T. IkappaB-zeta: an inducible regulator of nuclear factor-kappaB. Vitam Horm. 2006;74:301–16.
O'Shea JM, Perkins ND. Regulation of the RelA (p65) transactivation domain. Biochem Soc Trans. 2008;36(Pt 4):603–8.
Okamoto K, Iwai Y, Oh-Hora M, Yamamoto M, Morio T, Aoki K, et al. IkappaBzeta regulates T(H)17 development by cooperating with ROR nuclear receptors. Nature. 2010;464(7293):1381–5.
Totzke G, Essmann F, Pohlmann S, Lindenblatt C, Janicke RU, Schulze-Osthoff K. A novel member of the IkappaB family, human IkappaB-zeta, inhibits transactivation of p65 and its DNA binding. J Biol Chem. 2006;281(18):12645–54.
Wu Z, Zhang X, Yang J, Wu G, Zhang Y, Yuan Y, et al. Nuclear protein IkappaB-zeta inhibits the activity of STAT3. Biochem Biophys Res Commun. 2009;387(2):348–52.
Yamamoto M, Yamazaki S, Uematsu S, Sato S, Hemmi H, Hoshino K, et al. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. Nature. 2004;430(6996):218–22.
Yamazaki S, Takeshige K. Protein synthesis inhibitors enhance the expression of mRNAs for early inducible inflammatory genes via mRNA stabilization. Biochim Biophys Acta. 2008;1779(2):108–14.
Yamazaki S, Muta T, Takeshige K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. J Biol Chem. 2001;276(29):27657–62.
Yamazaki S, Muta T, Matsuo S, Takeshige K. Stimulus-specific induction of a novel nuclear factor-kappaB regulator, IkappaB-zeta, via Toll/Interleukin-1 receptor is mediated by mRNA stabilization. J Biol Chem. 2005;280(2):1678–87.
Yamazaki S, Matsuo S, Muta T, Yamamoto M, Akira S, Takeshige K. Gene-specific requirement of a nuclear protein, IkappaB-zeta, for promoter association of inflammatory transcription regulators. J Biol Chem. 2008;283(47):32404–11.

IkB-zeta

▶ IkBz

IL-1 Inducible Nuclear Ankyrin-Repeat Protein (INAP)

▶ IkBz

IL6

Akihiro Kimura[1], Tetsuji Naka[2] and Tadamitsu Kishimoto[1]
[1]Laboratory of Immune Regulation, Osaka University Graduate School of Frontier Biosciences, Osaka, Japan
[2]Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan

Synonyms

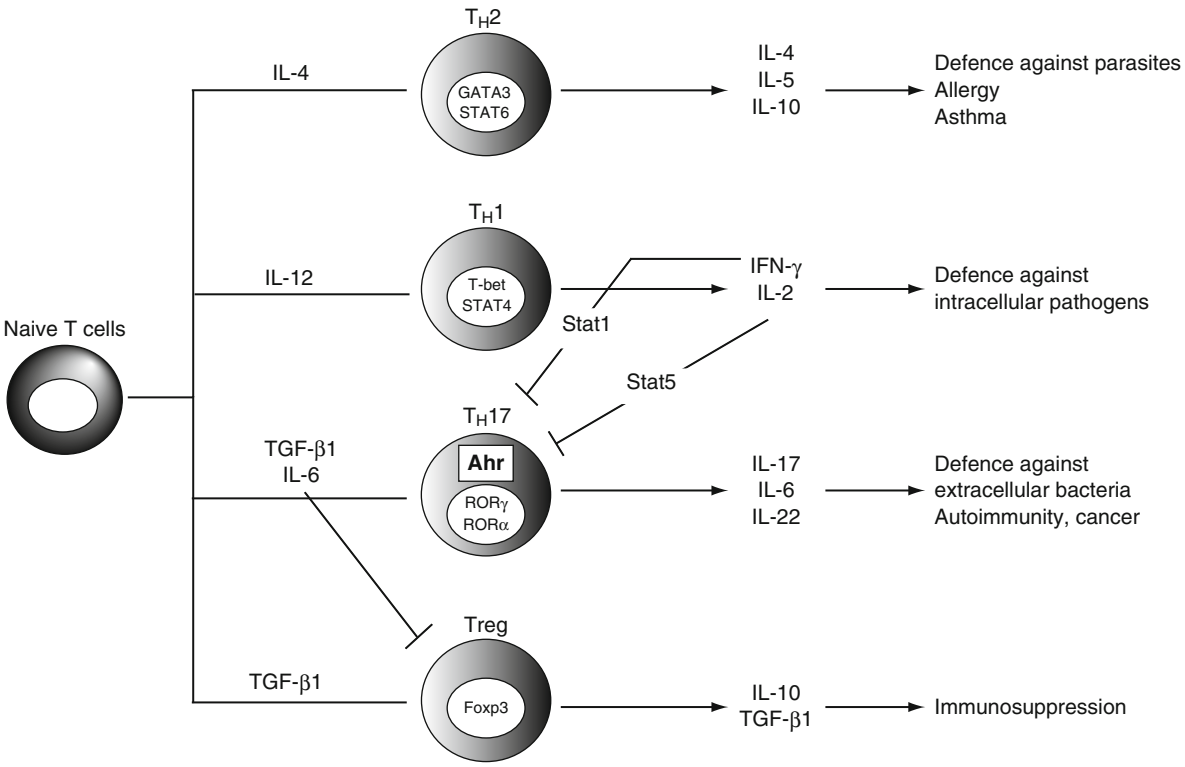
B cell stimulatory factor-2 (BSF-2); IFN-β2

Historical Background

IL-6 was cloned as B cell stimulatory factor-2 (BSF-2) in 1986 (Hirano et al. 1986). This molecule has various biological activities; a strong stimulatory effect on growth of murine plasmacytoma and human myeloma, its functions as a hepatocyte stimulating factor, and the induction of acute phase reaction. IL-6 knockout (KO) mice revealed the inhibition of the antiviral antibody response after immunization with a vesicular stomatitis virus, compared with wild-type (WT) mice. Thus, IL-6 is a pleiotropic cytokine that is involved in the physiology of virtually every organ system.

Introduction

$CD4^+$ T cells (Th) are essential regulators of immune responses and inflammatory diseases. They can be divided into different subsets such as Th1, Th2, and regulatory T (Treg) cells, whose development is specified by the transcription factors T-bet, GATA3, and fork head box p3 (Foxp3), respectively (Fig. 1). The development of Th1 cells, which activate macrophages and are highly effective in clearing intracellular pathogens, is coupled to the sequential actions of interferon-γ (IFN-γ) and interleukin-12 (IL-12). Th2 cells, which differentiation is driven by IL-4, are important for the production of immunoglobulin E and the clearance of extracellular organisms. In addition to these effector subsets, $CD4^+$ T cells can differentiate into distinct regulatory subsets (Treg), which express the fork head/winged helix transcription factor Foxp3. Transforming growth factor-β1 (TGF-β) promotes



IL6, Fig. 1 Th cell differentiation. Naïve T cells can differentiate into several subsets. Th17 cells were recently identified as a novel $CD4^+$ lineage. They are induced by TGF-β plus IL-6. RORγt and RORα act as the master transcriptional factors for Th17 cells. (See text for details about their differentiation)

the differentiation of Treg cells, which suppress adaptive T cell responses and prevent autoimmunity.

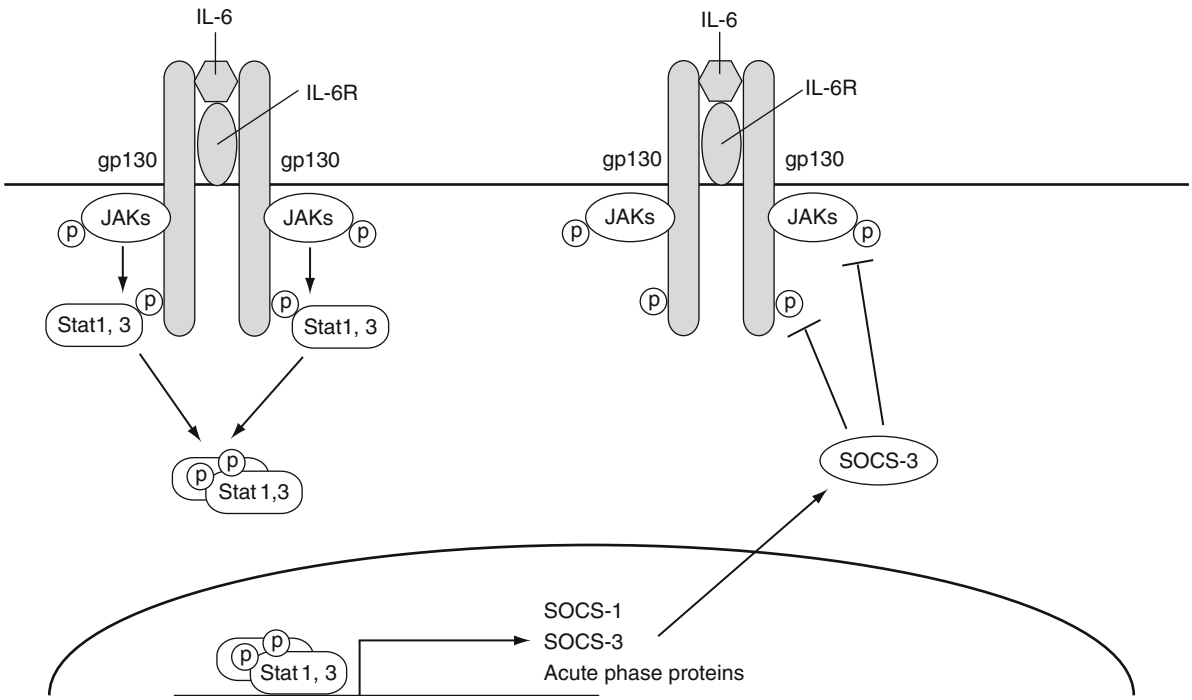
Until recent years, it has been believed that Th1 cells mainly dominate the induction and progression of many autoimmune diseases. However, IFN-γ deficient (KO) mice do not show resistance to autoimmunity. On the contrary, they are even more susceptible to autoimmunity, which led us to hypothesize that there may be an additional Th subset that is distinct from Th1 cells. Recently, a new subset of Th cells that produces IL-17 (Th17) has been identified and was shown to have a crucial role in the induction of autoimmune diseases, such as rheumatoid arthritis and experimental autoimmune encephalomyelitis (EAE), and allergen-specific responses. The differentiation of Th17 cells from naïve T cells requires stimulation by IL-6 and TGF-β, which induces the master transcriptional factors of the Th17 subset such as retinoid-related orphan receptor γt (RORγt) and RORα (Fig. 1) (Korn et al. 2009).

Interleukin-6 (IL-6)

IL-6 Receptors and Signaling

The IL-6 receptor (IL-6R) system consists of two polypeptide chains: an 80 kDa IL-6 receptor and a 130 kDa signal transducer (gp130) (Kishimoto 2005). IL-6R (80 kDa) exists in both a transmembrane form and a soluble form. IL-6 binds to both of these forms, which can then interact with gp130 and trigger signal transduction and gene expression. Gp130 is expressed ubiquitously in tissues. In addition to being a component of the IL-6R, gp130 is a component of receptors for other cytokines, such as leukemia inhibitory factor (LIF), IL-11, oncostatin M (OM), and cardiotropin-1 (CT-1), which explains the functional redundancy of IL-6 superfamily cytokines.

Although gp130 has no intrinsic kinase domain, members of the Janus kinase (JAK) family, such as JAK1, JAK2, and tyrosine kinase 2 (TYK2), are constitutively associated with gp130 (Kishimoto 2005). Complexes of IL-6, IL-6R, and gp130 phosphorylate



IL6, Fig. 2 IL-6 signaling and negative feedback regulation by SOCS. Upon IL-6 interaction, the IL-6R/gp130 complex phosphorylates JAKs and then activates STAT1 and STAT3. Activation of STAT1 and STAT3 induces *SOCS1* and *SOCS3* gene expression. SOCS1 binds primarily to phosphorylated JAK proteins through its SH2 domain, whereas the SH2 domain of SOCS3 binds to phosphorylated tyrosine residues in the cytoplasmic domain of receptors. These interactions terminate STAT activation and suppress downstream gene expression

these kinases and then activate the cytoplasmic transcriptional factors, signal transducers, and activators of transcription 1(STAT1) and STAT3 (Fig. 2) (Ihle and Kerr 1995). In addition, IL-6 activates mitogen-activated protein kinase (MAPK), which phosphorylates the nuclear factor for IL-6 (NF-IL6). Thus, IL-6 activates these kinases and transcriptional factors through IL-6R/gp130 complexes, which leads to gene expression.

Negative Feedback Regulation of IL-6 Signaling

Although IL-6 is essential for the regulation of the immune process, overproduction of the cytokine causes inflammation and autoimmune diseases such as rheumatoid arthritis, systemic juvenile arthritis, and Crohn's disease. Therefore, negative feedback regulation of IL-6 signaling is required for immune homeostasis. Cytokine signaling, such as IL-6 signaling, is negatively regulated by the suppressor of cytokine signaling (SOCS) and the protein inhibitor of activated STATs (PIAS). The SOCS family is composed of eight members: cytokine inducible SRC homology 2 (SH2)-domain-containing protein (CIS) and SOCS1 to SOCS7. SOCS-1, also called STAT-induced STAT inhibitor-1 (SSI-1) and JAK-binding protein (JAB), was initially identified as an intracellular negative-feedback molecule that inhibits JAK-STAT signaling initiated by various stimuli, including IFN-g, IL-4, IL-6, and leukemia inhibitory factor (LIF) (Krebs and Hilton 2000). SOCS-1 mainly inhibits IFN-γ signaling in vivo by binding to JAKs to inhibit its following signal transduction. However, SOCS-1 also negatively regulates innate immune responses such as lipopolysaccharide (LPS) -Toll-like receptor 4 (TLR4) signaling. Thus, SOCS-1 acts as an essential negative regulator in not only cytokine signaling but also TLR signaling.

Although SOCS-3 is also induced by cytokines such as IFN-γ and IL-6, and it can inhibit JAK activation as well as SOCS-1, SOCS-3 binds the cytokine receptor through its SH2 domain (Fig. 2). The activation of Stat1 and Stat3 induced by IL-6 is prolonged in SOCS-3-deficient tissues and cells, but not in

SOCS-1-deficient tissues and cells, which indicates that SOCS-3 is a pivotal regulator of IL-6 signaling in vivo.

IL-6 and Immune Diseases

IL-6 is involved in many diseases such as rheumatoid arthritis, systemic-onset juvenile idiopathic arthritis, systemic lupus erythematosus, Crohn's disease, and inflammatory bowel disease (Kishimoto 2005). In addition, IL-6 is involved in multiple sclerosis (MS), which is a chronic inflammatory disease affecting the central nervous system (CNS) white matter. Patients with MS exhibit higher mean levels of IL-6 in their cerebrospinal fluid than normal controls, and the treatment with an anti-IL-6 receptor monoclonal antibody (anti-IL-6R mAb) inhibited the development of EAE, which is a murine model of human MS that shares many pathological and histological characteristics with human MS.

IL-6 blockade seems to be an innovative treatment strategy for the numerous immune diseases that are impacted by IL-6 overproduction. Actually, blocking IL-6 signaling with a humanized anti IL-6R monoclonal antibody (tocilizumab) is an effective treatment for patients with autoimmune diseases such as Castleman's disease and systemic onset juvenile arthritis (Venkiteshwaran 2009). Although many proinflammatory cytokines including IL-6, TNF-α, and IL-1β are increased in patients with autoimmune diseases such as rheumatoid arthritis and MS, the discovery of Th17 cells has defined IL-6 blockade as the potent and dominant treatment for these diseases. The details of the relationship between IL-6 and Th17 cells in autoimmune diseases will be discussed in section IL-6 and Th17 Cells in Autoimmune Diseases.

IL-17-Producing Helper T Cell (Th17)

Th17 cells produce IL-17A (IL-17), IL-17F, IL-22, IL-6, and TNF-α. The IL-17 family is composed of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. IL-17 is a potent inflammatory cytokine (Korn et al. 2009). As stated above, autoimmune diseases were previously assumed to be associated with dysregulated Th1 responses. However, IFN-γ deficiency did not attenuate some models of autoimmune diseases like EAE; on the contrary, IFN-γ deficiency worsened the disease. It was recently demonstrated that Th17 cells are dominantly associated with human and mouse autoimmune diseases such as rheumatoid arthritis, MS, and inflammatory bowel disease. In fact, IL-17 KO mice are resistant to the development of collagen-induced arthritis (CIA) and EAE, and IL-17 blockade by IL-17-blocking antibody prevents the development of EAE.

Th17 Cell Differentiation

Although initial reports claimed that IL-23 is required for the generation of Th17 cells from naïve T cells, it was subsequently demonstrated that IL-23R is not expressed on naïve T cells and that IL-23 acts as a survival signal for Th17 cells. At present, it is believed that Th17 cell differentiation is driven by the combination of IL-6 and TGF-β (Kimura and Kishimoto 2010). The orphan nuclear receptors, RORγt and RORα, are the key transcription factors that determine the differentiation of the Th17 lineage. IL-6 together with TGF-β induces these transcription factors, whereas IL-6 inhibits TGF-β-induced expression of Foxp3, a master transcriptional factor for Treg. The levels of RORγt and RORα are significantly reduced in Stat3-deficient T cells, but not in Stat1-deficient T cells under Th17-polarizing conditions, which indicates that Th17 cell differentiation is dependent on Stat3. In contrast to Stat3 activation, Stat1 activation inhibits the development of Th17 cells (discussed below). Although IL-6 activates both Stat3 and Stat1, Stat3 activation is maintained while Stat1 activation is suppressed in Th17 cells.

Conditioned medium from LPS-stimulated bone marrow-derived dendritic cells (DCCMs) can induce the production of IL-17 in naïve T cells. Interestingly, IL-17 was produced by DCCM even with the addition of anti-gp130 antibody or DCCM from IL-6 KO mice, which indicates that there is an IL-6-independent pathway in Th17 commitment. Although several cytokines including TNF-α and IL-23 participate in Th17 cell development, they are not required for the initiation of Th17 differentiation. What, then, is required for Th17 differentiation besides IL-6? It has been demonstrated that IL-21 acts as an initiator for Th17 commitment independently of IL-6. IL-21 is a novel cytokine produced by activated T cells and natural killer T (NKT) cells, and its receptor complex is composed of the common IL-2 receptor γ chain (γc) and IL-21 receptor (IL-21R). IL-21 and IL-6 inhibit TGF-β-induced Foxp3 expression and induce RORγt and RORα in a Stat3-dependent manner such that naïve T cells differentiate into Th17 cells.

Other Factors Involved in Th17 Cell Differentiation

The combination of IL-6 and TGF-β is unable to sustain the activation of Stat1 in Th17 cells, although it can sustain Stat3 activation. On the other hand, both Stat1 and Stat3 remained activated in Th17 cells induced by DCCM. These findings provide a novel and unknown basis for Th17 cell differentiation from naïve T cells. In fact, transcriptional factors such as Interferon-regulatory factor 4 (IRF4) and T-bet act as the positive and negative regulator for Th17 commitment, respectively. It has been also reported that retinoic acid inhibits Th17 cell development, and dioxin, a ligand of Aryl hydrocarbon receptor, promotes the generation of Th17 cells. Thus, IL-6 plays a central role in Th17 cell differentiation, whereas various factors regulate Th17 cell development.

Negative Regulation of Th17 Cell Differentiation

There is a negative regulatory system for Th17 cell differentiation. IL-27 and IFN-γ are responsible for the inhibition of its development in a Stat1-dependent manner. IL-27, another IL-12 family member, uses a receptor complex composed of IL-27R and gp130 to transduce its signal and activates both Stat1 and Stat3. Although both IL-6 and IL-27 transduce their signals via the gp130-JAK-STAT axis, IL-6 initiates Th17 commitment dependently on Stat3 activation, and IL-27 inhibits its development dependently on Stat1 activation. Additionally, IL-27 augments T-bet, the master transcriptional factor for Th1 cells, which indicates that IL-27 has both pro- and anti-inflammatory properties in Th cell differentiation.

It has been demonstrated that IL-2 also inhibits Th17 cell development. IL-2 cannot inhibit Th17 cell differentiation in Stat5-deficient T cells, and that there are Stat5 binding sites in the IL-17 promoter region; these findings suggest that Stat5 serves as a repressor. Thus, STAT family members activated by various cytokines provide positive and negative regulation of Th17 cell differentiation. However, the mechanisms of the regulation have not been elucidated.

Aryl Hydrocarbon Receptor: A New Player in Th17 Cells

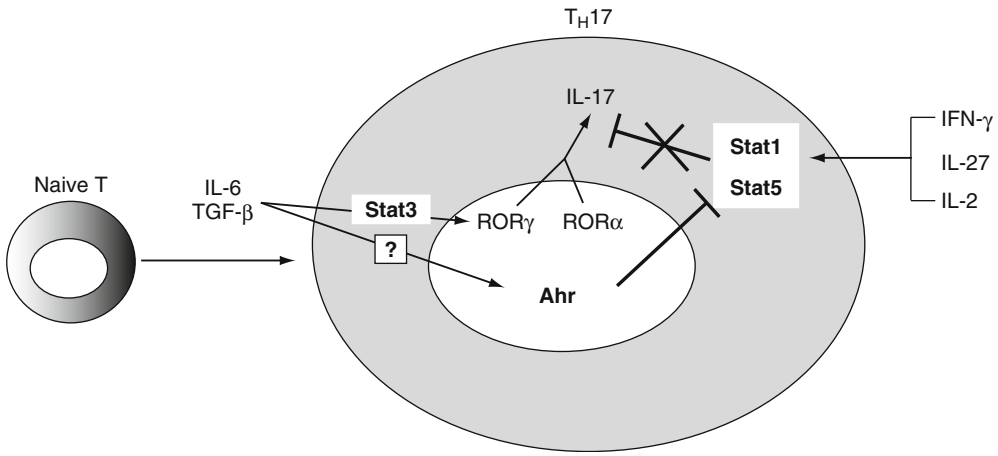
Aryl Hydrocarbon Receptor

Aryl hydrocarbon receptor (Ahr), also known as dioxin receptor, is a ligand-activated transcription factor that belongs to the basic-helix-loop-helix-PER-ARNT-SIM family. Ahr is present in the cytoplasm, where it forms a complex with heat shock protein (HSP) 90, Ahr-interacting protein (AIP), and p23. Upon binding with a ligand, Ahr undergoes a conformational change, translocates to the nucleus, and dimerizes with Ahr nuclear translocator (Arnt). Within the nucleus, the Ahr/Arnt heterodimer binds to a specific sequence, the xenobiotic responsive element (XRE), which causes a variety of toxicological effects. Interestingly, it has been recently reported that Ahr is a ligand-dependent E3 ubiquitin ligase, which implies that Ahr has dual functions in controlling intracellular protein levels, serving both as a transcriptional factor to promote the induction of target proteins and as a ligand-dependent E3 ubiquitin ligase to regulate selective protein degradation. Ahr activated by ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), regulates the generation of regulatory T cells (Tregs) and modulates the Th1/Th2 balance. In addition, it has been recently demonstrated that Ahr participates in Th17 cell differentiation. These data collectively demonstrate the importance of Ahr in the differentiation of T cell subsets.

Ahr Functions in Th17 Cells

Ahr is specifically induced in naïve T cells under Th17-polarizing conditions such as TGF-β plus IL-6 or TGF-β plus IL-21. Although the molecular mechanism of Ahr expression in Th17 development is not known, it is possible that its induction may be regulated downstream of Stat3 by IL-6 and TGF-β because Ahr expression was induced by TGF-β plus IL-6 in Stat1-deficient naïve T cells.

As stated above, Th17 differentiation is positively regulated by IL-6 or IL-21 in combination with TGF-β and negatively regulated by IFN-γ or IL-27. The positive regulation is controlled by Stat3, while the negative regulation is controlled by Stat1. It was found that Ahr binds to Stat1 and Stat5, but not to other members of the Stat family, in Th17 cells; this result suggests that Ahr may regulate the generation of Th17 cells by modifying the activation of Stat1 and Stat5, which negatively regulate Th17 generation. Indeed, Ahr deficiency prolonged Stat1 activation 24 h after stimulation with TGF-β plus IL-6, whereas its activation was relatively transient and returned to the basal level in WT naïve T cells during the same period. On the other hand, Stat3 activation was equally maintained in both Ahr WT and KO naïve T cells. The mechanism by which Ahr interacts with Stat1 and Stat5 and negatively regulates their activation in Th17 cell



IL6, Fig. 3 Ahr functions in Th17 cells. Ahr is induced under Th17-polarizing conditions. Ahr participates in Th17 cell differentiation by regulating Stat1 activation, which suppresses the development of Th17 cells. Ahr may regulate Stat1 activation by functioning as a ligand-dependent E3 ubiquitin ligase that degrades activated Stat1

differentiation is not yet understood. Given that Ahr serves both as a transcriptional factor and as a ligand-dependent E3 ubiquitin ligase, it is possible that Ahr marks activated Stat1 for degradation via its ubiquitin ligase function in Th17 cells (Fig. 3).

IL-6 and Th17 Cells in Autoimmune Diseases

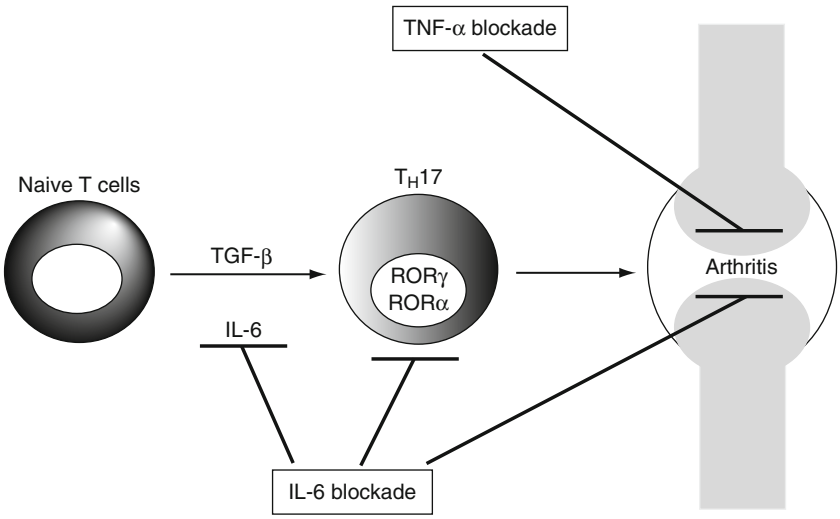
The overproduction of IL-6 or abnormalities in its signal transduction is causative factors in autoimmune disorders including rheumatoid arthritis. At present, there is increasing clarification of the role of IL-6 in diseases such as RA in which Th17 cells are considered to be the primary cause of pathology. Humanized anti-IL-6 receptor antibodies (Tocilizumab) are currently being used clinically as an IL-6-blocking therapy for several autoimmune diseases. This section focuses on the relationship between IL-6 and Th17 cells in the pathogenesis of inflammatory diseases and an effective approach by Tocilizumab for the treatment of several autoimmune diseases.

IL-6 and Th17 Cells in Mouse Autoimmune Disease Models (CIA, EAE)

Previously, Th1 cells were considered to play a major role in pathogenesis of CIA and EAE. However, in IFN-γ-deficient mice and IFN-γ receptor-deficient mice, CIA and EAE symptoms are not ameliorated but rather exacerbated. In contrast, these diseases are suppressed in IL-17-deficient mice, and are alleviated by treatment with IL-17-neutralizing antibodies. These results indicate that in diseases such as CIA and EAE, Th17 cells are actually the major population in their pathogenesis and the in vivo differentiation and propagation of Th17 cells can be used as an index of these disease models.

Previous analyses revealed that IL-6 deficient mice are resistant to CIA and EAE. However, the reason for this resistance has been poorly understood. In recent years, it has been clarified that Th17 cells are induced from naïve CD4 T cells by TGF-β and IL-6 in vitro; therefore, it is conceivable that the impaired Th17 cell differentiation in these mice is the major cause for the resistance to CIA and EAE. However, in genetically deficient mice, their susceptibility to diseases such as CIA and EAE may be influenced by possible intrinsic defects in immune cells or nonuniform genetic backgrounds. Therefore, the administration of anti-IL-6 receptor antibodies to congenic wild-type mice with CIA or EAE is required to investigate the in vivo action of IL-6 in Th17 differentiation.

CIA is induced by administering type II collagen together with an adjuvant on Day 0 and Day 21 in mice. Interestingly, although anti-IL-6 receptor antibodies administered on Day 0 suppressed the induction of Th17 cells in the regional lymph nodes and the development of arthritis, antibodies administered on Day 14 did not suppress Th17 cells or arthritic symptoms. These results indicate that the inhibition of Th17 differentiation caused by anti-IL-6 receptor antibodies is necessary for CIA suppression and that for CIA in the in vivo environment; IL-6 is required for the initial differentiation from naïve T cells to Th17 cells, but



IL6, Fig. 4 Different mechanisms between anti-IL-6 receptor antibodies and TNFR-Fc therapies in RA. Anti-IL-6 receptor antibodies inhibit IL-6 at inflammation sites, although they mainly act to suppress the onset of disease by suppressing the initial differentiation phase of Th17 cells. By contrast, TNF inhibition does not suppress the initial differentiation phase of Th17, but it is believed to inhibit TNF-α at inflammation sites

not for the maintenance of Th17 cells after differentiation. Additionally, it was investigated whether a suppressing effect of Th17 cell development is observed with TNF inhibitor therapy in CIA. Intriguingly, when a TNF-soluble receptor (TNFR-Fc) was administered during the initial CIA induction period (Days 0–14), arthritis and Th17 differentiation could not be suppressed. However, when TNFR-Fc was administered after Day 21, arthritis is substantially suppressed without any effects on Th17 cell development. These results suggest that IL-6 inhibitor treatment in CIA acts primarily on initial CD4 T cell response including Th17 cell differentiation, rather than on the effector phase including angiogenesis and osteoclast differentiation. By contrast, it is suggested that the main point of action in TNF inhibitor therapy is different from that in IL-6 inhibitor therapy; it does not play a role in initial Th17 differentiation but it does act in the effector phase.

EAE is induced by administering the myelin sheath framework protein myelin oligodendrocyte glycoprotein (MOG) peptide together with an adjuvant and pertussis toxin. When anti-IL-6 receptor antibodies were administered immediately after antigen stimulation, the occurrence of EAE could be suppressed in the same manner as for CIA. In EAE models treated with anti-IL-6 receptor antibodies, no Th17 cells were found in draining lymph nodes or the spinal cord. Moreover, immune cells such as T cells, B cells, and macrophages were hardly observed in the lesion of spinal cord. On the other hand, the effect of anti-IL-6 receptor antibodies on Th17 cells and the disease onset was abolished when their administration was delayed. Thus, IL-6 is required for the initial differentiation phase for Th17 in the EAE model, and it appears that IL-6 also acts on cells other than Th17 cells. These results show that IL-6 inhibitor therapy is highly effective in suppressing the occurrence of EAE. Analyses of CIA and EAE indicate that initial Th17 differentiation phase in these autoimmune diseases is fundamentally dependent on IL-6, which suggests that IL-6 is a promising therapeutic target for autoimmune diseases involving Th17 cell inflammatory functions.

IL-6-Blocking Therapy in Human Autoimmune Diseases

In humans, the therapy by Tocilizumab has become a novel therapeutic strategy for some inflammatory and autoimmune diseases, including RA, systemic-onset juvenile idiopathic arthritis (JIA), Crohn's disease (CD), Castleman's disease, multiple myeloma, and systemic lupus erythematosus (SLE) (Venkiteshwaran 2009). Tocilizumab can block the IL-6 signals induced by the interaction of IL-6 and IL-6R. In the RA patients, Tocilizumab significantly improved the symptoms and ACR (American College of Rheumatology) improvement scores 20, 50, and 70 were 89%, 70%, and 47%, respectively, and normalized CRP and SAA in the patients within 6 weeks. Although a role of Th17 in RA is less clear, it has been reported that IL-17 is detected in the synovial fluid from RA patients and acts as a potent stimulator of osteoclastogenesis. Given that IL-6 is important for Th17 cell differentiation not

only in mice but also in humans, Tocilizumab may improve the symptoms of RA through regulating the development of Th17 cells. As described above, it has been shown that IL-6 blockade by Tocilizumab is therapeutically effective for other inflammatory diseases such as Castleman's disease, JIA, and CD; however, it is also still controversial whether Tocilitumab can inhibit Th17 cell differentiation in the improvement of these autoimmune diseases. It is required to demonstrate how Tocilizumab contributes to the treatment for above autoimmune diseases and whether Tocilizumab can also bring the therapeutic benefits for other autoimmune disorders.

TNF-inhibitor therapies (infliximab, etanercept) as well as anti-IL-6 receptor antibodies are also clinically effective for the treatment of autoimmune diseases such as RA, JIA, and Crohn's disease. Although both TNF-α and IL-6 are conventional inflammatory cytokines, it was suggested that they clearly have different roles in Th17 differentiation and that their therapeutic effects are different in CIA (Fig. 4). Detailed elucidation of the relationship between Th17 and pro-inflammatory cytokines such as IL-6 and TNF-α in human autoimmune disorders will provide important information when considering the proper use and switchover of biological agents.

Summary

In the past two decades, the knowledge on IL-6 has advanced from basic science to medicine. IL-6 is a pleiotropic cytokine that plays a major role in immune response, inflammation and hematopoiesis and its levels are increased in various autoimmune diseases. Tocilizumab is a humanized antihuman IL-6R antibody that inhibits the biological activities of IL-6 by blocking the binding of IL-6 to IL-6R. IL-6 blockade holds therapeutic value in autoimmune diseases, including Castleman's disease, JIA, and RA. However, the precise reason why IL-6 blockade leads to the improvement of RA and other human autoimmune disorders is not well understood. The discovery of Th17 cells sheds light on the novel function of IL-6 and helps to address the above question. Th17 cells are IL-17-producing helper T cells and IL-17 is involved in the development of several autoimmune diseases. IL-6 is an essential factor for Th17 cell development, which is one of the reasons why targeting IL-6 activities is an effective approach. In the next phase, the potential of IL-6-targeting therapies in the treatment of various autoimmune diseases will be fully clarified.

Acknowledgments We thank our colleagues in our laboratory for helpful discussion. This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation and Chugai-Roche Pharmaceutical Co. Ltd, Tokyo, Japan.

Cross-References

► IL6

References

Hirano T, Yasukawa K, Harada H, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature. 1986;324:73–6.
Ihle JN, Kerr IM. Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet. 1995;11:69–74.
Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. Eur J Immunol. 2010;40:1830–5.
Kishimoto T. Interleukin-6: from basic science to medicine–40 years in immunology. Annu Rev Immunol. 2005;23:1–21.
Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485–517.
Krebs DL, Hilton DJ. SOCS: physiological suppressors of cytokine signaling. J Cell Sci. 2000;113:2813–9.
Venkiteshwaran A. Tocilizumab. MAbs. 2009;1:432–8.

IL7

Benedict Seddon
Division of Immune Cell Biology, MRC National Institute for Medical Research, Mill Hill, London, UK

Synonyms

Interleukin-7

Historical Background

Interleukin (IL)-7 is a secreted soluble globular protein 25 kDa in size encoded by the IL7 gene. It was first

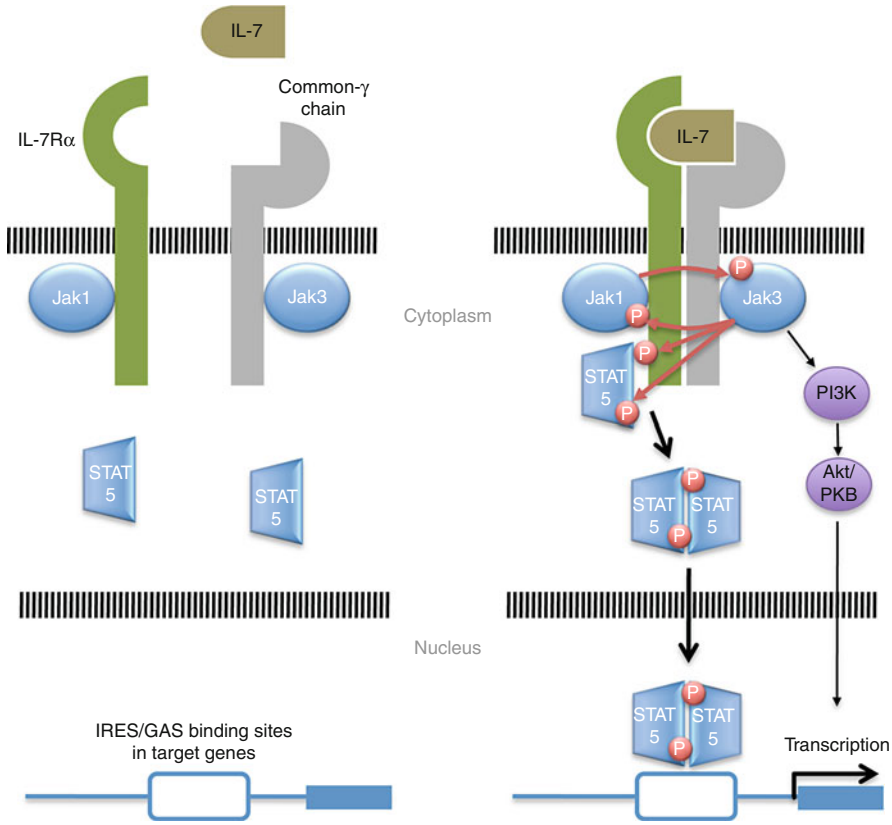
identified as a growth factor for murine B cell precursors found in the bone marrow. Other researchers found that mRNA encoding IL-7 was enriched in the thymus, the lymphoid organ in which T cells are made. Addition of recombinant IL-7 to in vitro cultures was found to promote survival and growth of thymocytes, the cells from the thymus that ultimately develop into mature T lymphocytes. These early experiments suggested that IL-7 was important for development of normal T lymphocytes in the thymus and B lymphocytes in bone marrow. The generation of genetically modified "knockout" mice, in which the IL-7 gene was specifically disrupted, ultimately confirmed these views (von Freeden-Jeffry et al. 1995). While these IL-7 deficient mice were grossly normal in appearance, the suggested importance of IL-7 for lymphocyte development was abundantly clear. These mice were virtually devoid of B lymphocytes and had greatly reduced numbers of T cells, but as will be discussed below, the highly distinctive phenotype of these mice revealed a number of other important functions of IL-7 in the immune system.

IL-7 Signaling

IL-7 belongs to a family of cytokines, termed the common gamma (γ)-chain family of cytokines (Rochman et al. 2009), that includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. These cytokines have been grouped in this way because their cell-surface receptors are either dimeric or trimeric complexes that all share a common component – the common γ chain (γc, CD132) of the high affinity IL-2 receptor. The IL-7 receptor is a heterodimeric complex consisting of the IL-7Rα chain (CD127), encoded by the gene IL7R, and the common γ chain, encoded by IL2RG. These two receptor components do not themselves have intrinsic enzymatic signaling properties. Signaling through the receptor complex is rather mediated by their association with members of the Janus kinase (JAK) family of non-receptor tyrosine kinases (Fig. 1). IL-7Rα is associated with JAK1, while the common γ chain is associated with JAK3. Binding of IL-7 to IL-7Rα promotes the heterodimerization of IL-7Rα and the common γ chain, thereby bringing the associated JAK1 and JAK3 kinases together (Fig. 1). JAK3 activates JAK1 and also phosphorlyates tyrosine docking sites on the cytoplasmic tail of IL-7Rα. This allows the recruitment of the transcription factor Signal transducers and activator of transcription (STAT)-5 to the IL-7Rα, where it is activated by tyrosine phosphorylation by the JAKs. Inactive STAT5 is monomeric. However, phosphorylation by JAKs promotes the homodimerization of STAT5 to become transcriptionally active. Active STAT5 dimers translocate to the nucleus and regulate expression of a wide range of genes, including those important in cell survival and cell division, such as Bcl2, Bcl-xL and members of the cyclin family. The serial activation of JAK kinases and STAT proteins is a signaling mechanism common not only to γ chain family members but also many other important cytokines and hormones, both in the immune system and other organ systems in the body (Shuai and Liu 2003). The well-characterized gene targets of activated STAT5 provide important insights into the cellular consequence of IL-7 signaling. Bcl-2 and Bcl-xL are anti-apoptotic factors that promote cell survival, while expression of cyclins is an essential step in cell cycle progression. Thus, in simple terms, IL-7 signaling is therefore expected to stimulate cell survival and cell division, as suggested by early studies describing IL-7 as a growth factor.

As well as activating the classical JAK-STAT signaling pathway, phosphorylation of the IL-7Rα cytoplasmic tail is also thought to allow recruitment and activation of Phosphoinositide 3-kinase (► PI3K). This kinase phosphorylates phosphoinositides (PIs), phospholipid components of the cell membrane, which in turn provide recruitment sites for proteins with pleckstrin homology (PH) domains. Phosphoinositide-dependent protein kinase 1 (PDK1) and Akt (also known as PKB) are kinases with PH domains that are recruited following PI3K activation. Importantly, PDK1 activates Akt. Akt is a serine/threonine protein kinase that has potential to regulate multiple cellular processes, including cell growth, glucose metabolism, apoptosis, cell division, and cell migration. Thus, activation of PI3K by IL-7 is known to be essential for cell growth and glucose metabolism, but not survival, of T lymphocytes.

An additional layer of complexity to IL-7R signaling that must also be considered is that the IL-7Rα chain is not exclusive to the IL-7R heterodimer complex. Interestingly, IL-7Rα can also couple with another membrane protein, TSLPR, to form a receptor for another cytokine, thymic stromal lymhopoietin (TSLP). While TSLP has distinct functions to IL-7,

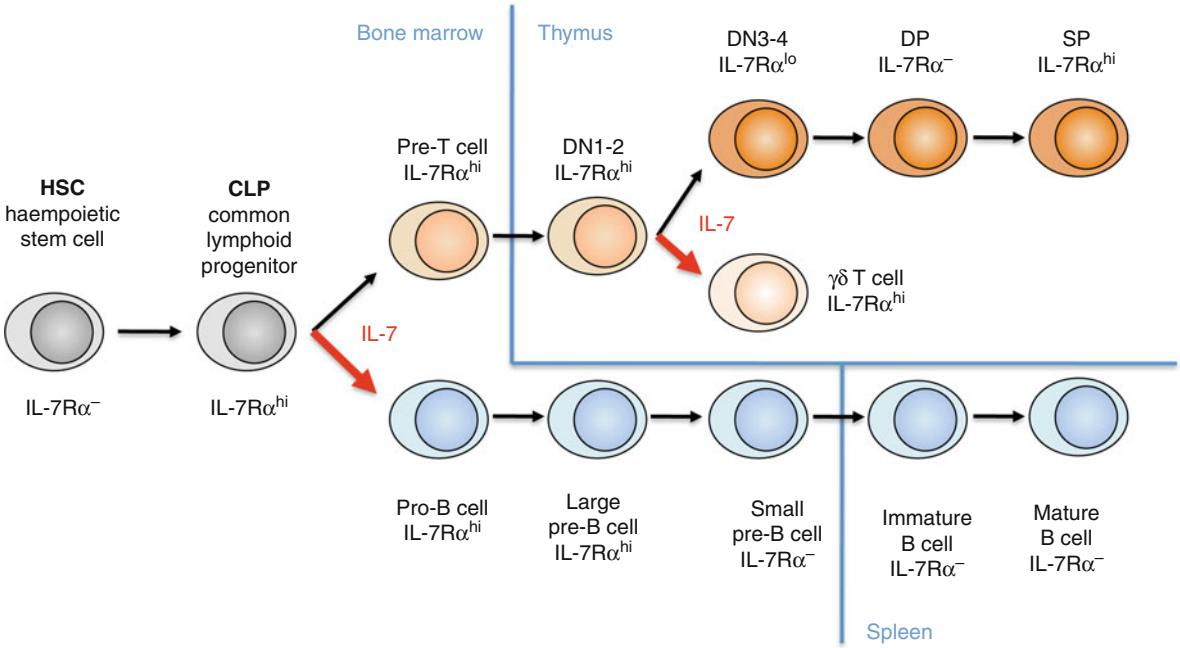


IL7, Fig. 1 Activation of the IL-7R complex by IL-7 binding

there are some instances, such as B-cell development in humans, in which TSLP shares a common function with IL-7, and is in fact even more important than IL-7 in supporting cell survival and development.

Developmental Functions of IL-7

Insights into the cellular roles of IL-7 can be made by examining where, and by which cells, the cytokine is made, and also by examining cells expressing the IL-7Rα chain that are in turn the targets of IL-7 activity. Studies of bone marrow chimeras, in which the complete bone marrow stem cell compartment of IL-7 knockout mice was replaced by normal bone marrow stem cells, revealed that such bone marrow transplantation could not cure the defects evident in IL-7 knockout mice. Conversely, similar chimeras in which normal mice had their bone marrow stem cells replaced with those from IL-7 knockout mice had none of the defects and problems inherent in the IL-7 knockout strains. These experiments clearly demonstrated that IL-7 is not produced by haematopoetic cells but must be produced by a stromal cell of mesenchymal origin. Recent studies have more clearly identified the cellular sources of IL-7 by generating mouse strains in which a specific reporter protein, in this case human CD25, is expressed from within the IL-7 gene locus (Repass et al. 2009). These studies show that IL-7 is indeed produced in the liver and small intestine, and is most highly expressed in the lymphoid organs of bone marrow, thymus, and lymph nodes (LNs). The cells that express IL-7 in these lymphoid organs are stromal cell components that also contribute to the formation of the three-dimensional framework of these organs. Thus, the significant sites of IL-7 production are also the organs in which lymphocytes are generated and where they reside as mature cells. The thymus is the organ in which T lymphocytes develop, while LNs are home to mature T and B lymphocytes and are the organs in which immune responses are initiated. The bone marrow is the main site of hematopoesis, in which pluripotent hematopoietic stem cells (HSCs) develop into all the different blood cell types.



IL7, Fig. 2 IL-7 signaling influences lineage decisions during lymphoid development. DN $CD4^{-}CD8^{-}$ double-negative thymocyte, DP $CD4^{+}CD8^{+}$ double-positive thymocyte, *SP* single-positive thymocyte

During the process of hematopoesis, HSCs develop into one of two main lineages – myeloid and lymphoid. Myeloid lineages include red blood cells, platelets, and cells of the innate immune system such as macrophages, monocytes, dendritic cells, eosinophils, and basophils. The lymphoid lineages include T lymphocytes, B lymphocytes, and NK cells. IL-7 plays an important role throughout the process of hematopoiesis, helping progenitors to make lineage fate decisions (summarized in Fig. 2). Researchers have made much progress in identifying bone marrow progenitor populations at different stages of hematopoiesis by their cell-surface phenotype. While there remains some controversy over the identity of the earliest lymphoid progenitor cell, the so-called common-lymphoid progenitor is in part characterized by its expression of IL-7Rα, and IL-7 signaling is thought to be important for lymphoid lineage specification, and in particular directing cells toward a B-lymphocyte fate in preference to a T-lymphocyte fate (Dias et al. 2005). While mature B cells do not express the IL-7R and therefore have no need for IL-7, developing B cells do express the receptor and require IL-7 for their growth, proliferation, and development.

Lymphoid progenitors destined to develop into T lineage cells leave the bone marrow and migrate to the thymus, another major site of IL-7 production. The thymus gives rise to numerous lymphoid lineages, including NK cells, γδ T cells, and the various αβ T lymphocyte lineages: CD4 T cells, CD8 T cells, regulatory T cells, and NK T cells, amongst others. IL-7 signaling in developing thymocytes influences lineage fates and cellular development. Pre-T thymocyte progenitors entering the thymus lack expression of CD4 and CD8 molecules and are termed double negative (DN). DN cells can be further subdivided into four populations (DN1-4) on the basis of expression of two markers, CD44 and CD25. Development of γδ T cells from DN2 thymocytes is absolutely dependent on IL-7, and strong IL-7 signaling is thought to specifically favor γδ T-cell development over αβ T cells (Riera-Sans and Behrens 2007). However, even in αβ T-cell development, IL-7 is essential as it provides both survival and proliferative signals to developing DN3 and DN4 thymocytes while they rearrange their T-cell antigen receptor (TCR) genes to generate mature TCRs. Crucially, thymocytes must test their newly generated TCR to ensure it is functional. This is achieved by up-regulating both CD4 and CD8 co-receptors to become double positive (DP), at which point DPs undergo the process of positive selection, in which only thymocytes with functional TCRs

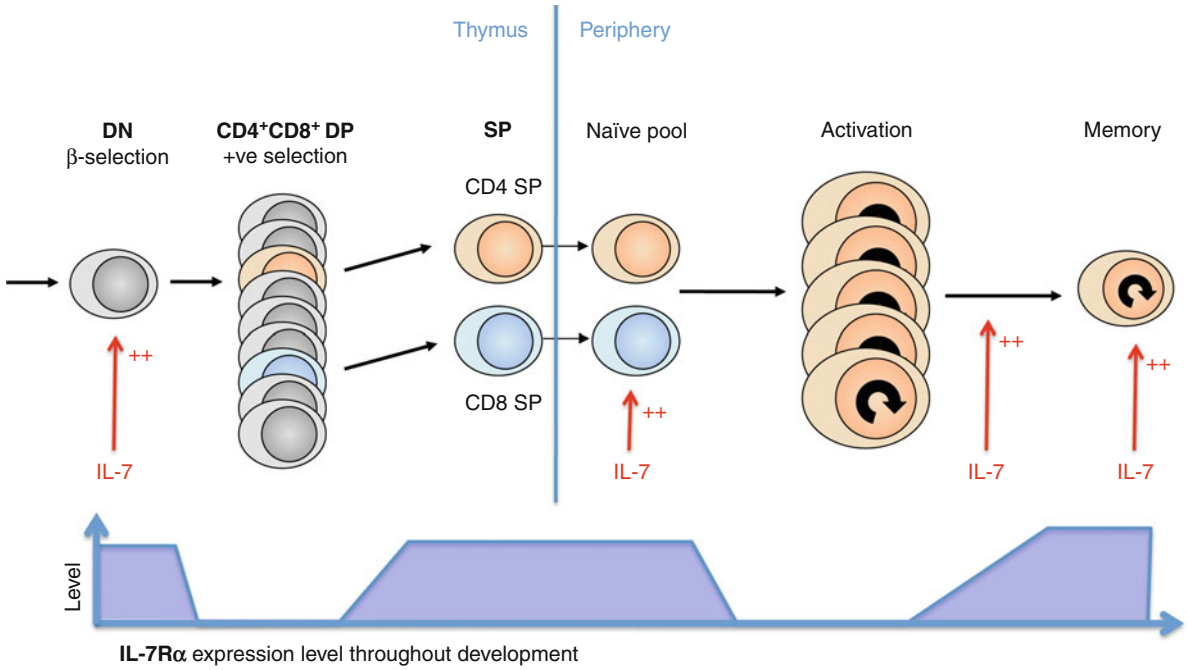
can survive and continue to develop into mature T lymphocytes. The criteria for a functional TCR are that it has the capacity to weakly recognize major histocompatibility complex (MHC) proteins on thymic epithelial cells. This proves to be the case for only a small minority of DP thymocytes, approximately 5%. It is therefore very significant that at the DP stage of thymocyte development, thymocytes completely lose IL-7R function. Doing so ensures that continued survival and development of thymocytes is entirely determined by their ability to successfully pass the stringent positive selection check point. This represents another important function of IL-7 during T lineage development, albeit a negative one. IL-7 signaling is essential throughout lymphoid development. However, loss of IL-7R function ensures that continued development depends solely on the generation of a functional and useful TCR and, therefore, ensures that the peripheral repertoire of T cells is mostly composed of cells with useful TCRs.

Interestingly, mice and humans have evolved different mechanisms to inactivate IL-7R signaling. In mice, loss of IL-7R function is achieved by the near complete loss of IL-7Rα surface expression coupled with high expression levels of suppressor of cytokine signaling (► SOCS) proteins that inhibit cytokine signal transduction. In contrast, human thymocytes express entirely normal levels of IL-7Rα but rather lose expression of STAT5 protein, the transcription factor target of IL-7 signaling. The fact that mice and humans have evolved different mechanisms of abating IL-7 signaling during thymic development may be viewed as an example of convergent evolution and may, therefore, represent a relatively recently evolved optimization of thymic selection. Cells that successfully pass the test of positive selection continue development to either CD4 or CD8 lineage T cells. DP thymocytes that successfully undergo positive selection by Class I MHC molecules lose expression of CD4 and become CD8 single positive (SP) thymocytes, while positive selection by Class II MHC induces commitment to the CD4 lineage and loss of CD8 expression so that cells become CD4 SP. In both cases, IL-7 function is restored in SP thymocytes, and mature T cells all express functional IL-7R. Thus, not only does IL-7 play a crucial role for development of both T and B lymphocytes, it is also important in determining the direction that developing progenitor populations take during the development of these lymphocyte lineages.

The analysis of IL-7 deficient mice revealed another developmental role for IL-7, but one which has only recently begun to be understood. In addition to its characterized role in the lymphopoiesis, IL-7 is also essential for normal organogenesis of secondary lymphoid structures. IL-7-deficient mice have extremely small LNs, and other lymphoid structures, such as the Peyer's patches that are located in the gut, are absent. While it was initially thought that the reduced LN size was merely secondary to the reduced numbers of lymphocytes in these mice, closer investigation revealed that the non-lymphocyte cellular structures that form the LNs were in fact defective. During embryonic development, LN structures actually form prior to development and generation of mature lymphocytes that only later populate the mature LNs. Formation of these early LN anlagen is initiated by the aggregation of so-called LN inducer (LNi) cells at the site of future LNs. These cells are hemopoetic in origin and express IL-7Rα. They, in turn, recruit a second population of cells, LN organizer (LNo) cells that are of non-hemopoetic mesenchymal origin. A final stage of LN formation occurs after population with mature lymphocytes. In IL-7-deficient mice, these structures initially form normally but fail to develop into normal LNs after birth, and it appears that IL-7 signaling either in the LTi or newly generated T cells that colonize the LNs is required for the final stage of LN formation (Repass et al. 2009).

Control of Homeostasis and Function of Mature Naïve T Cells by IL-7

Although vital for their generation in the bone marrow, mature B lymphocytes do not express IL-7R. Their survival and maintenance is rather dependent on tonic signaling processes through the B-cell antigen receptor and cytokines such as BAFF. In contrast, all αβ T-lymphocyte populations express IL-7R, suggesting it plays a continuing role in their function (Fig. 3). Mature peripheral T lymphocytes largely reside in what are termed the secondary lymphoid organs, which are comprised of LNs and the spleen. To understand why lymphocytes reside in secondary lymphoid organs and why this is important for the function of IL-7, it is important to understand the role of LNs and



IL7, Fig. 3 Control of T-cell homeostasis by IL-7 and its regulation through expression of IL-7Rα

the lymphatic system in the function of the immune system. Lymphatic vessels, similar in structure and size to veins that carry blood back to the heart, provide fluid drainage from all peripheral tissues, such as the skin and intestine. The system of lymphatics resembles the tributaries of a river as many smaller vessels merge to form larger vessels, which ultimately combine to a single lymphatic vessel in the thorax which drains, via the thoracic duct, directly into the left brachiocephalic vein. The progress of these lymphatic vessels from tissues to thoracic duct is not uninterrupted however. At major junction points in the neck, under the arms and in the groin, the lymphatic vessels swell to form spherical nodular structures that are the LNs. Lymphatic vessels draining into the LNs are termed afferent, while those that connect the LNs ultimately to the thoracic duct are termed efferent. In the event of an infection in the tissues or at mucosal sites such as the gut or respiratory tract, the lymphatic system provides conduits to transport antigens and antigen-bearing immune cells from the site of infection to the nearest LN. The spleen serves a similar purpose but for blood-born antigens rather than those from tissues. Once in the LN, the high concentration of lymphocytes present greatly increases the chances that a T or B cell that can recognize the pathogen will do so and initiate an immune response. Mature T cells that have not previously encountered antigen and been activated are termed "naïve," while T cells that have previously taken part in an immune response are termed "memory." Naïve T cells are the raw material of the adaptive immune system, and it is essential to maintain a large and diverse repertoire of these cells in order to ensure an effective immune response against new pathogens. Researchers studying the mechanisms by which the naïve T-cell repertoire is maintained at an optimal condition soon noticed that the total number of T cells is maintained at a constant level, both in humans and in laboratory rodents. This is somewhat remarkable when one considers that the pool of naïve T cells is extremely dynamic in its behavior. New T cells are continually generated by the thymus but not in a consistent manner as thymus size is reduced with age. Furthermore, naïve T cells are also lost as a consequence of being activated to generate memory cells. So despite the unpredictable and changing dynamics, the size of the naïve T-cell pool is largely the same throughout life, revealing that there must be strict homeostatic mechanisms to regulate the overall size of the T-cell pool.

Like other cells in the body, lymphocytes require specific signals to actively keep them alive. In the

absence of such signals, cells undergo the process of controlled cell death, known as apoptosis. All naïve T cells express IL-7R, and IL-7 is one of several essential signals required for the long-term survival of these lymphocytes. Within the lymphoid system, naïve T cells are not static but rather actively migrate around LNs seeking antigen, and also recirculate via efferent lymphatic vessels to the blood and, thence, to other LNs or the spleen. IL-7 is produced by stromal cells in LNs but is also detectable in high concentrations in the fluid in efferent lymphatics and in serum of blood. Naïve T cells are therefore constantly exposed to IL-7 during their recirculation through the blood and lymphatic system, providing them with the constant signal that they require to prevent them from undergoing apoptosis.

In providing survival signals to naïve T cells, IL-7 plays a key role as a master regulator of the size of the naïve T cell pool. Although the thymus is constantly generating new cells, this does not appear to be the limiting factor in setting the size of the naïve T-cell pool. This is particularly evident in experiments analyzing mice that can only make one of either CD4 or CD8 lineage T cells. In normal conditions, there are approximately two CD4 T cells for every one CD8 T cell. However, in mice that cannot make CD4 T cells, the total number of naïve T cells, all of which are CD8, is much the same as the total number of naïve T cells in normal mice, which comprise both CD4 and CD8 lineages. This is the case, even though the rate of CD8 T-cell production by the thymus remains unchanged regardless of whether CD4 T cells are produced or not. Thus, the space created by the absence of CD4 T cells is filled by more CD8 lineage cells. For similar reasons, mice that cannot make CD8 T cells also have normal T-cell numbers, but are all CD4 lineage. This ceiling on total naïve T-cell numbers is set by the amount of IL-7 available. Experimental mice that have artificially elevated IL-7 levels also have larger T-cell pools. Therefore, IL-7 is controlling T-cell homeostasis by its requirement as a survival resource. If there are too many T cells in an individual, they will not all get sufficient access to IL-7 signals and some will die, until the numbers of T cells reach a level that can be sustained by the amount of IL-7 available. As well as promoting T-cell survival, IL-7 signaling can also promote cell division, another property that lends itself well to the homeostatic role of this cytokine. In conditions of T-cell deficiency, as occurs in HIV-infected individuals or following radio- or chemo-ablative therapies, naïve T cells can sense the "space" in the T-cell compartment and start to undergo cell divisions in an effort to restore normal T-cell numbers. This response is highly dependent on IL-7 signaling. Such cell division is particularly important in humans, where recently generated naïve T cells undergo several cell divisions even in a normal individual (Jameson 2005).

IL-7 is thought to be produced at a constant rate by stromal cells in lymphoid organs. Whether these cells can dynamically regulate production in specific situations to modify T-cell homeostasis, for instance, during an immune response, is not at present known. However, the dependence on IL-7 for naïve T-cell survival goes a long way toward explaining how the immune system solves a difficult homeostatic problem. The size of other organs in the body, such as the kidneys, the liver, and the brain are controlled through strict regulation of developmental processes. In contrast, T cells do not reside in any one place but are spread throughout the body and undergo dynamic changes in their rates of production and loss throughout life. The reliance on a fixed resource such as IL-7 explains how the T-cell pool can be so precisely regulated to prevent over- or underpopulation.

Memory Formation and Persistence

During an immune response, T cells that recognize specific antigen become activated, undergoing cell division and developing specific effector functions such as cytokine secretion for CD4 T cells and development of cytolytic activity in the case of CD8 T cells. Following T-cell activation, T cells rapidly lose expression of IL-7R. Maintenance of IL-7R expression in peripheral T cells is dependent on the activity of the ▶ Foxo1 transcription factor (Kerdiles et al. 2009). The activity of Foxo transcription factors is negatively regulated by their phosphorylation by Akt. TCR stimulation by antigen activates the PI3K pathway, resulting in activation of Akt and, therefore, causes loss of IL-7Rα expression by the repression of Foxo1 by Akt. An ongoing immune response represents a scenario that is analagous to thymic selection (Fig. 3). During such a response, the presence of antigen is a key determinant of T-cell behavior, inducing proliferation and differentiation. For an effective

immune response, it is important that a large number of effector T cells is quickly generated. Therefore, it makes sense to uncouple effector T cells from the shackles of normal T-cell homeostatic control by IL-7, and switch to autocrine-regulated T-cell proliferation by factors such as IL-2, synthesized by T cells following their activation by antigen. IL-2 is also a potent inhibitor of IL-7Rα expression, which may be mediated by similar mechanisms as TCR signaling since IL-2 strongly activates PI3K. However, similar to thymic positive selection, it is important that differentiation of T cells is strictly dependent on continued TCR signaling, and not IL-7, since persistence of the immune response should be closely allied to the ongoing presence of antigen to indicate the need for the response. This is very neatly achieved through the direct repression of IL-7Rα expression by antigen-dependent PI3K activation. Following the resolution of a successful immune response, most effector cells will undergo apoptosis. However, some survive to become long-term memory cells, a key feature of the adaptive immune system. IL-7 signaling plays a key role in the formation of this memory population. In the absence of antigen, TCR signaled repression of IL-7Rα expression is reversed, and effector T cells can start to reexpress IL-7Rα. In some experimental viral infections in mice, the effector cells that first reexpress IL-7Rα are the precursors of the long-term memory cells, suggesting that IL-7 could be instructing effectors to develop into memory cells. However, there is also evidence that effector T cells are far more predisposed to undergoing apoptosis than naïve or memory T cells. While reexpression of IL-7R can slow their death, the effector pool still undergoes a significant contraction because the effector cells are less able to compete and survive in response to IL-7 signaling than other T cells (Buentke et al. 2006). Nevertheless, IL-7 signaling represents a key gateway into the memory T-cell pool, ensuring only the most fit effectors persist. Like their naïve precursors, both CD4 (Seddon et al. 2003) and CD8 memory T cells (Schluns et al. 2000) are dependent on IL-7 for their long-term survival, although CD8 memory cells also have a strong reliance on the cytokine IL-15. Both CD4 and CD8 memory cells express higher levels of IL-7Rα than their naïve counterparts. However, in contrast to naive T cells, memory cells can also migrate to extra-lymphoid tissue sites in order to patrol them for the presence of their specific pathogen, returning to lymph nodes via the afferent lymphatic vessels. It is unclear whether these cells can obtain survival signals in such sites as IL-7 expression is largely limited to lymphoid organs. Therefore, higher cytokine receptor expression levels may be important for these cells to gain stronger survival signals sufficient to maintain their survival whilst patrolling peripheral T tissues and before their return to lymphoid tissue for "refueling."

IL-7 and Disease

IL-7 has been implicated in a variety of different disease processes. There are several recognized severe combined immunodeficiencies (SCID) that arise from mutations in either the human IL7 or IL7R genes that affect production of IL-7 or signaling through IL-7R. Such patients have a phenotype similar to that described for IL-7 deficient mice, although one notable difference is that human B cells are less reliant on IL-7 than mouse B cells, and there appears to be some redundancy with TSLP. Therefore, IL-7RA mutations are more severe than IL-7 mutations. Given the potent survival and proliferative properties of IL-7, it is unsurprising that mutations in the IL7 gene are also implicated as susceptibility factors in certain T cell acute lymphoblastic leukemias (T-ALL), and experimental work has shown that IL-7 can accelerate disease progression of T-ALL transplanted into mice. In addition, there is evidence to suggest IL-7 may be involved in development of some autoimmune diseases. A recent genetic linkage study identified a point mutation in a noncoding region of the IL7R gene as strongly associated with development of multiple sclerosis (Gregory et al. 2007). Furthermore, recent experiments suggest that IL-7 signaling may exacerbate the T-cell mediated autoimmune process that is thought to contribute to the nerve damage in the disease.

IL-7 is also under consideration as a potential therapeutic. In HIV patients with active disease, reduction in T-cell counts is often accompanied with raised serum levels of IL-7, probably secondary to the reduction in T-cell numbers. However, IL-7 treatment has been tested in patients to see whether it could aid immune reconstitution in HIV patients with dwindling T-cell numbers. In some cases, a relatively short treatment with IL-7 was able to produce lasting increases in T-cell numbers. The potential for immune reconstitution by IL-7 is also considered in the context of aging.

In humans, maximal thymic productivity is reached very early, at about 1 year of age, after which the thymus undergoes gradual atrophy, producing ever-decreasing numbers of T cells. Homeostatic mechanisms like those discussed earlier are successful in maintaining a diverse T-cell repertoire until old age, when there appears to be a sudden collapse in T-cell diversity. The lack of new thymically generated T cells is thought to be a contributing factor. One of the causes of thymic atrophy is a loss of IL-7 production by thymic stromal cells. Administering IL-7 to mice can successfully overcome some of the effects of reduced IL-7 production locally in the thymus. Therefore, immuno-reconstitution by IL-7 treatment is starting to be considered, at least for some specific conditions.

Summary

In conclusion, it is evident that IL-7 has a broad array of biological functions. It is vital for development of nearly all lymphoid lineages, but also has a role to play in controlling the lineage decisions at different stages of progenitor differentiation. The absolute number of mature T cells that an individual has is critically dependent on IL-7 availability, and all the mature subsets and lineages of T cells have at least some reliance on IL-7 for their survival. Even the absence of IL-7 signaling has a fundamental role to play in shaping the T-cell repertoire and memory populations. One of the greatest challenges for the future will be to understand how this one cytokine can influence such a diverse range of cellular properties. Our understanding of the molecular mechanisms of IL-7 signaling remains crude, and it is unclear whether all the potential signaling pathways are activated in different in vivo situations, and if not, which ones are important for different biological outcomes. Such understanding will be essential if the potent biological properties of IL-7 are to be fully exploited clinically.

References

Buentke E, Mathiot A, Tolaini M, Di Santo J, Zamoyska R, Seddon B. Do CD8 effector cells need IL-7R expression to become resting memory cells? Blood. 2006;108(6):1949–56.

Dias S, Silva Jr H, Cumano A, Vieira P. Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. J Exp Med. 2005;201(6):971–9.

Gregory SG, Schmidt S, Seth P, Oksenberg JR, Hart J, Prokop A, et al. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. Nat Genet. 2007;39(9):1083–91.

Jameson SC. T cell homeostasis: keeping useful T cells alive and live T cells useful. Semin Immunol. 2005;17(3):231–7.

Kerdiles YM, Beisner DR, Tinoco R, Dejean AS, Castrillon DH, DePinho RA, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat Immunol. 2009;10(2):176–84.

Repass JF, Laurent MN, Carter C, Reizis B, Bedford MT, Cardenas K, et al. IL7-hCD25 and IL7-Cre BAC transgenic mouse lines: new tools for analysis of IL-7 expressing cells. Genesis. 2009;47(4):281–7.

Riera-Sans L, Behrens A. Regulation of alphabeta/gammadelta T cell development by the activator protein 1 transcription factor c-Jun. J Immunol. 2007;178(9):5690–700.

Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol. 2009;9(7):480–90.

Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol. 2000;1(5):426–32.

Seddon B, Tomlinson P, Zamoyska R. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. Nat Immunol. 2003;4(7):680–6.

Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. Nat Rev Immunol. 2003;3(11):900–11.

von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med. 1995;181(4):1519–26.

Immune IFN

▶ Interferon-Gamma

Immunity-Related GTPases (IRG)

Maria Traver and Gregory A. Taylor
Departments of Medicine; Molecular Genetics and Microbiology; and Immunology, Duke University, Durham VA Medical Center, Geriatric Research, Education, and Clinical Center, Durham, NC, USA

Synonyms

p47 GTPases

Historical Background

The IRGs are a family of proteins that play critical roles in innate immune responses to intracellular pathogens (Martens and Howard 2006; Shenoy et al. 2007; Taylor 2007). IRG cDNAs were discovered independently by several scientists who were conducting screens to identify mouse genes transcriptionally upregulated by ▶ interferon-γ and/or lipopolysaccharide (LPS). These IRGs were thus named and described piecemeal, but were later recategorized in a comprehensive study of the gene family across vertebrates (Bekpen et al. 2005). The standardized nomenclature places the proteins into subfamilies based on amino acid homology within the GTP-binding regions. IRG proteins in most subfamilies contain the canonical GKS sequence within the G1 motif in the GTP-binding region including the IRGA, IRGB, IRGC, and IRGD subfamilies. Proteins in the IRGM subfamily contain the noncanonical GMS sequence with a lysine to methionine substitution positioned in the GTP-binding pocket. IRG proteins that have received the greatest amount of study include the GKS proteins Irga6 (IIGP1), Irgb6 (TGTP), Irgb10, and Irgd (IRG-47) in the mouse, and the GMS proteins Irgm1 (LRG-47), Irgm2 (GTPI), and Irgm3 (IGTP) in the mouse, and IRGM in humans. The IRG genes are not evenly distributed across vertebrates – mice for example contain about 23 members, and humans only two – which may reflect relatively recent evolutionary events in some species to combat unique pressures from endemic pathogens.

Gene Expression

Expression of most mouse IRG genes is highly induced by interferons (IFNs), most robustly by type II IFN (IFN-γ), and more weakly by type I IFN (IFNα/β). This is a consequence of multiple γ-activated sequences (GAS) and/or interferon-stimulated response elements (ISRE) that are found in the promoters of the genes (Bekpen et al. 2005). It is notable, however, that the positions of these elements vary from gene to gene in the mouse, implying that there has been evolutionary pressure to maintain IFN-regulated expression. The transcriptional response to IFN-γ is rapid; for Irgm3, mRNA accumulates within 1 h of stimulation, reaching maximal levels within 3 h, and having a half-life of about 4.5 h (Taylor et al. 1996). Irgm3 protein accumulates within 3 h of induction and reaches maximal levels within 8 h that does not decrease in the continued presence of IFN-γ. The induction by IFN has been noted in a wide variety of tissues and cell types. Although IRG genes are also induced by LPS, this is thought to be an indirect response resulting from LPS-induced production of type I IFN that then acts autocrinely. In contrast, a variety of other cytokines do not induce expression of IFN-regulated IRG genes, underscoring the specificity of IFN in controlling expression of these genes (Sorace et al. 1995).

Despite the dominant role of IFN, control of IRG gene expression by IFN is not absolute. There are some IRG genes that are not induced by IFNs: These include mouse Irgc and human IRGC that are constitutively expressed in testes, and human IRGM that is constitutively expressed in several cell types (Bekpen et al. 2005). In addition, among those IRG that are regulated by IFN, there is evidence for basal, constitutive expression, including that for Irga6 in liver (Zeng et al. 2009).

Biochemical Properties

IRG proteins can be placed functionally within the larger dynamin protein superfamily (Praefcke and McMahon 2004). Dynamins are large GTPases that have a high affinity for lipid membranes, on which they are able to self-assemble into structures that are thought to contract in a GTP-dependent manner and mechanically alter the membranes. Dynamins are therefore distinct from the smaller Ras-like GTPases, in which the guanine nucleotide moiety functions as a biochemical switch to place the protein in an active state when GTP bound, and then in an inactive state when hydrolyzed to GDP. In contrast, dynamins are "mechanochemical" enzymes, with the energy released by the hydrolysis of GTP to GDP inducing conformational changes in the protein oligomers that drive overall protein function. Through their ability to disrupt and distort membranes, dynamins have been implicated in many cellular processes, including vesicle formation and transport, organelle division, cytokinesis, and cell motility. Two other families of IFN-regulated GTPases – the guanylate-binding proteins (GBP) and the Mx proteins – are also functionally related to dynamins (Martens and Howard 2006).

The abilities to bind and hydrolyze GTP to GDP have been confirmed for IRG proteins (Taylor et al. 1997; Uthaiah et al. 2003). Overall affinities for GTP and GDP, however, vary among IRG proteins. For instance, Irgm3 is largely GTP bound, while Irga6 has a higher affinity for GDP. These differences may or may not stem from the unique GMS sequence found in the Irgm3 GTP-binding pocket as previously discussed. Currently, no GTPase-activating proteins (GAPs) or guanine nucleotide exchange factors (GEFs) have been identified for IRG proteins.

IRG proteins, like other dynamins, have been shown to localize strongly to various cellular membranes, including the Golgi and endoplasmic reticulum, through different localization moieties (Martens et al. 2004; Taylor et al. 1997). For example, Irga6 is largely ER-localized through an N-myristoylation motif; Irgm1 localizes to the Golgi and late lysosomes through an amphipathic helix near its C-terminus; Irgm2 and Irgm3 target the Golgi and ER, respectively, through helices analogously placed within the protein though not amphipathic; and human IRGM targets mitochondria, though targeting motifs have not been identified (Singh et al. 2010). Where tested, targeting to these membranes does not appear to be dependent on GTP hydrolysis. However, many IRG proteins also target the membranes of phagosomes containing certain pathogens, with the targeting of these membranes requiring GTP hydrolysis. Like other dynamins, IRG proteins have been shown to self-assemble, both into homotypic complexes as shown in in vitro assays using purified protein, as well as into heterotypic complexes with other IRG proteins as determined by pull-down, co-immunoprecipitation, and yeast two-hybrid studies (Hunn et al. 2008). The requirement for GTP hydrolysis in order to target phagosomal membranes suggests that the IRG proteins probably exist in oligomeric complexes on these membranes, and in fact, data suggest that there is a distinct temporal order to which various IRG proteins assemble on the phagosomal membrane (Khaminets et al. 2010).

Functions in Innate Immunity

The creation and analysis of mice lacking IRG proteins – Irgm1, Irgm3, Irgd, and Irga6 – have established the prominent role that the GTPases play in innate immunity to multiple intracellular pathogens (Al-Zeer et al. 2009; Collazo et al. 2001; Henry et al. 2007; MacMicking et al. 2003; Taylor et al. 2000). The clear but distinct phenotypes of the IRG-deficient mouse strains have also emphasized that the genes are nonredundant and of varying importance depending on the pathogen.

Many studies have focused on the role for IRG proteins in resistance to *Toxoplasma gondii*. Mice lacking Irgm1 or Irgm3 demonstrate acute susceptibility to this protozoan parasite, on par with the susceptibility in IFN-γ-deficient mice, thus suggesting an essential role for the Irgm proteins in IFN-γ-induced resistance. In contrast, Irgd- and Irga6-deficient mice demonstrate weak susceptibility that becomes manifest later during the infection, implying a generally weaker role for GKS IRG proteins. The role of the IRGs in resistance is tied to their ability to provide cell autonomous resistance to *T. gondii*: Macrophages or astrocytes that lack IRG proteins demonstrate varying degrees of impaired IFN-γ-induced *T. gondii* killing activity (Butcher et al. 2005). Data from several studies have suggested a model in which GKS IRG proteins load onto the *T. gondii* vacuole, where they drive vesiculation of that vacuole, releasing the parasite into the cytosol of the cell where it is destroyed (Ling et al. 2006; Zhao et al. 2009). GMS IRG proteins do not load to the same extent on the vacuole; rather, they regulate the GKS proteins. In the absence of the GMS IRG proteins, the GKS IRG proteins form aberrant aggregates and are consequently unable to load as efficiently onto *T. gondii* vacuoles (Hunn et al. 2008). Absence of even one of the three mouse GMS IRG proteins leads to altered positioning of most or all of the cellular complement of GKS IRG proteins on the *T. gondii* vacuole, thus explaining the more prominent role of GMS IRG proteins, relative to that of any one GKS IRG protein, in controlling resistance to *T. gondii*. The generally pivotal function of IRGs in this context is further implicated by the fact that virulent strains of *T. gondii* have acquired the ability to phosphorylate IRGs and prevent their loading onto the *T. gondii* vacuole (Fentress et al. 2010; Steinfeldt et al. 2010).

The roles of IRG proteins in resistance to bacterial infections vary somewhat with those in resistance to *T. gondii* or other protozoa. Irgm1-deficient mice display increased susceptibility to a wide range of intracellular bacteria including *Listeria monocytogenes, Salmonella typhimurium, Mycobacterium tuberculosis,*

Mycobacterium avium and *Chlamydia trachomatis* (Taylor 2007). In contrast, Irgm3-deficient mice have increased susceptibility to only *C. trachomatis*, and the Irgd- or Irga6-deficient mice have little or no increased susceptibility. Irgm1, therefore, plays a particularly important role in bacterial resistance, and may well possess function(s) not shared with the other mouse IRG proteins. Similarly, not only does the closest human homologue of Irgm1, IRGM, play a role in resistance to *M. tuberculosis*, but polymorphisms in the IRGM gene have been linked with susceptibility to *M. tuberculosis* infection (King et al. 2011) and to Crohn's disease (McCarroll et al. 2008). While the mechanism through which Irgm1 and IRGM regulate resistance to bacteria has yet to be clearly defined, there is no evidence to suggest that the GTPases regulate vesiculation of bacterial phagosomes, as is the case with *T. gondii*; rather, accumulating evidence supports a role in regulating autophagy and mitophagy, and removal of bacteria through these pathways (Singh et al. 2006, 2010). Along these lines, regulation of autophagy also leads to the ability of Irgm1 to modulate T cell homeostasis during infection (Feng et al. 2008). In addition, other cellular functions that have been suggested for Irgm1 – including regulating acidification and/or lysosome fusion of the bacterial phagosome (MacMicking et al. 2003) and controlling motility of macrophages (Henry et al. 2010) – may well be manifestations of its ability to control autophagy.

Summary

IRGs are critical mediators of interferon-γ-mediated resistance to a variety of important intracellular pathogens. The family is part of the larger dynamin protein superfamily, as a consequence of its member's GTPase function, affinity for lipid membrane compartments, and ability to modulate those membranes. Two major subcategories of IRGs exist: the GMS and GKS IRGs. The GMS IRG proteins appear to play a more important role in innate immunity, as evidenced by the more striking susceptibility of Irgm-deficient mice to a variety of intracellular pathogens. The exact mechanisms whereby IRG proteins mediate immunity are not fully defined, but include their ability to vesiculate the membranes of vacuoles that contain *T. gondii*, and to promote autophagy in several contexts. Given the large number of important pathogens to which the IRG family confers immunity, the study and understanding of these proteins has significant consequences for human health.

References

Al-Zeer MA, Al-Younes HM, Braun PR, Zerrahn J, Meyer TF. IFN-gamma-inducible Irga6 mediates host resistance against *Chlamydia trachomatis* via autophagy. PLoS One. 2009;4:e4588.

Bekpen C, Hunn JP, Rohde C, Parvanova I, Guethlein L, Dunn DM, Glowalla E, Leptin M, Howard JC. The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. Genome Biol. 2005;6:R92.

Butcher BA, Greene RI, Henry SC, Annecharico KL, Weinberg JB, Denkers EY, Sher A, Taylor GA. p47 GTPases regulate *Toxoplasma gondii* survival in activated macrophages. Infect Immun. 2005;73:3278–86.

Collazo CM, Yap GS, Sempowski GD, Lusby KC, Tessarollo L, Woude GF, Sher A, Taylor GA. Inactivation of LRG-47 and IRG-47 reveals a family of interferon gamma-inducible genes with essential, pathogen-specific roles in resistance to infection. J Exp Med. 2001;194:181–8.

Feng CG, Zheng L, Jankovic D, Bafica A, Cannons JL, Watford WT, Chaussabel D, Hieny S, Caspar P, Schwartzberg PL, et al. The immunity-related GTPase Irgm1 promotes the expansion of activated CD4+ T cell populations by preventing interferon-gamma-induced cell death. Nat Immunol. 2008;9:1279–87.

Fentress SJ, Behnke MS, Dunay IR, Mashayekhi M, Rommereim LM, Fox BA, Bzik DJ, Taylor GA, Turk BE, Lichti CF, et al. Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii*-secreted kinase promotes macrophage survival and virulence. Cell Host Microbe. 2010;8:484–95.

Henry SC, Daniell X, Indaram M, Whitesides JF, Sempowski GD, Howell D, Oliver T, Taylor GA. Impaired macrophage function underscores susceptibility to *Salmonella* in mice lacking Irgm1 (LRG-47). J Immunol. 2007;179:6963–72.

Henry SC, Traver M, Daniell X, Indaram M, Oliver T, Taylor GA. Regulation of macrophage motility by Irgm1. J Leukoc Biol. 2010;87:333–43.

Hunn JP, Koenen-Waisman S, Papic N, Schroeder N, Pawlowski N, Lange R, Kaiser F, Zerrahn J, Martens S, Howard JC. Regulatory interactions between IRG resistance GTPases in the cellular response to *Toxoplasma gondii*. EMBO J. 2008;27:2495–509.

Khaminets A, Hunn JP, Konen-Waisman S, Zhao YO, Preukschat D, Coers J, Boyle JP, Ong YC, Boothroyd JC, Reichmann G, et al. Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. Cell Microbiol. 2010;12:939–61.

King KY, Lew JD, Ha NP, Lin JS, Ma X, Graviss EA, Goodell MA. Polymorphic allele of human IRGM1 is associated with susceptibility to tuberculosis in African Americans. PLoS One. 2011;6:e16317.

Ling YM, Shaw MH, Ayala C, Coppens I, Taylor GA, Ferguson DJ, Yap GS. Vacuolar and plasma membrane stripping and

autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. J Exp Med. 2006;203:2063–71.
MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science. 2003;302:654–9.
Martens S, Howard J. The interferon-inducible GTPases. Annu Rev Cell Dev Biol. 2006;22:559–89.
Martens S, Sabel K, Lange R, Uthaiah R, Wolf E, Howard JC. Mechanisms regulating the positioning of mouse p47 resistance GTPases LRG-47 and IIGP1 on cellular membranes: retargeting to plasma membrane induced by phagocytosis. J Immunol. 2004;173:2594–606.
McCarroll SA, Huett A, Kuballa P, Chilewski SD, Landry A, Goyette P, Zody MC, Hall JL, Brant SR, Cho JH, et al. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. Nat Genet. 2008;40:1107–12.
Praefcke GJ, McMahon HT. The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol. 2004;5:133–47.
Shenoy AR, Kim BH, Choi HP, Matsuzawa T, Tiwari S, MacMicking JD. Emerging themes in IFN-gamma-induced macrophage immunity by the p47 and p65 GTPase families. Immunobiology. 2007;212:771–84.
Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science. 2006;313:1438–41.
Singh SB, Ornatowski W, Vergne I, Naylor J, Delgado M, Roberts E, Ponpuak M, Master S, Pilli M, White E, et al. Human IRGM regulates autophagy and cell-autonomous immunity functions through mitochondria. Nat Cell Biol. 2010;12:1154–65.
Sorace JM, Johnson RJ, Howard DL, Drysdale BE. Identification of an endotoxin and IFN-inducible cDNA: possible identification of a novel protein family. J Leukoc Biol. 1995;58:477–84.
Steinfeldt T, Konen-Waisman S, Tong L, Pawlowski N, Lamkemeyer T, Sibley LD, Hunn JP, Howard JC. Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii*. PLoS Biol. 2010;8:e1000576.
Taylor GA. IRG proteins: key mediators of interferon-regulated host resistance to intracellular pathogens. Cell Microbiol. 2007;9:1099–107.
Taylor GA, Jeffers M, Largaespada DA, Jenkins NA, Copeland NG, Woude GF. Identification of a novel GTPase, the inducibly expressed GTPase, that accumulates in response to interferon gamma. J Biol Chem. 1996;271:20399–405.
Taylor GA, Stauber R, Rulong S, Hudson E, Pei V, Pavlakis GN, Resau JH, Vande Woude GF. The inducibly expressed GTPase localizes to the endoplasmic reticulum, independently of GTP binding. J Biol Chem. 1997;272:10639–45.
Taylor GA, Collazo CM, Yap GS, Nguyen K, Gregorio TA, Taylor LS, Eagleson B, Secrest L, Southon EA, Reid SW, et al. Pathogen-specific loss of host resistance in mice lacking the IFN-gamma-inducible gene IGTP. Proc Natl Acad Sci USA. 2000;97:751–5.
Uthaiah RC, Praefcke GJ, Howard JC, Herrmann C. IIGP1, an interferon-gamma-inducible 47-kDa GTPase of the mouse, showing cooperative enzymatic activity and GTP-dependent multimerization. J Biol Chem. 2003;278:29336–43.
Zeng J, Parvanova IA, Howard JC. A dedicated promoter drives constitutive expression of the cell-autonomous immune resistance GTPase, Irga6 (IIGP1) in mouse liver. PLoS One. 2009;4:e6787.
Zhao YO, Khaminets A, Hunn JP, Howard JC. Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFNgamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. PLoS Pathog. 2009;5:e1000288.

Immunoreceptor

▶ CLEC4E

Inap

▶ IkBz

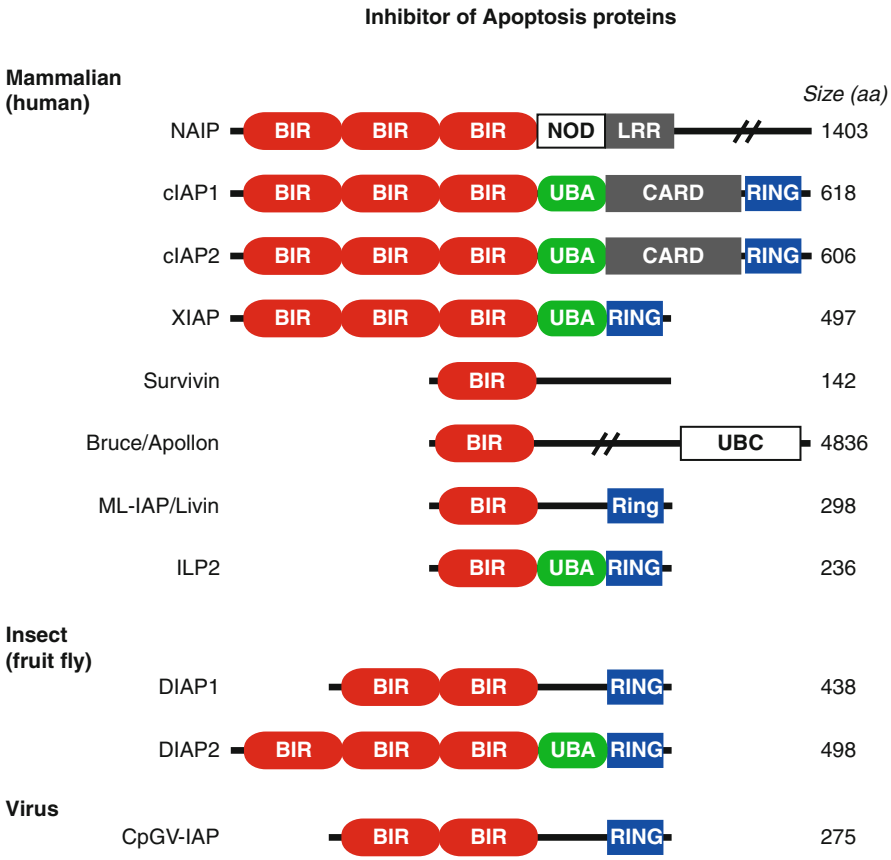
Inhibitor of Apoptosis (IAP) Proteins

Mads Gyrd-Hansen
Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Copenhagen, Denmark

Historical Background

Inhibitor of apoptosis (IAP) proteins were identified in 1993 in a cDNA-based screen for baculovirus genes that prevent the induction of apoptosis in *Spodoptera frugiperda* SF-21 insect cells after viral infection (Crook et al. 1993). *iap* genes were found in other baculoviruses, and further analysis of these IAP proteins led to the identification of their functional domains, namely, two baculovirus IAP repeat (BIR) domains and a C-terminal C3HC4-type Zn-finger, which is now known to be a *r*eally *i*nteresting *n*ew *g*ene (RING) domain (Vaux and Silke 2005) (Fig. 1).

In 1995 and 1996, the first IAPs were identified in insects and mammals, and based on the definition that BIR-containing proteins are designated as IAP proteins, eight IAPs are encoded in the human genome (Fig. 1). Most IAPs harbor several functional structural domains in addition to the BIR domains, and it is



Inhibitor of Apoptosis (IAP) Proteins, Fig. 1 *Schematic representation of IAP proteins*. The number of residues in each IAP as well as the functional motifs that they contain is shown. NAIP, survivin, BRUCE, ILP2, and ML-IAP are shown in this figure, but are not discussed in detail. In addition to DIAP1 and DIAP2, orthologues of survivin and BRUCE are also encoded in the genome of fruit flies (*Drosophila melanogaster*). Domain abbreviations used are as follows: *BIR* baculovirus IAP repeat, *UBA* ubiquitin-associated, *CARD* caspase-associated recruitment domain, *UBC* ubiquitin-conjugation, *NOD* nucleotide-binding oligomerization domain, *LRR* leucine-rich repeats

therefore not surprising that individual IAPs serve distinct biological functions. This entry will primarily go through what is currently known about three IAP proteins, cIAP1 (also termed BIRC2, MIHB, API1, and RNF48), cIAP2 (also termed BIRC3, MIHC, API2, and RNF49), and XIAP (also termed BIRC4, MIHA, API3, hILP, and XLP2), that are structurally related and share functions important for cellular signaling processes (Srinivasula and Ashwell 2008; Vaux and Silke 2005).

IAP Structural Domains

As mentioned above, the defining feature of an IAP protein is the presence of one or more BIR domains and mammalian IAPs contain either one or three copies. cIAP1, cIAP2, and XIAP all have three BIRs and a C-terminal RING domain (Fig. 1). In addition, they comprise an *ubi*quitin-*a*ssociated (UBA) domain, and cIAP1 and cIAP2 also contain a *c*aspase-*a*ssociated *r*ecruitment *d*omain (CARD). BIR domains are ca. 70 amino acids in size and mediate protein-protein interaction with partners that contain an IAP-binding motif (IBM) as well as other non-IBM-type protein interactions. IBMs are four amino acid motifs starting with an exposed N-terminal alanine. IBMs have been described in several proteins including the processed forms of the mitochondrial factors Smac/DIABLO and Omi/Htr2A and in cleavage-activated caspases. Through their RING domains, IAPs interact with ubiquitin-conjugating enzymes (E2s) and facilitate the transfer of activated ubiquitin from the bound E2 to lysine residues on target proteins (ubiquitylation). The UBA domain enables IAPs to directly interact with polyubiquitylated proteins (Gyrd-Hansen and Meier 2010). The CARD functions as a self-regulatory domain by keeping cIAP1 in a monomeric state, thus limiting its ubiquitin ligase activity (Lopez et al. 2011).

IAPs in Apoptosis

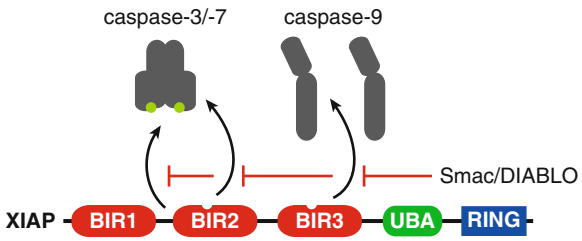
Apoptosis is executed by a family of proteases termed caspases and can be triggered by extrinsic signals such as ligands that activate death receptors on the cell surface (e.g., Fas Ligand (FasL; also known as CD95 ligand and APO-1 ligand) and tumor necrosis factor (TNF)) or by intrinsic insults including DNA damage, oncogene activation, oxidative stress, and growth factor deprivation (Meier and Vousden 2007). The extrinsic pathway leads to activation of caspase-8, which in turn activates downstream effector caspases (e.g., caspase-3 and caspase-7) either directly or via the mitochondrial apoptosis pathway. Activation of the mitochondrial pathway, which also is engaged by intrinsic insults, leads to the release of proapoptotic factors from the mitochondrial intermembrane space (e.g., cytochrome *c* and Smac/DIABLO). In the cytosol, cytochrome *c* associates with Apaf-1 and caspase-9 to form the caspase-9 activating platform, termed the apoptosome. In turn, the active caspase-9 activates effector caspases through proteolytic cleavage. The release of apoptogenic factors from the mitochondria is considered the "point of no return," and this process is tightly regulated by a network of pro- and antiapoptotic ▶ Bcl-2 family proteins including Bid, which links extrinsic signals and caspase-8 activation to the intrinsic mitochondrial pathway.

The strongest evidence that IAPs are important for regulation of apoptosis in vivo comes from studies of *Drosophila melanogaster* (fruit fly) IAP1 (DIAP1) (Ganesan et al. 2011; Orme and Meier 2009; Steller 2008). Genetic loss of DIAP1 results in spontaneous caspase activation causing widespread apoptosis and death of the embryo. Among the mammalian IAPs, XIAP is the only direct inhibitor of caspases, and overexpression of XIAP can inhibit caspase activation and apoptosis triggered by several stimuli (Eckelman et al. 2006; Srinivasula and Ashwell 2008). Conversely, cells that lack XIAP or are depleted for XIAP may become sensitized to apoptosis. Nonetheless, XIAP-deficient mice or mice expressing a truncated form of XIAP without the C-terminal RING domain are healthy and display no apparent defects, and cells isolated from these mice are not overtly sensitized to apoptosis except when treated with TNF in combination with cycloheximide (a protein synthesis inhibitor) (Gyrd-Hansen and Meier 2010). XIAP is therefore not essential for maintaining cell survival in mammals as DIAP1 is in flies, and the evidence rather suggests that XIAP modulates the apoptotic threshold under certain conditions and/or in certain cell types. This notion is supported by in vivo experiments showing that XIAP, in the absence of Bid, determines whether hepatocytes undergo apoptosis after exposure to FasL (Kaufmann et al. 2011). In wild-type mice, intraperitoneal injection of FasL results in massive apoptosis of hepatocytes in a manner that requires amplification of the caspase cascade through Bid-mediated release of apoptosis-promoting factors from the mitochondria. Bid-deficient mice are refractory to FasL treatment, but mice that are deficient for both Bid and XIAP are sensitive to FasL treatment and die from the injection of FasL akin to wild-type mice.

The inhibition of caspase activity/activation by XIAP involves different molecular mechanisms (Srinivasula and Ashwell 2008). By binding caspase-9 through its BIR3 domain, XIAP maintains caspase-9 in a monomeric inactive state. In contrast, caspase-3 and caspase-7 are inactivated primarily by the linker region immediately N-terminal to the XIAP BIR2, which fits the catalytic pocket of the active caspase in a backward orientation and blocks entry of substrates. The inhibition is, however, aided by an IBM-type interaction between BIR2 and the N-terminus of the processed caspase (Fig. 2). cIAP1 can also interact with active caspase-7 through an IBM-based interaction, but whether the BIR1-2 linker motif in cIAP1 contributes to caspase-regulation is not known.

IAPs may also regulate the activity of caspases through RING-dependent ubiquitylation (Broemer and Meier 2009; Vucic et al. 2011). The RING activity of DIAP1 is important for inhibition of caspase activity and proper regulation of cell death in the fruit fly. DIAP1 ubiquitylation of active effector caspases does not cause proteasomal degradation of the caspases but inhibits their activity. Similarly, XIAP can facilitate caspase-3 ubiquitylation in a RING-dependent manner to regulate its activity, although here the ubiquitylation is also reported to target caspase-3 for proteasomal degradation.

cIAP1 and cIAP2 are poor direct inhibitors of the catalytic activity of caspases but serve an important function in determining cell survival in response TNF receptor 1 (TNFR1) activation (Eckelman et al. 2006; Wu et al. 2007). This realization was sparked by the use of IAP-antagonistic compounds, so-called Smac



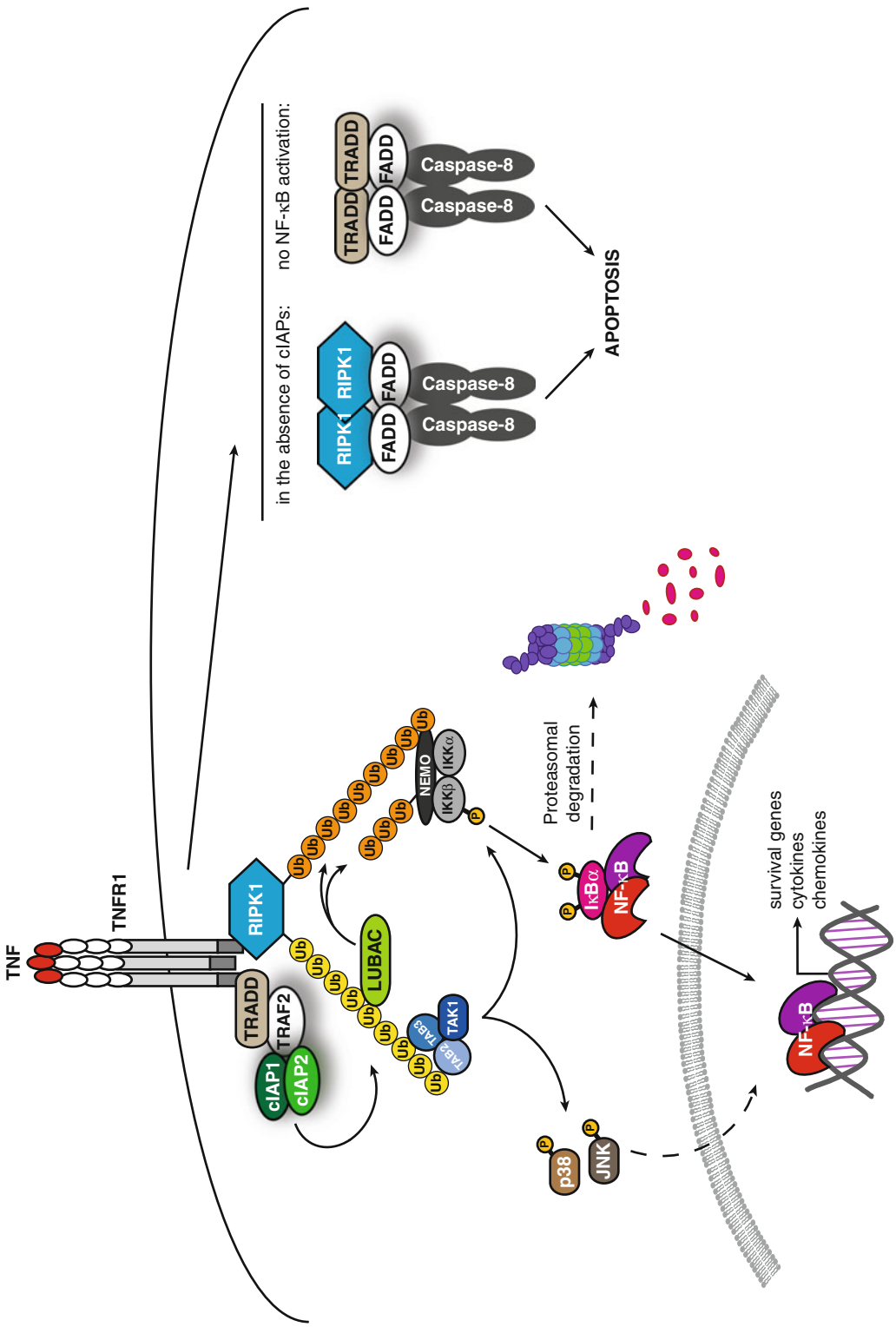
Inhibitor of Apoptosis (IAP) Proteins, Fig. 2 *XIAP-mediated inhibition of caspases*. The linker sequence between the BIR1 and BIR2 domains of XIAP occupies the catalytic pocket of caspase-3 and caspase-7 and thereby blocks substrate entry. In addition, the BIR2 domain interacts with the IBM of caspase-3 and caspase-7, which is exposed after their cleavage-mediated activation. XIAP inhibits caspase-9 activation by preventing dimerization, which is a prerequisite for initiator caspase activity. The N-terminal tetrapeptide IBM of processed Smac-DIABLO relieves caspase inhibition by competing for binding to BIR2 and BIR3. *Green circles* indicate the catalytic pocket in active caspase-3 and caspase-7

mimetics (SM), originally developed with the objective to interfere with the interaction between XIAP and active caspases and thereby render cells (in particular cancer cells) more susceptible to apoptosis. SMs mimic the structure of the Smac/DIABLO IBM (Ala–Val–Pro–Ile) and compete for binding with proteins that, like active caspases, contain IBMs (Fig. 2). SM compounds have proven effective in sensitizing cells to apoptosis (or induce cell death as a single treatment), but surprisingly, the mechanism behind this sensitization is primarily indirect and results from rapid proteasomal degradation of cIAP1 and cIAP2 rather than derepression of caspases (Vucic et al. 2011; Wu et al. 2007). From the use of SM compounds, cIAPs were found to prevent spontaneous activation of the so-called non-canonical ▶ NF-κB signaling pathway, which in some cells leads to secretion of TNF and thereby autocrine TNFR1 signaling. In turn, because cIAPs are absent, RIPK1 is not ubiquitylated by TNFR1 stimulation, and this interferes with NF-κB activation and favors the formation of a death-inducing signaling complex consisting of RIPK1, FADD, and caspase-8. This complex resembles the death-inducing complex formed when ▶ NF-κB activation is inhibited but contains RIPK1 instead of TRADD (Gyrd-Hansen and Meier 2010) (Fig. 3). In cells with high levels of the RIP family member RIPK3, TNF may also induce another type of programmed cell death termed necroptosis, which executes without caspase activity but rather is negatively regulated by caspase-8 (Declercq et al. 2009). Although mammalian IAPs under experimental settings either in cells or in vivo have been clearly demonstrated to have the capacity to determine cell survival, the physiological conditions where this occurs are less well established. Contrary to this, accumulating evidence now points to IAPs having a key function in innate immunity through regulation of inflammatory signaling pathways.

IAPs in Immune Signaling

The first evidence that IAP proteins regulate inflammatory signaling and activation NF-κB transcription factors came from genetic studies in fruit flies. Here, DIAP2 was found to be indispensable for NF-κB activation and innate immunity in response to Gram-negative bacteria (Ganesan et al. 2011; Lopez and Meier 2010). Infection with Gram-negative bacteria elicits an innate immune response by activating the *immune deficiency* (Imd) pathway, which is critically dependent on the ubiquitin ligase activity of DIAP2. DIAP2 conjugates lysine (K) 63-linked ubiquitin chains onto Imd, which facilitates activation of downstream of kinases that, in turn, activate the transcription factor Relish (a NF-κB-like transcription factor) (Ganesan et al. 2011; Lopez and Meier 2010). Accordingly, DIAP2-deficient flies fail to induce Relish-mediated expression of antimicrobial peptides and rapidly succumb to bacterial infections. Intriguingly, XIAP-deficient mice were recently found to be unable to efficiently clear bacterial infections and to ultimately die from the infection (Damgaard and Gyrd-Hansen 2011).

The domain organization of DIAP2 is very similar to that of the mammalian IAPs and akin to the role of DIAP2; cIAP1, cIAP2, and XIAP have emerged as regulators of the inflammatory response downstream of pattern recognition receptors (PRRs) and members of the TNF receptor superfamily (Lopez and Meier 2010). cIAPs regulate activation of NF-κB transcription factors downstream of TNFR1, Toll-like receptor 4 (▶ TLR4), and the cytosolic bacterial sensors NOD1 and NOD2. A unifying point in these pathways is the activation of TAK1 (TGFβ-activated kinase 1; also known as mitogen-activated protein kinase kinase kinase 7 (MAP3K7)) which in turn facilitates activation of ▶ MAP kinases and the IκB kinase (IKK)



Inhibitor of Apoptosis (IAP) Proteins, Fig. 3 (continued)

complex consisting of the catalytic subunits IKKα and IKKβ and the obligate regulatory subunit *N*F-κB *e*ssential *mo*difier (NEMO; also known as IKKγ). Once activated, IKK phosphorylates IκBα, an inhibitory subunit of NF-κB, which leads to its ubiquitylation and degradation by the proteasome. This enables nuclear translocation of ▶ NF-κB where it drives transcription of target genes required for the inflammatory response (Fig. 3). Activation of TAK1 and IKK is dependent on recruitment of the kinases to ubiquitin-modified proteins via the associated regulatory proteins TAB2/3 and NEMO, respectively. TAB2/3 and NEMO harbor ubiquitin-binding domains that mediate the recruitment to ubiquitylated proteins within the receptor complexes. In response to TNFR1 activation, ubiquitylation of RIPK1 and other components by cIAPs promotes the association of a trimeric ubiquitin ligase complex, termed LUBAC (linear ubiquitin chain assembly complex) (Walczak 2011). LUBAC expands ubiquitylation at the TNFR1 complex to enable efficient signaling and activation of the downstream kinases (Fig. 3). In addition, cIAPs are essential for signaling downstream of NOD1/2 where they are reported to ubiquitylate RIPK2 – a required adaptor similar to RIPK1. XIAP is also implicated in NOD2 signaling and was found to associate with RIPK2, but how XIAP contributes to signaling is currently unknown (Damgaard and Gyrd-Hansen 2011).

As mentioned above, cIAP1 and cIAP2 are required to prevent spontaneous activation the non-canonical NF-κB signaling pathway. This pathway is activated by receptors such as the B cell activating factor receptor (BAFF-R) and ▶ CD40 that are important for B cell function and lymphoid organ development. BAFF-R and ▶ CD40 stimulation leads to accumulation and self-activation of the NF-κB-inducing kinase (NIK). Upon activation, NIK activates an IKK complex consisting of IKKα dimers, and in turn, IKK facilitates activation of the NF-κB transcription factors (Bonizzi and Karin 2004).

The pathway is kept in check by a complex consisting of cIAP1, cIAP2, TRAF2, and TRAF3. TRAF3 links the complex to NIK, and the cIAP proteins ubiquitylate NIK (Lopez and Meier 2010). In response to CD40 or BAFF-R stimulation, cIAPs, through an unknown mechanism, shift target and instead ubiquitylate TRAF3, which leads to its degradation. This stabilizes NIK and allows activation of IKK and NF-κB. Treatment of cells with SM compounds results in a similar activation of NF-κB since cIAPs are degraded by the compound and no longer can mark NIK for degradation. This pathway is also activated by stimulation of the cytokine receptor Fn14 by its cognate ligand TWEAK. However, in this setting, Fn14 activation causes lysosomal degradation of the TRAF-cIAP complex, which thereby causes stabilization of NIK and subsequently activation of NF-κB.

cIAP-mediated ubiquitylation and degradation of TRAF3 also seems to contribute to TLR4 and RIG-I signaling (Lopez and Meier 2010). In response to TLR4 activation by LPS (a component of the bacterial cell wall), cIAPs are reported to ubiquitylate TRAF3 and cause its degradation which contributes to activation of MAP kinases, but not activation of NF-κB. RIG-I is an intracellular sensor of double-stranded viral RNA, and RIG-I activation leads to production of type I interferons such as IFNβ. cIAP-mediated degradation of TRAF3 after RIG-I activation is reported to be essential for IFNβ production after Sendai virus infection.

Inhibitor of Apoptosis (IAP) Proteins, Fig. 3 *cIAPs in TNFR1 signaling*. Activation of tumor necrosis factor receptor 1 (*TNFR1*) stimulates the formation of a signaling complex that consists of TNFR1, TNFR-associated via death domain (*TRADD*), RIPK1, TNFR-associated factor 2 (*TRAF2*), cellular inhibitor of apoptosis 1 (*cIAP1*), and cIAP2. cIAPs ubiquitylate (through K63-linked chains, shown in *yellow*) RIPK1, and probably other components of the receptor complex (not shown). This facilitates ubiquitin (*Ub*)-dependent recruitment of the linear ubiquitin chain assembly complex (*LUBAC*), transforming growth factor-β (*TGFβ*)-activated kinase (*TAK1*)/TAB2/TAB3 through their respective Ub-binding domains (*UBDs*). LUBAC conjugates linear ubiquitin chains (shown in *orange*) on to NEMO and RIPK1, which stabilizes the receptor complex, and allows efficient activation IKK and MAP kinases. This leads to activation of NF-κB transcription factors and induction of target genes important for inflammation and cell survival. In addition to stimulating TNF-mediated activation of nuclear factor-κB (*NF-κB*), cIAPs also suppress the formation of a death-inducing complex, which is the activation platform for caspase-8 that induces death by the extrinsic pathway. In the absence of cIAPs, this complex is formed by RIPK1 and Fas-associated via death domain (*FADD*), but in the absence of NF-κB activation, it is formed by TRADD and FADD

Other IAP Functions

IAPs are reported to negatively regulate the Ras-Raf-ERK signaling pathway. The pathway is activated by multiple growth factors and is frequently hyperactivated in cancer. XIAP, cIAP1, and cIAP2 were shown to interact with ▶ C-Raf and reduce the cellular levels of ▶ C-Raf in a ubiquitin-dependent manner, which involves the Hsp90 cochaperone and ubiquitin ligase CHIP (Gyrd-Hansen and Meier 2010). Accordingly, knockdown of XIAP or cIAPs led to an increase in cell motility and the invasive potential of HeLa cells in response to growth factor stimulation. Contrary to this, however, XIAP has been suggested to promote cell migration and metastasis in vivo in cooperation with survivin. The mechanism for this is not fully uncovered but appears to involve NF-κB activity.

XIAP is involved in the regulation of intracellular copper levels through its ubiquitylation of COMMD1 (copper metabolism gene MURR1 domain 1; also known as MURR1) (Srinivasula and Ashwell 2008). Defects in regulation of copper levels may cause severe pathologies such as Wilson's disease, an autosomal recessive condition that presents with neurological damage to the basal ganglia and cirrhosis due to accumulation of copper, particularly in brain and liver. XIAP binds COMMD1 via its BIR3 domain and ubiquitylates it in a RING-dependent manner, which targets COMMD1 for proteasomal degradation. The physiological relevance of this function of XIAP is supported by the finding that liver tissue and fibroblasts from XIAP-deficient mice have increased COMMD1 levels and decreased copper levels compared to wild-type controls (Srinivasula and Ashwell 2008).

IAPs in Human Diseases

Deregulation of several IAP family members has been observed in cancer. Although most links are correlative, there is evidence that cIAP1 and cIAP2, in particular, may contribute directly to cancer development. For example, the locus harboring *BIRC2*/*cIAP1* and *BIRC3*/*cIAP2* as well as *Yap1* and several *MMP* genes (11q21–q22) is found to be amplified in multiple human cancers including hepatocellular carcinoma (HCC), lung cancers, oral squamous cell carcinomas, medulloblastomas, glioblastomas, and pancreatic cancers (Gyrd-Hansen and Meier 2010; Srinivasula and Ashwell 2008). This is further supported by tumor models in mice where recurrent amplification of the 9qA1 locus, which is syntenic to the human 11q22 locus, was found in ▶ c-Myc-driven HCC in $p53^{-/-}$ mice and in spontaneously occurring osteosarcomas in $p53^{+/-}$ mice (Gyrd-Hansen and Meier 2010). In a separate report, cIAP1 was shown to positively regulate ▶ c-Myc activity by ubiquitylating the Myc-antagonistic protein, Max-dimerization protein-1 (Mad1), and facilitate its degradation (Vucic et al. 2011). The cIAP1-dependent decrease in Mad1 level correlated with increased proliferation and cell transformation in cell culture studies. However, whether this fully explains the role of cIAP1 in tumorigenesis in vivo needs further investigation.

Intriguingly, biallelic deletion of the locus encoding cIAP1 and cIAP2 is frequently observed in multiple myeloma (MM) together with other genes involved in regulation of ▶ NF-κB activity. MM is a B cell malignancy characterized by accumulation of antibody producing B cells (clonal) in the bone marrow. Combined, genetic lesions that cause deregulation of ▶ NF-κB activity were identified in ca. 20% of cases. Among the most frequent genetic alterations found were deletion of the *BIRC2*/*cIAP1* and *BIRC3*/*cIAP2* locus, deletion of *TRAF3*, *TRAF2*, and *CYLD*, and enhanced expression of lymphotoxin beta receptor (LTβR), ▶ CD40, TNFRSF13B/TACI, NFKB1, NFKB2, and NIK (Gyrd-Hansen and Meier 2010).

The mechanistic understanding of how amplification or deletion of the cIAP genes contributes to cancer development is currently not fully understood. In the case of MM, however, the identification of multiple genetic and expression abnormalities in MM that all affect genes involved in NF-κB signaling strongly suggests that unrestrained ▶ NF-κB activity contributes to driving these tumors. Analogous to this, chromosomal translocations that favor unrestrained NF-κB activity are frequent in MALT lymphoma (Ye et al. 2005). MALT lymphoma is an extranodal B cell lymphoma that arises in mucosa, primarily in the gastric tract and lungs, and is closely linked to *Helicobacter pylori* infections. The most common genetic lesion in MALT lymphomas is the reciprocal chromosomal translocation t(11;18)(q21;q21) that generates a fusion gene whose product consists of the N-terminal part of cIAP2 and the C-terminal portion of ▶ MALT1. The resulting chimeric protein cIAP2-MALT1 (also known as API2-MALT1) drives constitutive canonical

NF-κB signaling without requirement for activation of cell surface receptors (Ye et al. 2005). The cIAP2-MALT1 fusion bypasses the normal regulation of B cell receptor signaling by facilitating constitutive K63-linked ubiquitylation of NEMO by the ubiquitin ligase ▶ TRAF6. In turn, ubiquitylated NEMO is retained by the cIAP2 UBA domain present in cIAP2-MALT1 to enable activation of IKK and ▶ NF-κB (Gyrd-Hansen and Meier 2010).

Although XIAP is described to be overexpressed in many types of cancer, no genetic lesions affecting *XIAP* in human malignancies have been identified. In contrast, *XIAP* mutations have recently been reported in patients suffering from X-linked lymphoproliferative syndrome type 2 (XLP-2), a condition characterized by deregulation of the immune system and defined by hemophagocytic lymphohistiocytosis (Pachlopnik Schmid et al. 2011). Following viral infection, XLP-2 patients frequently develop cytopenia, fever, splenomegaly and hemorrhagic colitis, suggesting that mutations in *XIAP* predispose patients to the development of immunodeficiency (Pachlopnik Schmid et al. 2011). This is consistent with the experimental evidence which indicates that XIAP contributes to innate immunity to bacteria by facilitating NOD2 signaling, but whether XIAP also functions in other immune-related signaling paradigms is yet to be investigated (Damgaard and Gyrd-Hansen 2011).

Summary

The understanding of the cellular functions of individual IAP proteins has come a long way since the initial observation that expression of a viral IAP can maintain viability of cells after viral infection. In addition to their well-described role as gatekeepers of caspase activity and apoptosis, IAPs have now emerged as key regulators of signaling pathways important for inflammation and innate immunity. The molecular details of how IAPs impinge on these processes are still scarce, and in-depth understanding of these processes will undoubtedly be pursued in the years to come. Intriguingly, the realization that IAPs regulate immune-related processes may have unanticipated implications for the clinical applications of IAP-antagonistic compounds to also include treatment of immune-related syndromes as well as cancers driven by uncontrolled inflammatory signaling.

References

Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 2004;25:280–8.

Broemer M, Meier P. Ubiquitin-mediated regulation of apoptosis. Trends Cell Biol. 2009;19:130–40.

Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol. 1993;67:2168–74.

Damgaard RB, Gyrd-Hansen M. Inhibitor of apoptosis (IAP) proteins in regulation of inflammation and innate immunity. Discov Med. 2011;11:221–31.

Declercq W, Vanden Berghe T, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. Cell. 2009;138:229–32.

Eckelman BP, Salvesen GS, Scott FL. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO Rep. 2006;7:988–94.

Ganesan S, Aggarwal K, Paquette N, Silverman N. NF-kappaB/Rel proteins and the humoral immune responses of *Drosophila melanogaster*. Curr Top Microbiol Immunol. 2011;349:25–60.

Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer. 2010;10:561–74.

Kaufmann T, Strasser A, Jost PJ. Fas death receptor signalling: roles of Bid and XIAP. Cell Death Differ. 2011;19:42–50.

Lopez J, Meier P. To fight or die – inhibitor of apoptosis proteins at the crossroad of innate immunity and death. Curr Opin Cell Biol. 2010;22:872–81.

Lopez J, John SW, Tenev T, Rautureau GJ, Hinds MG, Francalanci F, Wilson R, Broemer M, Santoro MM, Day CL, Meier P. CARD-mediated autoinhibition of cIAP1's E3 ligase activity suppresses cell proliferation and migration. Mol Cell. 2011;42:569–83.

Meier P, Vousden KH. Lucifer's labyrinth–ten years of path finding in cell death. Mol Cell. 2007;28:746–54.

Orme M, Meier P. Inhibitor of apoptosis proteins in Drosophila: gatekeepers of death. Apoptosis. 2009;14:950–60.

Pachlopnik Schmid J, Canioni D, Moshous D, Touzot F, Mahlaoui N, Hauck F, Kanegane H, Lopez-Granados E, Mejstrikova E, Pellier I, et al. Clinical similarities and differences of patients with X-linked lymphoproliferative syndrome type 1 (XLP-1/SAP deficiency) versus type 2 (XLP-2/XIAP deficiency). Blood. 2011;117:1522–9.

Srinivasula SM, Ashwell JD. IAPs: what's in a name? Mol Cell. 2008;30:123–35.

Steller H. Regulation of apoptosis in *Drosophila*. Cell Death Differ. 2008;15:1132–8.

Vaux DL, Silke J. IAPs, RINGs and ubiquitylation. Nat Rev Mol Cell Biol. 2005;6:287–97.

Vucic D, Dixit VM, Wertz IE. Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol. 2011;12:439–52.

Walczak H. TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. Immunol Rev. 2011;244:9–28.

Wu H, Tschopp J, Lin SC. Smac mimetics and TNFalpha: a dangerous liaison? Cell. 2007;131:655–8.

I

Ye H, Gong L, Liu H, Hamoudi RA, Shirali S, Ho L, Chott A, Streubel B, Siebert R, Gesk S, et al. MALT lymphoma with t(14;18)(q32;q21)/IGH-MALT1 is characterized by strong cytoplasmic MALT1 and BCL10 expression. J Pathol. 2005;205:293–301.

Inhibitor of DNA Binding 4 (ID4)

Giovanni Blandino and Giulia Fontemaggi
Translational Oncogenomics Unit, Regina Elena Cancer Institute, Rome, Italy

Synonyms

bHLHb27; ID4; IDB4

Historical Background

Transcription factors containing a basic helix-loop-helix (bHLH) motif regulate expression of tissue-specific genes in a number of mammalian and insect systems, playing a key role in the differentiation processes. These proteins contain an HLH domain, which mediates homo- and heterodimerization, plus an adjacent DNA-binding region rich in basic amino acids. The bHLH proteins bind to a DNA sequence known as E-box (CANNTG).

Dominant-negative HLH proteins encoded by Id-related genes, such as Id4, also contain the HLH-dimerization domain but lack the DNA-binding basic domain. Consequently, Id proteins inhibit binding to DNA and transcriptional transactivation by heterodimerization with bHLH proteins (Pagliuca et al. 1995). In mammals, there are four known Id gene family members, known as Id1, Id2, Id3, and Id4. The identity between the HLH regions of Id proteins is very high, while the remaining regions of the proteins are not conserved. The N- and C-terminal fragments of Id proteins do not adopt a helical conformation, with the exception of Id4 fragment 27–64. This helix propensity is dictated by the presence of an Ala-rich motif between residues 39 and 57. It can be hypothesized that Id4 might exert unique functions through this structural feature (reviewed in Dell'Orso et al. 2010). Despite the high similarity in the HLH domain, the Id proteins bind different targets with different affinities; for example, Id2 is the only Id family member that recognizes the retinoblastoma protein.

Typically, Id proteins are highly expressed during embryogenesis and expressed at lower levels in mature tissues, with the exception of some stem cells and many cancers (reviewed in Perk et al. 2005). Id proteins were described initially as inhibitors of differentiation and more recently as regulators of cell cycle progression, senescence, apoptosis, and tumorigenesis. Concerning the ability of Id proteins to control the developmental processes, Id4 in particular was shown to be involved in the differentiation of neurons, adipocytes, and osteoblasts, and in the nervous system and mammary gland development. Deregulated Id4 expression is also frequent in tumors. Specifically, Id4 was found upregulated in glioblastoma multiforme (GBM) and breast cancer (BC) and amplified in ovarian carcinomas (Cancer Genome Atlas Research Network 2011). Conversely to what observed in GBM and BC, an Id4 underexpression due to promoter hypermethylation was observed in carcinomas of the gastrointestinal tract, indicating a possible role for Id4 in tumor suppression.

Id4 Regulates Neural Progenitor Proliferation and Differentiation

It is well documented that the expression of each Id gene occurs in many regions of the developing nervous system in a complex and dynamic manner. Id4 expression is essentially restricted to the developing nervous system, whereas expression of Id1-3 is much more widespread during mouse embryogenesis (Jen et al. 1997). Early in neurogenesis, Id4 expression is prominent in the ventricular zone (VZ) of specific regions of the central nervous system (CNS), including the developing forebrain. Later, Id4 expression is apparent in the cortical plate of the telencephalon and the subventricular zone (SVZ) of the basal ganglia (Jen et al. 1997). Id4 expression is also observed in the postnatal and adult brains (Andres-Barquin et al. 1999).

Studies on knockout mice revealed that Id4 is required for normal brain size and regulates neural stem cells' proliferation and differentiation. In particular, Id4 regulates lateral expansion of the proliferative zone in the developing cortex and hippocampus. Since Id4 is required for the normal G1/S transition in early cortical progenitors, the absence of its expression

compromises the proliferation of stem cells in the ventricular zone (Yun et al. 2004).

Id4 is expressed in oligodendrocyte precursor cells and may control the timing of oligodendrocyte differentiation. Enforced expression of Id4 in vitro stimulates proliferation and blocks differentiation of oligodendrocyte precursor cells (Bedford et al. 2005). Id4 was recently found in neural progenitor cells to directly interact with bHLH OLIG1 and OLIG2, two crucial transcription factors responsible for oligodendroglial differentiation. Id4 also mediates the inhibitory effects of bone morphogenetic protein-4 (BMP-4) on oligodendroglial differentiation that leads to astrocytic differentiation (Yun et al. 2004).

The molecular mechanism by which Id4 expression is downregulated during oligodendrocyte differentiation has remained unknown. Accumulating evidence however suggests that Id4 expression is repressed by DNA methylation at neighboring CpG islands. Interestingly, it was found that PRMT5, a type-II protein arginine methyltransferase, is required for maintaining the methylation status of CpG islands of Id2 and Id4, leading to gene silencing during glial cell differentiation (Huang et al. 2011). In addition, a transcriptional factor, Rp58, was very recently reported to negatively regulate all four Id genes (Id1–Id4) in developing cerebral cortex. Consistently, Rp58 knockout (KO) mice demonstrated enhanced astrogenesis accompanied with an excess of neural stem cells (NSCs). Rp58 KO phenotypes were rescued by the knockdown of all Id genes in mutant cortical progenitors but not by the knockdown of each single Id gene. These findings establish RP58 as a novel key regulator that controls the self-renewal and differentiation of NSCs and restriction of astrogenesis by repressing all Id genes during corticogenesis (Hirai et al. 2012).

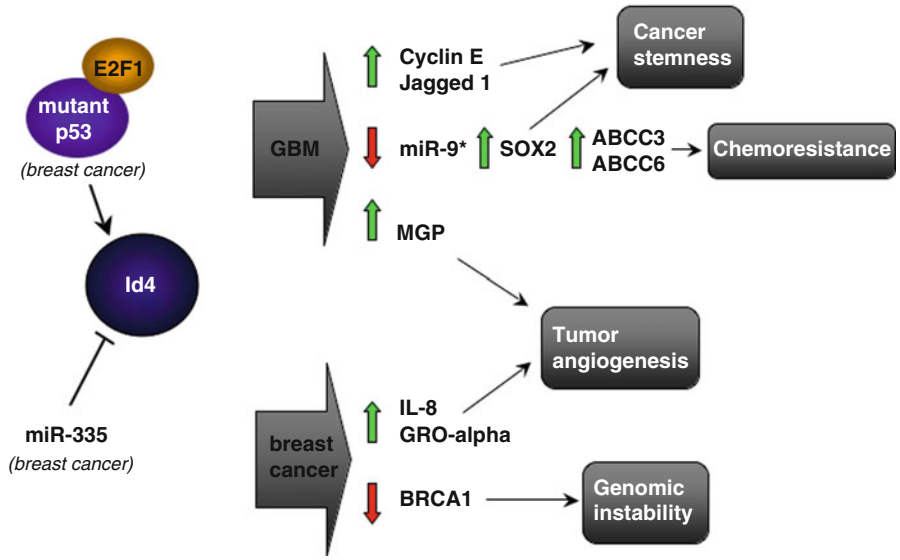
Id4 Maintains the Stem Cells Compartment in Glioblastoma Multiforme

The expression of Id proteins, which is very low in adult tissues, can be reactivated in human cancers. It has been proposed that deregulated Id signaling may promote multiple attributes of malignancy, like unrestricted proliferation, loss of differentiation (anaplasia), invasiveness, and neoangiogenesis (Perk et al. 2005). Elevated levels of Id proteins have been reported in several malignancies (carcinoma, squamous cell carcinoma, adenocarcinoma, neural tumors, melanoma, sarcoma, seminoma, and leukemia). In some cases, high levels are associated with tumor stage/grade and with prognosis. Analysis of genetic alterations of Id genes in human tumors has found no mutations. This may indicate that Id genes are not common oncogenes. Nevertheless, the overexpression of Id genes in cancer is frequently governed by bona fide oncogenes, such as ▶ MYC-driven Id2 expression in neuroblastoma cells, ▶ beta-catenin-driven Id2 expression in colon cancer cells, and mutant ▶ p53-driven Id4 expression in breast cancer cells.

Id4 was found overexpressed in glioblastoma multiforme (GBM) when compared to normal brain tissue. The effects of Id4 overexpression in GBM are summarized in Fig. 1. In GBM, it shows a robust expression with heterogenous staining pattern within the same tumor tissue. In particular, Id4 is preferentially expressed in cells of astrocytic lineage in oligodendroglioma and oligoastrocytoma tumors (Liang et al. 2005). Interestingly, the analysis of Id4 protein expression in human GBM specimens evidenced that the majority of Id4-positive cells resides near the vasculature, a location postulated to be the niche for brain tumor stem cells (Jeon et al. 2008).

Enforced Id4 expression can drive malignant transformation of primary murine Ink4a/ARF−/− astrocytes via deregulation of cell cycle and differentiation control. Id4 indeed increases the levels of both cyclin E (that leads to a hyperproliferative state) and Jagged1 to drive astrocytes into a neural stem-like cell state. These findings highlight the role of Id4 in controlling the "stemness" of neural cells during development of the central nervous system (Jeon et al. 2008). Further studies showed also that Id4 expression contributes to the chemoresistance of glioma stem cells. In particular, Id4 suppresses the expression of microRNA-9*, leading to the de-repression of SOX2, a crucial player in cancer stem cells. SOX2 upregulation contributes, on one hand, to the maintenance of cancer stem cells compartment, and on the other hand, to glioma chemoresistance, through the transcriptional induction of drug resistance genes, such as those encoding the ATP-binding cassette transporters ABCC3/ABCC6 (Jeon et al. 2011).

Moreover, human glioblastoma xenografts overexpressing Id4 are characterized by higher tumor sizes compared to controls. This is not due to a higher proliferation rate of Id4-overexpressing cell clones but instead due to the better vascularization of the derived xenograft tumors compared to control xenografts.



Inhibitor of DNA Binding 4 (ID4), Fig. 1 *Id4 network in cancer*. Deregulated Id signaling has been reported in glioblastoma multiforme (GBM) and breast cancer and may promote multiple attributes of malignancy. In glioblastoma multiforme, Id4 was shown to contribute to the maintenance of stem cells compartment (through the induction of cyclin E, jagged1, and SOX2), to chemoresistance (through the induction of ABC transporters), and to angiogenesis (through the release of MGP in the extracellular matrix). In proliferating breast cancer cells carrying mutant p53, the protein complex mutp53/E2F1/p300 assembles on specific regions of Id4 promoter and positively controls Id4 expression. The newly synthesized Id4 protein binds to mRNAs encoding proangiogenic factors, like IL8 and GRO-alpha, causing their stabilization and enhancement of translation. This results in an increase of the angiogenic potential of cancer cells expressing mutant p53. In breast cancer cells, Id4 mRNA is targeted by miR-335. Low miR-335 levels correlate with high Id4 expression which in turn results in downregulation of BRCA1 and genomic instability

Superficial blood vessels in xenografts expressing high Id4 levels are larger and more numerous than vessels in control tumors. The Id4-dependent mediator responsible for the enhanced angiogenesis is matrix GLA protein (MGP), a member of vitamin-K-dependent family of proteins, which includes prothrombin (Kuzontkoski et al. 2010).

Id4 Controls Mammary Gland Development and Cancer

During the normal mammary gland development, Id4 expression is required for ductal expansion and branching morphogenesis as well as cell proliferation induced by estrogen and/or progesterone. p38 MAPK is activated in Id4-null mammary cells and this activation is required for the reduced proliferation and increased apoptosis observed in Id4-ablated mammary glands. Therefore, ID4 promotes mammary gland development by suppressing p38 MAPK activity (Dong et al. 2011).

A positive role for Id4 in mammary and ovarian tumorigenesis was also proposed (reviewed in Dell'Orso et al. 2010). The modulation of Id4 expression in these systems indeed resulted in inversely regulated expression of BRCA1 (Fig. 1). An increase of Id4 expression was associated with the ability of ovarian and breast cancer cells to exhibit anchorage-independent growth, while its depletion determined morphological change to a large and flat epithelial phenotype. The expression of Id4 and BRCA1/ER (► estrogen receptor) inversely correlated in sporadic breast cancers. Id4 3'-UTR was also recently found to be targeted by microRNA-335 in breast cancer cells, resulting in BRCA1 induction and decrease in genomic instability. Accordingly, Id4 and miR-335 expression are inversely correlated in breast cancer specimens (Heyn et al. 2011).

The expression of Id4, as well as that of Id2, can be induced by mutant ► p53 proteins (Fontemaggi et al. 2009, 2010; Dell'Orso et al. 2010). In breast cancer cell lines, the transcriptional transactivation of Id4 promoter is exerted by the complex mutant p53/E2F1/p300. Accordingly, Id4 protein expression is enriched in breast cancer tissues, showing p53

overexpression (predicting the presence of p53 mutations). This association is particularly evident in the HER2 subtype, where Id4 is expressed in nearly 80% of p53-positive cases, compared to 40% of the p53-negative cases. HER2-overexpressing breast cancer subtype presents very high frequency of ▶ TP53 mutations, like all ER-negative breast cancers. The net biological output of the transcriptional activation of Id4 gene by mutant ▶ p53 is the increase of the angiogenic potential of mutant ▶ p53-carrying tumor cells. The mutant ▶ p53/Id4 axis promotes endothelial cells' proliferation and migration in vitro. In addition, the analysis of human breast cancer cases revealed that a higher microvessel density is present in the Id4-positive population than in Id4-negative one.

At the molecular level, Id4 protein binds to the mRNAs of proangiogenic factors like CXCL8 (IL8) and CXCL1 (GRO-alpha) (Fig. 1), containing AU-rich (ARE) elements in their 3'UTR, resulting in an increased stability and a higher rate of translation of these transcripts (Fontemaggi et al. 2009). Significantly, the most expressed cytokines in HER2 tumors, displaying the highest correlation between p53 and Id4 expressions among all breast cancer subtypes, are just CXCL8 and CXCL1.

Studies performed in 4 T1 invasive mouse mammary cancer cells also evidenced the ability of Id4 to promote features typical of the cancer stem cells, such as the tumor sphere-forming ability (Park et al. 2011). In addition, Id4 is involved in the induction of the expression of ABCC3, belonging to the family of ABC transporters.

Summary

Id4 is a member of the inhibitor-of-DNA-binding family of HLH proteins that comprises four members (Id1-4). The Id group of proteins was reported to promote proliferation and inhibit differentiation in several cell types. Because they lack a DNA-binding domain at the N-terminus, Id proteins are generally thought to exert their function by forming heterodimers with bHLH proteins, preventing these other proteins from forming transcriptionally active homodimers or heterodimers with the ubiquitous E proteins on consensus E-boxes present on target promoters. The gene encoding Id4 is required for neuroprogenitor cell proliferation and proper differentiation. The expression of Id proteins, which is very low in adult tissues, can be reactivated in human cancers and deregulated Id signaling may promote multiple attributes of malignancy. Id4 is overexpressed in glioblastoma multiforme, where it promotes the maintenance of cancer cell stemness and neovascularization. A proangiogenic role of Id4 was also reported in breast cancer, where mutant p53 proteins induce Id4 promoter transactivation.

References

Andres-Barquin PJ, Hernandez MC, Israel MA. Id4 expression induces apoptosis in astrocytic cultures and is down-regulated by activation of the cAMP-dependent signal transduction pathway. Exp Cell Res. 1999;247:347–55.

Bedford L, Walker R, Kondo T, van Crüchten I, King ER, Sablitzky F. Id4 is required for the correct timing of neural differentiation. Dev Biol. 2005;280:386–95. doi:10.1016/j.ydbio.2005.02.001.

Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. Nature. 2011;474:609–15. doi:10.1038/nature10166.

Dell'Orso S, Ganci F, Strano S, Blandino G, Fontemaggi G. ID4: a new player in the cancer arena. Oncotarget. 2010;1:48–58.

Dong J, Huang S, Caikovski M, Ji S, McGrath A, Custorio MG, Creighton CJ, Maliakkal P, Bogoslovskaia E, Du Z, Zhang X, Lewis MT, Sablitzky F, Brisken C, Li Y. ID4 regulates mammary gland development by suppressing p38MAPK activity. Development. 2011;138:5247–56. doi:10.1242/dev.069203.

Fontemaggi G, Dell'Orso S, Trisciuoglio D, Shay T, Melucci E, Fazi F, Terrenato I, Mottolese M, Muti P, Domany E, Del Bufalo D, Strano S, Blandino G. The execution of the transcriptional axis mutant p53, E2F1 and ID4 promotes tumor neo-angiogenesis. Nat Struct Mol Biol. 2009;16:1086–93. doi:10.1038/nsmb.1669.

Fontemaggi G, Dell'Orso S, Muti P, Blandino G, Strano S. Id2 gene is a transcriptional target of the protein complex mutant p53/E2F1. Cell Cycle. 2010;9:2464–6.

Heyn H, Engelmann M, Schreek S, Ahrens P, Lehmann U, Kreipe H, Schlegelberger B, Beger C. MicroRNA miR-335 is crucial for the BRCA1 regulatory cascade in breast cancer development. Int J Cancer. 2011;129:2797–806. doi:10.1002/ijc.25962.

Hirai S, Miwa A, Ohtaka-Maruyama C, Kasai M, Okabe S, Hata Y, Okado H. RP58 controls neuron and astrocyte differentiation by downregulating the expression of Id1-4 genes in the developing cortex. EMBO J. 2012. doi:10.1038/emboj.2011.486.

Huang J, Vogel G, Yu Z, Almazan G, Richard S. Type II arginine methyltransferase prmt5 regulates gene expression of inhibitors of differentiation/DNA binding Id2 and Id4 during glial cell differentiation. J Biol Chem. 2011;286(52):44424–32. doi:/10.1074/jbc.M111.277046.

Jen Y, Manova K, Benezra R. Each member of the Id gene family exhibits a unique expression pattern in mouse

gastrulation and neurogenesis. Dev Dyn. 1997;208:92–106. doi:10.1002/(SICI)1097.
Jeon HM, Jin X, Lee JS, Oh SY, Sohn YW, Park HJ, Joo KM, Park WY, Nam DH, DePinho RA, Chin L, Kim H. Inhibitor of differentiation 4 drives brain tumor-initiating cell genesis through cyclin E and notch signaling. Genes Dev. 2008;22:2028–33. doi:10.1101/gad.1668708.
Jeon HM, Sohn YW, Oh SY, Kim SH, Beck S, Kim S, Kim H. ID4 imparts chemoresistance and cancer stemness to glioma cells by derepressing miR-9*-mediated suppression of SOX2. Cancer Res. 2011;71(9):3410–21. doi:10.1158/0008-5472.CAN-10-3340.
Kuzontkoski PM, Mulligan-Kehoe MJ, Harris BT, Israel MA. Inhibitor of DNA binding-4 promotes angiogenesis and growth of glioblastoma multiforme by elevating matrix GLA levels. Oncogene. 2010;29(26):3793–802. doi:10.1038/onc.2010.147.
Liang Y, Bollen AW, Nicholas MK, Gupta N. Id4 and FABP7 are preferentially expressed in cells with astrocytic features in oligodendrogliomas and oligoastrocytomas. BMC Clin Pathol. 2005;5:6. doi:10.1186/1472-6890-5-6.
Pagliuca A, Bartoli PC, Saccone S, Della Valle G, Lania L. Molecular cloning of ID4, a novel dominant negative helix-loop-helix human gene on chromosome 6p21.3-p22. Genomics. 1995;27(1):200–3. doi:10.1006/geno.1995.1026.
Park SJ, Kim RJ, Nam JS. Inhibitor of DNA-binding 4 contributes to the maintenance and expansion of cancer stem cells in 4T1 mouse mammary cancer cell line. Lab Anim Res. 2011;27(4):333–8. doi:10.5625/lar.2011.27.4.333.
Perk J, Iavarone A, Benezra R. Id family of helix-loop-helix proteins in cancer. Nat Rev Cancer. 2005;5:603–14. doi:10.1038/nrc1673.
Yun K, Mantani A, Garel S, Rubenstein J, Israel MA. Id4 regulates neural progenitor proliferation and differentiation in vivo. Development. 2004;131:5441–8. doi:10.1242/dev.01430.

Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

Michael A. Grillo and Peter Koulen
Department of Ophthalmology and Department of Basic Medical Science, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Synonyms

IP3 receptor–associated cGMP kinase substrate a; IRAG; Iraga; JAW1-related protein isoform a; MRV integration site 1; Mrv1; Mrv1a; Mrvi1; Murine retrovirus integration site 1 homolog

Historical Background

Inositol 1, 4, 5-trisphosphate (IP_3) receptor–associated cyclic GMP (cGMP) kinase substrate (IRAG) is a 125-kD type 2 integral endoplasmic reticulum (ER) membrane protein. IRAG is 900 amino acids long and encoded by the Mrvi1 gene. Two splice variants exist in both mouse and humans with the IRAGa isoform containing an extra 84 amino acids in the N-terminus. Both variants contain a C-terminal hydrophobic domain, α-helix coiled-coil domain, basic N-terminal region, and multiple glycosylation and phosphorylation sites (Ammendola et al. 2001; Schlossmann et al. 2000; Shaughnessy et al. 1999). IRAG forms a complex with the Inositol 1, 4, 5-trisphosphate receptor (IP_3R) and cGMP kinase I-β (cGKIβ), and is involved in nitric oxide (NO),cGMP, and cGKIβ signaling pathways. IRAG plays a role in transcription modulation, smooth muscle contraction, platelet aggregation, cell growth, differentiation, and possibly tumorigenesis.

Expression Pattern

Expression of IRAG is found in the heart, lungs, liver, pancreas, smooth muscle, colon, small intestines, pancreas, trachea, hippocampus, nucleus motorius, and nucleus trigemini (Graham et al. 2008). Perinuclear immunostaining and immunoprecipitation with resident ER proteins suggests IRAG localization to the ER membrane (Ammendola et al. 2001; Fritsch et al. 2004; Geiselhöringer et al. 2004a; Schlossmann et al. 2000; Shaughnessy et al. 1999). Downregulation of IRAG coincides with siRNA knockdown of the transcription factor BTF3 in pancreatic cancer cell lines (Kusumawidjaja et al. 2007), and knockout of Ca^{2+}-dependent K^+(BK) channel in trachea causes an upregulation in IRAG protein concentration (Sausbier et al. 2007). Deletion of exon 12 coding for the N-terminus coiled-coil domain of IRAG resulted in a 25% decrease in protein concentration in mouse intestine (Geiselhöringer et al. 2004).

Interactions with Ligands and Other Proteins

IRAG co-immunoprecipitates with both IP_3R1 and cGKIβ/cGKI in smooth muscle from bovine trachea (Schlossmann et al. 2000). Interaction with IP3R1

possibly occurs at the IRAG N-terminus coiled-coil domain. Deletion of this region of the protein in mice shows disruption in IRAG/IP_3R coupling in aortic and intestinal smooth muscle (Geiselhöringer et al. 2004). Interactions between IRAG and cGKIβ occur between amino acids 152 and 184 of IRAG, through possible electrostatic interactions, and the N-terminal leucine zipper motif, amino acids 1–53, of cGKIβ but not cGKIα or cGKII (Ammendola et al. 2001; Casteel et al. 2005). Upon cGMP stimulation, cGKIβ phosphorylates IRAG at ser 644 and ser 677 in human platelets and ser 696 in bovine trachea cells (Schlossmann et al. 2000; Antl et al. 2007). Phosphorylation of IRAG causes a decrease in IP_3R1 function (Schlossmann et al. 2000), with ser 696 phosphorylation being essential for this response (Schlossmann et al. 2000). In COS-7 cells, expression of phospholambin, IP_3R1, cGKIβ, and IRAG resulted in co-immunoprecipitation of these proteins in complex (Koller et al. 2003).

Protein Function

As previously stated, upon phosphorylation, IRAG decreases calcium release from IP3R-mediated stores (Schlossmann et al. 2000). The interaction of IRAG and cGKIβ has been shown to mediate translocation of cGKIβ to the nucleus, thus attenuating cAMP-response element–dependent genes in hamster kidney cells (Casteel et al. 2008). Deletion of the IRAG/IP3R association in platelets caused NO/cGMP inhibition of platelet aggregation and control of thrombosis to be lost (Antl et al. 2007), and this loss of association in smooth muscle caused a loss in cGMP-dependent relaxation of hormone receptor smooth muscle contraction (Hoffman et al. 2004). In colonic smooth muscle, siRNA interference of IRAG caused a loss of NO-induced relaxation (Fritsch et al. 2004), and deletion ablated cGMP relaxation (Frei et al. 2009). Deletion of IRAG causes a loss in atrial natriuretic peptide (ANP) and NO relaxation of smooth muscle tone (Desch et al. 2010). Osteoclast attachment is impaired in siRNA knockouts of the IRAG protein (Yaroslavskiy et al. 2010).

Summary

IRAG is involved directly or indirectly in many signaling pathways. IRAG regulates the intracellular calcium concentration through an interaction with IP_3R (Schlossmann et al. 2000), affects transcription through its anchoring of cGKIβ (Casteel et al. 2008), and controls NO, ANP, and cGMP relaxation of smooth muscle in multiple tissues throughout the body (Hoffman et al. 2004; Fritsch et al. 2004; Desch et al. 2010). Due to its essential role in NO/cGMP signaling, IRAG provides an interesting target for diseases involving cardiovascular dysfunction.

Acknowledgments This study was supported in part by grants EY014227 from NIH/NEI, RR022570 from NIH/NCRR and AG010485, AG022550 and AG027956 from NIH/NIA, by the Felix and Carmen Sabates Missouri Endowed Chair in Vision Research (PK). We thank Margaret, Richard, and Sara Koulen for their generous support and encouragement.

References

Ammendola A, Geiselhöringer A, Hofmann F, Schlossmann J. Molecular determinants of the interaction between the inositol1,4,5-trisphosphate receptor associated cGMP kinase substrate (IRAG) and cGMP kinase Ibeta. J Biol Chem. 2001;276(26):24153–9.

Antl M, von Brühl ML, Eiglsperger C, Werner M, Konrad I, Kocher T, Wilm M, Hofmann F, Massberg S, Schlossmann J. IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation. Blood. 2007;109(2):552–9.

Casteel DE, Boss GR, Pilz RB. Identification of the interface between cGMP-dependent protein kinase I beta and its interaction partners TFII-I and IRAG reveals a common interaction motif. J Biol Chem. 2005;280(46):38211–8.

Casteel DE, Zhang T, Zhuang S, Pilz RB. cGMP-dependent protein kinase anchoring by IRAG regulates its nuclear translocation and transcriptional activity. Cell Signal. 2008;20(7):1392–9.

Desch M, Sigl K, Hieke B, Salb K, Kees F, Bernhard D, Jochim A, Spiessberger B, Höcherl K, Feil R, Feil S, Lukowski R, Wegener JW, Hofmann F, Schlossmann J. IRAG determines nitric oxide- and atrial natriuretic peptide-mediated smooth muscle relaxation. Cardiovasc Res. 2010;86(3):496–505. Epub 2010 Jan 15.

Frei E, Huster M, Smital P, Schlossmann J, Hofmann F, Wegener JW. Calcium-dependent and calcium-independent inhibition of contraction by cGMP/cGKI in intestinal smooth muscle. Am J Physiol Gastrointest Liver Physiol. 2009;297(4):G834–G9 [Epub ahead of print].

Fritsch RM, Saur D, Kurjak M, Oesterle D, Schlossmann J, Geiselhöringer A, Hofmann F, Allescher HD. InsP3R-associated cGMP kinase substrate (IRAG) is essential for nitric oxide-induced inhibition of calcium signaling in human colonic smooth muscle. J Biol Chem. 2004;279(13):12551–9.

Geiselhöringer A, Gaisa M, Hofmann F, Schlossmann J. Distribution of IRAG and cGKIisoforms in murine tissues. FEBS Lett. 2004a;575(1–3):19–22.

I

Geiselhöringer A, Werner M, Sigl K, Smital P, Wörner R, Acheo L, Stieber J, Weinmeister P, Feil R, Feil S, Wegener J, Hofmann F, Schlossmann J. IRAG is essential for relaxation of receptor-triggered smooth muscle contraction by cGMP kinase. EMBO J. 2004b;23(21):4222–31.

Graham SM, Longoria S, Sarkar P, Koulen P. Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate. UCSD Nat Molecule Pages. 2008. doi:10.1038/mp.a001275.01.

Koller A, Schlossmann J, Ashman K, Uttenweiler-Joseph S, Ruth P, Hofmann F. Association of phospholamban with a cGMP kinase signaling complex. Biochem Biophys Res Commun. 2003;300(1):155–160.

Kusumawidjaja G, Kayed H, Giese N, Bauer A, Erkan M, Giese T, Hoheise JD, Friess H, Kleeff J. Basic transcription factor 3 (BTF3) regulates transcription of tumorassociated genes in pancreatic cancer cells. Cancer Biol Ther. 2007;6(3):367–6.

Sausbier M, Zhou XB, Beier C, Sausbier U, Wolpers D, Maget S, Martin C, Dietrich A, Ressmeyer AR, Renz H, Schlossmann J, Hofmann F, Neuhuber W, Gudermann T, Uhlig S, Korth M, Ruth P. Reduced rather than enhanced cholinergic airway constrictionin mice with ablation of the large conductance Ca2+−activated K+ channel. FASEB J. 2007;21:812–22.

Schlossmann J, Ammendola A, Ashman K, Zong X, Huber A, Neubauer G, Wang GX, Allescher HD, Korth M, Wilm M, Hofmann F, Ruth P. Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. Nature. 2000;404(6774):197–201.

Shaughnessy JD, Largaespada DA, Tian E, Fletcher CF, Cho BC, Vyas P, Jenkins NA, Copeland N. Mrvi1, a common MRV integration site in BXH2 myeloid leukemias, encodes a protein with homology to a lymphoid-restricted membrane protein Jaw1. Oncogene. 1999;18(12):2069–84.

Yaroslavskiy BB, Turkova I, Wang Y, Robinson LJ, Blair HC. Functional osteoclast attachment requires inositol-1,4,5-trisphosphate receptor-associated cGMP-dependent kinase substrate. Lab Invest. 2010;90(10):1533–42 [Epub ahead of print].

Inositol 3,4,5,6-Tetrakisphosphate 1-Kinase

► ITPK1 (Inositol 1,3,4-Triphosphate 5/6 Kinase)

Inositol Polyphosphate-5-Phosphatase, 145 kDa

► SHIP

Inositol Trisphosphate 5/6-Kinase

► ITPK1 (Inositol 1,3,4-Triphosphate 5/6 Kinase)

INP10

► CXCL10

Inpp5d

► SHIP

Insulin-Like Growth Factor Receptor Type I (IGF1R)

► Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation

Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation

Alexander Annenkov
Bone and Joint Research Unit, William Harvey Research Institute, Queen Mary University of London, London, UK

Synonyms

Insulin-like growth factor receptor type I (IGF1R)

Historical Background

The IGF1R, a Protein Tyrosine Kinase Receptor, is expressed in many tissues and can be activated by its physiological ligands IGF-I and IGF-II, which are available systemically and from local sources. Activation of the IGF1R promotes cell growth and survival in a cell-autonomous manner. Because of a wide distribution of IGF1R and good bioavailability of its ligands, under pathological conditions this cytoprotective intracellular signaling pathway may be activated in cells that also receive stimulation by pathology-specific factors. Cross-regulation between IGF1R signaling and pathways activated by

inflammation has recently been in the focus of attention in several laboratories because pro-inflammatory factors are a common cause of cellular pathology in many different conditions (O'Connor et al. 2008).

Resident parenchymal cells activated by pro-inflammatory cytokines in tissues affected by inflammation are characterized by several features, including increased activity of the stress-related Mitogen-Activated Kinases (MAPK) and transcription factors of nuclear factor-κB (▶ NF-κB) family, stress of the endoplasmic reticulum (ER), and generation of reactive oxygen species (ROS). Several components in inflammation-activated pathway may be cross-regulated by IGF1R-activated signaling, and there are signaling molecules in the IGF1R pathway that are subject to cross-regulation by pro-inflammatory factors. Here IGF1R signaling has been reviewed and biochemical events that may facilitate cross-regulation of this pathway and those activated by inflammation have been elucidated. The generic signaling networks presented here are based on molecular interaction shown in non-transformed nonimmune cells. There is a considerable variation between cells of different lineages and differentiation states in usage of these networks.

IGF1R Signaling Cascade

IGF1R Activation

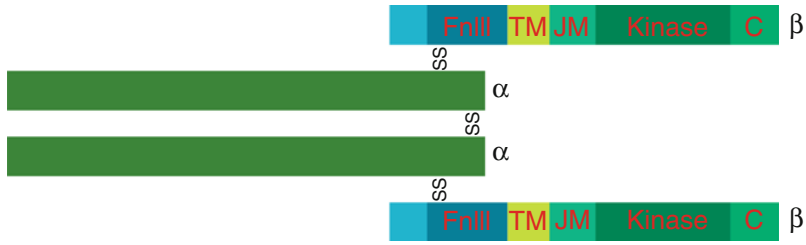
The IGF1R (Adams et al. 2000) consists of two α- and two β-chains (Fig. 1). The α-chains are extracellular and associated by disulfide bonds. They form a ligand-binding pocket, which can accommodate one ligand molecule. A ligand that has the highest affinity to the IGF1R is IGF-I. Two other ligands, IGF-II and insulin, can also bind to the receptor with, respectively, 6–8 and 100 times lower affinity than IGF-I. A single disulfide bond binds the α-chain to the extracellular fibronectin type III homology domain of the β-chain. Therefore, the receptor consists of two αβ heterodimers covalently linked by the disulfide bonds between the α-chains (Fig. 1).

The β-chain is transmembrane, with its cytosolic portion containing the catalytic kinase domain flanked with the juxtamembrane region and the C-terminal tail. In the absence of ligand, the autocatalytic activity of the IGF1R is inhibited by conformational constraints imposed by the extracellular and juxtamembrane portions of the β-chain. The autoinhibition is relieved when ligand binds to the α-chains, facilitating phosphorylation of three tyrosines in the activation loops of the first kinase domain by the second kinase domain and vice versa (transphosphorylation, Fig. 2). This is followed by phosphorylation of several additional tyrosines in the IGF1R and other signaling proteins. Three serine residues in the C-terminal tail of the receptor are also phosphorylated upon ligand binding via an incompletely understood mechanism. Different segments of the IGF1R signaling portion appear to be linked to different arms of the IGF1R signaling pathway and different cellular responses.

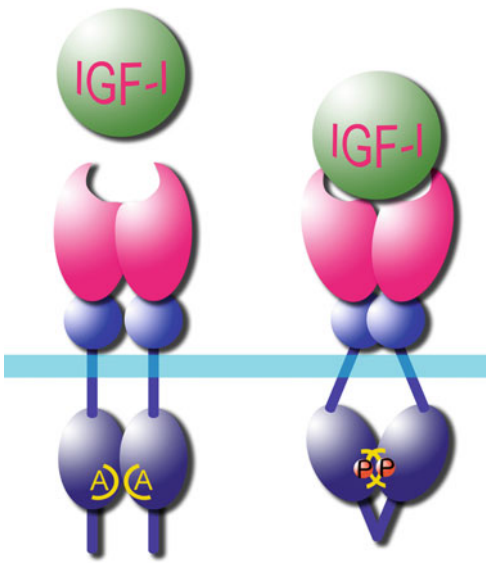
IGF1R Signaling Complex

Ligand binding initiates formation of a protein complex consisting of kinases and adaptor proteins around the IGF1R. An important component of this complex is Insulin Receptor Substrate (IRS), a scaffolding protein containing N-terminal Pleckstrin Homology (PH) domain, the Src Homology 2 (SH2) domain, and several tyrosine and serine residues that can be substrates of protein kinases. Isoforms 1–4 of IRS have been implicated in IGF1R signaling, with IRS-1 and -2 being the most widely distributed. The SH2 domain of IRS-1 binds to tyrosine phosphorylated Asn-Pro-X-Tyr motifs in the juxtamembrane region of the IGF1R. Another signal positioning IRS near the activated IGF1R is the interaction between PH domain of IRS and 3'-phosphorylated phosphoinositides formed in the vicinity of the activated IGF1R as described below.

Phosphotyrosines of IRS provide docking sites for several SH2 domain-containing proteins, associating IGF1R activation with downstream signaling pathways. One of these pathways is initiated by binding of the regulatory p85 subunit of ▶ Phosphatidylinositol 3 Kinase (▶ PI3K) to IRS (Fig. 3). This releases the catalytic subunit of PI3K p110 from p85-mediated inhibition. PI3K converts phosphatidylinositol lipids to 3-phosphorylated forms, most importantly phosphatidylinositol 3,4,5 triphosphate (PIP3). PIP3 provides docking sites for IRS and promotes activation of other PH domain-containing molecules, including serine/threonine kinases v-akt murine thymoma viral oncogene homolog (Akt), 3-Phosphoinositide-Dependent Protein Kinase (Pdk) 1 and 2, and Protein Kinase C ζ (PKCζ). Pdk is an upstream kinase of several signaling molecules, including Akt and PKCζ. Pdk phosphorylates Akt on Thr^{308}, and full activation of Akt requires phosphorylation on Ser^{473},



Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 1 *Subunit structure of the IGF1R*. α α-chain, β β-chain, ss disulfide bonds, FnIII fibronectin type III homology domain, TM transmembrane domain, JM juxtamembrane domain, Kinase kinase domain, C C-terminal tail



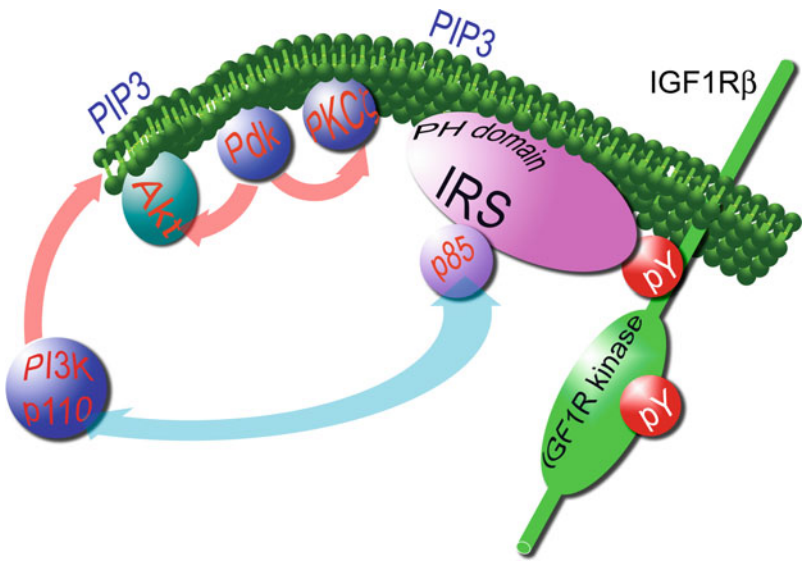
Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 2 *IGF1R activation*. Without ligand, the activation (A) loop in the kinase domain of the receptor has conformation that is unfavorable for transphosphorylation. This may be a result of constraints imposed by the extracellular portion of the β-chain. Binding of IGF-I to the α-chains causes conformational changes relieving these constraints and facilitating kinase domain transphosphorylation (P)

which is achieved by autophosphorylation or mediated by other kinases.

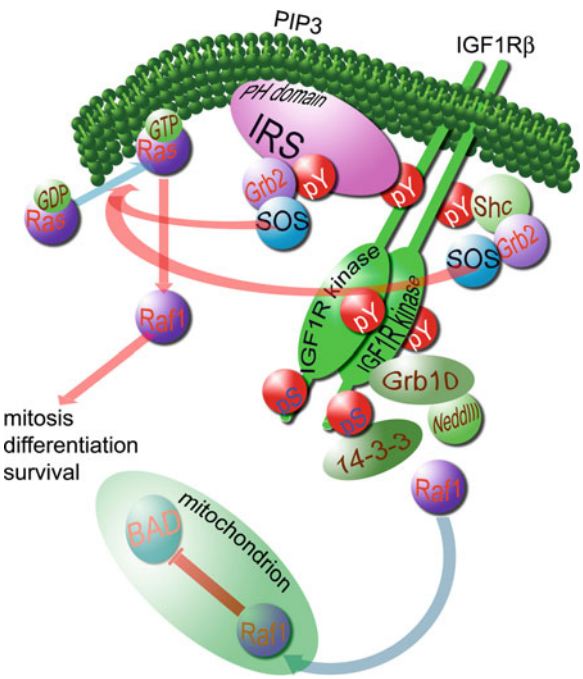
Another adaptor protein, Shc, is recruited to the tyrosine-phosphorylated signaling domain of the IGF1R with a slower binding kinetics than IRS-1. Tyrosine phosphorylation of Shc induced by binding to the receptor facilitates its interaction with the adaptor protein growth factor receptor-binding protein 2 (Grb2). Grb2 may also bind to phosphorylated IRS-1 in some cell types (Fig. 4). The SH2 domain-containing protein tyrosine phosphatase Shp2, another binding partner of IRS, is required for optimal association of the IGF1R with downstream targets and its deficiency is associated with reduced activation of MAPK, although Shp2 inactivates IRS-1 by dephosphorylation. Stoichiometry of the protein complex formed around the activated IGF1R may vary depending on the cell type.

To various degrees, all four groups of conventional MAPK are involved in signal transmission from the activated IGF1R. These include Erk1/2, c-jun NH_2-terminal protein kinase (JNK) 1/2/3, p38α/β/γ/δ, and Erk5. MAPK are activated by dual specificity MAPK kinases (MKK or MAP2K) phosphorylating Thr and Tyr residues within a conserved Thr-X-Tyr motif in the MAPK activation loop. Upstream of MAP2K are their activators MAP2K kinases (MKKK or MAP3K) (Cargnello et al. 2012). Signal wiring within the MAPK cascade is dependent on substrate specificity of second and third tire MAPK and participation of scaffolding proteins. Increased signaling activity of MAPK is associated with their nuclear translocation.

Erk1 and Erk2, the first MAPK to be cloned and characterized in early 1990, are most frequently associated with mitogenic effects of the activated IGF1R and other receptor tyrosine kinases in different cell lineages (Cargnello et al. 2012). The Erk1/2 module of the MAPK cascade includes the MAP2K MEK1 and MEK2, as well as the MAP3K ▶ Raf1. The serine/threonine kinase Raf1 is activated by the small guanosine triphosphate (GTP)-binding protein Ras. Ras is inactive when bound to guanosine diphosphate (GDP) and becomes activated when associated GDP is replaced by GTP. This exchange is promoted by the guanine nucleotide exchange factor (GEF) son of sevenless, which is activated by recruitment to the IGF1R signaling complex via interaction with Grb2



Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 3 *PI3K activation*. IRS binds to the juxtamembrane portion of the activated IGF1R and activates PI3K, which generates PIP3. Akt and PKCζ, as well as their kinase Pdk, bind to PIP3 and become activated. Only β-chain of the IGF1R is shown (IGF1Rβ). Here and in other figures bars show inhibitory interactions and arrows show activating interactions or transition between different states



Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 4 *MAPK and ubiquitous adaptor proteins in IGF1R signaling*. Shc and IRS bind to specific phosphotyrosine-containing motifs (pY) in the activated IGF1R. Grb2 is recruited to the IGF1R signaling complex by binding to Shc or IRS. The GEF son of sevenless (SOS) is activated by binding to Grb2 and induces exchange of Ras-bound GDP to GTP, thereby activating Ras, which activates Raf1. In addition, phosphorylated serines (pS) at the C-terminus of the receptor may bind 14-3-3. This and Grb10-mediated recruitment of NeddIII is required for mitochondrial translocation of Raf1

(Fig. 4) and a mechanism that requires Shp2. In addition, IGF1R-agonist-induced Raf1 activation may occur independently of IGF1R autocatalytic activity, and Raf1-independent Erk1/2 activation in response to IGF1R ligation has been shown.

The stress MAPK p38 and JNK, which are usually associated with apoptosis and cell differentiation, as well as Erk5, are also involved in IGF1R signaling, but less frequently than Erk1/2. IGF1R-induced JNK activation may be dependent on receptor-interacting protein, a death domain-containing serine/threonine kinase with a well-documented function of linking JNK activation to the Tumor Necrosis Factor Receptor (TNFR) type 1.

Ubiquitously Expressed Adaptors

The activation status of the IGF1R signaling cascade is a subject to modulation by several ubiquitously expressed scaffolding or adaptor proteins. IRS family proteins belong to the group classified by some authors as docking proteins (Brummer et al. 2010) on the basis of having a N-terminal PH domain and SH2 domain, as well as multiple tyrosine residues. In addition to IRS, docking proteins participating in IGF1R signaling include members of two families, Grb-associated binder (Gab) and downstream of tyrosine kinases (Dok). ▶ Gab1, one of the best studied docking proteins of Gab family, binds to several components of the IGF1R signaling complex, including Shp2, p85, and Grb2, and its deficiency is associated with reduced proliferation in response to IGF-I.

Another adaptor, Grb10, binds to a diverse range of partners through SH2 and PH domains, including the IGF1R, Raf1, MEK, Akt, the regulatory subunit of PI3K and Gab1, and has the capacity to positively and negatively regulate IGF1R signaling. Grb10 may promote Ras-independent activation of Raf1 in response to IGF1R activation. Grb10 also provides a link between the IGF1R and Nedd4, an E3 ubiquitin ligase, which is required for optimal IGF1R signaling (Fig. 4).

Among molecules interacting with Grb10 is the ubiquitously expressed adaptor 14-3-3 which has affinity to phosphorylated serine and threonine residues. Binding partners of 14-3-3 isoforms are found in different intracellular signaling pathways. Regulatory effects of 14-3-3 isoforms are mediated by restricting subcellular localization of client proteins and shielding them from interaction with other molecules (Darling et al. 2005). Several signaling molecules in IGF1R- and inflammation-activated pathways, including IGF1R, IRS-1, PI3K, ASK1-interacting protein 1 (AIP1), as well as the third tire MAPK Raf, apoptosis signal-regulating kinase 1 (ASK1), and MEKK3, may generate binding sites for this adaptor by phosphorylation of serine and threonine residues. Therefore, serine/threonine phosphorylation of a signaling molecule in a given pathway by a kinase activated in another signaling pathway may be a 14-3-3-dependent mechanism of cross-talk between these pathways.

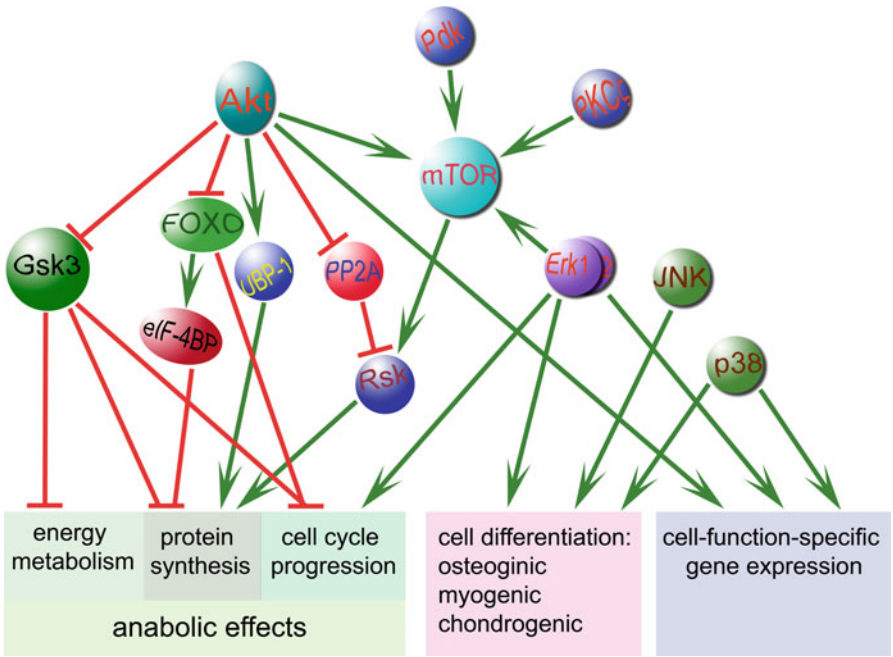
Grb10 and 14-3-3 may be involved in one of the IGF1R-dependent anti-apoptotic pathways. Association of 14-3-3 with IGF1R C-terminal serines, phosphorylated during receptor activation by a mechanism that might involve autophosphorylation, may induce translocation of the MAP3K Raf1 to the mitochondrion where it inhibits the pro-apoptotic ▶ Bcl-2 Family protein BAD. The effect of Raf1 is independent of Erk1/2 activation and may require interaction of Raf1 with Grb10 and Nedd4 (Fig. 4). This may provide cytoprotection against mitochondrial cell death, which is often induced by pro-inflammatory conditions in resident cells.

Cellular Responses to IGF1R Activation and Intracellular Signaling Pathways Involved

IGF1R activation has anabolic effect in many different cell lineages. In the absence of other growth factors, IGF1R-dependent signaling alone is sufficient for maintenance of cellular energy metabolism and protein synthesis. Akt is an important mediator of anabolic response to IGF1R activation because this serine/threonine kinase modulates activation status of several effector kinases and transcription factors that suppress or promote cell growth (Manning and Cantley 2007) (Fig. 5). One of the Akt targets is the serine/threonine kinase Glycogen Synthase Kinase (Gsk)-3, an inhibitor of cell metabolism, growth, and differentiation. Another target is forkhead transcription factors (FOXO), a subclass of transcription factors inhibiting cell growth and survival. Their effects include activation of Eukaryotic Initiation Factor 4E binding protein 1, a global translational repressor, as well as activation of pro-apoptotic pathways and inhibition of cell cycle progression (Hedrick 2009). FOXO protein nuclear translocation is regulated by multiple types of post-translational modifications, with Akt-dependent phosphorylation resulting in their nuclear exclusion and transcriptional inactivation.

Another IGF1R-dependent effector is mammalian target of rapamycin (▶ mTOR), which positively regulates protein translation by activating p70 Ribosomal S6 Kinase (Rsk), a kinase activating the 40S ribosomal protein. Akt and other kinases in the IGF1R signaling cascade, including Pdk-1, PKCζ, and Erk1/2, can activate mTOR and Rsk, thereby promoting protein synthesis (Kuemmerle 2003). Finally, IGF1R signaling activates upstream binding factor 1, which regulates cell size by transcriptionally activating RNA polymerase I, a limiting factor in ribosome biosynthesis (Fig. 5).

By promoting cell growth IGF1R signaling indirectly promotes cell proliferation. This pathway also has a direct mitogenic effect on selected lineages, for example, myoblasts, by modulating activity of proteins regulating cell cycle progression, such as Cyclins, their kinases and c-▶ Myc, effects that are often dependent on Erk1/2. Furthermore, Akt and the aforementioned 14-3-3/Raf1-dependent mechanism mediate anti-apoptotic effects of activated IGF1R by targeting BAD. In addition, IGF-I and -II are differentiation factors for several cell lineages, including osteoblasts, chondroblasts, and myoblasts, because in these cells they induce expression of lineage-specific genes. Finally, IGF1R signaling may lead to cell activation and increased functional competence in several cell types, for example, steroidogenesis in ovarian cells or expression of cytokines in various immune and non-immune cells (Fig. 5).



Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 5 *Downstream effectors of cellular responses to IGF1R activation*. GSK3 and FOXO transcription factors, negative regulators of cell growth, are inhibited by Akt-mediated phosphorylation. mTOR and its effector Rsk, are maintained in the activated state by phosphorylation on multiple serine and threonine residues mediated by Akt, Pdk, PKCζ and Erk1/2. Akt prevents inhibitory dephosphorylation of Rsk mediated by protein phosphatase 2A (PP2A). eIF4E-BP – eukaryotic initiation factor 4E binding protein

Cross-Regulation Between IGF1R and Inflammation-Activated Intracellular Signaling Pathways

ASK1

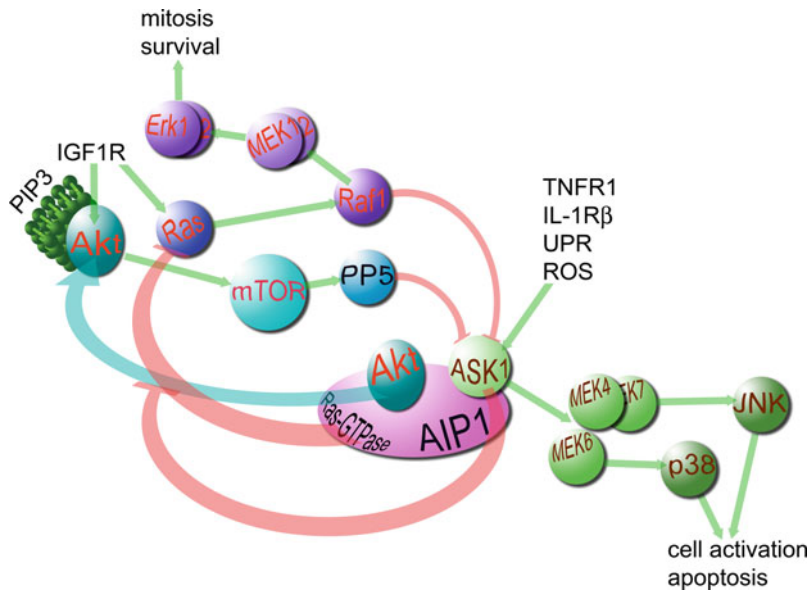
While Erk1/2 associates IGF1R activation with cell growth and differentiation, cytotoxic effects of inflammation are often mediated by the stress kinases JNK and p38 (Fig. 6). Preferential use of different MAPK modules by IGF1R and inflammation-associated pathways, however, is not absolute. Inflammation-activated MAP3K of the JNK and p38 MAPK modules include ASK1, MEKK2, MEKK3, and Mixed Lineage Kinase (MLK). Activation of ASK1 and its downstream effector MAPK is an evolutionarily conserved pathway mediating cellular pathology in inflammatory and other diseases (Takeda et al. 2008). Inflammation-related pathways of ASK1 activation include the pro-inflammatory cytokines TNFα and IL-1β, ROS, and cellular response to ER stress.

Perhaps unsurprisingly, cytoprotective effects of the IGF1R pathway are in part mediated by inhibition of ASK1 (Fig. 6). ASK1 may be inhibited by recruitment to the IGF1R signaling complex via a mechanism that requires Raf1. In addition, two modifications of ASK1 molecule that lead to its inactivation can be induced by the IGF1R signaling pathway. They are dephosphorylation of Thr^{838} by protein phosphatase 5 (Fig. 6), which is activated by mTOR, and Akt-induced phosphorylation on Ser^{83}.

On the other hand, ASK1 may inhibit IGF1R signaling. This may involve AIP1. Under inflammatory conditions, AIP1 is activated in response to cytokines and ER stress. AIP1 has the ability to inhibit Ras because it has Ras-GTPase activity. It also contains domains that bind ASK1 and Akt, bringing them together. This sequesters Akt in cytosol, preventing its translocation to the plasma membrane and interaction with PIP3. The Akt-inhibiting function of AIP1 is increased by phosphorylation at Ser^{604} induced by ASK1 (Xie et al. 2009). Therefore, AIP1 inhibits both Erk1/2 activation and the PI3K/Akt pathway. These properties implicate AIP1 in the inhibition of IGF1R signaling during inflammation (Fig. 6).

Stress of the ER

Proteins are synthesized and folded in ER. Overloading of ER with nascent protein molecules or decrease in its functional capacity may lead to an adaptive cellular response, known as unfolded protein response (UPR), which attempts to restore ER functions. The major adaptive effect of UPR is inhibition of translation, resulting from inactivation of eukaryotic initiation factor 2 by double-stranded RNA-activated protein kinase (► PKR) and PKR-like ER kinase.



Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 6 *ASK1 in cross-regulation of IGF1R and inflammation-activated signaling pathways*. ASK1 may be inhibited by IGF1R signaling via Akt- and MAPK-dependent pathways. ASK1 interaction with AIP1 negatively regulates IGF1R signaling because docking to AIP1 and phosphorylation by ASK1 sequesters Akt in the cytosol preventing its translocation to PIP3 in the plasma membrane. Furthermore, because of its Ras-GTPase activity AIP1 inhibits Ras

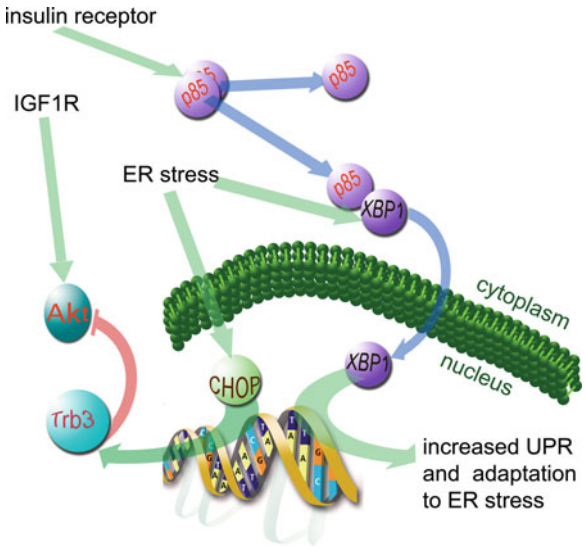
These kinases are activated together with the other sensors of ER stress, which include activating transcription factor 6 and inositol-requiring enzyme-1 (Ire1). Activated Ire1α subunit removes an internal fragment from X box-binding protein 1 (XBP1) transcript, generating thereby a stable transcriptionally active splice variant of XBP1 (Zhang and Kaufman 2008). UPR leads to the induction of gene expression programs that restore protein folding capacity of ER. Therefore, adaptive role of UPR is mediated by relieving ER overload, increasing its protein folding capacity and sparing cellular resources.

UPR is a common feature of cellular pathology in inflammation. Moreover, since IGF1R activation has a potent anabolic effect, it leads to increased loading of ER with nascent proteins, also predisposing cells to ER stress. This, however, does not compromise IGF1R-dependent cytoprotection because IGF1R signaling can promote cell adaptation to ER stress, regardless of its causes, by inducing expression of chaperones, such as Grp78 (Pfaffenbach and Lee 2012), and via several other mechanisms.

Failure to resolve ER stress by UPR results in the activation of cell death pathways. UPR-specific transcription factor, core/emopamil binding protein homologous protein, activates several genes in pro-apoptotic pathways. One of its targets is the intracellular pseudokinase ▶ Tribbles-related protein 3 (Trb3), which is expressed in response to ER stress in different cell types, including neuronal cells, chondrocytes, skeletal muscles, and cardiac myocytes. Trb3 inhibits the PI3K/Akt pathway by dephosphorylation of Akt, but its effect may be counteracted by IGF1R activation (Fig. 7). IGF1R role in UPR enhancement may also be mediated by increased nuclear translocation of XBP-1 induced by monomeric p85 subunits of PI3K, which are released from dimers when PI3K becomes activated (Fig. 7). Furthermore, UPR enhancement by IGF1R activation has been shown that is independent of any known arm of the IGF1R signaling cascade (Novosyadlyy et al. 2008). Therefore, increased adaptation to ER stress may play a role in cytoprotective action of IGF1R in inflammation.

NF-κB Signaling Pathway

NF-κB family of transcription factors transduce signals from several receptors activated by pro-inflammatory factors, including the receptors of TNFα and IL-1β, as well as TLR, receptors recognizing pathogen molecular patterns (Wajant and Scheurich 2012). The five members of this family of transcription factors form homo- and heterodimers maintained in transcriptionally inactive state by interaction with an inhibitor of κB (IκB). NF-κB dimers are activated when IκB is inhibited by phosphorylation mediated by IκB kinase complex (IKK). The sequence of events leading to nuclear translocation of NF-κB dimers after they have been released from IκB-mediated inhibition is known as the classical pathway of NF-κB activation. This pathway is characterized by



Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 7 *IGF1R signaling and UPR*. PI3K activation results in dissociation of p85 dimers, and monomeric p85 enhances nuclear translocation of XBP1, promoting adaptation to ER stress. At the same time, ER stress may inhibit Akt by induction of Trb3 expression. This may be counteracted by IGF1R activation. CHOP core/emopamil binding protein homologous protein

predominant formation of heterodimers consisting of p50 and p65 proteins of NF-κB family (Wajant and Scheurich 2012). Activation of the classical NF-κB pathway in resident nonimmune cells at sites of inflammation may induce production of pro-inflammatory cytokines and improve cell survival, which in many instances results from inhibition of JNK-induced apoptosis.

NF-κB does not appear to be a major signaling pathway mediating cellular responses to IGF1R activation, but nuclear translocation of p65 and NF-κB-mediated transcriptional control have been implicated in a diverse range of biological effects elicited by IGF1R signaling, including stimulation of endothelial cell migration, chondroblast proliferation, lung fibroblast differentiation, cytoprotection of keratinocytes from UV, neuroprotection, and MHC I promoter activation. IGF1R-induced NF-κB activation is dependent on MAPK and Akt, but, unlike NF-κB activation in response to pro-inflammatory factors, may not require IκB phosphorylation.

NF-κB signaling activated by pro-inflammatory factors may be inhibited or modified by co-activation of the IGF1R, as shown in untransformed cells of different lineages, including keratinocytes, astrocytes, and vascular smooth muscle cells. Co-activation of the IGF1R may change the composition of NF-κB dimers formed in response to pro-inflammatory factors. This may inhibit nuclear translocation of NF-κB or change the array of genes activated by this pro-inflammatory pathway. These effects of IGF1R signaling might be in part mediated by phosphorylation of NF-κB subunits.

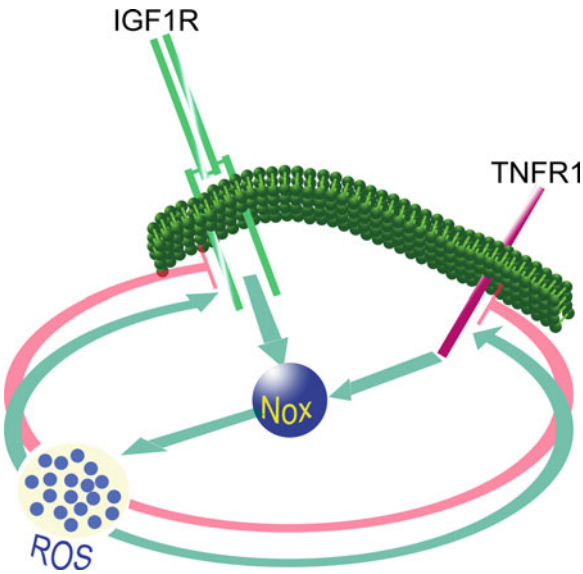
Oxidative Stress

ROS are generated during oxygen metabolism, predominantly in mitochondria by electron transfer from nicotine amid dinucleotide phosphate (NADPH) to O_2 by NADPH oxidases (Nox). Pro-inflammatory cytokines, as well as insulin and IGF-I, have the capacity to increase cellular oxidative potential by inducing increased Nox activity (Meng et al. 2008). Nox are regulated by changes in the phosphorylation status of their regulatory subunits. The association of IGF1R activation with increased ROS production may also be mediated by Rac1, a member of Rho Family GTPases (Meng et al. 2008).

ROS may modify intracellular signal transduction pathways of several receptors. Enhanced signaling of the IGF1R, as well as receptors of several pro-inflammatory cytokines and insulin, may result from inhibition of protein phosphatases by reversible oxidation of cysteine residues in the signature motif (Vardatsikos et al. 2009). Whether oxidants inhibit or activate the IGF1R signaling cascade may be dose- and cell-type-dependent, because ROS transactivated IGF1R in vascular smooth muscle cells, increasing IGF1R-depednent activation of Erk1/2 and Akt, but in microglia and neurons IGF1R-dependent Akt phosphorylation was inhibited by ROS. Therefore, ROS may be involved in cross-regulation of IGF1R and inflammation-activated signaling pathways (Fig. 8).

IRS as a Target of Inflammation-Activated Pathways

IRS-1 is a substrate of several serine/threonine kinases found in the signaling pathways activated by the IGF1R, as well as those activated during inflammation, such as Erk1/2, JNK, IKK, mTOR, Rsk, PKR, Protein Kinase C δ, and PKCζ. Serine phosphorylation of IRS inhibits its function as a signaling molecule in the IGF1R complex by inducing dephosphorylation of IRS tyrosines, which results in IRS dissociation from the IGF1R and plasma membrane. Targeting IRS by serine kinases activated in response to IGF-I may



Insulin-like Growth Factor Receptor Type I (IGF1R) Signaling and Inflammation, Fig. 8 *ROS in cross-talk between IGF1R and inflammation*. Agonist-dependent activation of the IGF1R and TNFR1 leads to increased activity of Nox and ROS production. ROS may positively or negatively regulate activation status of the receptors

mediate negative feedback, whereas IRS inhibition by inflammation-activated kinases may reduce cellular sensitivity to cytoprotective effects of IGF-I under inflammatory conditions. IRS inhibition, together with some other mechanisms, implicates low-level chronic inflammation in the development of systemic insulin resistance (Hotamisligil 2010). Dephosphorylation of tyrosines by protein phosphatases is also inhibitory for IRS-1.

Few serine phosphorylation sites are found in IRS-2, making this isoform less amenable to regulation by differential phosphorylation. IRS-2, however, may be transcriptionally inhibited during ER stress.

The IGF1R Signaling Pathway and Inflammatory Diseases

Inflammatory mechanisms are involved in tissue degeneration in many diseases. These include a group of chronic tissue-specific autoimmune conditions, which are characterized by inflammation-dependent tissue loss followed by functional deficit, with examples including demyelination in multiple sclerosis, cartilage destruction in rheumatoid arthritis, and inflammation-mediated degeneration of pancreatic β-cells in type I diabetes. Both IGF1R-mediated cytoprotection and inhibition of IGF1R signaling by pro-inflammatory factors have been shown in these conditions (Glass 2005; Ye et al. 2007). It remains to be elucidated to what extent cytoprotective mechanisms mediated by endogenous IGF-I and -II modify the course of these inflammatory diseases.

Pro-inflammatory factors, including cytokines, ER stress, and ROS, contribute to the pathogenesis of type 2 diabetes and the metabolic syndrome by suppression of intracellular signal transmission from insulin receptor and IGF1R. Interplay between IGF1R signaling and inflammation may also play an important and complex part in pathogenesis of atherosclerosis. IGF1R signaling is required for maintenance of vascular smooth muscle cells in differentiated status, which is an atherosclerosis-inhibiting effect, but dedifferentiated vascular smooth muscle cells proliferate and migrate in response to IGF1R activation, thereby promoting atherosclerotic lesion formation. Furthermore, tissue hyperplasia in several inflammatory conditions, such as Crohn's disease and allergic airway inflammation, may be dependent on IGF1R-mediated signaling.

Testing experimentally all possible interactions mediating cross-regulation of the IGF1R signaling cascade by inflammation-activated signaling and vice versa may be inefficient because of the complexity of these intracellular signaling networks. Computational modeling is, therefore, an important approach for understanding network dynamics and underlying mechanisms. In one of the pioneering studies in this area, a discrete modeling approach in combination with experimental data analysis has been applied in order to analyze the involvement of PKR in cross-regulation of the insulin receptor signaling network (Wu et al. 2009).

Summary

Cells of different lineages express the IGF1R beginning from early developmental stages into adulthood. This receptor mediates effects of the growth factors IGF-I and -II. Cellular responses to IGF1R activation include increased cell growth, inhibition of apoptosis, cell division, differentiation and acquisition of new phenotypic features required for functional competence. Depending on the cell type these responses are activated in various combinations. Effects of IGF1R ligation are mostly mediated by activation of MAPK family proteins and PI3 kinase. Phosphatidylinositol 3,4,5 triphosphate generated by PI3K from plasma membrane lipids activates Akt and several other

pleckstrin homology domain containing proteins, which regulate cell growth and survival by targeting several effectors, including GSK3, FOXO transcription factors, and mTOR. As IGF1R is so widely distributed, it is important to understand how this cytoprotective signaling cascade interacts with signaling pathways activated by pathological processes leading to tissue degeneration, in particular, inflammation-activated pathways. IGF1R activation may counteract cytotoxic effects of pro-inflammatory factors by inhibiting some of the components in their signaling pathways, including the stress MAPK JNK and p38, NF-κB transcription factors, pro-apoptotic Bcl-2 family members, and mediators of endoplasmic reticulum stress. On the other hand, inflammation-activated signaling pathways may inhibit IGF1R signaling, for example, by targeting IRS and Akt. Reactive oxygen species, which are generated during cell activation by IGF1R ligands and pro-inflammatory factors, may also mediate cross-regulation between the IGF1R and inflammation-activated signaling pathways. This cross-talk, rather than an isolated response to pro-inflammatory factors, may drive a number of pathological processes, including tissue-specific autoimmune diseases, atherosclerosis, and the metabolic syndrome.

References

Adams TE, Epa VC, Garrett TP, Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci. 2000;57(7):1050–93.

Brummer T, Schmitz-Peiffer C, Daly RJ. Docking proteins. FEBS J. 2010;277(21):4356–69.

Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. 2012;75(1):50–83.

Darling DL, Yingling J, Wynshaw-Boris A. Role of 14-3-3 proteins in eukaryotic signaling and development. Curr Top Dev Biol. 2005;68:281–315.

Glass DJ. Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol. 2005;37(10):1974–84.

Hedrick SM. The cunning little vixen: Foxo and the cycle of life and death. Nat Immunol. 2009;10(10):1057–63.

Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell. 2010;140(6):900–17.

Kuemmerle JF. IGF-I elicits growth of human intestinal smooth muscle cells by activation of PI3K, PDK-1, and p70S6 kinase. Am J Physiol Gastrointest Liver Physiol. 2003;284(3):G411–22.

Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129(7):1261–74.

Meng D, Lv DD, Fang J. Insulin-like growth factor-I induces reactive oxygen species production and cell migration through Nox4 and Rac1 in vascular smooth muscle cells. Cardiovasc Res. 2008;80(2):299–308.

Novosyadlyy R, Kurshan N, Lann D, Vijayakumar A, Yakar S, LeRoith D. Insulin-like growth factor-I protects cells from ER stress-induced apoptosis via enhancement of the adaptive capacity of endoplasmic reticulum. Cell Death Differ. 2008;15(8):1304–17.

O'Connor JC, McCusker RH, Strle K, Johnson RW, Dantzer R, Kelley KW. Regulation of IGF-I function by proinflammatory cytokines: at the interface of immunology and endocrinology. Cell Immunol. 2008;252(1–2):91–110.

Pfaffenbach KT, Lee AS. The critical role of GRP78 in physiologic and pathologic stress. Curr Opin Cell Biol. 2012;23(2):150–6.

Takeda K, Noguchi T, Naguro I, Ichijo H. Apoptosis signal-regulating kinase 1 in stress and immune response. Annu Rev Pharmacol Toxicol. 2008;48:199–225.

Vardatsikos G, Sahu A, Srivastava AK. The insulin-like growth factor family: molecular mechanisms, redox regulation, and clinical implications. Antioxid Redox Signal. 2009;11(5):1165–90.

Wajant H, Scheurich P. TNFR1-induced activation of the classical NF-kappaB pathway. FEBS J. 2012;278(6):862–76.

Wu M, Yang X, Chan C. A dynamic analysis of IRS-PKR signaling in liver cells: a discrete modeling approach. PLoS One. 2009;4(12):e8040.

Xie D, Gore C, Zhou J, Pong RC, Zhang H, Yu L, et al. DAB2IP coordinates both PI3K-Akt and ASK1 pathways for cell survival and apoptosis. Proc Natl Acad Sci U S A. 2009;106(47):19878–83.

Ye P, Kollias G, D'Ercole AJ. Insulin-like growth factor-I ameliorates demyelination induced by tumor necrosis factor-alpha in transgenic mice. J Neurosci Res. 2007;85(4):712–22.

Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. Nature. 2008;454(7203):455–62.

int-2

► FGF (Fibroblast Growth Factor)

Integrin Alpha 11

Cédric Zeltz and Donald Gullberg
Department of Biomedicine, University of Bergen, Bergen, Norway

Historical Background

Integrin α11 is the last member of the integrin family to be discovered. This integrin subunit was initially named α_{mt}, since it was first identified on cultured

human fetal myotubes (Gullberg et al. 1995). In 1995, Velling et al. identified the β1-associated α11 as a collagen-binding integrin. Two years later, Tiger et al. (2001) described the α11β1 integrin as a collagen receptor involved in cell migration and collagen reorganization. The generation of α11 integrin $^{-/-}$ mice was a major advance in determination of α11 integrin functions (Popova et al. 2007).

Gene and Protein Structure of the α11 Integrin

The human α11 integrin gene (*ITGA11*) is localized on chromosome 15q23 and consists of 130 kb, whereas the mouse α11 integrin gene (*Itga11*; length of 106 kb), has been mapped to chromosome 9. Both the human and mouse genes contain 30 exons and 29 introns.

ITGA11 encodes a mature protein of 1,166 amino acids. The extracellular domain contains seven FG-GAP (Phe-Gly – Gly-Ala-Pro) repeats and a 195-amino acid-long I domain inserted between the repeats 2 and 3. The I domain presents a metal ion-dependent adhesion site (MIDAS) motif and three potential divalent cation-binding motifs. The short cytoplasmic tail of 24 amino acids contains the motif Gly-Phe-Phe-Arg-Ser (GFFRS) instead of the conserved Gly-Phe-Phe-Lys-Arg (GFFKR) sequence described in most of α subunits. A 23-amino acid-long transmembrane domain links the extracellular and cytoplasmic domains (Velling et al. 1999).

The mouse α11 integrin shows an 89% identity with human α11 at the protein level, 97% in the I domain (Popova et al. 2004).

Expression and Regulation of the α11 Integrin

Distribution

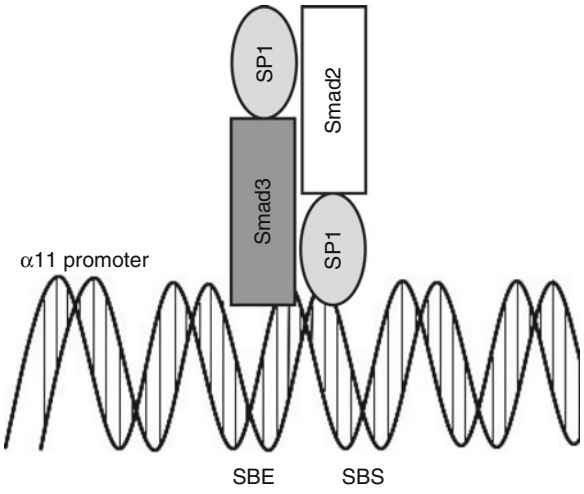
The expression of integrin α11 was first described in cultured human fetal muscle cells in vitro (Gullberg et al. 1995). In human adult tissue, α11 mRNA was expressed in high levels in uterus and heart and in intermediate levels in skeletal muscle (Velling et al. 1999). However, in human and mouse embryos, no expression of α11 was detected in muscle cells (Tiger et al. 2001; Popova et al. 2004). Later it was shown that in muscle tissue α11 is expressed in fibroblasts. α11 is present in fibroblasts around ribs, vertebrae, in intervertebral disks, and in keratocytes of the cornea of 8-week human embryos. In the mouse embryo, α11 is prominent in the ectomesenchyme in the head including the periodontal ligament, in tendons and intestinal villi fibroblasts. The α11 chain expression appears to be specific to mesenchymal non-muscle cells in vitro and in vivo. α11 expression has also been reported in tumor tissue from melanoma and lung carcinoma (Vuoristo et al. 2007; Zhu et al. 2007). The high levels of α11 integrin expression in lung carcinoma in situ are derived from the cancer-associated fibroblasts and is thus in the lung not contributed by the cancer cells. Recent RNA data indicate that α11 might be involved in epithelial mesenchymal transition (Ke et al. 2008).

Regulation

The α11 integrin proximal promoter, which is important for basal transcription, contains some regulatory elements. The presence of two Sp1 sites and an Ets-1 site is necessary to the control of the α11 integrin gene expression (Lu et al. 2006). Cytokines are able to regulate the α11 integrin expression by inducing signaling molecules, which regulate transcription factor binding to these responsive elements.

Thus, TGF-β1 was shown to up-regulate the α11 expression in HT1080 fibrosarcoma cell line as well as in primary fibroblasts and MRC-5 human myofibroblasts (Honda et al. 2010; Lu et al. 2010). The responsiveness to TGF-β1 is dependent on Smad2/3 and Sp1-regulated transcription. The Smad-binding element SBE2 and the Sp1-binding site SBS1 are located in close region on the proximal promoter (nt –182/–176 and –140/–134, respectively). This proximity could promote a possible interaction between the Smad and Sp1 proteins (Fig. 1). Activin A, which belongs to the TGF-β family, is involved in the up-regulation of α11 in mouse embryonic fibroblasts (MEFs), in a mechanosensitive manner (Carracedo et al. 2010). This induction of α11 expression requires the Smad3 protein. It is also not excluded that collagen could activate α11 expression via the induction of the Smad signaling pathway, independently of the TGF-β ligand (Garamszegi et al. 2010). Since this crosstalk signaling requires β1-integrins, α11β1, like α2β1, might also exert a positive feedback on its expression using this pathway.

Type I interferons, including IFN-α and IFN-β, have also been described to regulate the α11



Integrin Alpha 11, Fig. 1 The schematic representation shows the Smad-binding element (*SBE*) and the Sp1-binding site (*SBS*) of the *ITGA11* promoter. Sp1 is predicted to form a dimer with Smad2. Direct binding of Smad3 to SBE is predicted to occur. This interaction might also cooperate with Sp1 binding to SBS

expression. IFNs are able to stimulate α11 mRNA and protein expression in glioblastoma-derived cell line T98G (Leomil Coelho et al. 2006).

Up to now, down-regulation of α11 was only reported in mesenchymal stem cells treated with ▶ FGF-2 (Varas et al. 2007). However, the responsive elements involved in the down-regulation have not yet been determined in the α11 promoter.

Integrin α11 Functions

In Vitro Functions

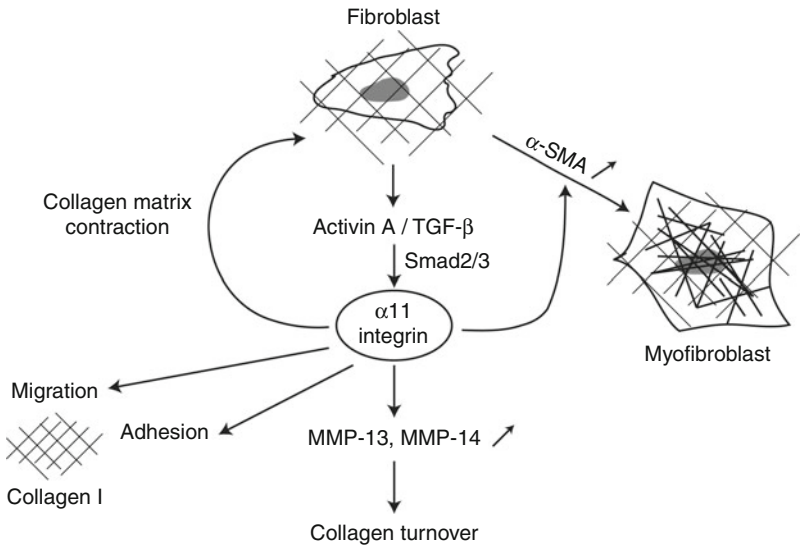
The α11 integrin chain is exclusively associated with the β1 subunit at the cell surface, to form the α11β1 integrin. Tiger et al. (2001) have demonstrated that α11β1 promoted cell attachment to collagen I. Integrin α11β1 displays certain collagen specificity, since it binds preferentially type I collagen, whereas it interacts with collagen IV with a low affinity. The α11 I domain recognizes the triple-helical GFOGER (where single letter amino acid is used, O = hydroxyproline) sequence present in collagen I as well as the GLOGER motif (Zhang et al. 2003; Siljander et al. 2004). Thus, α11β1-mediated cell responses could differ depending on the GxxGER collagen motif that interacts with this integrin. Another study has identified the GLPGER motif of the recombinant Scl1 protein, a prokaryotic collagen, as an α11β1 binding sequence (Caswell et al. 2008). The interaction between the cell surface streptococcal Scl1 and the human α11β1 integrin might increase host colonization by pathogenic bacteria, but the details of this process remains to be determined.

The role of α11β1 in platelet-derived growth factor (PDGF)-stimulated cell migration on collagen I coating seems to be cell type dependent. The C2C12 mouse satellite cells, stably transfected with human α11 integrin cDNA, showed a stronger chemotactic response to PDGF-BB, compared to C2C12 wild-type cells, which lack endogenous collagen receptors (Tiger et al. 2001). In contrast, MEFs depleted in α11β1 migrated more on collagen I in comparison to wild-type embryonic fibroblasts (Popova et al. 2004). However, in this last case, a compensatory mechanism, involving other collagen receptors, cannot be excluded.

α11β1 also showed an ability to contract collagen lattices, an important function which contributes to the reorganization of collagen matrices (Tiger et al. 2001).

Under certain conditions, fibroblasts can be activated and differentiated into so-called myofibroblasts. Myofibroblasts are characterized by α-smooth muscle actin (α-SMA) incorporated into stress fibers. Corneal fibroblasts, under action of TGF-β, overexpress α-SMA. Since siRNA directed against the α11 integrin completely abrogated α-SMA up-regulation, it was shown that α11β1 also plays a role in myofibroblast differentiation (Carracedo et al. 2010). The regulation of myofibroblast differentiation by α11β1 could be relevant in pathological processes such as tumor–stroma interactions and fibrosis, where myofibroblasts are involved.

Integrin turnover is an essential process involved in cell adhesion and migration. Generally, integrins present on the cell surface are either released and used in new adhesion sites or internalized by endocytosis. Rab proteins, including Rab21, regulate the traffic of endocytotic vesicles via interaction with the cytoplasmic tail of α integrin subunit, as shown for ▶ integrin α2β1 (Pellinen et al. 2006). The C-terminal part of Rab21 was also able to bind to the cytoplasmic domain of α11 integrin, thus suggesting that α11β1 could be regulated by endocytosis. Since Rab21 activity has been shown to regulate the motility of breast and prostate cancer cells, it could be interesting to examine if an association between this small GTPase and α11β1 occurs in cancer-associated fibroblasts and if it might have an impact on the tumor progression.



Integrin Alpha 11, Fig. 2 α11 is induced in a mechanosensitive manner via activin A. It promotes cell adhesion and migration on type I collagen and participates in the contraction of collagen lattices. α11 is also involved in the regulation of collagen turnover and in myofibroblast differentiation. The different functions of α11 occur in various contexts

In Vivo Functions

The in vivo function of the α11 integrin was partially elucidated using the knockout mouse model. The α11-deficient mice were smaller and displayed increased mortality compared to the heterozygous and wild-type mice (Popova et al. 2007). Dwarfism observed in these α11-deficient mice was not due to structural defects in forming cartilage or bone. Instead, the smaller size and malnutrition of α11-deficient mice appear to correlate with delayed incisor eruption and altered tooth shape. The incisor periodontal ligament (PDL), which plays a central role during rodent incisor eruption, showed increased thickness due to increased amount of collagen. In this mutant tissue, a decrease of MT1-MMP and MMP-13 mRNA level was also noted. A reproducible result was obtained in vitro, where MEFs isolated from α11-deficient embryos showed reduced MT1-MMP and MMP-13 mRNA expression, whereas MMP-2 and MMP-9 activities were not affected. These observations suggest that α11 could be involved in the regulation of metalloproteinases as MMP-13 and -14, thus controlling the collagen turnover in PDL (Fig. 2).

As described above, α11β1 has been reported to be up-regulated in some tumor forms. It might be used as marker for diagnosis, as suggesting in human non-small cell lung cancer (Chong et al. 2006). α11 was also shown to enhance tumorigenicity of this type of tumor by regulation of the IGF2 expression, a potent growth stimulator of epithelial tumor cells (Zhu et al. 2007). However, the exact role of α11 in the tumor stroma during TGF-β1-dependent myofibroblast differentiation, tumor growth, and tumor metastasis remains to be determined.

Summary

Integrin α11β1 is expressed in mesenchymal non-muscle cells in vitro and in vivo at sites where collagens are organized in a highly ordered manner. It appears as a multifunctional integrin in different contexts (Fig. 2). However, little is known about the detailed molecular mechanisms involved in α11β1 functions, and thus, the major signaling pathways utilized by α11β1 remain to be determined.

References

Carracedo S, Lu N, Popova SN, Jonsson R, Eckes B, Gullberg D. The fibroblast integrin alpha11beta1 is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation. J Biol Chem. 2010;285:10434–45.

Caswell CC, Barczyk M, Keene DR, Lukomska E, Gullberg DE, Lukomski S. Identification of the first prokaryotic collagen sequence motif that mediates binding to human collagen receptors, integrins alpha2beta1 and alpha11beta1. J Biol Chem. 2008;283:36168–75.

Chong IW, Chang MY, Chang HC, Yu YP, Sheu CC, Tsai JR, Hung JY, Chou SH, Tsai MS, Hwang JJ, Lin SR. Great potential of a panel of multiple hMTH1, SPD, ITGA11 and COL11A1 markers for diagnosis of patients with non-small cell lung cancer. Oncol Rep. 2006;16:981–8.

Garamszegi N, Garamszegi SP, Samavarchi-Tehrani P, Walford E, Schneiderbauer MM, Wrana JL, Scully SP. Extracellular matrix-induced transforming growth factor-beta receptor signaling dynamics. Oncogene. 2010;29:2368–80.
Gullberg D, Velling T, Sjöberg G, Sejersen T. Up-regulation of a novel integrin alpha-chain (alpha mt) on human fetal myotubes. Dev Dyn. 1995;204:57–65.
Honda E, Yoshida K, Munakata H. Transforming growth factor-beta upregulates the expression of integrin and related proteins in MRC-5 human myofibroblasts. Tohoku J Exp Med. 2010;220:319–27.
Ke XS, Qu Y, Goldfinger N, Rostad K, Hovland R, Akslen LA, Rotter V, Øyan AM, Kalland KH. Epithelial to mesenchymal transition of a primary prostate cell line with switches of cell adhesion modules but without malignant transformation. PLoS ONE. 2008;3:e3368.
Leomil CLF, Mota BE, Sales PC, Marques JT, de Oliveira JG, Bonjardim CA, Peregrino FPC, Kroon EG. Integrin alpha 11 is a novel type I interferon stimulated gene. Cytokine. 2006;33:352–61.
Lu N, Heuchel R, Barczyk M, Zhang WM, Gullberg D. Tandem Sp1/Sp3 sites together with an Ets-1 site cooperate to mediate alpha11 integrin chain expression in mesenchymal cells. Matrix Biol. 2006;25:118–29.
Lu N, Carracedo S, Ranta J, Heuchel R, Soininen R, Gullberg D. The human alpha11 integrin promoter drives fibroblast-restricted expression in vivo and is regulated by TGF-beta1 in a Smad- and Sp1-dependent manner. Matrix Biol. 2010;29:166–76.
Pellinen T, Arjonen A, Vuoriluoto K, Kallio K, Fransen JA, Ivaska J. Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. J Cell Biol. 2006;173:767–80.
Popova SN, Rodriguez-Sánchez B, Lidén A, Betsholtz C, Van Den Bos T, Gullberg D. The mesenchymal alpha11beta1 integrin attenuates PDGF-BB-stimulated chemotaxis of embryonic fibroblasts on collagens. Dev Biol. 2004;270:427–42.
Popova SN, Barczyk M, Tiger CF, Beertsen W, Zigrino P, Aszodi A, Miosge N, Forsberg E, Gullberg D. Alpha11 beta1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor. Mol Cell Biol. 2007;27:4306–16.
Siljander PR, Hamaia S, Peachey AR, Slatter DA, Smethurst PA, Ouwehand WH, Knight CG, Farndale RW. Integrin activation state determines selectivity for novel recognition sites in fibrillar collagens. J Biol Chem. 2004;279:47763–72.
Tiger CF, Fougerousse F, Grundström G, Velling T, Gullberg D. alpha11beta1 integrin is a receptor for interstitial collagens involved in cell migration and collagen reorganization on mesenchymal nonmuscle cells. Dev Biol. 2001;237:116–29.
Varas L, Ohlsson LB, Honeth G, Olsson A, Bengtsson T, Wiberg C, Bockermann R, Järnum S, Richter J, Pennington D, Johnstone B, Lundgren-Akerlund E, Kjellman C. Alpha10 integrin expression is up-regulated on fibroblast growth factor-2-treated mesenchymal stem cells with improved chondrogenic differentiation potential. Stem Cells Dev. 2007;16:965–78.
Velling T, Kusche-Gullberg M, Sejersen T, Gullberg D. cDNA cloning and chromosomal localization of human alpha(11) integrin. A collagen-binding, I domain-containing, beta(1)-associated integrin alpha-chain present in muscle tissues. J Biol Chem. 1999;274:25735–42.
Vuoristo M, Vihinen P, Vlaykova T, Nylund C, Heino J, Pyrhönen S. Increased gene expression levels of collagen receptor integrins are associated with decreased survival parameters in patients with advanced melanoma. Melanoma Res. 2007;17:215–23.
Zhang WM, Kapyla J, Puranen JS, Knight CG, Tiger CF, Pentikainen OT, Johnson MS, Farndale RW, Heino J, Gullberg D. Alpha 11beta 1 integrin recognizes the GFOGER sequence in interstitial collagens. J Biol Chem. 2003;278:7270–7.
Zhu CQ, Popova SN, Brown ER, Barsyte-Lovejoy D, Navab R, Shih W, Li M, Lu M, Jurisica I, Penn LZ, Gullberg D, Tsao MS. Integrin alpha 11 regulates IGF2 expression in fibroblasts to enhance tumorigenicity of human non-small-cell lung cancer cells. Proc Natl Acad Sci USA. 2007;104:11754–9.

Integrin Alpha 4

► Integrin Alpha 4 (Itga 4)

Integrin Alpha 4 (Itga 4)

Maria Mittelbrunn[1] and Francisco Sánchez-Madrid[2]
[1]Centro Vascular Biology and Inflamation Department, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain
[2]Servicio de Inmunología, Hospital Universitario La Princesa Instituto Investigación Sanitaria Princesa, Madrid, Spain

Synonyms

CD49d; Integrin alpha 4; Itga4

Historical Background

Integrins are heterodimeric glycoproteins composed of non-covalently associated α and β subunits, and constitute one of the *largest* families of adhesion molecules. Integrins mediate cell-to-cell and cell-to-extracellular matrix interactions and are involved in a wide range of physiological processes such as development, immune regulation, and hemostasis.

Mammalian genomes contain 18 α subunit and 8 β subunit genes which generate 24 different αβ heterodimer combinations.

The α_4 integrin subunit is expressed on the cell surface associated with β_1 or β_7 integrin chains (Hemler et al. 1987; Sanchez-Madrid et al. 1986). These α_4 integrins ($\alpha_4\beta_1$, also known as VLA-4, and $\alpha_4\beta_7$) are involved in important cell differentiation processes such as neural and muscle cell differentiation and hematopoiesis. The importance of α_4 integrins in development is demonstrated by the embryonic lethality of disruption of the α_4 integrin gene in mice; a fraction of these embryos die due to a defect in the development of the placenta, while the rest die after hemorrhage in the heart region. The generation of chimeric mice revealed that α_4 is essential for hematopoiesis and also for epicardial development (Arroyo et al. 1996). α_4 integrins also play a key role in the adhesion interaction between stem/progenitor cells and bone marrow stromal cells and their matrix (Miyake et al. 1991). The most well-documented role of α_4 integrins is in leukocyte adhesion, motility, and extravasation during the inflammatory response. All of these functions are mediated through the binding of α4 integrins to their ligands, which include fibronectin, and the Ig Superfamily members VCAM-1, JAM-B, and MadCAM.

α4 Integrin Expression

The α_4 integrin subunit is expressed as an integral membrane protein on the surface of several cell types, mostly restricted to the hematopoietic lineage in adults (Hemler et al. 1987; Sanchez-Madrid et al. 1986). Bone marrow progenitor cells and several mature cells, including mononuclear leukocytes and eosinophils, express α_4, mainly as $\alpha_4\beta_1$. Myocytes, thymocytes, osteoclasts, cardiac cells, placental cells, erythrocytes, neutrophils, and fibroblasts also express α_4, but expression on these cells depends on developmental stage and stimulation. Melanoma and neural-crest-derived tumor cell lines express $\alpha_4\beta_1$. $\alpha_4\beta_7$ is expressed at high levels by a small subset of circulating memory T cells that preferentially localize to gut-associated lymphoid tissue. This homing is mediated by the interaction of this integrin with MAdCAM-1, an addressin molecule selectively expressed on intestinal endothelial cells (Holzmann and Weissman 1989).

α4 Integrin Ligands

The most important $\alpha_4\beta_1$ integrin ligands include the extracellular matrix molecules fibronectin (FN; (Wayner et al. 1989)), osteopontin and thrombospondin, the immunoglobulin superfamily member VCAM-1 (Elices et al. 1990), which is expressed by activated endothelium, and the junctional adhesion molecule JAM-B (also known as JAM-2) (Cunningham et al. 2002), which localizes at endothelial cell–cell junctions. $\alpha_4\beta_1$, like other β_1 integrins, also interacts with the bacterial coat protein invasin (Isberg and Leong 1990), mediating bacterial entry into mammalian cells. $\alpha_4\beta_7$ integrin binds to fibronectin, VCAM-1, and the gut addressin MAdCAM-1 (Berlin et al. 1995).

α4 Integrin Function

α_4 integrins are important for the motility of many types of leukocytes, including hematopoietic stem cells, eosinophils, mast cells, and T and B lymphocytes. During inflammatory responses leukocytes interact with the activated endothelium in order to migrate from the bloodstream to peripheral tissue. This extravasation process consists of the steps of tethering and rolling, firm adhesion, and diapedesis. α4 integrins, by interacting first with VCAM-1 (vascular cell adhesion molecule-1) and then with JAM-B (junctional adhesion molecule-B), participate in all of these steps (Berlin et al. 1995; Elices et al. 1990). α_4 integrins are involved in the recruitment of bone marrow-derived endothelial progenitors to the neovasculature and mediate their adhesion to VCAM-1 on endothelial and mural cells in neovessels (Garmy-Susini et al. 2005). Integrin $\alpha_4\beta_1$ is enriched at the immune synapse during activation of T lymphocytes and acts as a co-stimulatory molecule driving T helper (T_H)1/T_H2 cell differentiation (Mittelbrunn et al. 2004). Integrin $\alpha_4\beta_7$, through its interaction with MAdCAM-1 expressed on intestinal endothelial cells (Holzmann and Weissman 1989), acts as a homing receptor for a subset of memory T cells that preferentially migrate to gut-associated lymphoid tissue.

Regulation of α_4 Integrin Function

The capacity of α_4 integrins to bind their ligands is regulated both by intracellular signaling mechanisms and by extracellular stimuli. Intracellular signals induce conformational changes (integrin activation) that result in increased ligand-binding affinity, while

extracellular stimuli modify the expression and activation status as well as the level of receptor aggregation/clustering on the plasma membrane. The conformational change of integrin activation involves the cytoplasmic domains of the α and β subunits. Under resting conditions, these domains associate with each other. This association is thought to function as a "clasp" that keeps integrins in a low ligand-affinity bent conformation. When the cells are activated, the cytoplasmic domains dissociate and the extracellular domains form an open conformation that allows interaction with ligands. The association of the α and β cytoplasmic domains is regulated by the GFFKR sequence, a well-preserved motif in the α4 cytoplasmic domain.

The signaling pathway that underlies the inside-out activation mechanism of $\alpha_4\beta_1$ upon stimulation of the B-cell receptor has been described, and involves the consecutive activation of Lyn, Syk, phosphatidylinositol-3′ hydroxy kinase, Bruton's tyrosine kinase (Btk), phospholipase C (PLC)γ_2, Ins(1,4,5)P_3-receptor-mediated Ca^{2+} release, and protein kinase C (Spaargaren et al. 2003). The affinity of α_4 integrins is also regulated by divalent cations; Mn^{2+} and Mg^{2+} modulate the strength of binding to VCAM-1 and MAdCAM-1, while Ca^{2+} facilitates the binding of $\alpha_4\beta_1$ to VCAM-1. The small GTPase Rap 1 and its effector RAPL play a role in the activation of β_1 and β_2 integrins induced by the T-cell antigen receptor (TCR), CD31, and cytokines (Katagiri et al. 2004). Clustering of integrins on the cell membrane also increases their adhesiveness as a result of cooperative binding (Hogg et al. 2003). An additional factor that might contribute to α_4 integrin function is their lateral association with CD44 and the tetraspanins CD53, CD63, CD81, and CD82, which might facilitate integrin receptor clustering.

Signaling

The cytoplasmic domain of the α_4 subunit can bind directly to paxillin, a signaling adaptor molecule that has been reported to regulate the function of α_4 integrins in immune cells (Liu et al. 1999). Paxillin binds to the α_4 cytoplasmic domain upon dephosphorylation of Ser988. The paxillin–α_4 interaction inhibits the formation of focal adhesions, stress fibers, and lamellipodia by triggering the activation of various tyrosine kinases, such as focal adhesion kinase (FAK), Pyk2, Src, and Abl. The paxillin–α_4 complex inhibits the formation of stable lamellipodia by sequestering ADP-ribosylation factor (Arf)-GTPase-activating protein, thereby decreasing Arf activity and inhibiting Rac. It has recently been reported that dissociation of ▶ Vav1 from talin generates alpha4beta1-talin complexes, resulting in high-affinity alpha4beta1 conformations and efficient integrin activation (Garcia-Bernal et al. 2009). Moreover, recent evidence suggests that Kindlins, a group of 3 structurally related adaptors, cooperate with talin in activating integrins through binding to the integrin β subunits (Montanez et al. 2008).

Therapeutic Applications

It is well recognized that inflammatory responses form part of the pathogenesis of many disorders, including autoimmune diseases. The integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ are fundamental for the adhesion and migration of leukocytes to different inflammatory scenarios and have therefore been investigated as potential therapeutic targets. Administration of anti-α_4 mAb or various synthetic blockers has shown beneficial effects in several animal models, including experimental allergic encephalomyelitis, adjuvant-induced arthritis, diabetes mellitus of NOD mice, experimental graft-versus-host disease, allograft rejection, and immediate hypersensitivity reactions (Yusuf-Makagiansar et al. 2002).

In a clinical setting, the humanized anti-α_4 mAb natalizumab (Tysabri) has been approved for treatment of the relapsing forms of multiple sclerosis and Crohn's disease (Gonzalez-Amaro et al. 2005). Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system that is presumably caused by activated T cells specific for myelin antigens. Crohn's disease is an inflammatory bowel disease (IBD), a group of chronic systemic diseases involving inflammation of the gastrointestinal tract. Patients with multiple sclerosis or Crohn's disease show significant improvements with natalizumab (Gonzalez-Amaro et al. 2005).

Another recently suggested application for α_4 blocking agents is the treatment and prevention of epilepsy. Recent findings indicate that seizure activity is associated with leukocytic inflammatory changes in the central nervous system vasculature. α_4-specific antibodies decrease leukocyte adhesion to the brain vessel wall and have anti-epileptogenic properties, reducing and preventing subsequent seizures (Fabene et al. 2008).

It has been assumed that the therapeutic effect of this type of mAb is a consequence of the inhibition of the interaction of α_4 integrins with VCAM-1 and MAdCAM-1, thus inhibiting leukocyte extravasation to inflammatory foci. However, the complex role of α_4 in immune cells, including its involvement in leukocyte activation and T_H1/T_H2 polarization (Mittelbrunn et al. 2004), might lead to undesired side effects with anti-α_4 mAbs. This possibility should be considered, especially in relation to the treatment of autoimmune and inflammatory diseases associated with T_H1/T_H2 imbalance. Further studies are needed to minimize risks and optimize benefits of long-term maintenance therapy with these antibodies.

Summary

Integrins are one of the largest families of adhesion molecules that mediate cell–cell and cell–matrix interactions. α_4 integrins are expressed on many types of hematopoietic cells, including stem/progenitor cells, and are critical regulators of hematopoiesis and leukocyte trafficking.

Inhibition of leukocyte trafficking by antibody blockade of α4-integrins is a validated therapeutic approach for the treatment of multiple sclerosis and Crohn's disease. It remains unclear, however, why anti-α_4-based therapies are effective in some chronic autoimmune and inflammatory conditions but not in others, and this is an area that deserves further research. The onset of demyelinating diseases such as progressive multifocal leukoencephalopathy (PML) in some antibody-treated patients suggests that the mechanism of action of these drugs is not fully understood and forces us to carefully consider the potential risks associated with long-term administration of anti- α_4-integrin antibodies. These risks might include alterations to T helper cell differentiation, thereby distorting the population profile of T helper cells and subverting the type of immune response. Further studies are therefore needed to define the regulatory mechanisms that mediate α_4 function. The development of a new generation of modulators of α_4 integrin function, small molecules that block interaction with ligands or intracellular-associated proteins, is likely to lead to novel therapies not only for multiple sclerosis and Crohn's disease but also for others inflammatory/autoimmune diseases.

References

Arroyo AG, Yang JT, Rayburn H, Hynes RO. Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. Cell. 1996;85:997–1008.

Berlin C, Bargatze RF, Campbell JJ, von Andrian UH, Szabo MC, Hasslen SR, Nelson RD, Berg EL, Erlandsen SL, Butcher EC. Alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell. 1995;80:413–22.

Cunningham SA, Rodriguez JM, Arrate MP, Tran TM, Brock TA. JAM2 interacts with alpha4beta1. Facilitation by JAM3. J Biol Chem. 2002;277:27589–92.

Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell. 1990;60:577–84.

Fabene PF, Navarro Mora G, Martinello M, Rossi B, Merigo F, Ottoboni L, Bach S, Angiari S, Benati D, Chakir A, et al. A role for leukocyte-endothelial adhesion mechanisms in epilepsy. Nat Med. 2008;14:1377–83.

Garcia-Bernal D, Parmo-Cabanas M, Dios-Esponera A, Samaniego R, Hernán-P de la Ossa D, Teixidó J. Chemokine-induced Zap70 kinase-mediated dissociation of the Vav1-talin complex activates alpha4beta1 integrin for T cell adhesion. Immunity. 2009;31:953–64.

Garmy-Susini B, Jin H, Zhu Y, Sung RJ, Hwang R, Varner J. Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. J Clin Invest. 2005;115:1542–51.

Gonzalez-Amaro R, Mittelbrunn M, Sanchez-Madrid F. Therapeutic anti-integrin (alpha4 and alphaL) monoclonal antibodies: two-edged swords? Immunology. 2005;116:289–96.

Hemler ME, Huang C, Takada Y, Schwarz L, Strominger JL, Clabby ML. Characterization of the cell surface heterodimer VLA-4 and related peptides. J Biol Chem. 1987;262:11478–85.

Hogg N, Laschinger M, Giles K, McDowall A. T-cell integrins: more than just sticking points. J Cell Sci. 2003;116:4695–705.

Holzmann B, Weissman IL. Peyer's patch-specific lymphocyte homing receptors consist of a VLA-4-like alpha chain associated with either of two integrin beta chains, one of which is novel. EMBO J. 1989;8:1735–41.

Isberg RR, Leong JM. Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell. 1990;60:861–71.

Katagiri K, Ohnishi N, Kabashima K, Iyoda T, Takeda N, Shinkai Y, Inaba K, Kinashi T. Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. Nat Immunol. 2004;5:1045–51.

Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, Pfaff M, Ginsberg MH. Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. Nature. 1999;402:676–81.

Mittelbrunn M, Molina A, Escribese MM, Yanez-Mo M, Escudero E, Ursa A, Tejedor R, Mampaso F, Sanchez-Madrid F. VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses. Proc Natl Acad Sci USA. 2004;101:11058–63.

Miyake K, Weissman IL, Greenberger JS, Kincade PW. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J Exp Med. 1991;173:599–607.
Montanez E, Ussar S, Schifferer M, Bösl M, Zent R, Moser M, Fässler R. Kindlin-2 controls bidirectional siganling of integrins. Genes Dev. 2008;22(10):1325–30.
Sanchez-Madrid F, De Landazuri MO, Morago G, Cebrian M, Acevedo A, Bernabeu C. VLA-3: a novel polypeptide association within the VLA molecular complex: cell distribution and biochemical characterization. Eur J Immunol. 1986;16:1343–9.
Spaargaren M, Beuling EA, Rurup ML, Meijer HP, Klok MD, Middendorp S, Hendriks RW, Pals ST. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. J Exp Med. 2003;198:1539–50.
Wayner EA, Garcia-Pardo A, Humphries MJ, McDonald JA, Carter WG. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J Cell Biol. 1989;109:1321–30.
Yusuf-Makagiansar H, Anderson ME, Yakovleva TV, Murray JS, Siahaan TJ. Inhibition of LFA-1/ICAM-1 and VLA-4/VCAM-1 as a therapeutic approach to inflammation and autoimmune diseases. Med Res Rev. 2002;22:146–67.

Integrin Alpha V (*ITGAV*)

Bethany A. Kerr and Tatiana V. Byzova
Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Lerner Research Institute, The Cleveland Clinic, Cleveland, OH, USA

Synonyms

Alpha v integrin; CD51; Vitronectin receptor alpha (VNRA); Vitronectin receptor, alpha polypeptide

Historical Background

The term "integrin" was coined by R.O. Hynes in 1986 to describe a family of adhesion proteins which integrate the cytoskeleton with the extracellular matrix and external stimuli. These integrins are composed of alpha and beta subunits to form a complete signaling molecule. The α_v subunit forms a complete integrin complex through heterodimerization with one of five β subunit binding partners: beta 1 (β_1), beta 3 (β_3), beta 5 (β_5), beta 6 (β_6), or beta 8 (β_8). The first α_v integrin characterized was $\alpha_v\beta_3$, which was originally termed the "vitronectin receptor," as it bound to plasma-derived vitronectin. The $\alpha_v\beta_3$ integrin was purified from plasma in 1985 by R. Pytela and then cloned and sequenced in 1986 by S. Suzuki. The name "vitronectin receptor" was quickly proven to be a misnomer as $\alpha_v\beta_3$ displays highly promiscuous binding (Table 1). The other α_v integrins were then identified over the next decade, and their binding to various extracellular ligands (Table 1), expression sites, and physiologic and pathologic functions are still being characterized.

Expression Sites

Integrin α_v is essential for embryonic and perinatal development, with a complete knockout being lethal. Approximately 80% of integrin α_v-null mice die between embryonic day (E) 10 and E12 due to placental defects, while the remaining 20% die perinatally from massive hemorrhages or cleft palates. Using conditional knockouts, the α_v integrin was proven to be important for the development of cardiomyocytes, eye basal epithelium, glial cells, sebaceous glands, pancreatic cells, the palate, and limbs (Fig. 1a). In adult tissues, the integrin α_v regulates angiogenesis, wound healing, tumorigenesis, bone remodeling, vasculogenesis, inflammation, atherosclerosis, and neurogenesis (Fig. 1b) (Bouvard et al. 2001). The role of α_v integrin in these tissues is based on its ability to switch between active and inactive conformations, resulting in the activation of signaling cascades.

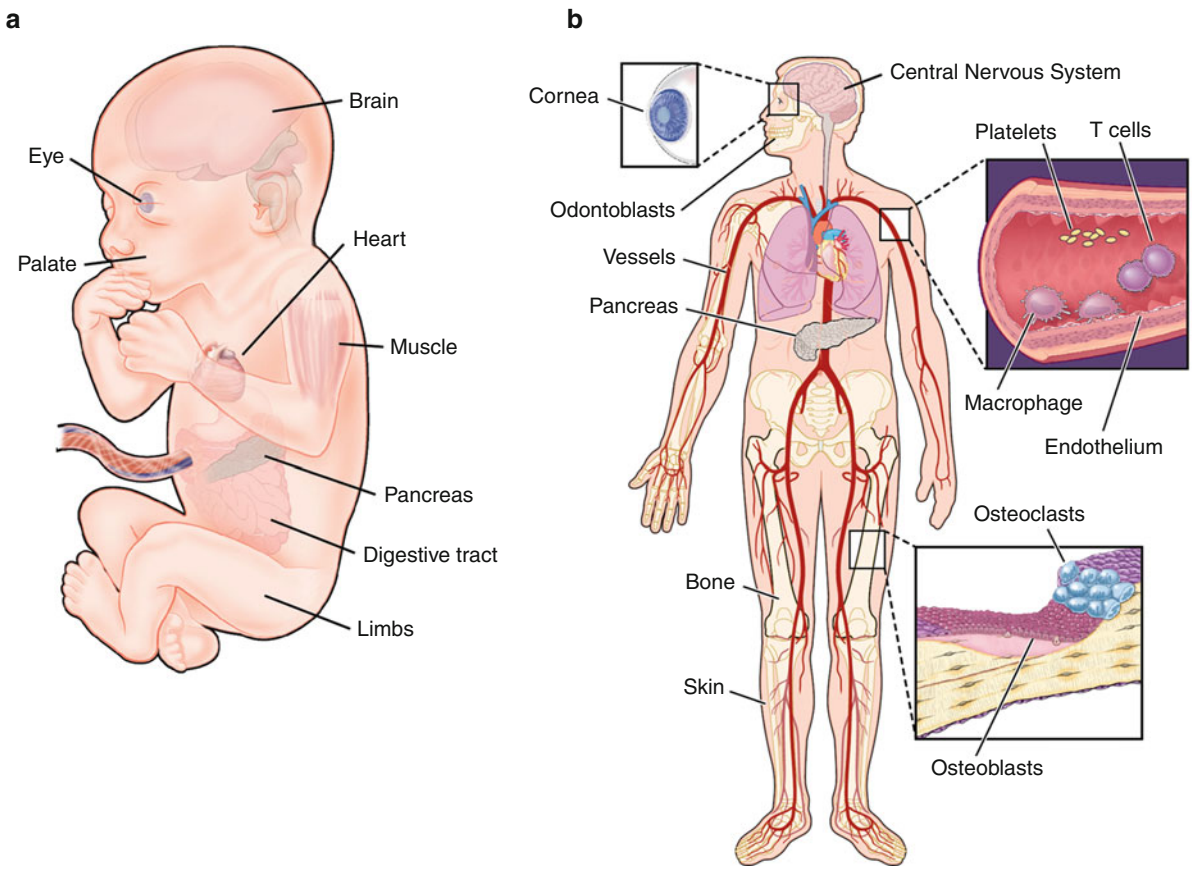
Alpha V Structure

The α_v subunit N-terminus contains a β-propeller domain consisting of seven 60-amino-acid repeats which fold to create a seven-bladed β-propeller structure (Fig. 2a). Blades 4–7 of this β-propeller each contain a Ca^{2+}, Mg^{2+}, or Mn^{2+} cation in the β-hairpin loop, which may interact with the upper leg to maintain rigidity of the integrin. This ligand-binding, seven-bladed head connects to the cell membrane by a ~170-Å "leg." The leg contains an Ig-like "thigh" domain and two large β-sandwich domains in the "calf" region. The second β-sandwich domain includes a proteolytic cleavage site. In the "knee" region of the α_v subunit leg, a metal ion is bound when the integrin is ligated (Mg^{2+}) or unligated (Ca^{2+}). Mn^{2+} binds to the

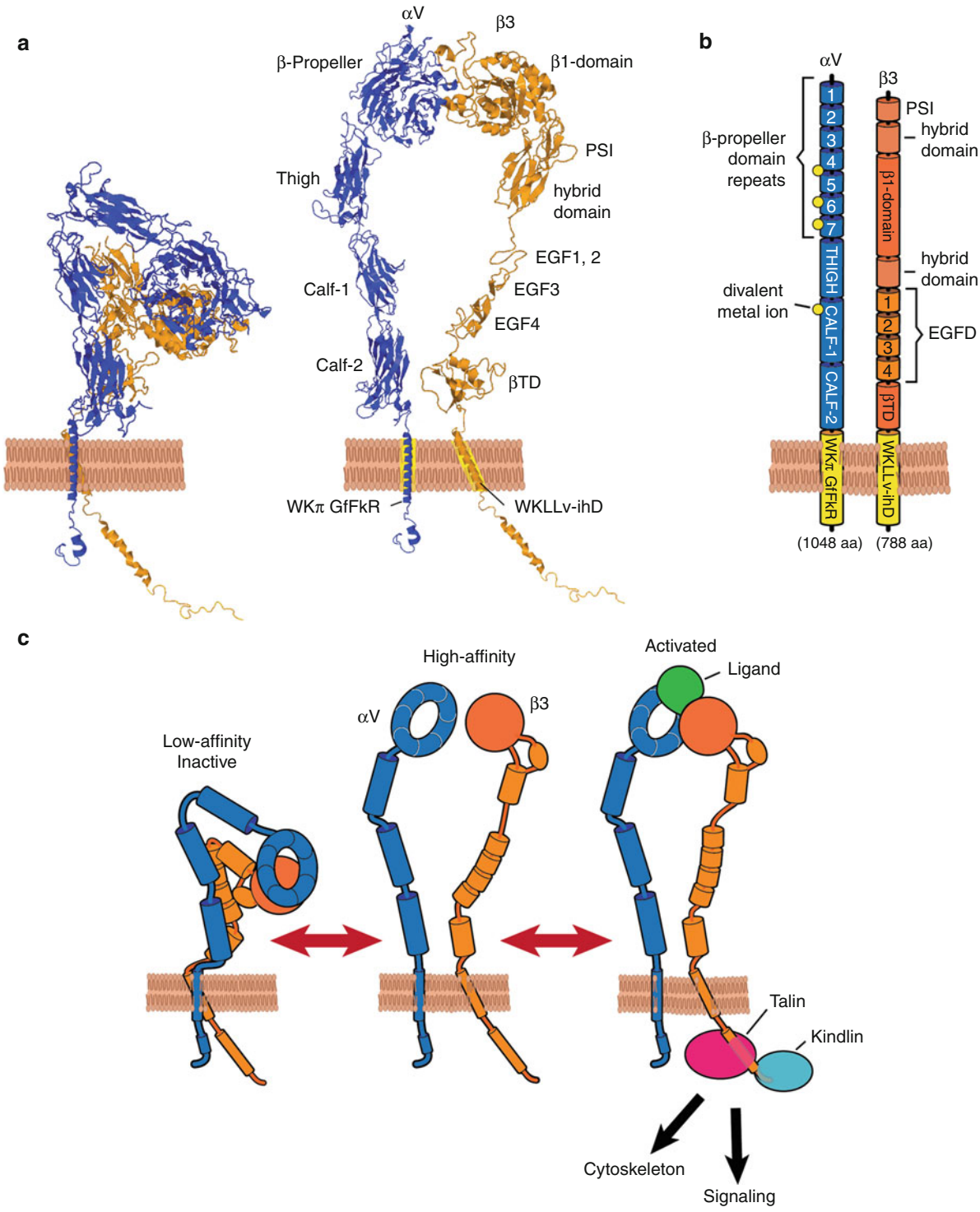
Integrin Alpha V (*ITGAV*), Table 1 The alpha v integrins binding a variety of extracellular ligands

α_v Integrin	Original name	CD designation	ECM ligands	Soluble ligands	Miscellaneous ligands
$\alpha_v\beta_1$	VNR	CD51/CD29	FN, VN, OPN, LN, denatured collagens	LAP-TGFβ, FGN	CD171, RGD
$\alpha_v\beta_3$	Platelet IIIa, VNR	CD51/CD61	FN, VN, OPN, LN, SPARC, periostin, canstatin, vWF, tenascin, BSP, COMP, tumstatin, denatured collagen type I	LAP-TGFβ, FGN, TSP, Cyr61, MMP-2, MMP-9, FGF-2, FGF-1, urokinase, plasmin, prothrombin	CD171, ADAM, fibrillin, PECAM-1, MGF-E8, ICAM-4, urokinase receptor, angiostatin, cardiotoxin, Del-1, RGD
$\alpha_v\beta_5$	VNR_{alt}	CD51/CD19	FN, VN, OPN, SPARC, periostin, canstatin, BSP	LAP-TGFβ, TSP, Cyr61	Del-1, CCN3, MGF-E8, RGD
$\alpha_v\beta_6$	VNR_β	CD51/—	FN, VN, OPN, tenascin	LAP-TGFβ, FGN	ADAM, RGD
$\alpha_v\beta_8$	—	CD51/—	FN, VN, LN	LAP-TGFβ	RGD

—Represents not available. *ADAM* disintegrin and metalloproteinase domain-containing proteins, *FGN* fibrinogen, *FGF* fibroblast growth factor, *FN* fibronectin, *ICAM* intracellular adhesion molecule, *LAP-TGFβ* TGFβ latency-associated peptide, *LN* laminin, *MGF-E8* milk fat globule-EGF factor 8 protein, *MMP* matrix metalloproteinase, *OPN* osteopontin, *PECAM* platelet endothelial cell adhesion molecule, *RGD* arginine-glycine-aspartic acid, *TSP* thrombospondin, *VN* vitronectin, *VNR* vitronectin receptor



Integrin Alpha V (*ITGAV*), Fig. 1 Major expression sites of integrin alpha v. (**a**) Integrin alpha v is expressed in the heart, eye, brain, pancreas, palate, muscles, digestive tract, and limbs during development. (**b**) In the adult, integrin alpha v is located on blood vessels, cornea (*top left inset*), odontoblasts, pancreas, skin, central nervous system; in the bone (on osteoblasts and osteoclasts) (*bottom right inset*); and in the blood (on the endothelium, monocytes, macrophages, T cells, and platelets) (*top right inset*) (Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved)



Integrin Alpha V (*ITGAV*), Fig. 2 (continued)

α_v integrin knee region and stimulates activation by imitating ligand binding. This knee region is also the site of a 135° hinge which is in a sharp bend when the integrin is inactive, but is linear in the activated integrin (Fig. 2a) (Arnaout et al. 2005; Byzova et al. 1998; Plow et al. 2000; Qin et al. 2004; Takada et al. 2007). Under non-reducing conditions, the α_v subunit has an apparent molecular weight of approximately 150 kDa. However, once reduced, the α_v subunit splits into a heavy chain of 120 kDa and a light chain of 25 kDa (Byzova et al. 1998). The α_v integrin subunit's tail associates with the β integrin subunit cytoplasmic tail through a conserved GFFKR motif proximal to the transmembrane region (Fig. 2b) (Takada et al. 2007). The α_v integrin complexes bind to a variety of extracellular ligands and cluster with growth factor receptors, tyrosine kinase receptors, and other membrane glycoproteins to mediate intracellular signaling.

Integrin Alpha V Activation and Signaling

Integrin Activation

Integrins on the plasma membrane exist in a low-affinity or high-affinity state (Fig. 2c). The low-affinity, resting state is maintained by interactions between the transmembrane domains and cytoplasmic tails of the α_v and β subunits. The state of the α_v integrin is partially controlled by divalent cations. Ca^{2+} association inhibits ligand binding of the α_v integrins, while the presence of Mg^{2+} is required for ligand binding. Association of Mn^{2+} with the α_v integrin imitates ligand binding leading to signaling. Once Mg^{2+} is associated, an extracellular ligand has bound, and intracellular signaling proteins are present, the integrin rapidly switches to a reversible high-affinity state with signaling between the cytoplasmic tail, the transmembrane domain, and the extracellular ligand binding pocket (Fig. 2c) (Plow et al. 2000; Qin et al. 2004). Conformational changes in the extracellular domains control integrin activation and are necessary for ligand binding. The transmembrane domains contribute to integrin activation through alterations in the interaction of their GxxG dimerization motifs. Separation of the transmembrane domains is proposed to be necessary for integrin activation and signaling (Fig. 2c). The cytoplasmic tails of the integrins have essential roles in regulating the signaling responsible for and in response to integrin activation.

Inside-Out Signaling

Activation signals can be transduced from the cytoplasmic tail to the extracellular domain in response to intracellular activation or in response to mechanical stress, known as inside-out signaling. Conversely, the binding of integrins to soluble or fixed ligands stimulates intracellular cascades, known as outside-in signaling (Arnaout et al. 2005; Qin et al. 2004). In inside-out signaling, integrins are activated by signals from G protein–coupled receptors, by growth factor receptors, or due to mechanical stresses which activate the α_v subunit. Integrin activation and intracellular signaling molecules lead to phosphorylation of the β subunit cytoplasmic tail and its interaction with cytoskeletal adaptor proteins. The cytoskeletal adaptor proteins talin and kindlin regulate integrin activation and affinity. The talin head region binds the β subunit cytoplasmic tail and results in increased ligand affinity of the α_v integrin (Qin et al. 2004; Takada et al. 2007). In addition, kindlin proteins facilitate talin binding and coordinate control of integrin affinity, cell spreading, and cell adhesion (Plow et al. 2009). The adaptor proteins involved inside-out signaling are described in greater detail below.

Outside-In Signaling

Outside-in integrin signaling results in clustering of integrins and their binding partners on the plasma membrane and the transmission of signals to the cytoplasmic tail leading to cytoskeletal reorganization

Integrin Alpha V (*ITGAV*), Fig. 2 The structure and activation of integrin alpha v. (**a**) Ribbon structure of the inactive and active integrin $\alpha_v\beta_3$, where integrin alpha v is *on the left*. (**b**) Domain structure of integrin alpha v and integrin beta 3. Divalent cation binding sites are indicated by *small circles* on the extracellular domains. The transmembrane and intracellular regions depict binding motifs for several signaling pathways and proteins. The GFFKR domain, which interacts with the beta subunit, is highlighted. (**c**) Model of integrin activation showing integrin alpha v in an inactive, low-affinity state on the *left*, at a high-affinity state in the *center*, and the ligand-bound, activated state on the *right*. Integrin activation is marked by conformational changes and the transition of the transmembrane domains from crossed to separate (Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved)

(Arnaout et al. 2005; Qin et al. 2004). The various proteins involved in membrane clustering and signal transduction are described in detail below. Once in the high-affinity state, integrins cluster together with other cellular receptors to transmit signals. The final activation step is increased avidity of integrins for their ligands. The inactivation of integrins has not been elucidated. Integrins must separate from their ligands and intracellular partners to terminate signaling or for cell migration. Adhesion complexes can disassemble, remodel, or slide by changes in integrin affinity and association. Competitor binding and proteolysis of binding partners and β cytoplasmic tails can also return integrins to their inactive state (Qin et al. 2004).

Clustering

Integrin activation results in the clustering of integrins with growth factor receptors on the plasma membrane. This interaction facilitates signaling and leads to specificity and synergistic effects on several signaling pathways. The α_v integrins modulate transforming growth factor β (TGFβ) by binding to the TGFβ latency–associated peptide (LAP-TGFβ) and by directly binding to the TGFβ receptor (TGFβR). This clustering activates TGFβRII resulting in the recruitment of TGFβRI and stimulation of the TGFβ pathway. Integrin $\alpha_v\beta_3$ is also involved in another cell-surface complex with urokinase-type plasminogen activator (uPA), ▶ uPA receptor (uPAR), and the low-density lipoprotein receptor. The activation of ▶ uPAR and $\alpha_v\beta_3$ results in activation of TGFβ, initiating an autocrine activation loop. Thus, these receptors are internalized and recycled after activation to stop the autocrine loop and ▶ uPAR is inactived by the plasminogen activator inhibitor protein. Integrin $\alpha_v\beta_3$ also interacts with the vascular endothelial growth factor (VEGF) receptors (VEGFR2) on blood vessels to coordinate and amplify angiogenesis (Somanath et al. 2009). VEGF stimulates the formation of a complex between VEGFR2 and $\alpha_v\beta_3$ on endothelial cells. Further, VEGFR2 phosphorylation is diminished in the absence of $\alpha_v\beta_3$. In addition, $\alpha_v\beta_3$ directly interacts with the receptors: platelet-derived growth factor (PDGF) receptor, fibroblast growth factor receptor (FGFR3), insulin receptor substrate (IRS)-1, and Met. The IRS-1 complexes with $\alpha_v\beta_3$ integrin with Grb2 and phosphoinositide-3 kinase (PI3k). Interactions between vitronectin and hepatocyte growth factor stimulates the clustering of $\alpha_v\beta_3$ integrin with Met. Integrin $\alpha_v\beta_3$ also binds insulin-like growth factor-1 (IGF-1) directly and can also cluster and bind to the IGF-1 receptor (IGF1R) (Somanath et al. 2009). This binding triggers IGF1R phosphorylation and stimulation of the Akt and ERK1/2 pathways and enhances cell proliferation. The IGF-1, IGFR1, and $\alpha_v\beta_3$ integrin were shown to cluster in a ternary complex on the cell surface (Somanath et al. 2009). In addition to integrin $\alpha_v\beta_3$, integrin $\alpha_v\beta_5$ has been shown to cross-talk with the epidermal growth factor (EGF) receptor (EGFR). The EGFR and α_v integrins cooperate to control cell-cycle progression. EGFR stimulates Raf, while integrins stimulate Ras. These two proteins can then interact to induce ERK signaling (Rüegg and Mariotti 2003).

In addition, activated integrins can associate with lipid membrane rafts and membrane glycoproteins. Membrane rafts are composed of sphingolipids tightly packed with cholesterol and are ringed by flexible phospholipid-rich regions. Integrin $\alpha_v\beta_3$ localizes to membrane rafts, where it interacts with CD47 (integrin associated protein, IAP), a membrane glycoprotein. CD47 also binds thrombospondin to control the function of $\alpha_v\beta_3$ in cell migration and spreading. The binding of these two proteins recruits the Gαi subunit and diminishes intracellular cAMP leading to stimulation of G protein signaling (Rüegg and Mariotti 2003). CD47 also activates $\alpha_v\beta_3$ directly through the Gαi. These two membrane proteins may also act synergistically to effect osteoclast function, endothelial cell attachment, and cancer metastasis. The clustering of α_v integrins with membrane proteins results in synergistic effects on signaling and increased binding avidity of intracellular proteins.

Intracellular Binding Partners

Integrins bind several classes of intracellular proteins: structural, adaptor, and signaling proteins, which form focal adhesions. A majority of integrin binding to intracellular partners is through the β-subunit; however, the α_v subunit directly binds caveolin, calreticulin, and Rack-1. The binding of caveolin to the α_v subunit occurs within the transmembrane region. Caveolin physically links the α_v integrins to signaling proteins, such as Fyn, leading to the recruitment of Shc. Shc then induces signal transduction through pathway such as MAPk to promote DNA synthesis and cell-cycle progression. In addition, α_v binds directly with calreticulin through the GFFKR

motif to induce signaling pathways (Fig. 2b). Calreticulin is a luminal endoplasmic reticulum calcium-binding protein, which may modulate cell adhesion and signal transduction. Receptor for activated protein kinase C (Rack-1) interacts with the α_v subunit. Rack-1 also binds to several of the β subunits.

In α_v integrin activation, actin-binding proteins are recruited to focal adhesions and bind the cytoplasmic tail of the β subunits. These actin-binding proteins include talin, filamin, and kindlin. Talin binding to the proximal NxxY motif of the β tail induces conformational changes of the integrin subunits resulting in activation of the α_v integrin (Rüegg and Mariotti 2003). Talin also binds several of the signaling kinases that function downstream of integrins. Recruitment of talin to integrins is stimulated by the unmasking of the integrin binding site, FERM (4.1, ezrin, radixin, and moeisin) domain, in the talin head by the PKC-Rap1-RIAM pathway, ▶ calpain, or PIP2. The PKC pathway and calpain activity may control the activation state of $\alpha_v\beta_3$ on endothelial and smooth muscle cells through their effects on talin binding. The FERM domain of talin binds tyrosine 747 in the β_3 subunit tail to induce signaling (Arnaout et al. 2005). In addition, talin functions as an initial contact between integrins and the actin cytoskeleton. Talin is responsible for the association of vinculin and Arp2/3 complex to actin, resulting in the stabilization and attachment of actin filaments to the focal adhesion complexes. In addition, the interaction between talin and the $\alpha_v\beta_3$ integrins is required for mechanotransduction.

Filamins also associate with the β subunit of the α_v integrins. Filamins can be found in the focal adhesions with integrins, but are mainly found along the cortical actin cytoskeleton and along stress fibers. The recruitment of filamin to focal adhesions can be stimulated by mechanical stress. Filamin also binds signaling proteins that control the actin cytoskeleton, including Rho, Rac, Cdc42, and MAPK signaling proteins (Plow et al. 2009).

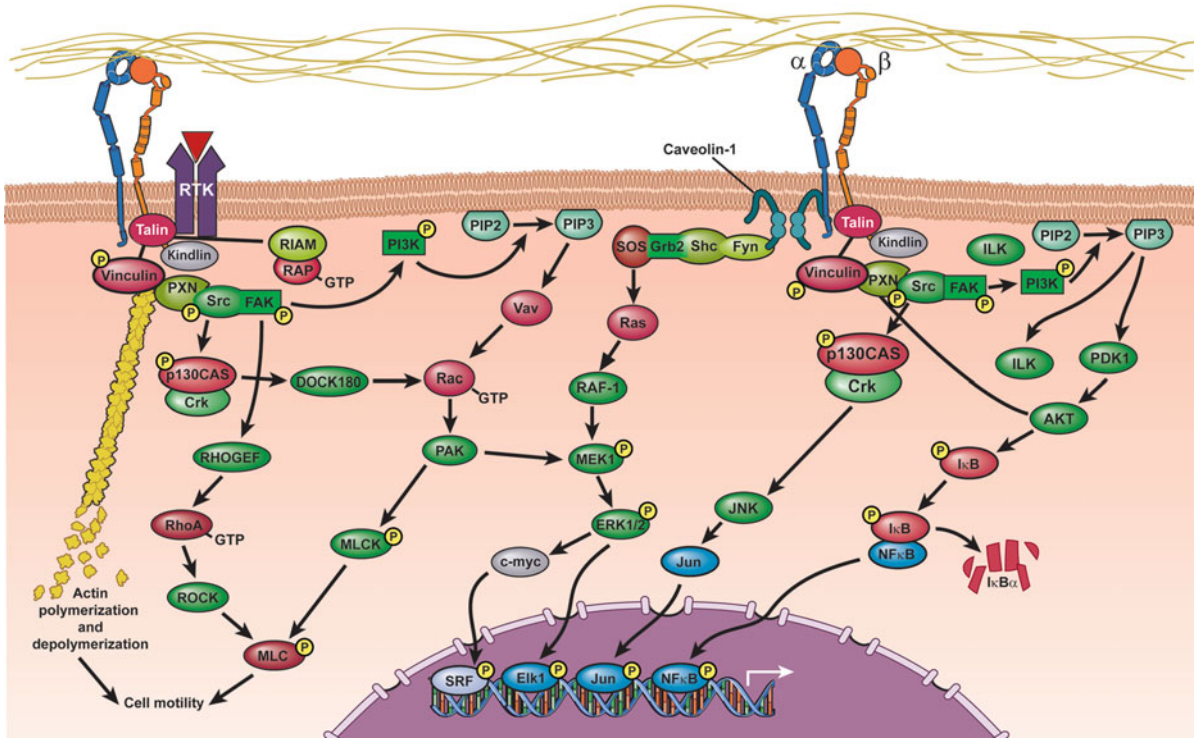
Recently, additional integrin-binding proteins, the kindlin protein family, have been identified, although their complete functions remain to be elucidated. The kindlins interact with the cytoplasmic tails of β_1 and β_3, which localize with α_v integrin. The kindlin-binding domain is located at the C-terminus of the β subunit and is separate from the domain used by talin. Kindlin binds the distal NxxY motif (tyrosine 795) of the β subunit. Kindlin and talin cooperate to control integrin affinity, with kindlins aiding talin function (Plow et al. 2009). Interestingly, the absence of kindlin-3 results in the dysfunction of integrin signaling resulting in severe defects in platelet and leukocyte function, as well as osteopenia (Malinin et al. 2010). Kindlins do not have known catalytic domains, but interact with other adaptor and signaling proteins, such as integrin-linked kinase.

The α_v integrins cross-talk with proteins involved in cell–cell junctions, such as adherens junctions. One molecule in adherens junctions is nectin, which associates with the actin cytoskeleton through afadin. Nectin creates cell–cell adhesions and stimulates c-Src-mediated activation of Rap1, Cdc42, and Rac. Nectins 1 and 2 associate with $\alpha_v\beta_3$ through their extracellular domains at cell–cell adhesion sites.

Intracellular Signaling

Integrin signal transduction requires the binding of signaling proteins including protein kinases, lipid kinases, small GTPases, and phosphatases (Fig. 3). Many of these pathways are also activated by growth factors, and through their clustering with integrin, α_v may have synergistic effects. The main protein kinase that is activated by the α_v integrins is focal adhesion kinase (FAK). FAK localizes to focal adhesions, where it interacts with the β subunit of the α_v integrins. The phosphorylation and tyrosine kinase activity of FAK is stimulated by integrin activation and binding to the extracellular matrix. FAK can also be phosphorylated by the clustering of isolated β subunit tails. FAK binds directly to talin and can also interact with vinculin and paxillin to control cell signaling and actin cytoskeletal formation. FAK activation recruits ▶ Src or Fyn, ▶ PI3k subunit p85, or PLCγ. These interactions stimulate the signaling cascades of Ras/Erk, ▶ PI3k/Akt, and Crk/Dock180/Rac (Rüegg and Mariotti 2003).

Further, the α_v integrins bind integrin-linked kinase (ILK), a serine/threonine kinase that binds directly to β_1 and β_3 cytoplasmic tails of the α_v integrins in focal adhesions (Fig. 3). The C-terminal of ILK binds the integrin β subunit, while the N-terminal domain binds PINCH, a LIM domain protein. PINCH binding to other adaptor proteins and ILK stimulation leads to the activation of several signaling pathways and actin polymerization. Further, ILK activation recruits several binding partners which result in the interaction of ILK with growth factor receptors (Siebers et al. 2005).

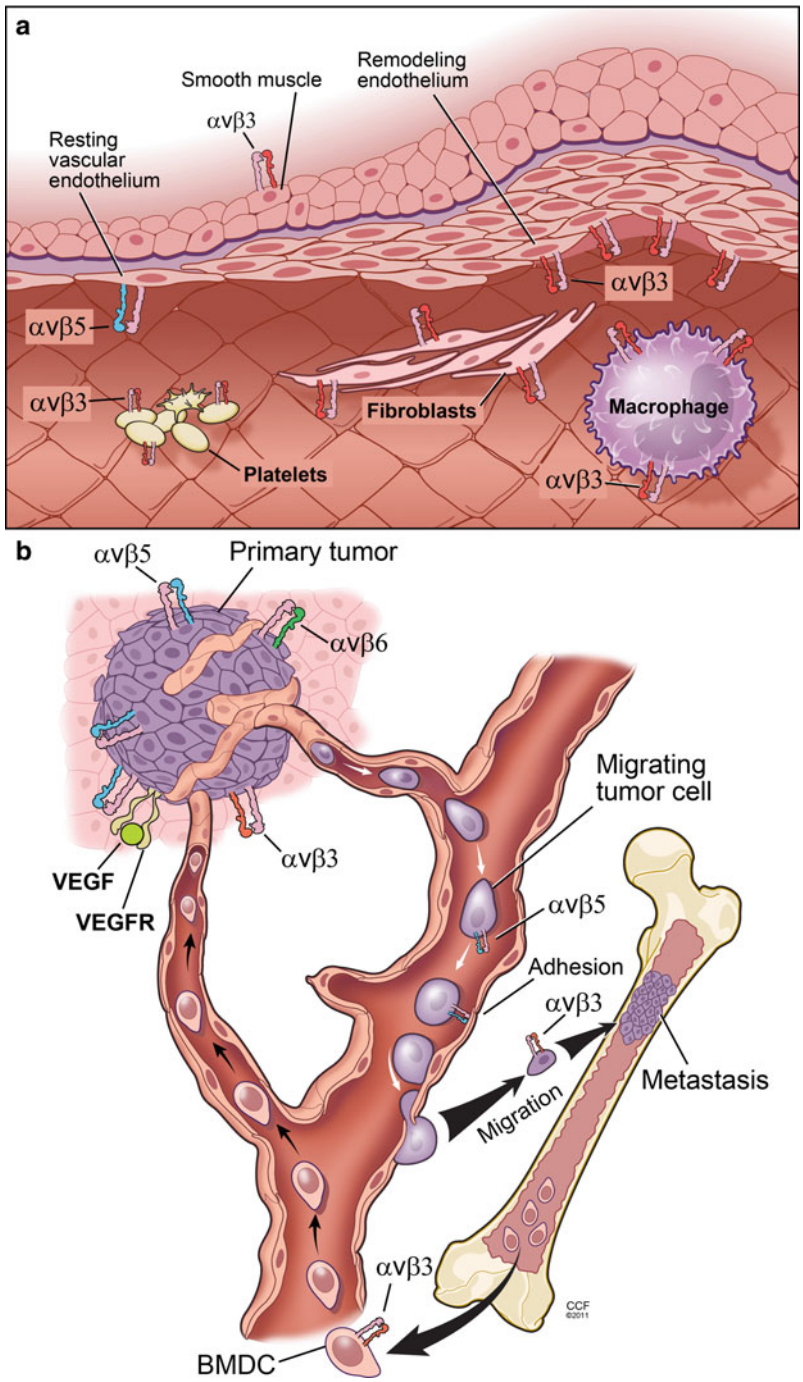


Integrin Alpha V (*ITGAV*), Fig. 3 Integrin alpha v activation stimulates signaling pathways controlling cytoskeletal organization, cell proliferation, and integrin modulation. The alpha v integrin is either bound to the actin cytoskeletal proteins or clusters with other integrins, growth factors, and membrane proteins to promote signaling through talin, vinculin, and caveolin resulting in the activation of the Src kinases (Shc, Fyn, Grb2), MAP kinases, FAK, PI3 kinase, Rho family, or Ras family signaling pathways (Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved)

The stimulation of ILK, FAK, and the α_v integrin β subunits results in the activation of a variety of intracellular signaling pathway including ▶ PI3k/Akt, Ras/▶ MAP kinase, ▶ Src family kinases, and the Rho GTPases: Rac, Rho, and Cdc42 (Fig. 3) (Rüegg and Mariotti 2003; Somanath et al. 2009). The ▶ PI3k/Akt pathway regulates gene transcription, cell proliferation, migration, and survival. The Ras-MAPK signaling pathway may mediate the effects of integrins on cell proliferation, survival, and migration. Integrin stimulation of the MAPK pathways can regulate ▶ p53, $p21^{WAF1/CIP}$, ▶ Bcl-2, and Bax, thus controlling cell-cycle progression and cell survival in response to α_v activation (Rüegg and Mariotti 2003). The ▶ Src family kinases play important roles in integrin and growth factor interaction and signaling. The signaling pathways of the ▶ Src kinases further cross-talk with the other integrin responsive pathways (Somanath et al. 2009). The Rho GTPase family, Rho, Rac, and Cdc42, is responsible for actin polymerization, the formation of focal adhesions, cell proliferation, and regulation of gene expression. Thus, through the regulation of various signaling pathways, the α_v integrins control cell survival, migration, and function within tissues (Rüegg and Mariotti 2003).

Integrin Alpha V in Angiogenesis and Wound Healing

Conditional knockout mice have demonstrated that integrin α_v is not necessary for vascular development; however, it is important in adult vascular remodeling (Fig. 4a). On the resting endothelium, $\alpha_v\beta_3$ is minimally expressed, while it is upregulated during development and on vascular cells in tumors and ischemic tissues. Expression of $\alpha_v\beta_3$ on endothelial cells can also be induced by cytokines and growth factor exposure or by shear stress. Prothrombin is responsible for $\alpha_v\beta_3$ adhesion of endothelial and smooth muscle cells,



Integrin Alpha V (*ITGAV*), Fig. 4 Integrin alpha v supports angiogenesis and tumorigenesis. (**a**) The $\alpha_v\beta_5$ integrin is expressed on resting endothelium, while the $\alpha_v\beta_3$ integrin is only expressed on remodeling endothelium and smooth muscle cells. Integrin $\alpha_v\beta_3$ is also expressed on platelets, macrophages, and fibroblasts involved in angiogenesis. (**b**) Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ are involved in tumorigenesis. Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ are expressed on primary tumor cells. In addition, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed on migrating tumor cells during metastasis. Integrin $\alpha_v\beta_3$ is also integral to adhesion at metastatic sites. Further, $\alpha_v\beta_3$ is located on bone marrow–derived cells (BMDC) which are recruited to tumors to support tumor growth (Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved)

thus controlling hemostasis (Byzova et al. 1998). Knockin mice (DiYF) with $\alpha_v\beta_3$ phosphorylation ablated demonstrate impaired angiogenesis. Endothelial cells from these DiYF mice display defective cell adhesion, spreading, migration, and capillary tube formation (Mahabeleshwar et al. 2006). In addition, $\alpha_v\beta_5$ integrins are located on quiescent endothelial cells. Integrin $\alpha_v\beta_5$ binds Del-1, a pro-angiogenic factor, leading to the activation of a pro-angiogenic expression program. This angiogenic program

includes inducing $\alpha_v\beta_3$ and ▶ uPAR expression. Partially, through its effects on angiogenesis, the α_v integrin regulates wound healing.

Integrin $\alpha_v\beta_3$ expression is increased during wound healing, including on platelets, endothelial cells, macrophages, and fibroblasts responsible for repairing the dermis. Interestingly, mice deficient in $\alpha_v\beta_3$ demonstrate enhanced wound healing, which appears to be due to increased fibroblast recruitment and elevated TGFβ signaling. In addition, $\alpha_v\beta_5$ and $\alpha_v\beta_6$ have increased expression during wound closure of the epidermis. Integrin $\alpha_v\beta_6$ is not expressed in healthy epithelia, but is upregulated during wound healing. In epithelial cells, integrin $\alpha_v\beta_6$ bind the LAP-TGFβ promoting the interaction of TGFβ with its receptors by inducing a conformational change. Thus, expression of $\alpha_v\beta_6$, through its effects on TGFβ, controls inflammation and the response to local injury (Nemeth et al. 2007). The control that the α_v integrins exert over angiogenesis and wound healing is recapitulated in tumorigenesis.

Integrin Alpha V in Cancer and Metastasis

Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ regulate tumor formation and progression in a variety of cancers (Fig. 4b) (Nemeth et al. 2007). Integrin $\alpha_v\beta_3$ mediates the tumor growth, migration, and metastasis of several cancers including breast and prostate. The adhesion of these cancers to bone is $\alpha_v\beta_3$ dependent and is integral to the development of bone metastases. Further, a variety of cancer-derived cell lines, including those from bone marrow aspirates, express $\alpha_v\beta_3$ (De et al. 2005). In addition, $\alpha_v\beta_3$ integrin expression increases the recruitment of bone marrow–derived cells (BMDCs) to angiogenic sites within tumors and wounds. Integrin $\alpha_v\beta_3$ activation on the BMDC mediates their adhesion and migration through the endothelial layer (Fig. 4b) (Feng et al. 2008). This BMDC recruitment is mediated by platelets and is required for continued tumor growth. Further, $\alpha_v\beta_3$ integrin expression is integral to prostate cancer metastasis, where it is required for tumor growth and for tumor-induced bone formation. The activation state of the $\alpha_v\beta_3$ integrin controls the recognition of bone matrix proteins by prostate cancer cells, thus controlling metastasis (Mahabeleshwar et al. 2006; McCabe et al. 2007). In prostate cancer cells, $\alpha_v\beta_3$ also mediates cell adhesion and migration on vitronectin and osteopontin. Further, in response to EGF activation, $\alpha_v\beta_3$ stimulates the ▶ PI3k/Akt pathway in prostate cancer cells. EGF activation signaling also cross-talks with the $\alpha_v\beta_5$ integrin–induced pathways. Stimulation of carcinoma cells by EGF induces cell migration mediated by the $\alpha_v\beta_5$ integrin. EGFR induces ▶ Src activity and can mediate the $\alpha_v\beta_5$ integrin control of metastasis. ▶ Src activity also mediates the $\alpha_v\beta_3$-controlled metastatic progression of pancreatic cells. Integrin $\alpha_v\beta_5$ has also been implicated in prostate cancer metastasis to bone. Expression of VEGF and its receptor VEGFR2 on prostate cancer cells results in activation of $\alpha_v\beta_3$ and $\alpha_v\beta_5$. These integrins then stimulate the migration of prostate cancer cells toward SPARC protein in bone. The activation of $\alpha_v\beta_5$ induces augmented VEGF expression creating a positive-feedback loop stimulating further prostate cancer migration (Fig. 4b) (De et al. 2003). Further, integrin $\alpha_v\beta_5$ is involved in carcinoma cell invasion and metastasis. IGF-1 can cooperate with the $\alpha_v\beta_5$ integrin to induce pulmonary metastases. Additionally, expression of $\alpha_v\beta_6$ is increased in carcinomas of the colon, ovary, lung, breast, pancreas, stomach, salivary gland, and also skin and oral squamous cell carcinomas. The $\alpha_v\beta_6$ integrin promotes carcinoma progression by stimulating invasion, inhibiting apoptosis, regulating matrix metalloproteinase (MMP)-2 and −9 expressions, and activating TGF-β. Lastly, α_v is highly expressed in ovarian carcinomas, while β_1 is detected in both the tumor and stroma (Nemeth et al. 2007). Thus, the α_v integrin expression and function are important targets in cancer research. Correspondingly, several therapeutic agents targeting the alpha v integrin are in clinical development (Table 2).

Integrin Alpha V in the Bone

The α_v integrins are expressed on several skeletal cells: osteoblasts, osteoclasts, and odontoblasts, during various stages of development. Expression of $\alpha_v\beta_1$ has been described at a low level on osteoclasts (Nakamura et al. 2007), while integrin $\alpha_v\beta_3$ is expressed on both osteoclasts and osteoblasts (Siebers et al. 2005; Takada et al. 2007). In the skeleton, the $\alpha_v\beta_3$ controls osteoclast migration and resorption ring formation through its effects on actin cytoskeletal organization (Bouvard et al. 2001; Nakamura et al. 2007). Osteoclasts with

Integrin Alpha V (*ITGAV*), Table 2 Integrin alpha v therapeutic agents in clinical development

Therapeutic	Agent	Developer	α_v Target	Pathological target
LM609 (Vitaxin, MEDI 522, Abegrin)	Antibody	MedImmune	$\alpha_v\beta_3$	Melanoma, prostate carcinoma
CNTO 95	Antibody	Centocor	α_v	Solid tumors
Cilengitide (EMD 121974)	Small cyclic RGD peptide	Merck	$\alpha_v\beta_3$	Pancreatic adenocarcinoma, melanoma, lymphoma
ATN-161	Peptide	Attenuon	$\alpha_v\beta_3$ (also $\alpha_5\beta_1$)	Solid tumors
SM256	Small molecule agonist	Bristol-Myers Squibb[a]	$\alpha_v\beta_3$	Tumor neovascularization
SD983	Small molecule agonist	Bristol-Myers Squibb[a]	$\alpha_v\beta_5$	Tumor neovascularization
Abciximab (ReoPro)	Chimeric antibody	Centocor	$\alpha_v\beta_3$ (also $\alpha_{II}\beta_3$)	Coronary intervention, psoriasis, stroke

[a]These agents were developed by DuPont Pharmaceuticals, which has since been acquired by Bristol-Myers Squibb

mutated $\alpha_v\beta_3$ integrins do not from a ruffled membrane and are unable to resorb bone, resulting in hypocalcemia (Bouvard et al. 2001). Conversely, osteoblasts overexpressing $\alpha_v\beta_3$ integrin proliferate more quickly, uptake less calcium, and have impaired mineral deposition. In addition, increased $\alpha_v\beta_3$ expression in osteoblasts correlated with increased ERK and AP-1 activity and decreased JNK activity. Interestingly, blocking $\alpha_v\beta_3$ interaction with the extracellular matrix also results in decreased mineralization. This expression of the α_v integrins on bone cells regulates their attachment to the extracellular matrix and orthopedic implants.

The α_v integrins are expressed on osteoblasts and osteoclasts, which are often in contact with synthetic substrates covering orthopedic implants. The success or failure of these implants is contingent upon osseointegration. The composition and topography of the implant surface can influence integrin expression and cell behavior and thus dictate the failure rate. Osteoblasts express α_v integrins when exposed to titanium alloy, polystyrene (PS), or cobalt-chrome-molybdenum. In addition, implants are often coated with a variety of substrates derived from extracellular matrix proteins. Integrin α_v is upregulated on osteoblasts attaching to implants coated with laminin, but not fibronectin or collagen type I (Siebers et al. 2005), while integrin α_v is expressed by osteoclasts attaching to PS, collagen-coated PS, laminin-coated PS, titanium, and cobalt-chrome. In addition, the α_v and β_1 subunits are expressed by osteosacromas when attaching to Thermanox, uncoated titanium, hydroxyapatite, and hydroxyapatite-coated titanium. Further, Arg-Gly-Asp (RGD) peptides derived from extracellular matrix proteins have been used to coat implants improving osseointegration. Osteoblast binding to RGD peptides is dependent on the α_v integrin, and synthetic RGD motifs can induce cell attachment mimicking that found on parental molecules (Lebaron and Athanasiou 2000). Different RGD peptides stimulate attachment of different integrins and can thus be used to control the attachment of specific cell types. Cyclic RGD-coated ceramic implants or hydrogel disks promote bone regeneration via osteoblast attachment to the RGD peptide through the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Hersel et al. 2003). The coating of titanium with RGD peptides induces osteoblast attachment, stimulates differentiation, and inhibits apoptosis. In addition, RGD motifs have been used to promote nerve regeneration, spinal cord repair, and corneal tissue repair in vivo (Hersel et al. 2003). Thus, RGD-based and other biomimetic coatings stimulate the adhesion of α_v-expressing cells, enhancing tissue responses to implants and stimulating tissue regeneration.

Summary

The widely expressed α_v integrin subunit heterodimerizes with several β-subunits to create an integrin complex capable of recognizing a plethora of extracellular ligands. These activated integrin complexes can then cluster with different growth factor receptors, including IGF, PDGF, IRS, EGF, VEGF, TGFβ, uPA, and ▶ FGF, to stimulate several families of signaling cascades: ▶ PI3k/Akt, MAPK, Rho GTPase family, and ▶ Src family kinases. Thus, α_v integrin activation is integral to cell survival, proliferation, and function under physiological and pathological conditions. Integrin α_v activation contributes to angiogenesis, wound healing, tumorigenesis, and

bone remodeling. Targeting the α_v integrin provides an important therapeutic approach for cancer research, regenerative medicine, and tissue engineering.

References

Arnaout MA, Mahalingam B, Xiong J-P. Integrin structure, allostery, and bidirectional signaling. Annu Rev Cell Dev Biol. 2005;21:381–410.

Bouvard D, Brakebusch C, Gustafsson E, Aszódi A, Bengtsson T, Berna A, et al. Functional consequences of integrin gene mutations in mice. Circ Res. 2001;89:211–23.

Byzova TV, Rabbani R, D'Souza SE, Plow EF. Role of integrin $\alpha_v\beta_3$ in vascular biology. Thromb Haemost. 1998;80:726–34.

De S, Chen J, Narizhneva NV, Heston W, Brainard J, Sage EH, et al. Molecular pathway for cancer metastasis to bone. J Biol Chem. 2003;278(40):39044–50.

De S, Razorenova O, McCabe NP, O'Toole T, Qin J, Byzova TV. VEGF-integrin interplay controls tumor growth and vascularization. Proc Natl Acad Sci USA. 2005;102(21):7589–94.

Feng W, McCabe NP, Mahabeleshwar GH, Somanath PR, Phillips DR, Byzova TV. The angiogenic response is dictated by β_3 integrin on bone marrow-derived cells. J Cell Biol. 2008;183(6):1145–57.

Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials. 2003;24:4385–415.

Lebaron RG, Athanasiou KA. Extracellular matrix cell adhesion peptides: functional applications in orthopedic materials. Tissue Eng. 2000;6(2):85–103.

Mahabeleshwar GH, Feng W, Phillips DR, Byzova TV. Integrin signaling is critical for pathological angiogenesis. J Exp Med. 2006;203(11):2495–507.

Malinin NL, Plow EF, Byzova TV. Kindlins in FERM adhesion. Blood. 2010;115(20):4011–7.

McCabe NP, De S, Vasanji A, Brainard J, Byzova TV. Prostate cancer specific integrin $\alpha_v\beta_3$ modulates bone metastatic growth and tissue remodeling. Oncogene. 2007;26:6238–43.

Nakamura I, Duong LT, Rodan SB, Rodan GA. Involvement of $\alpha_v\beta_3$ integrins in osteoclast function. J Bone Miner Metab. 2007;25:337–44.

Nemeth JA, Nakada MT, Trikha M, Lang Z, Gordon MS, Jayson GC, et al. Alpha-v integrins as therapeutic targets in oncology. Cancer Invest. 2007;25:632–46.

Plow EF, Haas TA, Zhang L, Loftus J, Smith JW. Ligand binding to integrins. J Biol Chem. 2000;275:21785–8.

Plow EF, Qin J, Byzova TV. Kindling the flame of integrin activation and function with kindlins. Curr Opin Hematol. 2009;16(5):323–8.

Qin J, Vinogradova O, Plow EF. Integrin bidirectional signaling: a molecular view. PLoS Biol. 2004;2(6):e169.

Rüegg C, Mariotti A. Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. Cell Mol Life Sci. 2003;60:1135–57.

Siebers MC, ter Brugge PJ, Walboomers XF, Jansen JA. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. Biomaterials. 2005;16:137–46.

Somanath PR, Ciocea A, Byzova TV. Integrin and growth factor receptor alliance in angiogenesis. Cell Biochem Biophys. 2009;53:53–64.

Takada Y, Ye X, Simon S. The integrins. Genome Biol. 2007;8(5):215.

Integrin α1 (ITGA1)

Jyrki Heino
Department of Biochemistry and Food Chemistry, University of Turku, Turku, Finland

Synonyms

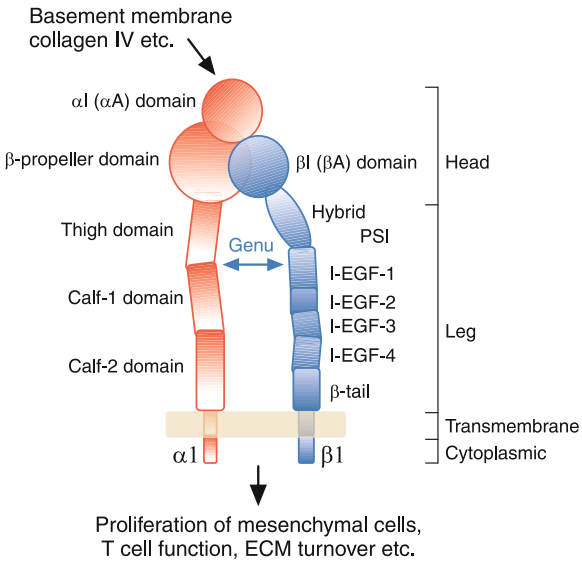
CD49a; Very late activation antigen 1 (VLA-1)

Historical Background

Integrin α1 subunit forms a heterodimer with integrin β1 subunit and acts as a cellular collagen receptor. α1β1 was one of the first integrins found and it was originally described as T lymphocyte very late activation antigen 1 (VLA-1). Today the human integrin family is known to contain 24 α/β heterodimers that mediate adhesion to extracellular matrix (ECM), plasma proteins, or counter receptors on other cells. The cytoplasmic domains of integrin α and β subunits are connected to cytoskeletal filaments and also to multiple cellular signaling pathways (Hynes 2004).

Structure

Integrins are single-pass type I membrane glycoproteins. Integrin α1 subunit contains short intracellular domain, transmembrane anchor, and an extracellular part, composed of α1I, β-propeller, thigh, calf-1, and calf-2 domains (Fig. 1). Thus, α1 belongs to a subgroup of integrins that contain an αI domain (inserted domain that is often called as A domain, based on its structural similarity with von Willebrand factor A domain). Integrin αI domain adopts a dinucleotide-binding or Rossmann fold and binds to ligands using a magnesium-containing metal ion dependent coordination site (MIDAS). The formation of α1–β1 complex is based on the non-covalent binding of β-propeller



Integrin α1 (ITGA1), Fig. 1 Domain structure of integrin α1β1, a collagen receptor. αI (αA) domain is a high affinity binding site for basement membrane collagen IV, but the receptor has been reported to bind numerous other ligands, as well. Ligand binding leads to signaling that regulates for example cell proliferation, differentiation, and survival

domain (α subunit) to β1I domain (β-subunit). Together with the α1I these two domains form the "head" part. The "leg" part of α1 subunit is composed of thigh, calf-1, and calf-2 domains, while hybrid, PSI, four I-EGF (1–4), and β-tail domains form the β1 leg part. In many integrins the leg parts can be bended in "knees" between thigh and calf-1 domain (α subunit) or between hybrid and I-EGF-1 domain (β subunit). This may also be the case with α1β1 integrin, but direct evidence is still lacking (Luo et al. 2007; Arnaout et al. 2007).

Evolution and Tissue Distribution

Based on their structure and phylogeny human integrin α subunits can be divided into four different subfamilies. Nine out of 18 human integrin α subunits belong to the group of the αI domain integrins. Integrin αI domains are only found in Chordates. The αI domain integrins can be further divided into leukocyte integrins and collagen receptor integrins. Integrin α1β1 forms together with three other heterodimers, namely, α2β1, α10β1, and α11β1, the collagen receptor subfamily. The biological functions of the integrin-type collagen receptors are often related to immunity and tissue regeneration. (Popova et al. 2007; Johnson et al. 2009)

Integrin α1 is expressed on mesenchymal cells, including fibroblasts, chondrocytes, osteoblasts, endothelial cells, and smooth muscle cells. It cannot exist on cell surface alone, but it is always in complex with integrin β1 subunit. Many inflammatory cells, including activated T lymphocytes, natural killer cells, and monocytes/macrophages are α1 positive (Ben-Horin and Bank 2004).

Ligands

Integrin α1β1 is a high avidity receptor for several collagen subtypes. The collagen family contains 28 members, and for most of the subtypes detailed information about receptor interactions is not available. Many published reports have emphasized the role of α1β1 as a receptor for basement membrane type IV collagen, whereas the receptor seems to have a relative low avidity to fibril-forming collagen subtypes (I, II, III, V). Thus, its preference to collagen subtypes seems to be different when compared to the other major collagen receptor, α2β1 integrin. Like all collagen receptor integrins α1β1 can recognize the triple helical motif formed by peptides containing a GFOGER (O = hydroxyproline) sequence or similar GLOGER and GASGER motifs. In addition to the GFOGER site α1β1 binds with high affinity to a separate triple helical motif found in collagen IV. In this site α1I domain recognizes two aspartic acid and one arginine residue, all located in different α chain. Thus, α1β1 may bind to fibril-forming and basement membrane collagens with a different mechanism. α1β1 has also been reported to recognize network-forming collagens (VIII), fibril-associated collagens with interruptions in triple helix (FACITs; IX, XVI), and transmembrane collagens (XIII). Presently, it is not possible to estimate what collagen subtypes are acting as the main α1β1 ligands during different physiological processes linked to this receptor.

Proteolytic fragments of ECM proteins have been reported to regulate biological processes. The cellular responses are often mediated by integrin-type receptors. The NC1 domain of α1(IV) collagen α-chain, also called as arresten, can be proteolytically released from collagen IV and has anti-angiogenic properties.

Arresten has been reported to bind to α1β1 and this may explain the mechanism of its function (Heino 2007; Leitinger 2011; Herr and Farndale 2009).

Other ligands of α1β1 integrin include various laminin subtypes. Laminin are the main structural proteins in basement membranes. Matrilin-1, a cartilage ECM protein, and Galectin-8, a β-galactoside binding lectin, can also bind to α1β1. Semaphorin 7A (Sema7A) is a glycosylphosphatidylinositol-linked membrane associated protein, known to have two receptors, plexin C1 and α1β1 integrin. Sema7A is expressed on neural cells, activated T cells, platelets, skin keratinocytes, and fibroblasts. In axon guidance, T cells activate cytokine production in monocytes and macrophages, as wells as in the attachment of skin melanocytes the effects of SemaA7 are dependent on β1-integrins. In addition to physiological ligands, snake venom KTS/RTS disintegrins can block α1β1 function (Eble 2010; Ivaska and Heino 2011).

Signaling Function

Like all integrins, also α1β1 may have several different conformation based functional states (Luo et al. 2007; Arnaout et al. 2007). Proteins, such as talin or kindlins can bind to the cytoplasmic domain of β1 subunit and activate the integrin. In some integrins the activation is associated to a conformational change from a bent to an extended state, in which the integrin is standing tall on cell surface. Ligand binding to αI domain is considered to lead to the opening of the domain, further changes in the conformation of the β1 subunit and finally to the separation of α and β legs. After binding to ECM α1β1 is concentrated to focal adhesion sites. The cytoplasmic domain of β1 subunit binds to cytoskeleton associated proteins, including talin, filamin, ▶ myosin, and tensin (Legate and Fässler 2009). Signaling and adapter proteins, such as focal adhesion kinase (FAK), integrin linked kinase (ILK), Yes, and Lyn can bind to β1 cytoplasmic domain and trigger further signaling events (Legate and Fässler 2009).

Detailed mutation-based analysis has revealed the important role of α1 cytoplasmic tail for α1β1 mediated cellular functions and for the activation of p38 and ERK MAP-kinase pathways. T-cell protein tyrosine phosphatase (TCPTP) is reported to bind to α1 cytoplasmic domain and negatively regulate epidermal growth factor (EGF) receptor function (Ivaska and Heino 2011).

Integrin α1β1 has been reported to regulate several signaling proteins and pathways. It can activate tyrosine kinases Fyn and Lck, which lead to phosphorylation of Shc. In arteries α1β1 plays an important role in the shear stress induced activation of Akt and ▶ PI 3-kinase. Furthermore, the production of reactive oxygen species (ROS) is controlled by α1β1 (Ivaska and Heino 2011).

In addition to their own signaling function the integrins have the ability to modify the signaling by many growth factor receptors. Often integrins are essential for the formation of molecular platforms that are needed for growth factor-dependent activation of growth factor receptors. In some cases integrins can directly activate the growth factor receptors in a growth factor independent manner. Furthermore, integrins may also regulate the recycling of growth factor receptors and regulate their number on cell surface (Ivaska and Heino 2011).

Function In Vivo

The in vivo function of α1β1 integrin has been analyzed by using knockout mice or by studying the effect of function blocking antibodies in various mouse models. In general α1 null mice are viable and fertile and harbor no remarkable developmental defects. Still, these animals are not fully normal since their skin is hypocellular, proposing that stem cell proliferation may be affected. The turnover rate of the skin collagenous matrix is also altered, confirming the in vitro observations that α1β1 is a negative feedback regulator of collagen synthesis. Despite the increased collagen synthesis, α1 knockout animals develop no obvious fibrotic disease, because the lack of α1 also seems to lead to increased production of matrix metalloproteinase. Conforming the affected regulation of collagen synthesis, α1 deficient mice are more sensitive to adriamycin-induced kidney fibrosis than their normal littermates.

The healing of bone fractures is compromised in α1 null mice due to the defected proliferation of bone marrow mesenchymal stem cells. Accordingly, α1 deficient animals seem to develop aging dependent osteoarthritis. These observations support the idea that one of the main functions of α1β1 integrin is to support the proliferation of specific mesenchymal stem cells populations. In α1 null animals also cancer

related angiogenesis, normal kidney function and retina development are affected (Heino 2007; Pozzi et al. 2009; Johnson et al. 2009; Ivaska and Heino, 2011).

Antibodies against α1 integrin can inhibit angiogenesis, lymphangiogenesis, and inflammation. α1β1 seems to augment T cell activation and proliferation and the important role of α1β1 integrin for cell mediated immunity has been shown in numerous animal experiments, including models for graft-versus-host disease, arthritis, colitis, allergen-induced bronchoconstriction, and glomerulonephritis. Integrin α1β1 may also play an important role in the pathogenesis of Alport syndrome, since in Alport mice collagen XIII in kidney endothelial cells mediates the selective recruitment of α1β1 integrin-positive monocytes (Ben-Horin and Bank 2004).

The important role of α1β1 integrin in the maintenance of bone metabolism is also proposed based on the observation that polymorphism in integrin α1 gene is associated with osteoporosis and related fracture risk in Korean females.

Summary

Integrin α1 subunit forms a heterodimer with integrin β1 subunit. Integrin α1β1 is an abundantly expressed mesenchymal collagen receptor that predominantly mediates cellular interactions with basement membrane collagen IV. Based on in vivo experiments with specific inhibitors and knockout animals α1β1 has multiple physiological roles. First, α1β1 supports the proliferation of cells, e.g., mesenchymal stem cells. Second, α1β1 regulates extracellular matrix turnover, including the synthesis of collagen and matrix metalloproteinases. Third, inflammatory cells, especially T cells and monocytes, use this collagen receptor. Integrin α1β1 is a potential target molecule for drug development in inflammation and fibrosis.

References

Arnaout MA, Goodman SL, Xiong JP. Structure and mechanics of integrin-based cell adhesion. Curr Opin Cell Biol. 2007;19:495–507.

Ben-Horin S, Bank I. The role of very late antigen-1 in immune-mediated inflammation. Clin Immunol. 2004;113:119–29.

Eble JA. Matrix biology meets toxinology. Matrix Biol. 2010;29:239–47.

Heino J. The collagen family members as cell adhesion proteins. Bioessays. 2007;29:1001–10.

Herr AB, Farndale RW. Structural insights into the interactions between platelet receptors and fibrillar collagen. J Biol Chem. 2009;284:19781–5.

Hynes RO. The emergence of integrins: a personal and historical perspective. Matrix Biol. 2004;23:333–40.

Ivaska J, Heino J. Cooperation between integrins and growth factor receptors in signaling and endocytosis. Annu Rev Cell Dev Biol. 2011;27:291–320.

Johnson MS, Lu N, Denessiouk K, Heino J, Gullberg D. Integrins during evolution: evolutionary trees and model organisms. Biochim Biophys Acta. 2009;1788:779–89.

Legate KR, Fässler R. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. J Cell Sci 2009; 122:187–98.

Leitinger B. Transmembrane collagen receptors. Annu Rev Cell Dev Biol. 2011;27:265–90.

Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. Annu Rev Immunol. 2007;25:619–47.

Popova SN, Lundgren-Akerlund E, Wiig H, Gullberg D. Physiology and pathology of collagen receptors. Acta Physiol (Oxf). 2007;190:179–87.

Pozzi A, Voziyan PA, Hudson BG, Zent R. Regulation of matrix synthesis, remodeling and accumulation in glomerulosclerosis. Curr Pharm Des. 2009;15:1318–33.

Integrin α2 (ITGA2)

Jyrki Heino
Department of Biochemistry and Food Chemistry, University of Turku, Turku, Finland

Synonyms

CD49b; Extracellular matrix receptor II (ECMR II); Platelet glycoprotein Ia; Very late activation antigen 2 (VLA-2)

Historical Background

The integrin family of cellular receptors contains 24 α/β heterodimers that mediate adhesion to extracellular matrix (ECM), plasma proteins, or counter receptors on other cells. The cytoplasmic domains of integrin α and β subunits are linked to cytoskeleton and cellular signaling pathways. Integrin α2 subunit forms a heterodimer with integrin β1 subunit and acts as a cellular collagen receptor. Before this receptor was

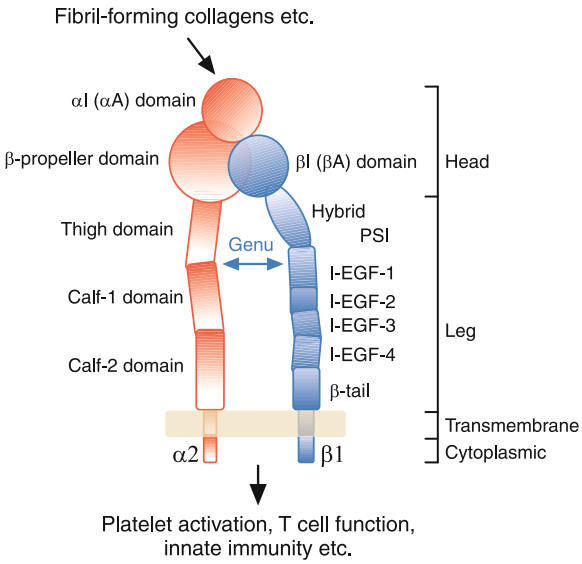
realized to be a member of the integrin family, the α2 subunit had already been named as the α subunit of T lymphocyte very late activation antigen 2 (VLA-2), platelet collagen receptor (glycoprotein Ia), and the larger subunit of collagen-binding extracellular matrix receptor II. Integrin α2β1 forms together with three other heterodimers, namely, α1β1, α10β1, and α11β1, the collagen receptor subfamily of the integrins (Hynes 2004).

Structure

Integrins are single-pass type I membrane glycoproteins. α2 belongs to a subgroup of integrins that contain an inserted domain (I domain that is often called as A domain, based on its structural similarity with von Willebrand factor A domain) (Fig. 1). Integrin αI domains recognize and bind ligands using a magnesium-containing metal ion dependent coordination site (MIDAS). Integrin α2 subunit contains short intracellular domain, transmembrane anchor, and an extracellular part, composed of α2I, β-propeller, thigh, calf-1, and calf-2 domains. In α2–β1 complex the β-propeller domain (α subunit) binds to β1I domain (β-subunit) in a non-covalent manner. These two domains, together with the α2I domain, form the "head" part of the heterodimer. Thigh, calf-1, and calf-2 domains form the "leg" part of α2 subunit, while β1 leg is composed of hybrid, PSI, four I-EGF (1–4), and β-tail domains. The leg parts of many integrins can be bended in "knees" between thigh and calf-1 domain (α subunit) or between hybrid and I-EGF-1 domain (β subunit) (Arnaout et al. 2007; Luo et al. 2007).

Evolution and Tissue Distribution in Human

Based on their structure and phylogeny human integrin α subunits can be divided into four different subfamilies, namely, RGD-motif binding integrins, α4/α9 integrins, laminin binding integrins, and αI domain integrin. While all metazoans have integrins, integrin αI domains exist in Chordates only. Accordingly, the collagen receptor integrins, including α2β1, are not found in non-vertebrates. The evolutionary history of the collagen receptors is in accordance with the fact that their biological functions are often related to immunity and tissue regeneration (Johnson et al. 2009)



Integrin α2 (ITGA2), Fig. 1 Domain structure of integrin α2β1, a collagen receptor. αI (αA) domain is a high affinity binding site for fibril-forming collagen, but the receptor has been reported to bind numerous other ligands, as well. Ligand binding leads to signaling that regulates, for example, cell proliferation, differentiation, and survival

Integrin α2β1 is abundantly expressed in human tissues. This integrin is considered to predominantly be epithelial, but also fibroblasts, chondrocytes, osteoblasts, and endothelial cell are α2 positive. Integrin α2β1 is one of the collagen receptors on platelets. Activated T lymphocytes (e.g., Th17 cells), mast cells, and neutrophils express this receptor, as well (Eckes et al. 2006; Heino 2007; Johnson et al. 2009; McCall-Culbreath and Zutter 2008; Popova et al. 2007).

Ligands

Distinct members of the collagen family are the major ligands for α2β1 integrin. In collagenous ligands α2I domain interacts with special triple-helical motifs, containing amino acid sequences such as GFOGER (O = hydroxyproline), GLOGER, GASGER, GROGER, and GLOGEN. The collagen family contains 28 different members and many of them are only known at nucleic acid level and, therefore, it has not been possible to test them in binging assays with integrins. At least fibril-forming collagens (I, II, III, V, XI), basement-membrane collagen (IV), fibril-associated collagens with interruptions in triple-helix (FACITs, IX, XIV), network-forming collagens

(VIII, X), beaded-filament forming collagens (VI), and anchoring filaments forming collagens (VII) are ligands for α2β1. Collagen XIII (a transmembrane collagen) seems to be a low affinity ligand, and negative binding results have been published with collagens XIV (FACIT) and XVII (a transmembrane collagen; COL15 domain). In general α2β1 integrin is considered to favor fibril-forming collagens over other collagen subtypes. Integrin α2β1 can also bind to collagen I fibrils. However, in tissues collagen fibrils are often covered with other proteins and proteoglycans and it is not clear, whether in vivo α2β1 or other collagen receptors mediate cell adhesion directly to the collagen fibrils. Instead, some in vitro observations suggest that α2β1 could regulate the formation of new collagen fibrils. The binding preference of α2β1 to collagen subtypes seems to be different when compared to the other major collagen receptor, α1β1 integrin. However, α11β1 may be more similar to α2β1 as a receptor for fibril-forming collagens (Heino 2007; Herr and Farndale 2009; Leitinger 2011)

In addition to collagens α2β1 can recognize a large number of other proteins, including different members of the laminin family and proteoglycans, such as decorin, endorepellin (a fragment of perlecan), and lumican. Tenascin C, chondroadherin, matrix metalloproteinase 1, E-cadherin, and collectin-family members (C1q complement protein, mannose-binding lectin, surfactant protein A) have been reported to be ligands for α2β1 integrin (Heino 2007; Herr and Farndale 2009; McCall-Culbreath and Zutter 2008).

Pathogens binding to α2β1 include echovirus-1 and rotavirus. These viruses use α2β1 as their cellular receptor and may take advantage of the integrin-mediated endocytosis mechanisms. Group A Streptococcus express a collagenous protein, named as Scl1, that is a ligand for α2β1. Snake venoms are known to contain toxins, such as EMS16, rhodocetin, and VP12, which block α2β1 function (Eble 2010).

Signaling Function

Like all integrins, also α2β1 can have several different conformation based functional states (Arnaout et al. 2007; Luo et al. 2007). Inactivated α2β1 is a low avidity collagen receptor, but can still attach to large ligands, for example human echovirus 1. Intracellular proteins, such as talin or kindlins can bind to the cytoplasmic domain of β1 subunit and activate the integrin (Legate and Fässler 2009). Ligand binding leads to the opening of the α2I domain, further changes in the conformation of the β1 subunit and finally to the separation of α and β legs (Arnaout et al. 2007; Luo et al. 2007).

After binding to extracellular matrix (ECM) α2β1 is concentrated to focal adhesion sites. The cytoplasmic domain of β1 subunit binds to cytoskeleton associated proteins, including talin, filamin, ▶ myosin, and tensin (Legate and Fässler 2009). Signaling and adapter proteins, such as focal adhesion kinase (FAK), integrin linked kinase (ILK), Yes, and Lyn can bind to β1 cytoplasmic domain and trigger further signaling events (Legate and Fässler 2009). Much less is known about the putative α2 cytoplasmic domain binding proteins. At least F-actin, calreticulin, Rap21, and vimentin have been reported to interact with α2 subunit. Deletion of α2 tail does not inhibit collagen binding, but leads to ligand-independent accumulation of the receptor to focal adhesion sites, suggesting that α2 tail may control the function and interactions of β1 cytoplasmic domain.

In various cell culture models α2β1 signaling has been connected to the activation of p38 and ERK MAP kinases, p27kip, Osf2, protein phosphatase 2A (PP2A), Akt, PI-3-kinase, Rac-1, and ▶ vav-2. In cell culture experiments α2β1 regulates cell proliferation, differentiation, migration, survival, ECM synthesis, and matrix degradation (Heino 2007; Ivaska and Heino 2011; McCall-Culbreath and Zutter 2008).

The role of α2β1 signaling in platelets has been studied in detail. The major platelet collagen receptors, glycoprotein VI and α2β1, seem to regulate each others activity and at least partially the same signaling proteins, including ▶ Src, Syk, ▶ SLP-76, and phospholipase Cγ2. Integrin α2β1 can also regulate Ca^{2+} concentration inside platelets. It is has been reported to induce specific rapid α-like peaks, but not longer-lasting γ-like peaks.

In general, the integrins have the ability to modify the signaling function of many growth factor receptors. They may create a proper environment for growth factor induced signaling or in some cases directly activate the growth factor receptors in a growth factor independent manner. Integrins may also orchestrate endocytosis and trafficking of growth factors receptors and regulate their number on cell surface (Ivaska and Heino 2011).

Function In Vivo

The in vivo function of α2β1 integrin has been analyzed by using knockout mice or by studying the effect of function blocking antibodies in various mouse models. The results from experiments utilizing null animals or specific inhibitors have in some cases produced conflicting results, which makes its sometimes difficult to estimate the actual biological function of α2β1 integrin. In human the role of α2β1 has been estimated in epidemiological studies that have been able to connect this receptor to specific human diseases.

In general α2 null mice are viable and fertile and harbor no obvious developmental defects. The architecture of mammary glands may be slightly altered. Their platelets show reduced response to collagen and slightly prolonged bleeding time. In addition, in an endothelial injury model, the lack of α2β1 integrin inhibits thrombosis. The small defects detected in the platelet function are in full agreement with the epidemiological data concerning human thrombotic diseases. Due to genetic polymorphism some individuals have elevated levels of α2β1 integrin on platelets. These persons may have elevated risk for myocardial infarction or cerebrovascular stroke. Furthermore, the medical literature knows one individual lacking α2β1 integrin on platelets and suffering from a mild bleeding disorder. Thus, α2β1 seems to be a functional collagen receptor on platelets. However, its relative importance, when compared to other collagen-binding mechanisms, namely, GPVI receptor or von Willebrand factor/GPIα system, has sometimes been challenged (Varga-Szabo et al. 2008).

In α2 deficient mice increased angiogenesis has been reported during wound healing. Similarly, cancer-related angiogenesis is enhanced. Paradoxically, antibodies against α2 can inhibit cancer-related angiogenesis. Furthermore, endorepellin, an inhibitor of angiogenesis seems to work in an α2β1 dependent manner. Similar controversy has been reported in the studies focused on the role of αV integrins in angiogenesis. The reason for this discrepancy is not clear, but integrin may orchestrate the endocytosis and trafficking of growth factor receptors and have indirect effects on many biological processes (Eckes et al. 2006; Ivaska and Heino 2011; Johnson et al. 2009; McCall-Culbreath and Zutter 2008).

Antibodies against α2β1 integrin can block the function of immunological cells and the null animals have defects in both native and acquired immunity (Eckes et al. 2006; Johnson et al. 2009; McCall-Culbreath and Zutter 2008).

In human cancer α2β1 integrin expression has been associated to the progression of melanoma, gastric, and ovarian cancer. Integrin α2β1 is a marker of prostate stem cells and it has been linked to prostate cancer invasion. However, in breast cancer α2β1 expression often decreases and α2β1 may even suppress metastasis.

Integrin α2β1 is considered to be a putative target for drug development in thrombosis related diseases, cancer, and inflammation.

Summary

Integrin α2 forms a heterodimer with integrin β1. α2β1 is an abundantly expressed receptor for various collagen subtypes as well as for many other ECM molecules. Based on the data from α2 null mice, as well from a human lacking α2 on platelets, the receptor participates in platelet binding to collagen. In thrombosis α2β1 acts together with another collagen receptor, namely, GPVI. Furthermore, α2β1 seems to participate in acquired as well as native immunity. The data related to the putative role of α2β1 during wound healing and angiogenesis is still incomplete. Polymorphism in ITG2A gene has been correlated to higher expression levels of α2β1 on platelets and increased risk to myocardial infarction and stroke. Integrin α2β1 is considered to be a potential drug target in thrombosis, cancer, and inflammation.

References

Arnaout MA, Goodman SL, Xiong JP. Structure and mechanics of integrin-based cell adhesion. Curr Opin Cell Biol. 2007;19:495–507.

Eble JA. Matrix biology meets toxinology. Matrix Biol. 2010;29:239–47.

Eckes B, Zweers MC, Zhang ZG, Hallinger R, Mauch C, Aumailley M, Krieg T. Mechanical tension and integrin α2β1 regulate fibroblast functions. J Investig Dermatol Symp Proc. 2006;11:66–72.

Heino J. The collagen family members as cell adhesion proteins. Bioessays. 2007;29:1001–10.

Herr AB, Farndale RW. Structural insights into the interactions between platelet receptors and fibrillar collagen. J Biol Chem. 2009;284:19781–5.

Hynes RO. The emergence of integrins: a personal and historical perspective. Matrix Biol. 2004;23(6):333–40.

Ivaska J, Heino J. Cooperation between integrins and growth factor receptors in signaling and endocytosis. Annu Rev Cell Dev Biol. 2011;27:291–320.
Johnson MS, Lu N, Denessiouk K, Heino J, Gullberg D. Integrins during evolution: evolutionary trees and model organisms. Biochim Biophys Acta. 2009;1788:779–89.
Legate KR, Fässler R. Mechanisms that regulate adaptor binding to β-integrin cytoplasmic tails. J Cell Sci. 2009;122:187–98.
Leitinger B. Transmembrane collagen receptors. Annu Rev Cell Dev Biol. 2011;265–90.
Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. Annu Rev Immunol. 2007;25:619–47.
McCall-Culbreath KD, Zutter MM. Collagen receptor integrins: rising to the challenge. Curr Drug Targets. 2008;9:139–49.
Popova SN, Lundgren-Akerlund E, Wiig H, Gullberg D. Physiology and pathology of collagen receptors. Acta Physiol (Oxf). 2007;190:179–87.
Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. Arterioscler Thromb Vasc Biol. 2008;28:403–12.

Integrin-Associated Protein

▶ CD47

Integrin-Associated Signal Transducer

▶ CD47

Interferon Regulatory Factor 5, IRF5

▶ IRF5

Interferon-Gamma

Fan-ching Lin and Howard A. Young
Laboratory of Experimental Immunology, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA

Synonyms

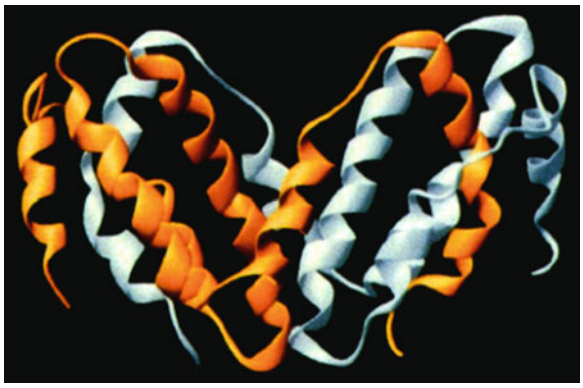
IFNG; IFN-gamma; IFN-γ; Immune IFN; Type II interferon

Historical Background

Interferons (IFNs) were first described in 1957 by Issacs and Lindenmann as substances that restrict viral replication (Issacs and Lindenmann 1957). In mid-1960, Wheelock reported that human leukocytes stimulated with phytohemagglutinin expressed IFN-like inhibitors (Wheelock 1965). However, these IFN-like substances had less resistance to heat and acid than the interferons described previously. In the 1970s, these substances were further characterized based on the inducing properties and cell-type expression patterns, and first named Immune IFN, then later Type II IFN. This nomenclature was originally a subject of some debate as the Type II IFN was thought to be physicochemically and also biologically different from Type I IFNs (IFN-α, IFN-β, IFN-ω, and IFN-τ) (Billiau 2009). In 1980, a panel of experts acknowledged the differences between Type I and Type II IFNs, and gave the Type II IFN the name IFN-γ.

IFN-γ and IFN-γ Receptor Complex

The major sources of IFN-γ are natural killer (NK) cells, T cells, and NKT cells. NK and NKT cells constitutively express IFN-γ mRNA retained in the nucleus, which allows rapid induction of IFN-γ upon stimulation. In T cells, the efficiency of IFN-γ induction is enhanced following activation (Hodge et al. 2002; Schoenborn and Wilson 2007). IFN-γ expression is induced by cytokines, e.g., Interleukin-12 (IL-12) and IL-18 secreted by antigen presenting cells (APC), and suppressed by IL-4, IL-10, transforming growth factor-β (TGF-β), and glucocorticoids. The IFN-γ mRNA contains a highly conserved AU-rich region in the 3′ untranslated region (UTR) that mediates the stability of the mRNA. Furthermore, Savan and colleagues have observed stabilization of the IFN-γ mRNA facilitated by microRNA 29 (Savan et al. unpublished data) resulting in increased protein expression. The active IFN-γ molecule, encoded by a single copy gene, consists of two antiparallel and intercalating polypeptides that then fold into a symmetrical twofold axis (Fig. 1). The dimer formation and the folding are important to the biological function and have been shown to be conserved among vertebrates (Savan et al. 2009).



Interferon-Gamma, Fig. 1 *IFN-γ structure*. The active IFN-γ molecule consists of two antiparallel and intercalating identical polypeptides that then fold into a symmetrical twofold axis. The structure was solved and published by Ealick et al. Science. 1991 May; 252(5006):698–702 (Printed with permission from Steven E. Ealick)

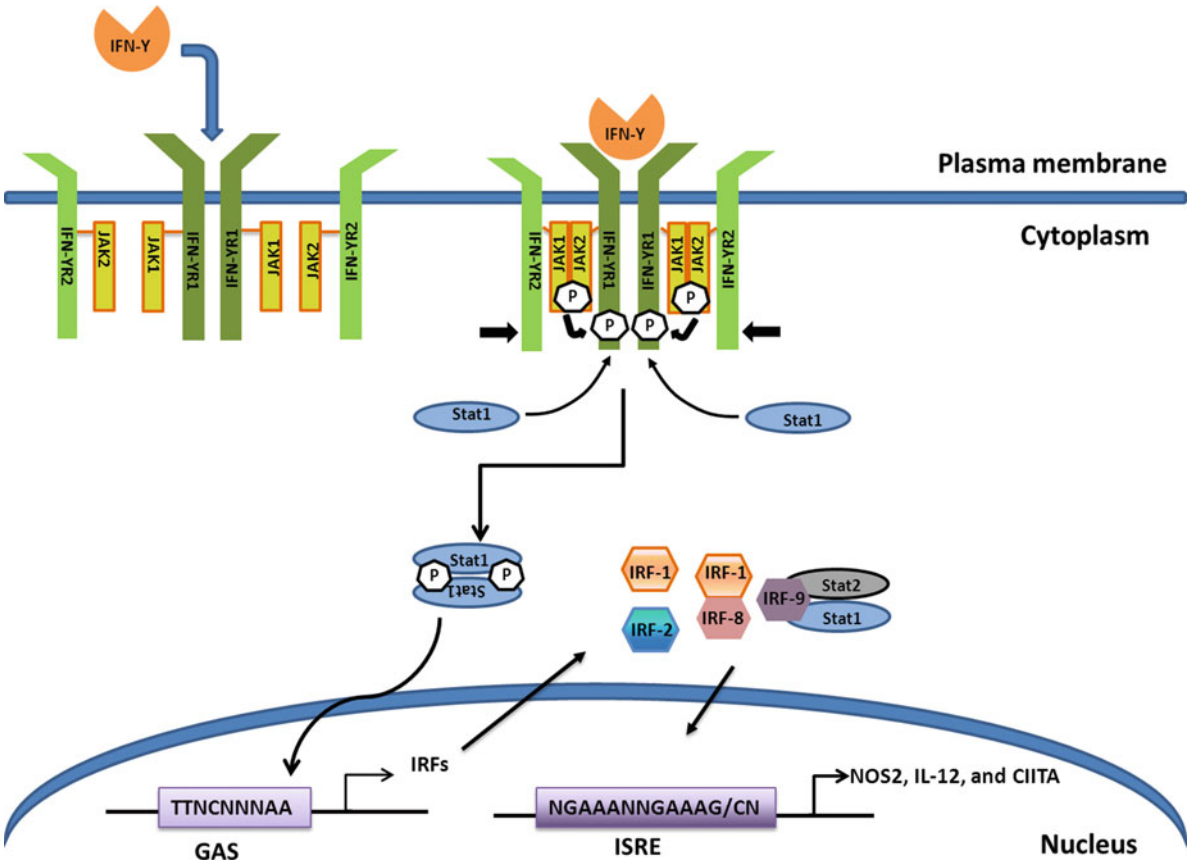
The biological activity of IFN-γ is initiated upon binding of the dimer to its receptor. The IFN-γ receptor consists of two ligand-binding IFN-γR1 chains and two signal-transducing IFN-γR2 chains. These proteins are encoded by separate genes (*IFNGR1* and *IFNGR2*, respectively) that are located on different chromosomes (Bach et al. 1997). Both chains are constitutively expressed on most cells. While the expression level of IFN-γR1 is usually strong, the expression level of IFN-γR2 is determined by the cell types, differentiation stages, and activation status of the cells. T cells have a lower IFN-γR2 expression level than B cells and monocytes. Compared to $CD4^+$ Th2 cells, Th1 cells have significantly lower expression of IFN-γR2. IFN-γ first binds to IFN-γR1, and the IFN-γ: IFN-γR1 oligomerization prompts its association with IFN-γ R2 that then initiates the downstream signaling events (Schroder et al. 2004). The interaction between IFN-γ: IFN-γR1 and IFN-γR1: IFN-γR2 has been shown to be species specific, e.g., mouse IFN-γ does not interact with the human IFN-γ receptors.

IFN-γ Signaling

The formation of the IFN-γ: IFN-γ receptor complex triggers the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Inactive JAK1 and JAK2 constitutively bind IFN-γR1 and IFN-γR2, respectively, through their N-terminal domains. After the formation of ligand receptor complex, the close proximity of JAK1 and JAK2 allows them to transactivate each other. The activated JAKs then phosphorylate the Tyr440 residue on each IFN-γ R1 chain to form the docking site for latent STAT1 monomers via src-homology 2 (SH2)-domains (Fig. 2). The recruited STAT1s are then phosphorylated at Tyr701 residue by the JAKs. The phosphorylation allows the two STAT1s to form a homodimer and dissociate from IFN-γR1. The homodimer STAT1 then translocates into the nucleus, binds to the gamma-activated sequence (GAS, TTCNNNGAA) in the promoter region of IFN responsive genes (ISGs; ISG database http://www.lerner.ccf.org/labs/williams/xchip-html.cgi), and initiates the transcription of the target genes (Saha et al. 2010). The activity of the STAT1 homodimer is significantly enhanced when the ser727 residue is phosphorylated by serine kinases such as p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (▶ PI3K) (Gough et al. 2008).

The primary gene products of IFN-γ stimulation are transcription factors, e.g., interferon-regulated factors (IRFs), which will then initiate the secondary transcription of ISGs. Both IRF-1 and IRF-2 interact with the interferon-stimulated response element (ISRE, NGAAANNGAAAG/CN) of ISGs. While IRF-1 initiates the transcription of genes, such as *NOS2*, *IL-12*, and *CIITA*, IRF-2 suppresses gene expression. Interestingly, IRF-8 and IRF-9 cannot bind to an ISRE and they regulate ISG transcription by binding to other transcriptional factors, e.g., IRF-8 forms a dimer with IRF-1 and IRF-9 forms a trimer with STAT1 and STAT2 (Fig. 2) (Savitsky et al. 2010).

IFN-γ also can activate STAT1-independent pathways, although it still requires the activation of JAKs. The profile of proteins recruited to IFN-γR1 shows that IFN-γ stimulation triggers the MAP kinase pathway. JAKs activate IFN-γR1 associated Raf1 and Rap1 serine kinases, which then phosphorylate ERK kinase. p38 MAP kinase is the other target of the MAP kinase pathway that has been shown to be activated after IFN-γ treatment. PI3K pathway is another pathway stimulated by IFN-γ that activates protein kinase C (PKC) α, δ, and ε isotypes. PKCα directly magnifies the transcription of ISGs. In contrast, PKCε amplifies transcription by phosphorylating MAP kinase, while PKCδ does so by phosphorylating ser727 on STAT1. It also has been shown that IFN-γ stimulation activates



Interferon-Gamma, Fig. 2 *IFN-γ signaling*. The IFN-γ: IFN-γR1 prompts its association to IFN-γR2 that results in the phosphorylation of JAKs. Activated JAKs then phosphorylate IFN-γR1 chains to form the docking sites for latent STAT1s. The recruited STAT1s are phosphorylated by the JAKs, form a homodimer and dissociate from IFN-γR1. The homodimer STAT1 translocates into the nucleus, and binds to GAS. The primary gene products, IRFs, then initiate the secondary transcription of ISGs by interacting with ISRE

► NF-κB pathway by degrading the inhibitor of κB (IκB) and triggering the phosphorylation of the IKKα and β subunits (Gough et al. 2008).

As stated above, the JAK-STAT pathway is the primary signaling pathway initiated by IFN-γ stimulation. With the activation of JAKs, the signal is further amplified by initiating other signal transduction pathways and maximizing STAT1 activity. To prevent undesirable outcomes, such as uncontrolled expression of specific genes resulting in tissue damage and autoimmunity, IFN-γ signaling needs to be tightly regulated. This is accomplished by the existence of a negative feedback mechanism that results in the IFN-γ induced expression of the suppressor of cytokine signaling genes (► SOCS). SOCS proteins negatively regulate IFN-γ signaling by inhibiting JAK catalytic activity. The IFN-γ signaling pathway is then suppressed within hours of IFN-γ treatment (Croker et al. 2008).

Biological Functions of IFN-γ

An antiviral effect was the first observed biological function of IFN-γ and thus accounts for its original designation as an interferon. IFN-γ exercises its antiviral activity by modulating both innate and adaptive immune responses. During virus infection, IFN-γ triggers the expression of protein kinase dsRNA-regulated and dsRNA-specific adenosine deaminase, proteins that inhibit viral protein synthesis. In addition, IFN-γ augments the antiviral state of the cells by enhancing Type I IFN expression and the formation of ISGF3, the main protein complex

involved in stimulating Type I IFN-induced gene expression. IFN-γ also plays a role in conveying antiviral signals from the innate to the adaptive immune response. Increased chemokine/chemokine receptors, induced by IFN-γ, recruit T cells to the infection site. Upon receiving the IFN-γ signal, APCs increase MHC class II: peptide complex and costimulatory molecule expression levels, hence facilitating peptide-specific $CD4^+$ T cell activation and initiation of the adaptive immune response against viral infection. IFN-γ can also limit viral infection by upregulating MHC class I pathway allowing cells to present a higher quantity and more diverse peptide repertoire to $CD8^+$ T cells (Schroder et al. 2004). In modulating adaptive immune responses, IFN-γ facilitates and maintains the commitment of $CD4^+$ T cells to the Th1 lineage that is crucial in controlling viral infection. IFN-γ also induces the expression of IL-12 by APCs. IL-12 not only activates NK cells, a major antiviral component of innate immunity, but also drives Th1 development. Additionally, IFN-γ signaling facilitates Th1 development and its own expression by inducing T-bet expression and suppressing the expression of GATA3, a protein that drives T cell Th2 differentiation. To further solidify the commitment to the Th1 lineage, IFN-γ signaling also inhibits the Th2 essential IL-4-STAT6 signaling pathway (Hu et al. 2008).

Another important biological function of IFN-γ is macrophage activation, a critical component in controlling microbial infection by the host. In fact, the original "Macrophage Activating Factor" was later determined to be IFN-γ. Once stimulated with IFN-γ, macrophages are primed and more responsive to pro-inflammatory mediators such as a tumor necrosis factor (TNF) and toll-like receptor (TLR) ligands. IFN-γ priming amplifies TLR signaling in macrophages by both increasing the expression of TLRs and activating the transcription factor NF-kB. As a result, macrophages increase the expression of inflammatory mediators and immune effectors including multiple cytokines and chemokines (Hu et al. 2008). IFN-γ suppresses the anti-inflammatory signaling triggered by IL-10 and TGF-β, thus IFN-γ primed macrophages are able to kill ingested pathogens through the generation of NADPH oxidase and nitric oxide synthase associated with the production of reactive oxygen species and reactive nitrogen intermediates, respectively (Saha et al. 2010). Furthermore, in response to IFN-γ, macrophages upregulate the expression of complement components which opsonize extracellular pathogens.

In contrast to the pro-inflammatory effects described above, modulation of an anti-inflammatory response is another major aspect of IFN-γ biological function. IL-17 is a pro-inflammatory cytokine produced by Th17 cells. IL-17 generates an inflammatory environment by enhancing the production of pro-inflammatory cytokines and chemokines. Thus, Th17 response is vital for protection against extracellular pathogens. However, if the response is not regulated, tissue damage may occur. IFN-γ inhibits the development of Th17 cells by inhibiting the effects of IL-6, IL-1, TGF-β, IL-21, and IL-23, all of which promote Th17 cell development. STAT1 inhibits STAT3, a critical component used by the IL-6, IL-23, and IL-21 signaling pathways. In addition, IFN-γ signaling downregulates the expression of both IL-23 and IL-1 receptors on Th17 cells (Bettelli et al. 2007). IFN-γ also plays a critical role in maintaining the homeostasis of the immune response. When stimulated with IFN-γ, $Foxp3^+$T cells upregulate the expression of T-bet. These Foxp3 + T-bet + regulatory T cells (Tregs) specialize in confining the Th1 immune response (Koch et al. 2009). IFN-γ also induces STAT1-dependent apoptosis in macrophages by upregulating caspases-1 to reduce the inflammation resulting from macrophage infiltration. For T cells, the apoptosis effect is associated with the strength of IFN-γ signaling. When treated with a high level of IFN-γ, T cells stop the internalization of IFN-γR2. The overexpression of IFN-γR2 then induces the expression of Fas ligand (FasL), promoting Fas-dependent apoptosis (Regis et al. 2006).

In summary, the data generated thus far clearly demonstrates that there are two arms of IFN-γ biological functions, i.e., pro- and anti-inflammatory pathways, and both arms are critical for a balanced immune response. The complex yet delicate signaling network allows IFN-γ to tailor the immune response either for defense against infection or toward maintaining the homeostasis of the host.

IFN-γ: Friend or Foe to Autoimmune Diseases

Due to its pro-inflammatory properties, IFN-γ has been associated with promoting different autoimmune

diseases. Systemic lupus erythematous (SLE) is a complex autoimmune disease and its main characteristic is the generation of autoantibodies by active B cells. The antibody-complement complex causes local and systemic inflammation and may result in kidney failure. In SLE patients, elevated pSTAT1 was observed in peripheral blood lymphocytes and similar results have also been seen in an SLE mouse model (Mozes and Sharabi 2010). Hodge and colleague has developed a mouse where the RNA stability element (ARE) in the 3′UTR of the IFN-γ mRNA has been removed. Without ARE-mediated decay of IFN-γ mRNA, the animals constitutively express low levels of IFN-γ. A significant finding resulting from the constitutive expression of low levels of IFN-γ is the rapid appearance of SLE-like symptoms (Hodge et al. unpublished data). While IFN-γ has not been shown to cause SLE (the pathogenesis of SLE remains unclear), the presence of IFN-γ exacerbates the disease. IFN-γ upregulates the expression of IFN-α whose signaling induces the transcription of the B-cell activation factor (BAFF). BAFF gives rise to the expansion of B cells and autoantibody production. Additionally, macrophages and fibroblasts increase the secretion of complement proteins in response to IFN-γ, resulting in complement deposition in the kidney. Furthermore, IFN-γ promotes widespread inflammation by inducing chemokines to recruit monocytes to different target organs. As a whole, various novel mouse models of lupus-like disease demonstrate that low levels of IFN-γ, if persistent, are capable of promoting autoimmunity.

In contrast to SLE, IFN-γ has been shown to mitigate the severity of disease in multiple sclerosis (MS). MS is an autoimmune disease caused by infiltration of lymphocytes into the central nervous system. As there is increased IFN-γ in the brain of MS patients, it was proposed that self-reactive Th1 cells maybe the culprit in promoting the destruction of the myelin sheath. However, the results of experiments using neutralizing anti-IFN-γ antibodies in an experimental autoimmune encephalomyelitis animal model showed otherwise. Injection with anti-IFN-γ antibodies did not ameliorate the symptoms, but actually aggravated the disease. Furthermore, recent reports have found multiple polymorphisms in the genes encoding IFN-γ/IFN-γR in MS patients. The defect in IFN-γ signaling may result in heightened Th1 immune responses due to the failure to generate Th1-specific Treg cells and induce apoptosis in active immune cells. Also, without the inhibition of IFN-γ, Th1 cells will commit to the Th17 lineage. Due to the defect in IFN-γ signaling found in MS patient, the involvement of Th17 cells in the pathogenesis of MS has been suggested (Billiau and Matthys 2009). However, IFN-γ treatment in MS patients often resulted in disease relapse. A recent report suggested the effect of IFN-γ on patients may differ based on the pathogenesis of demyelination. IFN-γ can be detrimental for patients with remyelination of oligodendrocytes (ODCs) but beneficial for those with ODC death (Lees and Cross 2007).

With respect to another autoimmune disease, rheumatoid arthritis (RA), the effect of IFN-γ depends on the stage of disease. RA is characterized by the accumulation of effector T cells that target the synovial membrane, cartilage, and bone. In the collagen-induced arthritis animal model, there was an acceleration of disease progression when treated with IFN-γ in the early phase of disease induction. As expected, the symptoms improved when animals were treated with anti-IFN-γ antibodies; however, symptoms became worse when the antibodies were given during the later stage of the disease (Saha et al. 2010). It is possible that IFN-γ promotes the activity of effector T cells during initiation of disease, and dampens the immune responses during disease progression by inducting apoptosis of effector T cells and generation of Tregs.

Autoimmune diseases are complex and the systems biology that affects disease development and progression is yet fully comprehended. Experimental studies thus far demonstrate that the effects of IFN-γ vary depending upon the types and progression status of disease. To utilize IFN-γ or antibodies to neutralize IFN-γ as a treatment for autoimmune disease, more work is needed to better understand disease pathogenesis.

IFN-γ and Cancer

The effect of IFN-γ on containing tumor progression and growth is well documented. IFN-γ augments the immunogenicity of tumor cells by increasing tumor antigen presentation to tumor-specific T cells and NK cells. IFN-γ also enhances the antitumor activity of T and NK cells. For example, it has been shown that IFN-γ can induce FasL on tumor cells which would promote Fas-dependent apoptosis. Furthermore, IFN-γ

inhibits the growth and proliferation of tumor cells by arresting the cell cycle (Saha et al. 2010). Also, depletion of IFN-γ promotes the growth of chemically induced tumors. However, a recent report suggested that the inflammatory environment driven by IFN-γ signaling may play a role in promoting cancer cell survival, immunoinvasion of the tumor cells, and immunosuppression of the host response in UVB-induced melanoma (Zaidi et al. 2011). The result implies that the effect of IFN-γ on cancer may be dependent on the local tumor environment.

Summary

IFN-γ is an extraordinarily pleiotropic cytokine. It can not only heighten both the innate and adaptive immune response against pathogens and tumors, but also has the ability to maintain immune homeostasis. IFN-γ has such diverse functions because its effect differs based on the cell type and the activation level of the response to receptor triggering. Utilizing IFN-γ for clinical trials in autoimmune disease and cancer therapy has been attempted, albeit with only modest success. The experimental data obtained thus far indicates that one needs to consider the fine balance between pro- and anti-inflammatory effects of IFN-γ when considering an effective clinical use for this important immunoregulatory protein. More than five decades after the discovery of IFN-γ, the complexity of the effects of IFN-γ has yet to be fully appreciated. Further investigation into the interactions among the different signal transduction pathways affected by IFN-γ will help to map out the network from a systems biology perspective. This knowledge will be beneficial in understanding the pathogenesis of multiple disease states, and will result in the identification of the appropriate clinical intervention strategy for either administering or neutralizing the effects of this key immunoregulatory protein.

Conflict of interest disclosure: The authors declare no competing financial interests.

References

Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. Annu Rev Immunol. 1997;15:563–91.

Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol. 2007; 8(4):345–50.

Billiau A. A brief history of interferon's trajectory to clinical application, and personal reminiscences of a large-scale human interferon production initiative. Verh K Acad Geneeskd Belg. 2009;71(1–2):15–42.

Billiau A, Matthys P. Interferon-gamma: a historical perspective. Cytokine Growth Factor Rev. 2009;20(2):97–113.

Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol. 2008;19(4):414–22.

Gough DJ, Levy DE, Johnstone RW, Clarke CJ. IFNgamma signaling-does it mean JAK-STAT? Cytokine Growth Factor Rev. 2008;19(5–6):383–94.

Hodge DL, Martinez A, Julias JG, Taylor LS, Young HA. Regulation of nuclear gamma interferon gene expression by interleukin 12 (IL-12) and IL-2 represents a novel form of posttranscriptional control. Mol Cell Biol. 2002; 22(6):1742–53.

Hu X, Chakravarty SD, Ivashkiv LB. Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. Immunol Rev. 2008;226:41–56.

Isaacs A, Lindernmann J. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci. 1957;147(927):258–67.

Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat Immunol. 2009;10(6):595–602.

Lees JR, Cross AH. A little stress is good: IFN-gamma, demyelination, and multiple sclerosis. J Clin Invest. 2007;117(2):297–9.

Mozes E, Sharabi A. A novel tolerogenic peptide, hCDR1, for the specific treatment of systemic lupus erythematosus. Autoimmun Rev. 2010;10(1):22–6.

Regis G, Conti L, Boselli D, Novelli F. IFNgammaR2 trafficking tunes IFNgamma-STAT1 signaling in T lymphocytes. Trends Immunol. 2006;27(2):96–101.

Saha B, Jyothi PS, Chandrasekar B, Nandi D. Gene modulation and immunoregulatory roles of interferon gamma. Cytokine. 2010;50(1):1–14.

Savan R, Ravichandran S, Collins JR, Sakai M, Young HA. Structural conservation of interferon gamma among vertebrates. Cytokine Growth Factor Rev. 2009;20(2):115–24.

Savitsky D, Tamura T, Yanai H, Taniguchi T. Regulation of immunity and oncogenesis by the IRF transcription factor family. Cancer Immunol Immunother. 2010; 59(4):489–510.

Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol. 2007;96:41–101.

Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004;75(2):163–89.

Wheelock EF. Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. Science. 1965;149(3681):310–1.
Zaidi MR, Davis S, Noonan FP, Graff-Cherry C, Hawley TS, Walker RL, et al. Interferon-gamma links ultraviolet radiation to melanomagenesis in mice. Nature. 2011;469(7331):548–53.

Interferon-Inducible eIF2α Kinase

▶ PKR

Interferon-Inducible RNA-Dependent Protein Kinase

▶ PKR

Interleukin-1 Signal Transducer

▶ TRAF6

Interleukin-7

▶ IL7

Intersectin

▶ ITSN

Ionotropic Glutamate Receptors (AMPA, Kainate and NMDA Receptors)

▶ Glutamate Receptors

IP-10

▶ CXCL10

IP3 Receptor–Associated cGMP Kinase Substrate A

▶ Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

IP_3 Receptors

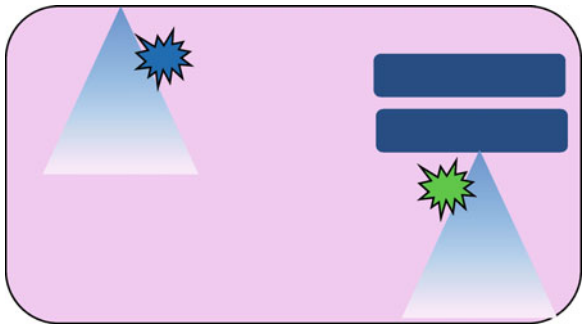
Colin W. Taylor
Department of Pharmacology, University of Cambridge, Cambridge, UK

Historical Background

All eukaryotic cells extrude Ca^{2+} from their cytosol. They have probably done so since the evolution of a phosphate-based energy economy, which preceded the appearance of eukaryotes, demanded a low cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) to avoid precipitation of calcium phosphate within the cell. The result of these selective pressures is that today, the free cytosolic Ca^{2+} concentration in most eukaryotic cells is about 100 nM. It is important to note the reference to *free* cytosolic $[Ca^{2+}]$ because up to 99% of the Ca^{2+} present in the cytosol is not free but bound to Ca^{2+} buffers. Aside from massively reducing the $[Ca^{2+}]_c$, these ubiquitous Ca^{2+} buffers also slow the rate at which Ca^{2+} diffuses in the cytosol. This occurs because for ~99% of the time each Ca^{2+} ion is bound to a large molecule (typically proteins like calmodulin), and big molecules diffuse more slowly than small ones. Slow diffusion of cytosolic Ca^{2+} is, as we shall see, a key feature without which spatially organized Ca^{2+} signaling would be impossible. Buffering alone is not enough to maintain the low $[Ca^{2+}]_c$; active extrusion of Ca^{2+} from the cytosol across the plasma membrane, the membranes of the endoplasmic reticulum (ER), and, to a lesser extent across the membranes of other intracellular organelles (secretory vesicles, Golgi, lysosomes and mitochondria, for example) is also required (Berridge et al. 2003). These active transport processes, mediated by both Ca^{2+} pumps and ion exchangers, ensure that every eukaryotic cell maintains steep $[Ca^{2+}]$ gradients across at least the plasma membrane and the membranes of the ER. Most

extracellular stimuli that evoke Ca^{2+} signals do so by opening Ca^{2+}-permeable channels in one or other of these membranes, allowing Ca^{2+} to flow rapidly down its concentration gradient into the cytosol. Slow diffusion of Ca^{2+} away from the mouths of these open Ca^{2+} channels ensures that local elevations of $[Ca^{2+}]_c$ can persist around them for long enough to be decoded by intracellular proteins. An important consequence is that Ca^{2+}-binding proteins in close proximity to one Ca^{2+} channel may be activated when it opens, while different proteins may be activated by other Ca^{2+} channels. Ca^{2+} entering the cell through different Ca^{2+} channels can thereby selectively regulate different processes (Berridge et al. 2003; Rizzuto and Pozzan 2006) (Fig. 1). This spatial organization of Ca^{2+} signals adds enormously to their versatility. Before turning to the details of these Ca^{2+} channels, it is worth considering why cells should have evolved signaling pathways that regulate Ca^{2+} fluxes across both the plasma membrane and membranes of the ER.

Many Ca^{2+}-permeable channels in the plasma membrane include within their oligomeric structures everything that is required to respond to an extracellular stimulus, a chemical messenger or a change in membrane potential, for example. These channels, and there is an enormous diversity of them, are the simplest and, presumably, the most ancient means of linking extracellular stimuli to changes in $[Ca^{2+}]_c$. Why then should cells also have evolved pathways that allow release of Ca^{2+} form intracellular stores? There are several, inevitably speculative, possibilities. First, it is less expensive to pump Ca^{2+} across the ER membrane (2 Ca^{2+} per ATP hydrolyzed), where there is a concentration gradient but no electrical gradient, than it is to pump Ca^{2+} across the plasma membrane against an electrical and concentration gradient (1 Ca^{2+} per ATP). A Ca^{2+} signal evoked by releasing Ca^{2+} from the ER is, therefore, cheaper than that evoked by Ca^{2+} entry across the plasma membrane. Second, the ER invades most of the cell and provides more abundant opportunities to deliver Ca^{2+} locally to specific intracellular targets. Third, because luminal Ca^{2+} within the ER is itself an important regulator of protein synthesis and folding, it seems likely that selective pressures had provided the machinery to maintain a stable source of Ca^{2+} within the ER before it was exploited as a reliable source of an intracellular messenger. Whatever the evolutionary history, it is clear that the ER is now the major intracellular source

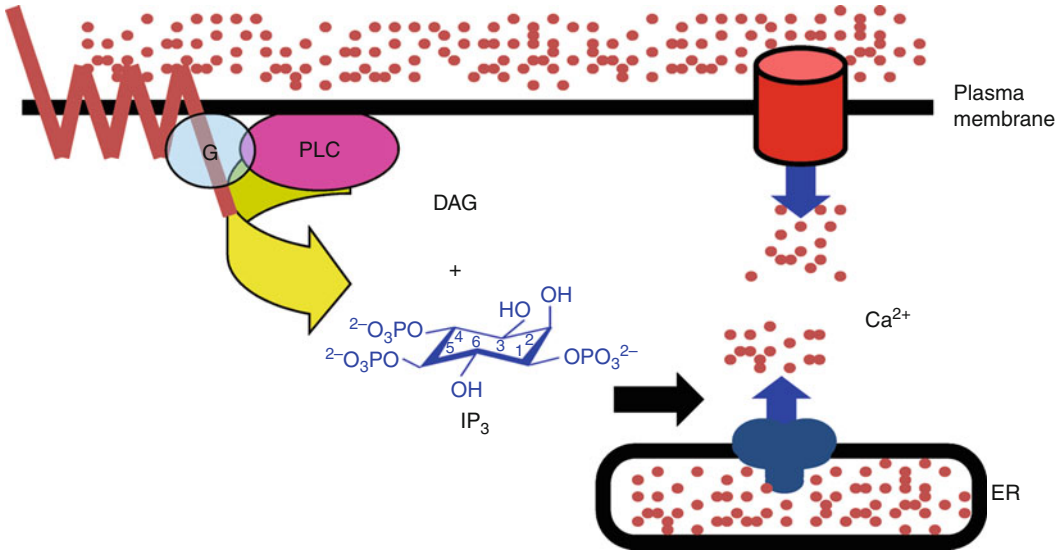


IP_3 Receptors, Fig. 1 Local decoding of Ca^{2+} signals. Because Ca^{2+} diffuses slowly in the cytosol, Ca^{2+} gradients persist around the mouths of open Ca^{2+} channels. Ca^{2+}-binding proteins positioned within these gradients can then respond selectively to opening of specific Ca^{2+} channels in, for example, the plasma membrane (*blue*) or ER (*green*)

of Ca^{2+} signals, although accumulating evidence suggests that the Golgi apparatus, secretory vesicles, the nuclear envelope, and lysosomes may also play more specialized roles. The inositol 1,4,5-trisphosphate receptors (IP_3R) that are the focus of this section are expressed primarily in the ER but occur also in some other intracellular organelles (e.g., Golgi apparatus, secretory vesicles, nuclear envelope) and even in the plasma membrane of some cells (Dellis et al. 2006). Although the diversity of intracellular Ca^{2+} channels does not approach that of Ca^{2+}-permeable channels in the plasma membrane, IP_3R are not the only intracellular Ca^{2+} channels. After IP_3R, their relatives, the ▶ ryanodine receptors, are probably the most abundant intracellular Ca^{2+} channels. Other intracellular Ca^{2+} channels that can mediate release of Ca^{2+} into the cytosol include those that respond to ▶ sphingosine-1-phosphate; the Ca^{2+}-activated channel, ▶ polycystin-2, and other transient receptor potential (▶ TRP) channels; and the two-pore channels (TPC) in acidic organelles that respond to NAADP (Patel et al. 2010). The interactions between IP_3R and some of these channels are considered later, but first it is instructive to recount briefly the discovery of IP_3 and its receptors.

Discovery of IP_3 and Its Receptors

Bob Michell, in a prescient review (Michell 1975), was the first person to propose a causal link between the ability of some receptors to stimulate hydrolysis of a minor class of membrane lipids, the phosphoinositides, and their ability to cause an increase in



IP_3 Receptors, Fig. 2 IP_3 links cell-surface receptors to release of Ca^{2+} from the ER. Receptors, like the G-protein-coupled receptor shown in the figure, stimulate PLC and, thereby, hydrolysis of phosphatidylinositol 4,5-bisphosphate into DAG and IP_3; both are important intracellular messengers. IP_3, via its receptor in the ER, stimulates release of intracellular Ca^{2+} stores, but it may also (directly or indirectly) stimulate Ca^{2+} entry across the plasma membrane (see text for details)

$[Ca^{2+}]_c$. At this stage, the thinking was that hydrolysis of phosphoinositides by phospholipase C (PLC) was linked directly to stimulation of Ca^{2+} entry across the plasma membrane, but as the story unfolded, it became clear that the link was with release of Ca^{2+} from intracellular stores mediated by IP_3 acting at its receptor. We know now that many extracellular stimuli acting via either G protein-coupled receptors or those with intrinsic ▶ protein tyrosine kinase activity stimulate PLCs, and these then catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce IP_3 and diacylglycerol (DAG) (Fig. 2). The signaling pathway is intriguing because the substrate (PIP_2) and both products of its hydrolysis (DAG and IP_3) are each important regulators of cellular activity. IP_3 is the soluble messenger that links the initial events at the plasma membrane to release of Ca^{2+} from intracellular stores. The salivary gland of the blowfly, in the hands of Mike Berridge, played a prominent role in the development of our present understanding. The blowfly spits digestive enzymes onto its food, and to do so quickly (and avoid being swatted!), its salivary glands must sustain impressive rates of fluid secretion. ▶ 5-hydroxytryptamine (5-HT) evokes this secretion via its ability to stimulate both formation of cyclic AMP (another important intracellular messenger) and an increase in $[Ca^{2+}]_c$. Berridge showed in the early 1980s that the *first* water-soluble product of 5-HT-stimulated PLC activity was IP_3, that IP_3 formation *preceded* the increase in $[Ca^{2+}]_c$, and that the formation of IP_3 was *required* for the Ca^{2+} signaling. Only later, and informed by these results from the uniquely favorable experimental attributes of the insect glands, were the findings extended to mammalian cells. That the seemingly arcane studies of a tiny gland from the common blowfly should have paved the way for unraveling a ubiquitous signaling pathway in eukaryotic cells illustrates how the most profound discoveries can come from the most unexpected places. The decisive experiment came in 1983, when IP_3, the first water-soluble product of PLC, was shown to release Ca^{2+} from non-mitochondrial intracellular Ca^{2+} stores of mammalian cells (Streb et al. 1983). Within months, the work had been repeated in countless cell types and, before long, it was in all the standard textbooks: IP_3 is the intracellular messenger that links many cell-surface receptors to release of Ca^{2+} from intracellular stores (Fig. 2).

Ca^{2+} Signals Evoked by IP_3 Receptors

IP_3R are the channels via which IP_3 regulates Ca^{2+} signals in animal cells, and they do so by controlling

both Ca^{2+} release and Ca^{2+} entry across the plasma membrane. Most IP_3R are expressed in the membranes of the ER, where they directly mediate Ca^{2+} release. In most cells, loss of Ca^{2+} from the ER then stimulates store-operated Ca^{2+} entry. Here, loss of Ca^{2+} from the luminal EF-hand of the STIM protein allows it to activate a Ca^{2+}-permeable channel, formed by Orai proteins, in the plasma membrane (Lewis 2007). In some cells, IP_3R can also more directly regulate Ca^{2+} entry either because the IP_3R is itself expressed in the plasma membrane (Dellis et al. 2006) or through interactions of intracellular IP_3R with other plasma membrane Ca^{2+} channels. A key point is that only rarely are release of Ca^{2+} from intracellular stores and Ca^{2+} entry entirely independent processes, more often complex interactions between Ca^{2+} channels in both membranes allow both sources of Ca^{2+} to contribute to the increase in $[Ca^{2+}]_c$. In this context, an important feature of many Ca^{2+} channels, including IP_3R, is that they are both Ca^{2+}-permeable and Ca^{2+}-regulated. IP_3R, for example, are rapidly stimulated by modest increases in $[Ca^{2+}]_c$ and more slowly inhibited by larger increases. The feedback interactions that this Ca^{2+} regulation provides allow regenerative propagation of Ca^{2+} signals by IP_3R, and it allows Ca^{2+} signals evoked by other Ca^{2+} channels to stimulate IP_3R. The interplay between different Ca^{2+} channels is an important and recurrent theme in Ca^{2+} signaling.

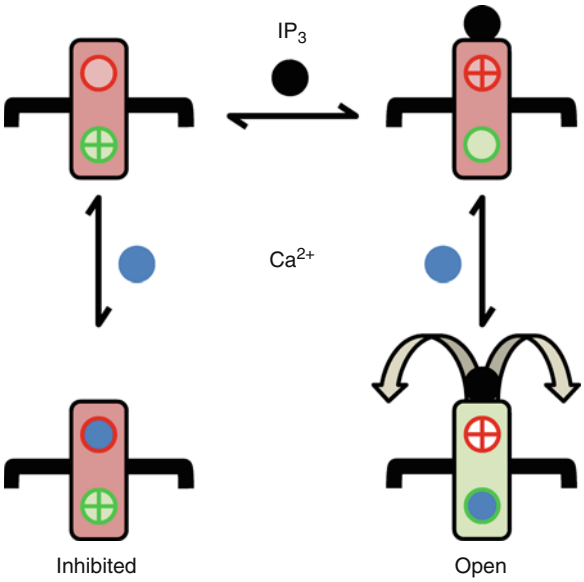
Optical imaging, using confocal or total internal reflection fluorescence (TIRF) microscopy, of cells loaded with fluorescent Ca^{2+} indicators has been used to resolve the openings of individual IP_3R in intact cells and the growth of these tiny Ca^{2+} signals into larger ones involving larger numbers of IP_3R (Smith et al. 2009). As the intracellular IP_3 concentration increases, it triggers a hierarchy of elementary Ca^{2+} release events, beginning with the openings of single IP_3R ("Ca^{2+} blips"), progressing to the coordinated openings of a cluster of several IP_3R ("Ca^{2+} puffs") and, finally, with sufficient IP_3, culminating in a regenerative Ca^{2+} wave invading the entire cell (Bootman et al. 1997) (Fig. 3). Current thinking, derived from both experimental and theoretical analyses, is that recruitment of these Ca^{2+} release events is mediated by the positive feedback provided by Ca^{2+} released by IP_3R. This conclusion focuses our attention on understanding how IP_3R are regulated by Ca^{2+} and IP_3, and on the processes that appropriately position IP_3R in ER membranes to allow interactions between them.

Regulation of IP_3 Receptors by IP_3 and Ca^{2+}

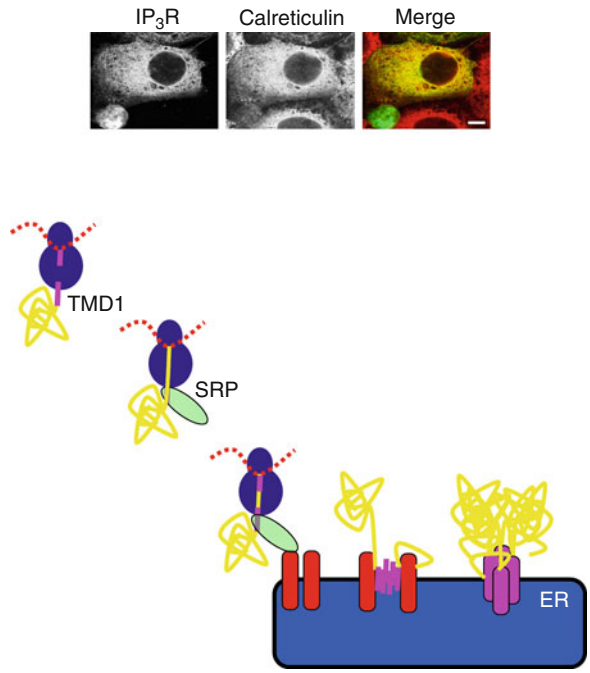
All IP_3R require both IP_3 and Ca^{2+} for their activation (Foskett et al. 2007). The consensus is that binding of IP_3 to the IP_3R is essential to initiate its activation. It is not, however, entirely clear whether all four subunits of the tetrameric IP_3R must bind IP_3. The effects of Ca^{2+} on IP_3R activity are probably mediated by two distinct Ca^{2+}-binding sites: one site promotes opening of the channel, and the other is inhibitory. Although details of the interactions between IP_3 binding and these Ca^{2+}-binding sites are not resolved, our use of methods that allow rates of Ca^{2+} release to be measured with very rapid temporal resolution (Adkins and Taylor 1999) provided a simple scheme that is consistent with most experimental observations. The essence of the scheme is that IP_3 controls IP_3R activation by regulating whether the stimulatory or inhibitory Ca^{2+}-binding site is accessible. IP_3 binding causes the inhibitory Ca^{2+}-binding site to become inaccessible, while simultaneously exposing the stimulatory Ca^{2+}-binding site. Binding of Ca^{2+} to the latter directly promotes channel opening. Others have presented more elaborate schemes (Foskett et al. 2007), but whatever the details, the key point is that Ca^{2+} ultimately controls gating of IP_3R; IP_3 works by priming IP_3R to respond to Ca^{2+} (Fig. 3).

Tuning of the Ca^{2+} regulation of IP_3R by IP_3 has important physiological consequences. It allows, IP_3 to set the gain on the regenerative propagation of Ca^{2+} signals by IP_3R. There is an apt analogy with a spreading bushfire: IP_3 can be envisaged as drying the tinder, while Ca^{2+} provides the spark. Dual regulation of IP_3R by IP_3 and Ca^{2+} may also allow IP_3R to behave as molecular coincidence detectors, opening only when IP_3 is provided by one signaling pathway (any of the receptors that stimulate PLC) and Ca^{2+} by another (Berridge 1998). The latter from either intracellular Ca^{2+} channels, like polycystin 2 or TPC2, or by Ca^{2+} channels in the plasma membrane.

Ca^{2+} and IP_3 are the essential regulators of IP_3R gating, but there are two additional important physiological influences. First, other organelles, most notably mitochondria closely abutting the ER, my rapidly sequester Ca^{2+} released by IP_3R and thereby modulate the relationship between released Ca^{2+} and its feedback effects on nearby IP_3R (Rizzuto et al. 2004). Second, many additional intracellular signals (like ATP, cyclic AMP, and calmodulin) or proteins,



IP_3 Receptors, Fig. 3 IP_3 activates IP_3 receptors by controlling Ca^{2+} binding. Binding of IP_3 (*black circle*) to the IP_3R determines whether a stimulatory (*green*) or inhibitory (*red*) Ca^{2+}-binding site is accessible. IP_3 binding causes the stimulatory site to become accessible and the inhibitory site to become hidden. Binding of Ca^{2+} (*blue circle*) to the former then triggers opening of the pore



IP_3 Receptors, Fig. 4 Co-translational targeting of IP_3 receptors to the ER. The upper panel shows confocal images of cells expressing EGFP-IP_3R1 and immunostained for calreticulin (a luminal ER protein). The colored overlay shows that IP_3R1 is expressed in the ER. Recognition of a signal sequence in TMD1 of IP_3R1 by SRP targets it to the ER membrane, where translation continues (see text for details)

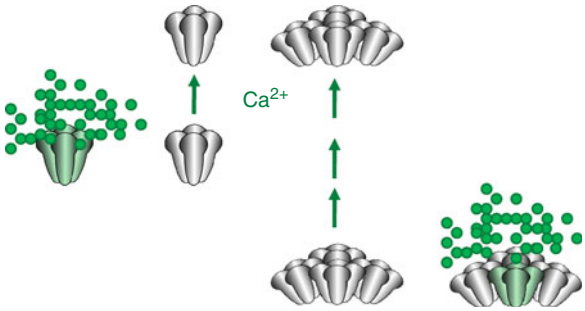
including protein kinases, also associate with IP_3R and modulate their sensitivity to IP_3, Ca^{2+}, or the links between their binding and opening of the pore. These interactions further endow IP_3R with an ability to process information arising from diverse signaling pathways and transduce it into an intracellular Ca^{2+} signal.

Putting IP_3 Receptors in the Right Place

IP_3R, like most integral ER membrane proteins, are co-translationally targeted to the ER (Fig. 4). This begins when the signal recognition particle (SRP) binds hydrophobic residues within a signal sequence on the nascent peptide chain. For IP_3R, the signal sequence lies within the first of the six transmembrane domains (TMD) of each subunit (Pantazaka and Taylor 2010). The SRP-ribosome-nascent chain complex is recognized by the SRP receptor in the ER membrane, allowing the growing protein to be threaded into a channel in the ER membrane, the translocon. The hydrophobic TMD initially remain within the translocon, while intervening hydrophilic loops are pushed into the cytosol or ER lumen. By this stage, the entire cytosolic N-terminal region of the IP_3R has folded. This is important in determining the final topology of the IP_3R because the massive N-terminal is too large to enter the translocon and so remains cytosolic and, thereby, dictates the orientation of successive TMD across the ER membrane. As remaining TMD are synthesized, they co-assemble and then pass through the wall of the translocon into the ER membrane (Fig. 4). A minority of IP_3R moves onward from the ER to the Golgi apparatus, secretory vesicles, or plasma membrane, but most remain within the ER. Pairs of TMD (TMD1-2, TMD3-4, or TMD5-6) provide ER retention or retrieval signals. There are, therefore, many effective signals working to keep IP_3R within the ER. This provides a satisfying explanation for the observation that most IP_3R are expressed in the ER but begs the question of how those IP_3R that do progress to later membrane compartments succeed in overcoming these powerful signals. Although the details lie beyond the scope of this review, IP_3R can

also be selectively directed to specific parts of the ER network: to one end of a polarized cell, for example, or become associated specifically with cell-surface receptors (via interaction with ▶ Homer, for example) or caveolae.

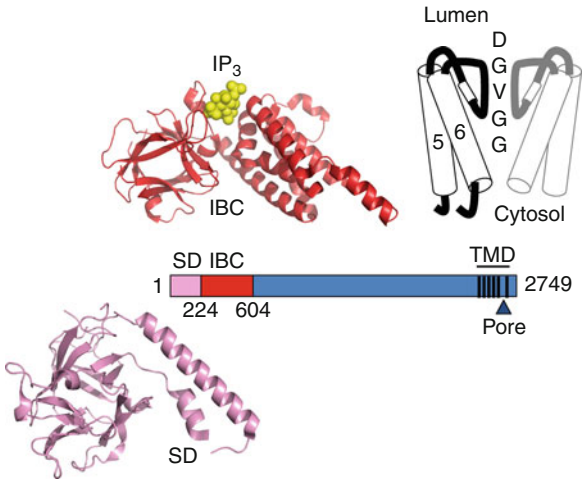
Fluorescence recovery after photobleaching (FRAP) studies have shown that most IP_3R within the ER are mobile. Our studies of IP_3R in the nuclear envelope, where they are accessible to electrophysiological analyses, has shown that IP_3 causes these mobile IP_3R rapidly and reversibly to assemble into small clusters of a few IP_3R (Rahman et al. 2009). Within these clusters, regulation of IP_3R by IP_3 and Ca^{2+} is retuned: lone and clustered IP_3 respond differently to IP_3 and Ca^{2+}. The functional significance of this clustering is twofold. First, it brings IP_3R together so that instead of being insulated from each others' activities by high concentrations of cytosolic Ca^{2+} buffers, they can immediately sense the Ca^{2+} released by a neighbor. Second, clustering at resting $[Ca^{2+}]_c$ reduces the sensitivity of IP_3R to IP_3, but this then reverses when $[Ca^{2+}]_c$ increases. Both effects serve to coordinate the openings of small groups of IP_3R by exaggerating the stimulatory effect of locally released Ca^{2+} (Rahman et al. 2009) (Fig. 5). We suggest, although others argue otherwise (Smith and Parker 2009), that IP_3, in addition to regulating opening of IP_3R, plays an essential role in assembling the small clusters of IP_3R responsible for the "Ca^{2+} puffs" observed in intact cells.



IP_3 Receptors, Fig. 5 IP_3-evoked clustering of IP_3R exaggerates the coordinating effect of Ca^{2+}. IP_3 causes IP_3R to form small clusters of about 4–5 IP_3R within which they become less sensitive to IP_3, but more susceptible to regulation by the Ca^{2+} released by a neighbor. Increased $[Ca^{2+}]_c$ reverses the inhibition caused by clustering and so increases the dynamic range for Ca^{2+} regulation (see text for details)

Structural Determinants of IP_3 Receptor Behavior

In vertebrates, three genes encode closely related IP_3R subunits, while invertebrates express only a single subtype. These subunits are amongst the largest of all ion-channel subunits, each comprising about 2,700 amino acid residues. All IP_3R are tetrameric assemblies of either the same or different subunits, each predicted to form a similar structure. Each subunit comprises three major regions: an N-terminal region to which IP_3 binds, the C-terminal portion with its six transmembrane regions (TMD), and a very large intervening sequence (Fig. 6). The outstanding problem is to explain how IP_3 binding leads to opening of the pore. We have some way to go before that explanation is fully in place.



IP_3 Receptors, Fig. 6 Major structural domains of the IP_3 receptor. The three key regions defined by the primary sequence of a single IP_3R subunit are highlighted: the N-terminal with its SD and IBC, the C-terminal region with its 6 TMD and the pore, and the large central region. Atomic structures of the SD and IBC with IP_3 bound are also shown. A possible structure of the IP_3R pore is shown with its luminal selectivity filter and a constriction formed by the tepee-like structure of TMD6. Only two of the four IP_3R subunits are shown

Several groups have published 3D structures of the entire IP_3R, all derived from rebuilding the structure from electron microscopy images of single IP_3R, but the resolution of these images is presently too low to provide much insight into the workings of IP_3R (Taylor et al. 2004). Instead, much of what we know

has come from analysis of mutant IP_3R, from single-channel analyses using patch-clamp methods and from high-resolution structures of fragments of IP_3R.

The pore of the IP_3R allows the selective passage of cations, although it discriminates only weakly between Ca^{2+} and K^+. Despite the lack of selectivity, IP_3R within the ER are likely to conduct mainly Ca^{2+} because only Ca^{2+} has an appreciable gradient across the ER membrane. The pore of the IP_3R is formed by the final pair of TMD (TMD5-6) and the luminal loop that links them from each of the four subunits (Fig. 6). The loop includes a sequence (GGVGD in IP_3R) similar to that of the selectivity filter of K^+ channels, consistent with the idea that the overall architecture of the pore region may be broadly similar to that of K^+ channels. This idea is consistent with mutagenesis of residues within this region affecting ion permeation, and with biophysical and structural analyses of the equivalent region of ryanodine receptors.

We know more about the IP_3-binding site. IP_3 binds to the so-called "IP_3-binding core" (IBC, residues 224–604) and a high-resolution structure shows IP_3 cradled within a clam-like structure in which the negatively charged phosphate groups of IP_3 are coordinated by basic residues (Bosanac et al. 2002) (Fig. 6). The two sides of the clam, the α- and β-domains, interact with the two essential 4- and 5-phosphate groups of IP_3, suggesting perhaps that IP_3 might pull the α- and β-domains together, causing the clam to close. We can envisage, therefore, that the first step in IP_3R activation requires IP_3 binding to the IBC to effectively cross-link its two domains and thereby initiate the processes that lead to opening of the pore. As discussed above, there are essential interactions between IP_3 and Ca^{2+} binding, with IP_3 proposed to reciprocally regulate the accessibility of stimulatory and inhibitory Ca^{2+}-binding sites, but the structural basis of these interactions is entirely unresolved. We do, however, have a high-resolution structure of the N-terminal of the IP_3R, the so-called "suppressor domain" (SD, residues 1–223) (Bosanac et al. 2005) (Fig. 6), and it is known to be essential for coupling IP_3 binding to opening of the pore (Rossi et al. 2009). We have shown that the conformational changes initiated by IP_3 binding to the IBC must pass entirely via the SD on their way to the pore (Rossi et al. 2009), but we can only speculate on how the SD might then communicate with either Ca^{2+}-binding sites or the pore. In the simplest of plausible, but speculative, schemes, we have suggested that the essential steps in IP_3R activation begin with IP_3 binding to the IBC, closure of the clam then initiates conformational changes that pass entirely via the SD. The SD may then directly contact a cytosolic helix linking TMD4 to TMD5 and so prise the pore open. Further work is urgently needed both to test and elaborate this, almost certainly over-simple, scheme.

Summary

IP_3 receptors are intracellular Ca^{2+} channels expressed in the membranes of the endoplasmic reticulum of all animal cells, where they mediate Ca^{2+} release in response to the many extracellular stimuli that evoke IP_3 formation. IP_3 receptors both release Ca^{2+} and respond to it, and this allows them to both initiate and regeneratively propagate intracellular Ca^{2+} signals. The resulting spatiotemporal complexity of the cytosolic Ca^{2+} signals underlies the versatility of Ca^{2+} as an intracellular messenger. Future challenges include resolving the means, whereby Ca^{2+} regulates IP_3 receptors and the processes that target IP_3 receptors to specific subcellular locations.

References

Adkins CE, Taylor CW. Lateral inhibition of inositol 1,4,5-trisphosphate receptors by cytosolic Ca^{2+}. Curr Biol. 1999;9:1115–8.

Berridge MJ. Neuronal calcium signaling. Neuron. 1998; 21:13–26.

Berridge MJ, Bootman MD, Roderick HL. Calcium signaling: dynamics, homeostasis and remodeling. Nat Rev Mol Cell Biol. 2003;4:517–29.

Bootman MD, Berridge MJ, Lipp P. Cooking with calcium: the recipes for composing global signals from elementary events. Cell. 1997;91:367–73.

Bosanac I, Alattia J-R, Mal TK, Chan J, Talarico S, Tong FK, Tong KI, Yoshikawa F, Furuichi T, Iwai M, Michikawa T, Mikoshiba K, Ikura M. Structure of the inositol 1,4,5-trisphosphate receptor binding core in complex with its ligand. Nature. 2002;420:696–700.

Bosanac I, Yamazaki H, Matsu-ura T, Michikawa M, Mikoshiba K, Ikura M. Crystal structure of the ligand binding suppressor domain of type 1 inositol 1,4,5-trisphosphate receptor. Mol Cell. 2005;17:193–203.

Dellis O, Dedos S, Tovey SC, Rahman TU, Dubel SJ, Taylor CW. Ca^{2+} entry through plasma membrane IP_3 receptors. Science. 2006;313:229–33.

Foskett JK, White C, Cheung KH, Mak DO. Inositol trisphosphate receptor Ca^{2+} release channels. Physiol Rev. 2007;87:593–658.

Lewis RS. The molecular choreography of a store-operated calcium channel. Nature. 2007;446(7133):284–7.
Michell RH. Inositol phospholipids and cell surface receptor function. Biochim Biophys Acta. 1975;415:81–147.
Pantazaka E, Taylor CW. Targeting of inositol 1,4,5-trisphosphate receptor to the endoplasmic reticulum by its first transmembrane domain. Biochem J. 2010;425:61–9.
Patel S, Marchant JS, Brailoiu E. Two-pore channels: regulation by NAADP and customized roles in triggering calcium signals. Cell Calcium. 2010;47:480–90.
Rahman TU, Skupin A, Falcke M, Taylor CW. Clustering of IP_3 receptors by IP_3 retunes their regulation by IP_3 and Ca^{2+}. Nature. 2009;458:655–9.
Rizzuto R, Pozzan T. Microdomains of intracellular Ca^{2+}: molecular determinants and functional consequences. Physiol Rev. 2006;86(1):369–408.
Rizzuto R, Duchen MR, Pozzan T. Flirting in little spce: the ER/mitochondria Ca^{2+} liaison. Sci STKE. 2004;215:re1.
Rossi AM, Riley AM, Tovey SC, Rahman T, Dellis O, Taylor EJA, Veresov VG, Potter BVL, Taylor CW. Synthetic partial agonists reveal key steps in IP_3 receptor activation. Nat Chem Biol. 2009;5:631–9.
Smith IF, Parker I. Imaging the quantal substructure of single IP_3R channel activity during Ca^{2+} puffs in intact mammalian cells. Proc Natl Acad Sci USA. 2009;106:6404–9.
Smith IF, Wiltgen SM, Shuai J, Parker I. Ca^{2+} puffs originate from preestablished stable clusters of inositol trisphosphate receptors. Sci Signal. 2009;2(98):ra77.
Streb H, Irvine RF, Berridge MJ, Schulz I. Release of Ca^{2+} from a nonmitochondrial store of pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature. 1983;306:67–9.
Taylor CW, da Fonseca PCA, Morris EP. IP_3 receptors: the search for structure. Trends Biochem Sci. 2004;29:210–9.

iPLA2

▶ Phospholipase A_2

IRAG

▶ Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

Iraga

▶ Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

IRF5

Betsy J. Barnes
Department of Biochemistry and Molecular Biology, New Jersey Medical School-University Hospital Cancer Center, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA

Synonyms

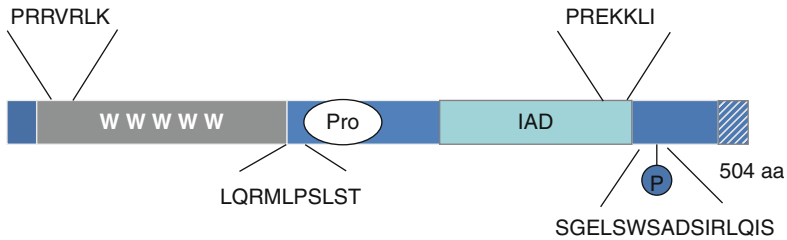
Interferon regulatory factor 5, IRF5

Historical Background

The transcription factor interferon regulatory factor 5 (IRF5) is one of the newer members of the IRF family to be characterized. All cellular family members share a region of homology in the amino-terminus, encompassing a highly conserved DNA binding domain consisting of five tryptophan repeats. By crystallography, this region has been shown to bind to conserved elements, termed "interferon (IFN)-stimulated response elements" (ISREs), in the promoters of target genes (Chen et al. 2008) thereby exerting the biological effects of IRF5. Given the nomenclature of this family, it is not surprising that the first function of IRF5 to be recognized was its ability to regulate type I *IFN* gene expression (Barnes et al. 2001). However, unlike other IRF family members, such as IRF3 and IRF7, the activity of IRF5 is regulated in a virus-specific manner leading to the induction of distinct *IFNA* genes (Barnes et al. 2001).

Regulation of IRF5 Biological Function

IRF5 is expressed primarily in human lymphoid tissues including the spleen, lymph nodes, peripheral blood lymphocytes, and bone marrow; low levels have been detected in the thymus and skeletal muscle (Barnes et al. 2001). High levels of IRF5 are constitutively expressed in purified immune cell subpopulations of activated B cells, natural killer cells, monocytes, plasmacytoid dendritic cells (PDC), and monocyte-derived dendritic cells (MDDC), suggesting an important role for IRF5 in the innate immune response



IRF5, Fig. 1 Domain structure of IRF5. Structural and functional domains involved in DNA binding, subcellular localization, posttranslational modification, and interaction with other proteins are shown. DNA binding domain is shown by the *gray box* containing the tryptophan repeat, the *white circle* represents a proline-rich region, the IAD (IRF activation domain) illustrates the protein-interacting domain, the circled P represents the carboxyl-terminal region, where phosphorylation occurs with residues shown below. *Top* two sequences are nuclear localization signals (NLS), *bottom* first sequence (behind the DNA binding domain) represents the nuclear export signal (NES)

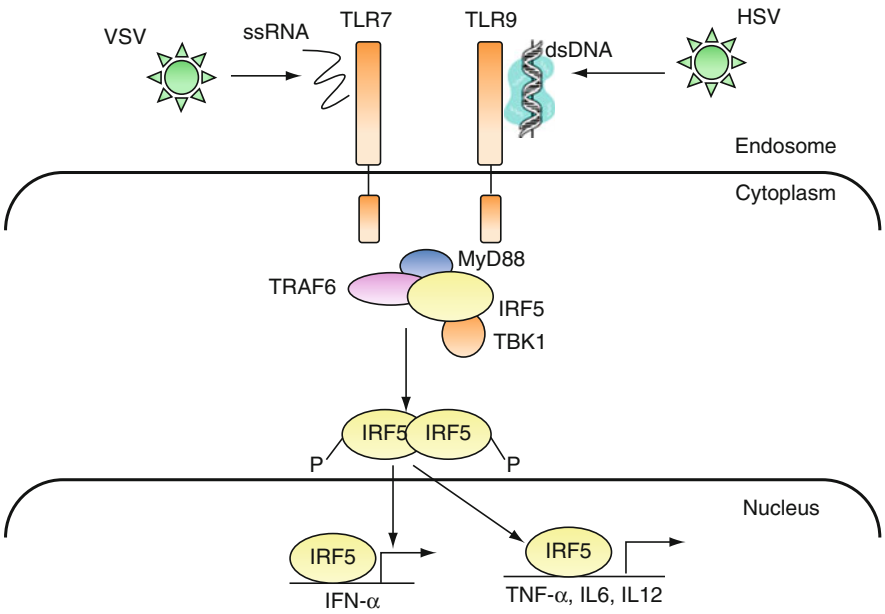
(Mancl et al. 2005). Expression of IRF5 can also be detected in other cell types after stimulation with type I IFN or other inducers.

IRF5 is generally localized to the cytoplasm of a cell in an inactivate state, and undergoes nuclear translocation upon "activation." The exact mechanism of IRF5 nuclear translocation in response to a given stimuli has not been fully elucidated but generally requires post-translational modification and homodimerization or heterodimerization with other IRF family members and proteins (Barnes et al. 2002; Cheng et al. 2006). IRF5 contains two functional nuclear localization signals (NLS), one in the amino-terminus and the other in the carboxyl-terminus of the protein (Barnes et al. 2002). IRF5 also contains a nuclear export signal (NES) that has been shown to regulate the dynamic shuttling of cellular IRF5 between the cytoplasm and the nucleus (Cheng et al. 2006). The molecular pathways leading to IRF5 activation include virus infection (Barnes et al. 2001, 2002), Toll-like receptor (TLR) signaling (Schoenemeyer et al. 2005; Takaoka et al. 2005), DNA damage (Hu et al. 2005), and death receptor signaling (Hu and Barnes 2009). Current data support the phosphorylation of IRF5 within the carboxyl-terminal autoinhibitory domain (Barnes et al. 2002; Chen et al. 2008) resulting in activation. The structural and functional domains of the IRF5 polypeptide are shown in Fig. 1.

Human *IRF5* exists as multiple alternatively spliced variants whose regulation is controlled at least in part by the presence of two functional promoters (Mancl et al. 2005). A number of these splice variants encode for functional polypeptides that have distinct cell type–specific expression, cellular localization, and biological function (Mancl et al. 2005). IRF5 expression may also be subject to regulation by hypermethylation as it contains a large CpG-rich island upstream of these promoters and has been shown to be regulated by hypermethylation in hepatocellular carcinoma tissues (Shin et al. 2010).

Role of IRF5 in the Innate Immune Response

In addition to coordinating the expression of type I IFNs in response to virus infection, IRF5 regulates the expression of a number of other cytokines and chemokines. Virus-infected B cells generated to overexpress human IRF5 were found to express a number of chemokines important for the recruitment of T lymphocytes to sites of inflammation such as RANTES, macrophage inflammatory protein (MIP1α) and monocyte chemoattractant protein 1 (MCP1) (Barnes et al. 2002). Data from microarray analyses further supported these findings and demonstrated a distinct role for IRF5 in the regulation of antiviral and early inflammatory genes (Barnes et al. 2004). Subsequently, IRF5 was shown to be a central mediator of TLR signaling (Schoenemeyer et al. 2005; Takaoka et al. 2005). Members of the TLR family are essential recognition and signaling components of mammalian antiviral host defense. Studies in both humans and mice reveal the critical role that IRF5 plays in the gene induction program activated by TLR4, TLR7, and TLR9 (Schoenemeyer et al. 2005; Takaoka et al. 2005) (Fig. 2). In hematopoietic cells from mice deficient in the *irf5* gene ($irf5^{-/-}$ mice), the induction of cytokines interleukin 6 (IL6), IL12, TNFα, and IFNα by various TLR ligands was severely impaired, along with type I IFNs in response to virus



IRF5, Fig. 2 Role of IRF5 in TLR signaling. IRF5 interacts with MyD88, TRAF6, and TBK1 in response to TLR7 and 9 activation. IRF5 becomes phosphorylated, homodimerizes, and translocates to the nucleus where it induces proinflammatory cytokine expression

infection (Takaoka et al. 2005; Paun et al. 2008). In human dendritic cells, IRF5 was shown to be required for late-phase TNF secretion through its direct and indirect binding to the TNF promoter (Krausgruber et al. 2010). While the mechanism of IRF5-mediated gene induction via virus is not well understood, induction by TLR ligands includes interaction and activation by MyD88, TRAF6 and TBK1 (Takaoka et al. 2005).

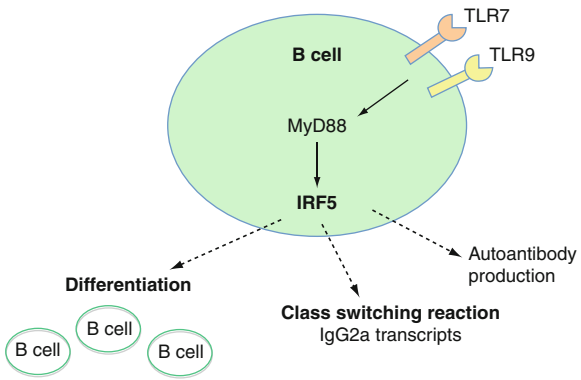
IRF5 as an SLE Susceptibility Gene

Using the genome-wide association approach, multiple laboratories have identified and confirmed *IRF5* gene variants with strong statistical association to SLE susceptibility (Kozyrev and Alarcon-Riquelme 2007). SLE is a complex systemic autoimmune disorder characterized by enhanced IFN production, loss of immune tolerance to self-antigens, persistent production of pathogenic autoantibodies, complement activation, immune complex (IC) deposition, inflammation, and end-organ damage. Identification of the *IRF5* gene in the susceptibility to develop SLE has marked an important breakthrough in the understanding of SLE pathogenesis since it has provided the first evidence that both the type I IFN and TLR signaling pathways are involved in disease pathogenesis. Association has now been convincingly replicated in SLE patients from multiple populations and distinct *IRF5* haplotypes that confer either susceptibility to (risk), or protection from, SLE in persons of varying ethnic ancestry have been identified (Kozyrev and Alarcon-Riquelme 2007). Genetic polymorphisms in the *IRF5* gene are thought to alter *IRF5* expression and/or lead to the expression of several unique isoforms (Kozyrev and Alarcon-Riquelme 2007).

Role of IRF5 in SLE Pathogenesis

IRF5 expression and alternative splicing are significantly upregulated in primary blood cells of SLE patients compared to healthy donors; enhanced transcript and protein levels are associated with an SLE risk haplotype suggesting a functional role for IRF5 in disease pathogenesis (Feng et al. 2010). Important insight on the potential function of IRF5 in SLE was gained by demonstrating an association between serum IFNα activity and *IRF5* risk haplotype in SLE patients (Niewold et al. 2008); however, no direct evidence in humans has shown that the observed upregulation of IRF5 expression in SLE patients causes enhanced IFN production. Recent studies in mice have begun to elucidate how the dysregulation of IRF5 expression, and therefore function, may alter disease pathogenesis. Mice that produce IFNα, IFNβ, and IL6 in response to sera or IgG-RNA IC from lupus patients were shown to be *tlr7*, *irf5*, and *irf7* dependent (Yasuda et al. 2007).

Additional data revealed that *irf5* is required for lupus development and autoantibody production in the $FcγRIIB^{-/-}Yaa$ and $FcγRIIB^{-/-}$ murine lupus models; however, no mechanism was provided (Richez et al. 2010). Subsequently, wild-type ($irf5^{+/+}$) and $irf5^{-/-}$ mice injected with pristane oil, which typically induces development of features characteristic of human SLE, was found to markedly reduce IgG glomerular deposits and antinuclear antibodies, and lack of IgG2a autoantibody secretion in $irf5^{-/-}$mice (Savitsky et al. 2010). Data support that IRF5 is responsible for the secretion of pathogenic IgG2a antibodies through class-switch recombination of the γ2a locus and intimates a critical role for IRF5 in B cell activation and/or responses to pathogens (Savitsky et al. 2010) (Fig. 3).



IRF5, Fig. 3 Role of IRF5 in B cell function associated with lupus pathogenesis. TLR7 and 9 are thought to signal in response to SLE immune complexes resulting in pathogenic autoantibody production. The dysregulation of IRF5 expression in SLE affects autoantibody production by altering B cell differentiation and/or class-switch recombination

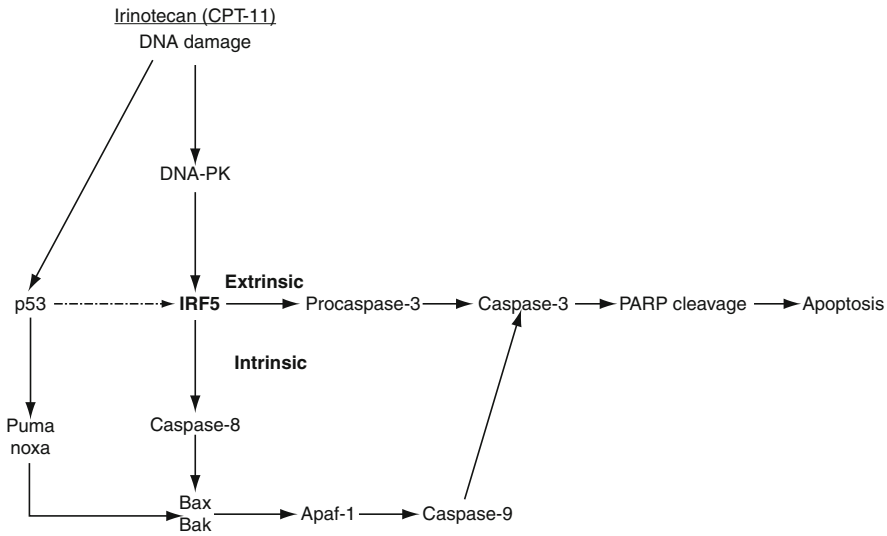
IRF5 in Cancer and as a Mediator of Apoptosis

Several lines of evidence support the notion that *IRF5* is a candidate tumor suppressor gene. IRF5 expression is absent in a variety of immortalized tumor cell lines of hematologic malignancies (Barnes et al. 2001) and was shown to be a direct target of the tumor suppressor p53 (Mori et al. 2002). Analyses of IRF5 expression in primary mononuclear cells from healthy donors and patients with acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), and acute monocytic leukemia (AML) confirmed a loss of IRF5 expression in cancers of hematologic origin (Barnes et al. 2003). Overexpression of IRF5 in a B cell lymphoma lacking functional p53 demonstrated for the first time the ability of IRF5 to recapitulate p53 tumor suppressor function (Barnes et al. 2003). Ectopic overexpression of IRF5 inhibited colony formation on soft agar and in vivo tumor cell growth in athymic nude mice (Mori et al. 2002; Barnes et al. 2003). In addition, IRF5 overexpression induced a G2/M cell cycle arrest and apoptosis by targeting genes with these functions (Barnes et al. 2003). Later studies in p53-deficient tumor cell lines demonstrated that IRF5 could sensitize them to DNA damage–induced apoptosis (Hu et al. 2005). Examination of the molecular mechanism(s) for sensitization revealed that DNA damage activated IRF5 by posttranslational modification resulting in nuclear translocation and induction of specific target genes (Hu et al. 2005). Data in $irf5^{-/-}$ mice have corroborated these findings in human cells demonstrating that loss of *irf5* makes cells resistant to DNA damage–induced apoptosis (Yanai et al. 2007). Furthermore, evidence is provided for its tumor suppressor function since $irf5^{-/-}$ mice are predisposed to tumorigenic transformation (Yanai et al. 2007). While the exact DNA damage–induced signaling pathway that IRF5 mediates is not known, studies in mice and humans support that it is acting on a pathway distinct from p53 (Fig. 4) (Hu et al. 2005; Yanai et al. 2007).

IRF5 has also been shown to regulate apoptosis in response to death receptor ligands Fas and TRAIL (tumor necrosis factor–related apoptosis-inducing ligand) thus supporting its critical role in the apoptotic response (Couzinet et al. 2008; Hu and Barnes 2009). While the mechanism of IRF5-mediated Fas-induced cell death is unknown, but appears to be cell type–specific (Couzinet et al. 2008), the mechanism of IRF5-mediated TRAIL-induced apoptosis has been worked out and supports the mechanism requiring IRF5 activation by post-translational modification and nuclear translocation (Hu and Barnes 2009).

Summary

IRF5 is a critical mediator of the cellular response to extracellular stressors including virus, DNA damage, pathogenic stimuli (i.e., TLR ligands), and death ligands. IRF5 functions downstream of these signaling pathways thereby providing a mechanism of cellular



IRF5, Fig. 4 IRF5 signals independent of p53 in response to DNA damage. DNA-PK is the candidate kinase for IRF5 activation in response to DNA damage induced by the chemotherapeutic agent Irinotecan (CPT-11). IRF5 target genes are shown, in addition to some known p53 target genes

protection in multiple cell types (i.e., immune cells, fibroblasts, epithelial cells). Once activated by post-translational modification, IRF5 translocates to the nucleus where it acts as a transcription factor regulating the expression of genes involved in innate immunity, cell growth regulation, and apoptosis. Given that IRF5 expression has been found to be dysregulated in a variety of cancers (most prominently in hematologic malignancies), combined with the fact that mice lacking *irf5* are susceptible to oncogene-induced tumor transformation and resistant to DNA damage–induced apoptosis, provides convincing support for its role as a new tumor suppressor gene. The fact that IRF5 mediates a DNA damage–induced signaling pathway that is distinct from p53 (Fig. 4) suggests that therapeutic strategies targeting this pathway will be useful for the treatment of p53-deficient cancers. Identifying the kinase(s) responsible for IRF5 activation in response to DNA damage, along with determining the p53-independent and IRF5-dependent signaling pathway induced by DNA damage, will be of critical importance for the design of agents that can upregulate and/or activate this pathway. Additionally, data support the inhibition of IRF5 activation and/or signaling in autoimmune disease. IRF5 expression is significantly upregulated in SLE patients; therefore, the design of therapeutic agents targeting the inhibition of IRF5 signaling in autoimmune diseases should prove beneficial. The ultimate challenge will be finding a balance in turning on and off IRF5 signaling that will not compromise a patients risk for cancer and autoimmune disease.

References

Barnes BJ, Moore PA, Pitha PM. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes. J Biol Chem. 2001;276:23382–90.

Barnes BJ, Kellum MJ, Field AE, Pitha PM. Multiple regulatory domains of IRF-5 control activation, cellular localization and induction of chemokines that mediate T-lymphocyte recruitment. Mol Cell Biol. 2002;22:5721–40.

Barnes BJ, Kellum MJ, Pinder KE, Frisancho JA, Pitha PM. IRF-5, a novel mediator of cell-cycle arrest and cell death. Cancer Res. 2003;63:6424–31.

Barnes BJ, Richards J, Mancl M, Hanash S, Beretta L, Pitha PM. Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection. J Biol Chem. 2004;279:45194–207.

Chen W, Lam SS, Srinath H, Jiang Z, Correia JJ, Schiffer CA, et al. Insights into interferon regulatory factor activation from the crystal structure of dimeric IRF5. Nat Struct Mol Biol. 2008;15:1213–20.

Cheng TF, Brzostek S, Ando O, Van Scoy S, Kumar KP, Reich NC. Differential activation of IFN regulatory factor (IRF)-3 and IRF-5 transcription factors during viral infection. J Immunol. 2006;176:7462–70.

Couzinet A, Tamura K, Chen HM, Nishimura K, Wang Z, Morishita Y, et al. A cell-type-specific requirement for IFN regulatory factor 5 (IRF5) in Fas-induced apoptosis. Proc Natl Acad Sci USA. 2008;105:2556–61.

Feng D, Stone RC, Elonranta ML, Sangster-Guity N, Singh S, Nordmark G, et al. Genetic variants and disease-associated factors contribute to enhanced interferon regulatory factor 5 expression in blood cells of patients with systemic lupus erythematosus. Arthritis Rheum. 2010; 62:562–73.

Hu G, Barnes BJ. IRF-5 is a mediator of the death receptor-induced apoptotic signaling pathway. J Biol Chem. 2009;284:2767–77.

Hu G, Mancl ME, Barnes BJ. Signaling through interferon regulatory factor-5 sensitizes p53-deficient tumor to DNA damage-induced apoptosis and cell death. Cancer Res. 2005;65:7403–12.
Kozyrev SV, Alarcon-Riquelme ME. The genetics and biology of Irf5-mediated signaling in lupus. Autoimmunity. 2007;40:591–601.
Krausgruber T, Saliba D, Ryzhakov G, Lanfrancotti A, Blazek K, Udalova IA. IRFS is required for late-phase TNF secretion by human dendritic cells. Blood. 2010;115:4421–30.
Mancl ME, Hu G, Sangster-Guity N, Olshalsky SL, Hoops K, Fitzgerald-Bocarsly P, et al. Two distinct promoters regulate the alternative-spliced human Interferon regulatory factor-5 variants: multiple variants with distinct cell-type specific expression, localization, regulation and function. J Biol Chem. 2005;280:21078–90.
Mori T, Anazawa Y, Iiizumi M, Fukuda S, Nakamura Y, Arakawa H. Identification of the interferon regulatory factor 5 gene (IRF-5) as a direct target for p53. Oncogene. 2002;21:2914–8.
Niewold TB, Kelly JA, Flesch MH, Espinoza LR, Harley JB, Crow MK. Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients. Arthritis Rheum. 2008;58:2481–7.
Paun A, Reinert JT, Jiang Z, Medin C, Balkhi MY, Fitzgerald KA, Pitha PM. Functional charcterization of murine interferon regulatory factor 5(IRF-5) and its role in the innate antiviral response. J Biol Chem. 2008;283:14295–308.
Richez C, Yasuda K, Bonegio RG, Watkins AA, Aprahamian T, Busto P, et al. IFN regulatory factor 5 is required for disease development in the FcgammaRIIB−/−Yaa and FcgammaRIIB−/− mouse models of systemic lupus erythematosus. J Immunol. 2010;184:796–806.
Savitsky DA, Yanai H, Tamura T, Taniguchi T, Honda K. Contribution of IRF5 in B cells to the development of murine SLE-like disease through its transcriptional control of the IgG2a locus. Proc Natl Acad Sci USA. 2010;107:10154–9.
Schoenemeyer A, Barnes BJ, Mancl ME, Latz E, Goutagny N, Pitha PM, et al. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. J Biol Chem. 2005;280:17005–12.
Shin SH, Kim BH, Jang JJ, Suh KS, Kang GH. Identification of novel methylation markers in hepatocellular carcinoma using a methylation array. J Korean Med Sci. 2010;25:1152–9.
Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature. 2005;434:243–9.
Yanai H, Chen HM, Inuzuka T, Kondo S, Mak TW, Takaoka A, et al. Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression. Proc Natl Acad Sci USA. 2007;104:3402–7.
Yasuda K, Richez C, Maciaszek JW, Agrawal N, Akira S, Marshak-Rothstein A, et al. Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. J Immunol. 2007;178:6876–85.

Isl 1

► LIMK

Itga4

► Integrin Alpha 4 (Itga 4)

ITPK1 (Inositol 1,3,4-Triphosphate 5/6 Kinase)

Yixing Zhou, Tobias M. H. Schenk and Stephen B. Shears
Laboratory of Signal Transduction, Inositol Signaling Section, National Institute of Environmental Health Sciences (NIEHS/NIH), NC, USA

Synonyms

Inositol 3,4,5,6-tetrakisphosphate 1-kinase; Inositol trisphosphate 5/6-kinase

Historical Background

The 6-kinase that stimulates the phosphorylation of inositol (1,3,4)P3 (Ins(1,3,4)P3) to Ins(1,3,4,6)P4 was first discovered in 1987 (Shears et al. 1987; Balla et al. 1987). It was this discovery of a pathway to InsP5 and InsP6 synthesis which prompted researchers (see York et al. 1999) to consider there might be other aspects to cell signaling by inositol phosphates in addition to calcium mobilization. Shortly after, a catalytic activity that phosphorylated Ins(3,4,5,6)P4 to Ins(1,3,4,5,6)P5 was discovered (Stephens et al. 1988). Interestingly, Ins(3,4,5,6)P4 and Ins(1,3,4)P3 were found to inhibit each other's phosphorylation. This observation led to the hypothesis that both activities were performed by two similar enzymes. Almost 10 years later, the gene encoding the 6-kinase reaction was cloned (Wilson and Majerus 1996). Subsequently, this single 46 kDa protein was found to phosphorylate both substrates, Ins(1,3,4)P3 and Ins(3,4,5,6)P4 (Yang and Shears 2000).

This enzyme was termed inositol trisphosphate kinase 1 (ITPK1) by HUGO. However, this HUGO-approved nomenclature does not reflect the versatile nature of ITPK1 to catalyze multiple phosphorylation reactions, whether as an Ins(1,3,4)P3 6-kinase, or an Ins(3,4,5,6)P4 1-kinase.

ITPK1 genes are well conserved from *Caenorhabditis elegans* to humans. To date, only yeast and drosophila genomes are known not to encode an ITPK1 gene (Seeds et al. 2004). Mammalian genomes only encode one ITPK1 gene, deletion of which is embryonic lethal; however, in plants there are often four to six genes encoding different isoforms of ITPK1 (Shears 2009). The reason for this diversity in plants is not well understood, but likely it reflects metabolic specialization of ITPK1.

Human *ITPK1* is comprised of 12 exons on chromosome 14. The mRNA transcripts of *ITPK1* were found to be ubiquitously expressed in tissues with highest expression levels in the brain and the heart (Wilson and Majerus 1996). Alternative splicing at the 5′ untranslated region is possible, although the significance is unknown (Yang and Shears 2000). ITPK1 is not only a key enzyme in inositol phosphate metabolism but also plays an important role in cell signaling to specifically regulate a CaMKII-activated cell surface chloride channel (ClC-3) to control neuronal plasticity, salt and fluid secretion, and endosomal ClC-3 to modulate inflammation, vesicular trafficking, and insulin secretion from the pancreatic β-cells.

It was thought for a while that ITPK1 was also a protein kinase (Wilson et al. 2001). Subsequent studies (Qian et al. 2005) have shown that the protein kinase activity associated with the recombinant ITPK1 expressed in insect cells is a persistent contaminant. When free of the contaminant, ITPK1 showed no protein kinase activity (Qian et al. 2005), yet the inositol kinase activity was similar to that observed by Wilson et al. (2001). A similar conclusion that ITPK1 is not a protein kinase can also be drawn from the crystal structure of the human ITPK1 (Chamberlain et al. 2007).

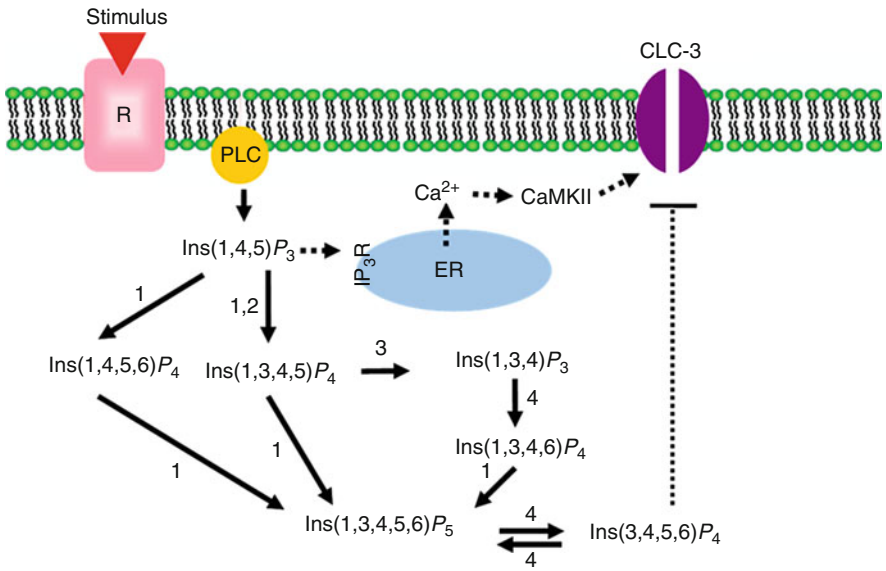
ITPK1 as an Important Metabolic Enzyme in the De Novo Synthesis of Inositol Phosphates

Inositol phosphates are freely diffusible intracellular signaling molecules which play many important roles in cell biology. ITPK1 is a multifunctional enzyme which is involved in both signaling and metabolic pathways. One important function of the Ins(1,3,4)P3 6-kinase activity of ITPK1, particularly in mammals, is to supply the precursor molecule, Ins(1,3,4,6)P4, for the synthesis of Ins(1,3,4,5,6)P5, as well as InsP6, and diphosphorylated inositol phosphates. Verbsky et al. (2005) have shown that overexpression of ITPK1 leads to increased levels of Ins(1,3,4,6)P4, Ins(3,4,5,6)P4, InsP5, and InsP6, whereas depletion of ITPK1 by RNAi results in decreased levels of these products. However, in some other species, such as slime molds, alternative synthetic routes are used as the major route for Ins(1,3,4,5,6)P5 synthesis. Yeast and *Drosophila* exclusively use an alternative synthetic route involving inositol polyphosphate multikinase (IPMK), since they lack the ITPK1 gene.

ITPK1 Regulates the Metabolism of the Ins(3,4,5,6)P4 Signaling Molecule

Another important function of ITPK1 is to control the signaling strength of the messenger molecule Ins (3,4,5,6)P4 which inhibits ClC-3 specific chloride current (Fig. 1). The key to this aspect of ITPK1 function is its phosphotransferase activity. ITPK1 not only phosphorylates Ins(3,4,5,6)P4 at the 1 position to produce InsP5, it can also remove the same phosphate so as to resynthesize Ins(3,4,5,6)P4 (Ho et al. 2002). In this manner, ITPK1 controls both the synthesis and the metabolism of Ins(3,4,5,6)P4 (Fig. 1).

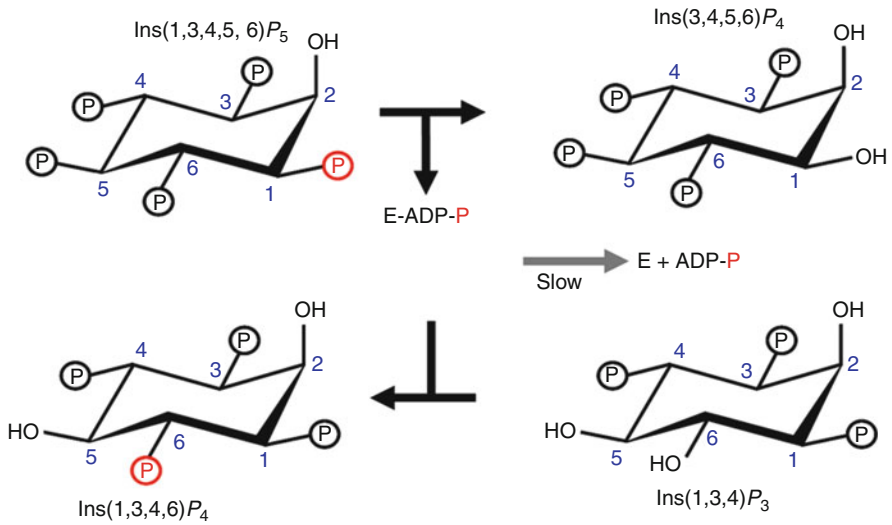
The phosphate group that is removed from Ins (1,3,4,5,6)P5 is not released into the bulk phase, instead it is retained by the enzyme and offered to Ins (1,3,4)P3, the alternative substrate of ITPK1 (Shears 2009). Therefore, elevations in the levels of Ins(1,3,4) P3 act in a phosphotransferase reaction to augment Ins (1,3,4,5,6)P5 dephosphorylation and competitively inhibit Ins(3,4,5,6)P4 phosphorylation (Fig. 2). Other experiments have shown that the addition of a cell-permeant analogue of Ins(1,3,4)P3 to cells leads to a specific increase in the cellular levels of Ins(3,4,5,6) P4 (Yang et al. 1999). This phosphotransferase activity of mammalian ITPK1 is a phenomenon that is unique in the inositol phosphate field (Michell 2002; Saiardi and Cockcroft 2008). This phosphotransferase activity provides the molecular mechanism by which Ins (3,4,5,6)P4 levels are coupled to receptor-regulated



ITPK1 (Inositol 1,3,4-Triphosphate 5/6 Kinase), Fig. 1 The metabolic pathway of receptor-dependent PLC activity and Ins(3,4,5,6)P4 synthesis in mammalian cells. The figure shows the most important metabolic reactions (*solid arrows*) in mammalian cells that link Ins(1,4,5)P3 to Ins(1,3,4,5,6)P5 through several possible pathways. Cell surface receptor activation of PLC generates the second messenger Ins(1,4,5)P3 which in turn is metabolized to Ins(1,3,4,5,6)P5. Ins(1,4,5)P3 binds to IP3 receptor (IP3R) at the ER to elicit intracellular calcium release (shown in *broken arrows*). Calcium together with calmodulin stimulates CaMKII which then activates the cell surface chloride channel ClC-3 (shown in *broken arrows*). On the other hand, ITPK1 converts Ins(1,3,4,5,6)P5 to Ins(3,4,5,6)P4 which specifically inhibits the very chloride channel that CaMKII activates (shown in *broken lines*). Numbers in the figure refer to various enzymes as follows: 1, Inositol phosphate multikinase (EC 2.7.1.151); 2, Ins(1,4,5)P3 3-kinase (EC 2.7.1.127); 3, Ins(1,4,5)P3/Ins(1,3,4,5)P4 5-phosphatase (EC 3.1.3.56); 4, ITPK1 (EC 2.7.1.134). Abbreviations used in the figure are listed as follows: R, receptor; PLC, phospholipase C; IP3R, Ins(1,4,5)P3 receptor; ER, endoplasmic reticulum; CaMKII, Calcium/calmodulin-dependent protein kinase II; ClC-3, type 3 member of the ClC gene family

phospholipase C (PLC) activity (Fig. 1) (Shears 2009). Upon cell surface receptor activation of PLC, cellular levels of Ins(1,3,4)P3 dramatically increase due to mass action effects of Ins(1,4,5)P3 metabolism. Receptor-dependent increases in Ins(1,3,4)P3 levels in turn stimulate the phosphotransferase activity of ITPK1 to increase the rate of Ins(1,3,4,5,6)P5 dephosphorylation, thereby generating increases in Ins(3,4,5,6)P4 levels (Fig. 1). Studies have confirmed that control over the levels of expression of the ITPK1 can regulate Ins(3,4,5,6)P4 signaling (Yang et al. 2006). More importantly, these studies also show that the strength and duration of the Ins(3,4,5,6)P4 signal is inevitably linked to the degree and duration of the receptor-dependent phospholipase C activity. This obligatory connection between Ins(3,4,5,6)P4 levels and PLC-dependent production of Ins(1,4,5)P3 is a vital component of this entire signaling system (Fig. 1).

Although the reaction mechanism of the phosphotransferase activity of ITPK1 has not been established, crystallographic structures have been obtained for the human and amoeboid forms of ITPK1 (Chamberlain et al. 2007; Miller et al. 2005). The determinants of ligand binding in human ITPK1 ensure that the enzyme's specificity depends upon the three-dimensional arrangement of phosphates and hydroxyls around the inositol ring, and also the three-dimensional stereochemistry at each position of the ring (Chamberlain et al. 2007). The amoeboid ITPK1 structure, however, reveals an unusually versatile catalytic cleft that was proposed not to impose any stereospecific constraints upon substrate binding (Miller et al. 2005). Interestingly, the phosphotransferase activity is found only in the mammalian ITPK1, but not in the amoeboid or soybean homologues (Chamberlain et al. 2007). That is, increases in Ins(1,3,4)P3 do not stimulate the



ITPK1 (Inositol 1,3,4-Triphosphate 5/6 Kinase), Fig. 2 The phosphotransferase activity of ITPK1. The graphic illustrates the proposed enzymatic reactions by which the 1-phosphate on Ins (1,3,4,5,6)P5 (*red*) is transferred to Ins(1,3,4)P3. The evidence for this reaction pathway came from HPLC analysis of the reaction products following the metabolism of [1-32P]-Ins (1,3,4,5,6)P5 by ITPK1 (Chamberlain et al. 2007). It has not yet been established whether or not a phosphoryl-enzyme (E-P) intermediate is involved, but this is a likely possibility (Adapted from Shears 2009)

dephosphorylation of Ins(1,3,4,5,6)P5 by amoeboid or soybean ITPK1. It is likely that mammalian ITPK1 has specifically evolved this signaling function. A substrate-bound human ITPK1 crystal has not yet been obtained, which contributes to the ambiguities concerning the phosphotransferase reaction mechanism. Nevertheless, a model has been put forth to suggest the structural determinants of ligand specificity for mammalian ITPK1 (Fig. 3). It is proposed that there are three modes of binding of inositol phosphates to mammalian ITPK1. Mode 1 binding is designated for Ins(3,4,5,6)P4 to allow 1-kinase activity; Mode 2 binding is designated for Ins(1,3,4)P3 to permit 6-kinase activity, and Mode 3 for Ins(1,3,4)P3 to permit 5-kinase activity (Shears 2004, 2009).

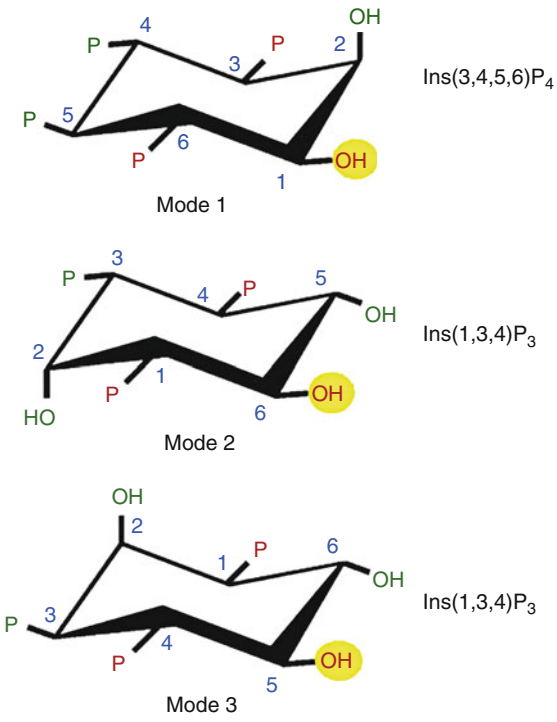
Simultaneous binding of both Ins(1,3,4)P3 and Ins (1,3,4,5,6)P5 to the same active site seems implausible from analysis of the enzyme's structure, but a sequential reaction model (Fig. 4) has been proposed in order to explain the transfer of a phosphate group between different substrates (Chamberlain et al. 2007). The key to this intersubstrate phosphate transfer is that the enzyme tightly binds nucleotide, either ATP or ADP, instead of releasing them into the bulk phase during this series of reactions.

Biological Significance of ITPK1

The biological significance of ITPK1 originates from the intracellular signaling molecule Ins(3,4,5,6)P4 and its ability to regulate chloride conductance in mammalian cells through inhibition of ClC-3 chloride channels (Mitchell et al. 2008). No other inositol phosphates are able to inhibit ClC-3, and ClC-3 is the only chloride channel known to be inhibited by Ins(3,4,5,6)P4 (Mitchell et al. 2008).

In hippocampal neurons, ClC-3 is responsible for the Ins(3,4,5,6)P4 regulated chloride conductance, which contributes to the overall regulation of the synaptic efficacy in generating action potentials. Therefore, Ins (3,4,5,6)P4 has the potential to affect electrical excitability in neurons via regulating chloride fluxes and hence has a direct influence on neuronal development (Ho et al. 2002; Mitchell et al. 2008; Shears 2009).

Because the regulation of ClC-3 by Ins(3,4,5,6)P4 also has an impact on salt and fluid secretion by epithelial cells, ITPK1 plays an important role in several environmentally influenced diseases of secretory epithelia, such as cystic fibrosis (CF), asthma, and bronchitis (Shears 2005, 2009). The nature of CF, for example, is primarily due to the mutation of cystic



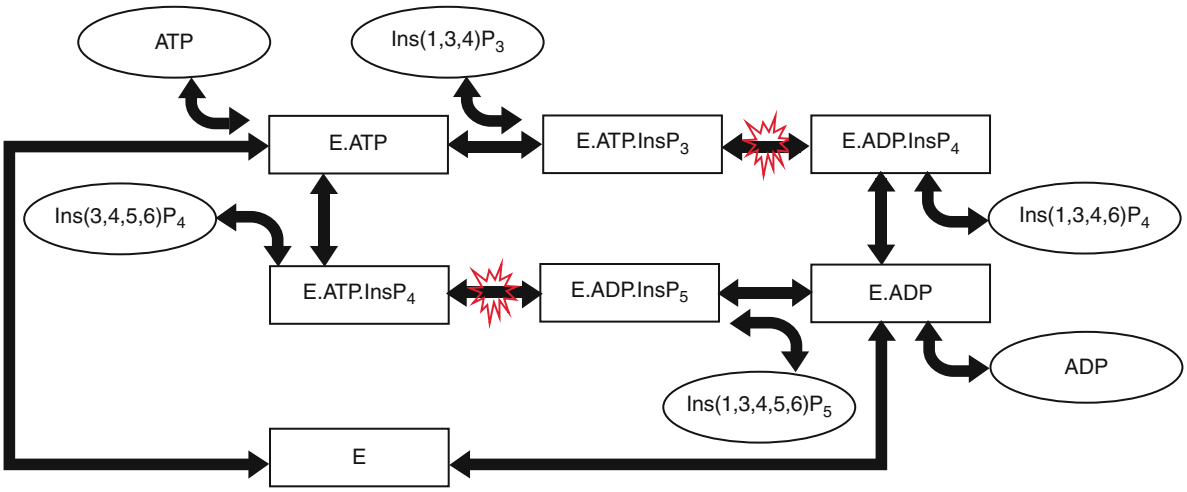
ITPK1 (Inositol 1,3,4-Triphosphate 5/6 Kinase), Fig. 3 A model for the structural determinants of ligand specificity for mammalian ITPK1. The figure depicts the proposal that there are three modes of binding of inositol phosphates to mammalian ITPK1. It can be illuminating to consider these different binding modes (i.e., "1", "2", and "3") as permitting 1-kinase, 6-kinase, and 5-kinase activities, respectively. The phosphorylation sites are marked with a *yellow circle*. Groups conserved in all three of these proposed binding modes are colored in *red*. Additional groups that contribute to structural specificity are colored in *green* (Adapted from Shears 2009)

fibrosis transmembrane regulator (CFTR) leading to reduction of Cl^- ion secretion from CF airway epithelia. This in turn perturbs the airway surface liquid volume and the height and composition of the mucus layer. This perturbation leads to impaired ciliary beating to expel pathogens. Thus, there is bacterial colonization of the airways, culminating in inflammatory responses and lung tissue death (Shears 2005). Cell surface receptor stimulated PLC activity mobilizes Ca^{2+}, thereby activating CaMKII, to stimulate ClC-3 (Fig. 1) to drive secretion of Cl^-. This mechanism aids CFTR in maintaining adequate airway liquid volume. Therefore, purinergic receptor agonists are potential drug candidate for CF patients. However, it needs to be taken into consideration that purinergic activation also leads to ITPK1-dependent formation of Ins (3,4,5,6)P4 that inhibits the very chloride channel that Ca^{2+}/CaMKII activates. Therefore, it could be useful to consider that the therapeutic efficacy of purinergic agonists might depend upon the expression levels of ITPK1 in individual patients. In other words, ITPK1 might be a modifier gene for the CF condition (Yang et al. 2006). In those with low ITPK1 expression, purinergic agonists would be beneficial to treat CF; on the other hand, for patients with high protein level of ITPK1 it might be of value to consider developing Ins(3,4,5,6)P4 antagonists (Shears 2005).

Ins(3,4,5,6)P4 not only regulates ClC-3 at the plasma membrane, it also inhibits ClC-3 channels at membranes of intracellular vesicles such as insulin granules and endosomal compartments. In these vesicles, chloride conductance through the ClC-3 serves as a charge neutralization shunt that supports acidification of these vesicles (Shears 2004). Intra-vesicular pH helps determine the fate of vesicular trafficking whether for recycling of plasma membrane proteins or exocytosis. The inhibition of chloride influx into vesicles by Ins(3,4,5,6)P4 leads to a more alkaline compartment (Mitchell et al. 2008). In the case of insulin granules in pancreatic β-cells, their alkalinization by Ins(3,4,5,6)P4 reduces insulin secretion (Renstrom et al. 2002). Abnormally elevated cellular Ins(3,4,5,6)P4 levels might contribute to hyperglycemia-dependent refractoriness of β-cells which typifies type2 diabetes.

Although ITPK1 has no obvious consensus sequences for membrane localization, it has been reported that ITPK1 is concentrated near the apical membrane of polarized mouse tracheal epithelial cells (Yang et al. 2006). It is not known yet how the compartmentalization of ITPK1 is achieved, whether by interaction with other membrane associated proteins, for example. This further leads to a question as to whether Ins(3,4,5,6)P4 could be compartmentalized in cells to locally affect a specific pool of ClC-3 channels. This idea contrasts with from the more general perception that Ins(3,4,5,6)P4 is uniformly distributed throughout the cell.

Note that ITPK1 knockout is embryonic lethal (Wilson et al. 2009), whereas the ClC-3 knockout is not (Dickerson et al. 2002; Yoshikawa et al. 2002). This suggests that there is more to the function of ITPK1 than just regulating ClC-3. Therefore, there may be other functions of ITPK1 that are independent from ClC-3. This idea awaits further exploration.



ITPK1 (Inositol 1,3,4-Triphosphate 5/6 Kinase), Fig. 4 Sequential intersubstrate phosphate transfer hypothesis for human ITPK1. The graphic suggests at the molecular level how a single active site of ITPK1 can accommodate two substrates. It is hypothesized that the phosphate from one substrate is not released into the bulk media (from dephosphorylation of Ins(1,3,4,5,6)P5); instead, it is retained by the enzyme or enzyme-nucleotide intermediate. This phosphate is then transferred to the other substrate of ITPK1, Ins(1,3,4)P3. The two enzymatic reactions occur in a sequential manner. Additionally, the presence of Ins(1,3,4)P3 stimulates the dephosphorylation of Ins(1,3,4,5,6)P5. The enzyme-bound nucleotide acts as the phosphate carrier. Unliganded ITPK1 is represented by E; phosphotransferase reactions are indicated by *red graphics*. The position of the [32P] group that is transferred between inositol phosphates is shown in *red* (Adapted from Chamberlain et al. 2007)

Summary

The synthesis and metabolism of Ins(3,4,5,6)P4 are solely regulated by a single multifunctional kinase and phosphotransferase, ITPK1. This enzyme dynamically couples the cell surface receptor activated PLC hydrolysis of phosphatidyl inositol lipids to the cellular levels of Ins(3,4,5,6)P4. This is a biologically significant event because Ins(3,4,5,6)P4 regulates a specific pathway for transmembrane chloride conductance that is involved in numerous cellular functions including synaptic efficacy, epithelial salt and fluid secretion, insulin release from pancreatic β-cells, and inflammatory responses.

References

Balla T, Guillemette G, et al. Formation of inositol 1,3,4,6-tetrakisphosphate during angiotensin II action in bovine adrenal glomerulosa cells. Biochem Biophys Res Commun. 1987;148(1):199–205.

Chamberlain PP, Qian X, et al. Integration of inositol phosphate signaling pathways via human ITPK1. J Biol Chem. 2007; 282(38):28117–25.

Dickerson LW, Bonthius DJ, et al. Altered GABAergic function accompanies hippocampal degeneration in mice lacking ClC-3 voltage-gated chloride channels. Brain Res. 2002; 958(2):227–50.

Ho MW, Yang X, et al. Regulation of Ins(3,4,5,6)P(4) signaling by a reversible kinase/phosphatase. Curr Biol. 2002;12(6): 477–82.

Michell RH. Inositol phosphates: a remarkably versatile enzyme. Curr Biol. 2002;12(9):R313–5.

Miller GJ, Wilson MP, et al. Specificity determinants in inositol polyphosphate synthesis: crystal structure of inositol 1,3,4-trisphosphate 5/6-kinase. Mol Cell. 2005;18(2):201–12.

Mitchell J, Wang X, et al. An expanded biological repertoire for Ins(3,4,5,6)P4 through its modulation of ClC-3 function. Curr Biol. 2008;18(20):1600–5.

Qian X, Mitchell J, et al. The Ins(1,3,4)P3 5/6-kinase/Ins (3,4,5,6)P4 1-kinase is not a protein kinase. Biochem J. 2005;389(Pt 2):389–95.

Renstrom E, Ivarsson R, et al. Inositol 3,4,5,6-tetrakisphosphate inhibits insulin granule acidification and fusogenic potential. J Biol Chem. 2002;277(30):26717–20.

Saiardi A, Cockcroft S. Human ITPK1: a reversible inositol phosphate kinase/phosphatase that links receptor-dependent phospholipase C to Ca2+-activated chloride channels. Sci Signal. 2008;1(4): pe5.

Seeds AM, Sandquist JC, et al. A molecular basis for inositol polyphosphate synthesis in Drosophila melanogaster. J Biol Chem. 2004;279(45):47222–32.

Shears SB. How versatile are inositol phosphate kinases? Biochem J. 2004;377(Pt 2):265–80.

Shears SB. Can intervention in inositol phosphate signalling pathways improve therapy for cystic fibrosis? Expert Opin Ther Targets. 2005;9(6):1307–17.

Shears SB. Molecular basis for the integration of inositol phosphate signaling pathways via human ITPK1. Adv Enzyme Regul. 2009;49(1):87–96.
Shears SB, Parry JB, et al. Metabolism of D-myo-inositol 1,3,4,5-tetrakisphosphate by rat liver, including the synthesis of a novel isomer of myo-inositol tetrakisphosphate. Biochem J. 1987;246(1):139–47.
Stephens LR, Hawkins PT, et al. L-myo-inositol 1,4,5,6-tetrakisphosphate (3-hydroxy)kinase. Biochem J. 1988; 249(1):283–92.
Verbsky JW, Chang SC, et al. The pathway for the production of inositol hexakisphosphate in human cells. J Biol Chem. 2005;280(3):1911–20.
Wilson MP, Majerus PW. Isolation of inositol 1,3,4-trisphosphate 5/6-kinase, cDNA cloning and expression of the recombinant enzyme. J Biol Chem. 1996;271(20): 11904–10.
Wilson MP, Sun Y, et al. Inositol 1,3,4-trisphosphate 5/6-kinase is a protein kinase that phosphorylates the transcription factors c-Jun and ATF-2. J Biol Chem. 2001;276(44): 40998–1004.
Wilson MP, Hugge C, et al. Neural tube defects in mice with reduced levels of inositol 1,3,4-trisphosphate 5/6-kinase. Proc Natl Acad Sci USA. 2009;106(24):9831–5.
Yang X, Shears SB. Multitasking in signal transduction by a promiscuous human Ins(3,4,5,6)P(4) 1-kinase/Ins(1,3,4)P(3) 5/6-kinase. Biochem J. 2000;351(Pt 3):551–5.
Yang X, Rudolf M, et al. Inositol 1,3,4-trisphosphate acts in vivo as a specific regulator of cellular signaling by inositol 3,4,5,6-tetrakisphosphate. J Biol Chem. 1999;274(27): 18973–80.
Yang L, Reece J, et al. Apical localization of ITPK1 enhances its ability to be a modifier gene product in a murine tracheal cell model of cystic fibrosis. J Cell Sci. 2006;119 (Pt 7):1320–8.
York JD, Odom AR, et al. A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science. 1999;285(5424):96–100.
Yoshikawa M, Uchida S, et al. CLC-3 deficiency leads to phenotypes similar to human neuronal ceroid lipofuscinosis. Genes Cells. 2002;7(6):597–605.

ITSN

Ameet S. Sengar[1], Michael W. Salter[1] and Sean E. Egan[2]
[1]Program in Neurosciences & Mental Health, The Hospital for Sick Children, Toronto, ON, Canada
[2]Program in Developmental & Stem Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada

Synonyms

Ese; Intersectin

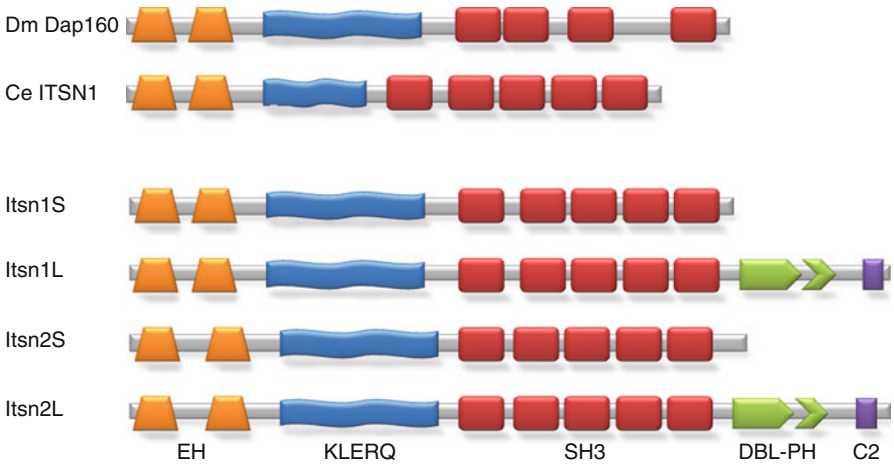
The Intersectin Family

The Intersectin family refers to a group of proteins that share N-terminal EH domains, a central coiled-coil domain, and multiple SH3 domains. In mammals, there are Intersectin1 and 2 genes (Itsn1 and Itsn2, respectively), whereas in invertebrates, only one gene appears to be present.

Historical Background

Intersectins are conserved modular scaffold proteins with two Eps15-homology (EH) domains, an extended KLERQ coiled-coil domain, four or five Src-homology 3 (SH3) domains, and, in some vertebrate splice variants, DBL/PH and C2 domains (Fig. 1). The Itsn proteins were discovered independently in a number of organisms and in a number of labs. For example, the single Itsn in *Drosophila melanogaster*, Dap160, or Dynamin-associated protein 160 kDa, copurified with the proline-rich C-terminus of Dynamin (Roos and Kelly 1998). Human *ITSN1* was cloned following identification of a trapped exon that coded for a novel EH domain (Guipponi et al. 1998). *Xenopus*, mouse and rat *Itsn1* genes were isolated in screens for proteins that bound to specific proline-rich sequences or to the coiled-coil protein Snap-25, respectively (Yamabhai et al. 1998; Okamoto et al. 1999; Sengar et al. 1999).

Drosophila Dap160 and vertebrate Itsn1 are very highly expressed in the nervous system (Roos and Kelly 1998; Hussain et al. 1999; Sengar et al. 1999). A second, widely expressed Itsn gene has also been identified. This gene, originally designated *Ese2*, is now known as *Itsn2* (Sengar et al. 1999; Pucharcos et al. 2000). There are many speculated functions for Itsn proteins in the literature. Based on protein domain structure and binding partners, as well as a series of overexpression experiments in cultured cells, it was accepted early on that Itsn proteins play a role in clathrin-mediated endocytic trafficking and in regulation of the actin cytoskeleton (Hussain et al. 1999; Sengar et al. 1999; Hussain et al. 2001). Tissue culture experiments have been used to assign biological context to the endocytic and signaling functions. Indeed, the number of processes where Itsn proteins are reported to function has continued to grow over the past decade, mostly based on in vitro or ex vivo experiments. The first genetic studies on Itsn were



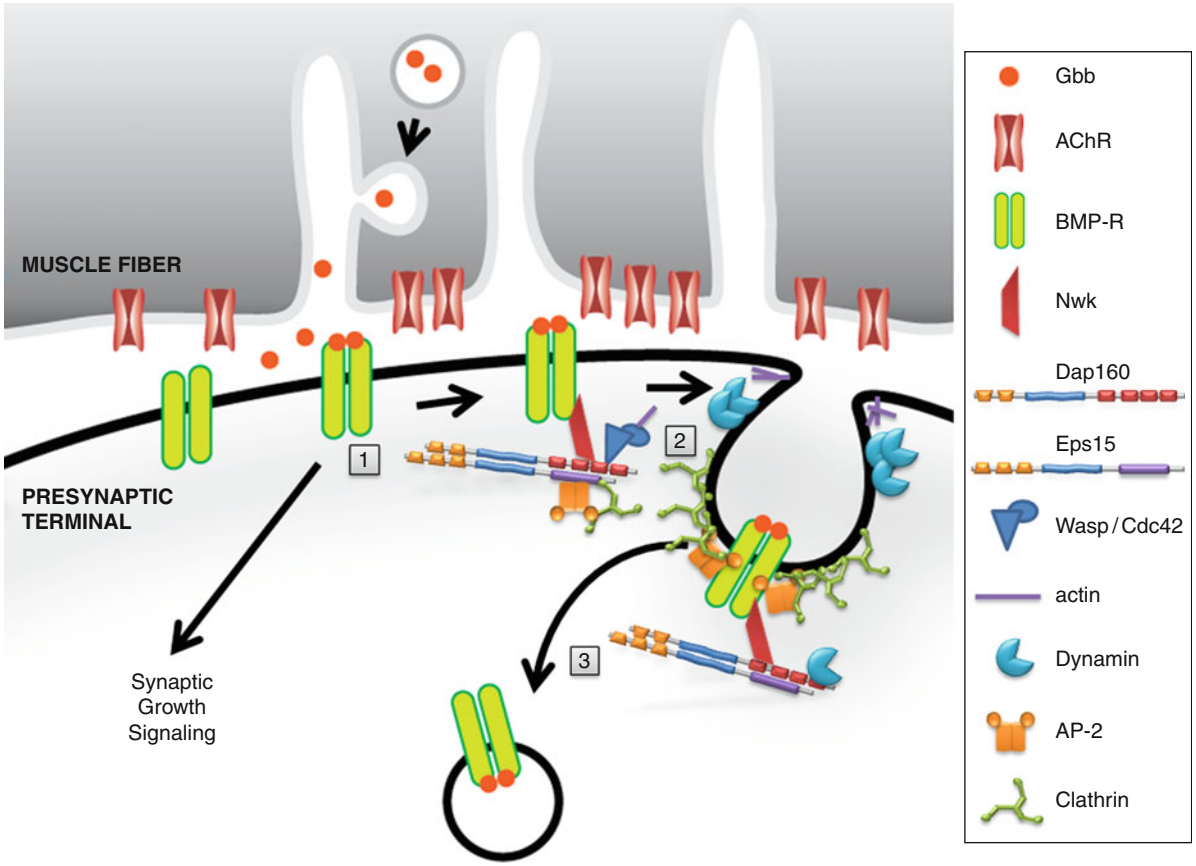
ITSN, Fig. 1 Schematic representation of *Drosophila* Dap160 and *C. elegans* Itsn1 proteins as well as major isoforms of Intersectin1 and 2 in mammals

performed in the fruit fly, where Dap160 was found to control synaptic structure and vesicle recycling at the neuromuscular synapse (see below) (Koh et al. 2004; Marie et al. 2004). Subsequent studies in nematodes have confirmed a role in synaptic vesicle recycling (Rose et al. 2007; Wang et al. 2008). Itsn1 mutant mice have also been reported (Yu et al. 2008). While these mice were born at the expected Mendelian frequency, some failed to thrive and died at a young age with evidence of enlarged endosomes in brain sections. These abnormalities were not seen in the majority of mutant animals, although exocytic and endocytic defects were observed in ex vivo cultures from the mice.

Synaptic Structure

Dap160 mutant flies show neuromuscular junction (NMJ) overgrowth, with an increased number of small sized satellite boutons (Koh et al. 2004; Marie et al. 2004). This phenotype is associated with disruption of Dap160 in the neuron, as opposed to the muscle target cell, and is therefore presynaptic. A similar mutant phenotype is also observed in other endocytic mutants, including mutants of Dynamin, Eps15, Endophilin, Synaptojanin, Rab11, and Spinster (Rikhy et al. 2002; Sweeney and Davis 2002; Verstreken et al. 2003; Khodosh et al. 2006; Majumdar et al. 2006). Interestingly, mutations in the Bone Morphogenic Protein (BMP) ligand, Glass Bottom Boat (Gbb), in its type I or II BMP receptors, or in the downstream Smad signaling proteins, cause the opposite effect, with undergrowth at the NMJ (McCabe et al. 2003). Indeed, BMP is a retrograde signal that stimulates growth and branching of presynaptic boutons. Dap160 and its partners inhibit this signal, likely through inhibition of BMP receptor signaling and/or recycling within the periactive zone (Fig. 2). The F-BAR/SH3 domain protein, nervous wreck (Nwk), facilitates this by binding to the cytoplamic domain of type I BMP receptors and also to Dap160 (O'Connor-Giles et al. 2008). This is mediated through interaction between a region of Nwk that maps near or within its second SH3 domain, and a region of Dap160 that maps to its third and fourth SH3 domains.

A number of studies in yeast have shown that endocytic proteins can regulate actin, and conversely, that actin regulatory proteins frequently control endocytosis. More recently, this phenomena has been demonstrated in higher organisms. Indeed, Wasp proteins, which regulate actin, also control internalization and trafficking of cell surface receptors. Consistent with this, mutants in *Drosophila* Wasp or its activator small GTPase, Cdc42, also enhance synaptic growth in a BMPR-dependent manner (Rodal et al. 2008; Nahm et al. 2010). Interestingly, Wasp and Dynamin are thought to compete for overlapping site(s) on Dap160, suggesting that Dap160-Wasp and Dap160-Dynamin complexes may perform distinct functions, perhaps during different steps of internalization and/or endocytic trafficking of BMPR (Rodal et al. 2008). Thus, at the periactive zone that surrounds each neuronal synapse, a region thought to perform functions in common with recycling endosomes of non-neuronal



ITSN, Fig. 2 Model of Dap160-dependent BMP receptor antagonism at the *Drosophila* neuromuscular junction. (1) Gbb ligand bound BMP receptors activate signaling pathways for synaptic growth. (2) Wasp/Cdc42 are recruited by Dap160 to promote actin polymerization, a necessary step for vesicle internalization. (3) Nwk links the Dap160/Eps15/Dynamin complex to BMP receptors to initiate vesicle scission

cells, Dap160/Itsn forms a complex with Wasp and Nwk to downregulate BMPR signaling (Fig. 2) (Rodal et al. 2008). In addition, Dap160/Itsn also forms complexes with Dynamin and Nwk, perhaps following dissolution of the Wasp complex (O'Connor-Giles et al. 2008). One or both of these complexes is likely associated with suppression of BMPR signaling, or recycling through the Rab11 compartment for return to the plasma membrane (Rodal et al. 2008). The Itsn partner Eps15 is also involved in these processes as Eps15 mutant neuromuscular junctions show the same overgrowth phenotype (Koh et al. 2007). In mammals, Itsn-1 also binds to TUC-4b, an orthologue of *C. elegans* UNC-33, which is thought to control neurite extension and branching (Quinn et al. 2003). Finally, in the differentiated synapse, Intersectins function within the periactive zone to coordinate events involved in efficient synaptic vesicle recycling. This function is independent of Nwk, and therefore involves distinct Itsn-protein complexes (see next section below).

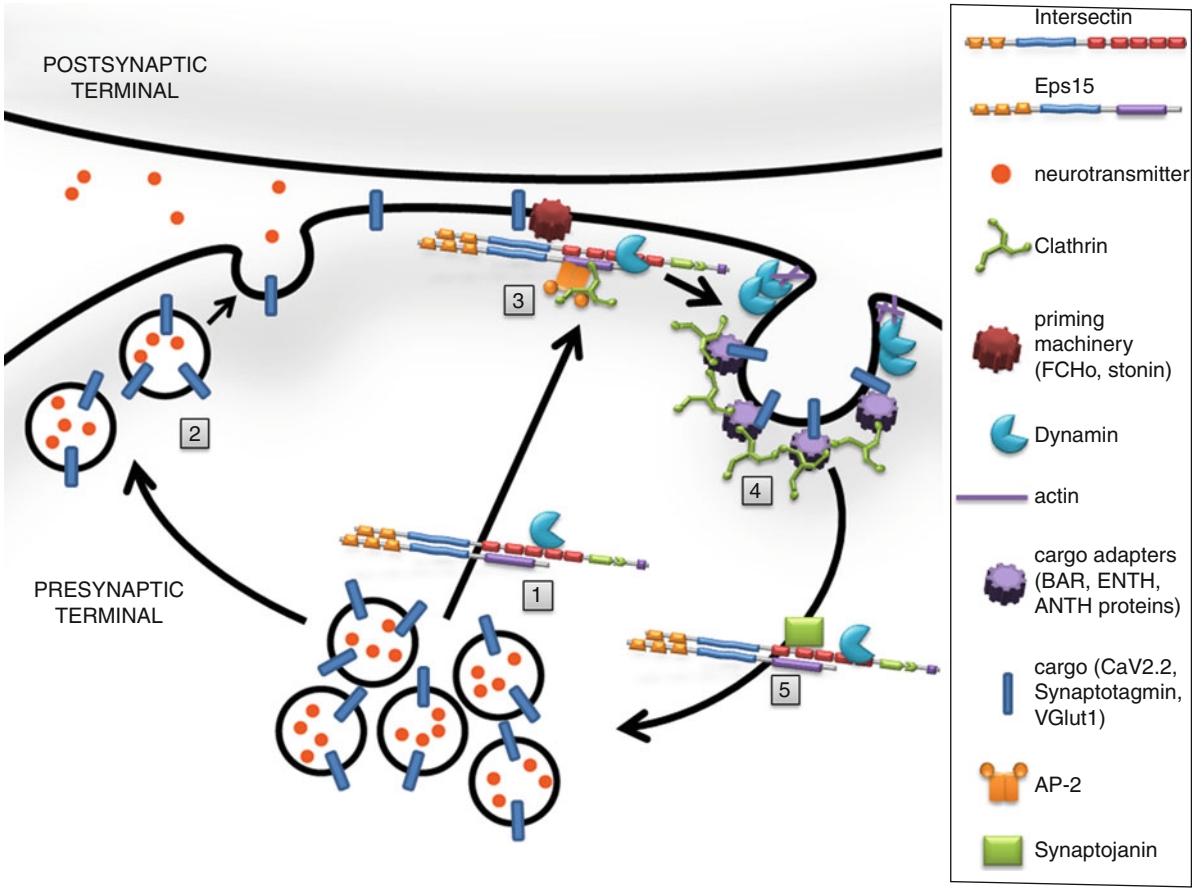
While genetic studies in flies have shown a very important role for Dap160 and its orthologues in control of pre-synaptic growth, other work has revealed post-synaptic functions for Itsn. For example, the N-terminal EH domain region of Itsn1L binds to the kinase domain of EphB2 receptors in cultured hippocampal neurons (Irie and Yamaguchi 2002). Activation of EphB2 receptor signaling thus leads to activation of the DBL/PH domain Cdc42 GEF activity coded within the C-terminus of Itsn1L. N-Wasp and the endocytic protein Numb are bound to distinct SH3 domains of Itsn1L in this complex (Nishimura et al. 2006). This helps alleviate intrinsic SH3-mediated repression of the Itsn GEF domain and thereby enhances GTP-loading of Cdc42. In cultured neurons,

Itsn1L colocalizes with F-actin at dendritic spines (Thomas et al. 2009). In turn, Cdc42-GTP and Itsn1L SH3 domains stimulate N-Wasp-mediated actin polymerization and dendritic spine formation, whereas Itsn1 siRNA knockdown disrupts spine maturation (Hussain et al. 2001; Thomas et al. 2009).

Synaptic Vesicle Recycling

A complex and specialized form of endocytosis is required in neurons to recover synaptic vesicle proteins and membranes in order to facilitate sustained synaptic transmission. This process appears to include fast and slow endocytic retrieval pathways that differ in their dependence on specific proteins, and that may also differ depending on whether they occur at small or large synapses, or in different synapses within the CNS. Based primarily on work in flies, a role for Itsn proteins in synaptic vesicle recycling has been definitively established (Pechstein et al. 2010b). Indeed, loss of Dap160 impaired membrane retrieval and caused altered localization and expression of key endocytic proteins Dynamin, Synaptojanin, Endophilin, LAP (Like-AP180) as well as the reserve pool marker Synapsin (Koh et al. 2004; Marie et al. 2004). Likewise, mutants of LAP in *Drosophila* show mislocalization of the presynaptic Ca^{++}-sensor and cargo protein Synaptotagmin as well as Dap160. Proteomic analysis of Synaptotagmin complexes from rat brain synaptosomes has identified a complex comprised of Synaptotagmin, Stonin2, AP180, Itsn1, Epsin, Clathrin, Dynamin, and AP-2 (Khanna et al. 2006). These findings in conjunction with in vitro identification of binding partners strongly support the notion that Itsn proteins act as scaffolds to regulate activity and localization of the endocytic machinery. For example, in giant reticulospinal synapses, Itsn1 is localized to synaptic vesicle clusters where it sequesters Dynamin prior to membrane depolarization. Following K^+ stimulation, however, Itsn1 redistributes to Clathrin-coated pits in the periactive zone where it recruits AP-2 to the budding vesicle and targets Dynamin to sites of vesicle fission (Evergren et al. 2007; Pechstein et al. 2010a). In the chicken calyx presynaptic terminal, an endocytic complex that includes Itsn1S and AP180 is tightly bound to CaV2.2 N-type calcium channels that cluster at transmitter release-sites. This complex also associates with a Dynamin, Clathrin, and Itsn1L containing subcomplex to facilitate rapid vesicle recovery (Khanna et al. 2007). Consistent with this conserved function, hippocampal neurons cultured from Itsn1 knockout mice show impaired synaptic vesicle recycling (Yu et al. 2008).

The diversity of synapses as well as the multiple distinct forms of synaptic vesicle recycling that occur in the nervous system have made it difficult, and perhaps inappropriate, to even try to establish one coherent model for the role of Itsn in synaptic vesicle recycling (Smith et al. 2008). Despite this, some general features have emerged from studies in different systems (Fig. 3). During very early steps of Clathrin-mediated endocytosis, including the specialized form involved in synaptic vesicle recycling, membrane bending FCHo1 and FCHo2 (F-BAR-domain-containing Fer/Cip4 homology domain-only) proteins, bind to $PI(4,5)P_2$ in the plasma membrane to establish endocytic hotspots (Henne et al. 2010). FCHo1/2 bind directly to Itsn1 and Eps15, thus forming a membrane-bound complex for recruitment of the AP-2 Clathrin adaptor complex and cargo (Henne et al. 2010). There are multiple forms of cargo that must be internalized together, in order to recreate synaptic vesicles of defined and consistent structure. Perhaps most important in this regard are the transmembrane Synaptotagmin proteins which link Ca^{++}-induced exocytosis to membrane retrieval. Synaptotagmin 1, following exocytosis and deposition into the plasma membrane, is bound by specialized adaptor proteins of the Stonin family (Maritzen et al. 2010). Interestingly, Stonins also bind Intersectins, Eps15 and AP-2. Thus, following establishment of endocytic hotpots in the periactive zone (with FCHo1/2, Intersectin and Eps15), these proteins are linked to Synaptotagmin cargo and even Clathrin, through Stonin and AP-2. Other cargo, including vesicular transporters or cotransporters like VGlut1, must also be recruited into Clathrin-coated pits to initiate synaptic vesicle recovery (Maritzen et al. 2010). Some cargo is recruited through alternative adaptor molecules including the N-BAR-domain protein, Endophilin. Indeed, a number of cargo adaptors and membrane-bending or curvature-sensing proteins with BAR-, ENTH-, or ANTH-domains are involved in linking cargo to Clathrin at endocytic hotspots. These proteins induce membrane bending and tubulation through an actin-dependent process (Ferguson et al. 2009).



ITSN, Fig. 3 Model of Itsn-dependent vesicle recovery at a central nervous system synapse. (1) Itsn-containing protein complex rest within the reserve pool during synaptic inactivity. (2) Neurotransmitters are released into the synaptic cleft following vesicle fusion. (3) Itsn complexes translocate to predetermined sites of internalization marked by FCHo proteins, where Itsn associates with cargo proteins (CaV2.2, Synaptotagmin, and VGlut1), Stonins, AP-2, and Clathrin. (4) Membrane-bending adapters (BAR-, ENTH-, and ANTH-domain proteins) force the membrane to invaginate while fission is induced after Dynamin recruitment to the neck of Clathrin-coated pits. (5) Itsn bound to Synaptojanin uncoats vesicles, thereby replenishing neurotransmitter reserve pools

Interestingly, the ANTH-domain and Itsn-binding protein AP180 is involved in defining the size of recovered synaptic vesicles. Finally, tubulation is terminated through vesicle scission by Dynamin, which is recruited to the neck of budding vesicles by Itsn1 (Evergren et al. 2007; Ferguson et al. 2009). As the vesicle buds from the plasma membrane, Itsn1L binds inositol-5-phosphatase, Synaptojanin 170, a protein involved in PI(4,5)P_2 turnover and vesicle uncoating (Pechstein et al. 2010a). Many of the steps involved in synaptic vesicle recycling are regulated by phosphatidylinositols. In non-neuronal cells, Itsn1S binds the Ship2 inositol 5′-phosphatase, likely to coordinate Clathrin-coated pit formation in a more general context (Xie et al. 2008). Indeed, the speed of membrane invagination is controlled by recruitment of inositol-5-phosphatases to the SH3 domains of Itsn1 (Xie et al. 2008; Nakatsu et al. 2010). Modulating the rate of vesicle formation may be important for ensuring efficient and complete loading of cargo. Thus, Itsn serves as a key scaffolding protein for early endocytic events, from assembly of the budding vesicle to fission to uncoating (Pechstein et al. 2010b).

While most efforts have focused on dissecting the role of Itsn in endocytosis, there is some evidence to suggest that Itsn may function in exocytosis as well. Indeed, Itsn1 can bind directly to the SNARE protein, Snap25 (Okamoto et al. 1999), while cultured

chromaffin cells from Itsn1 knockout mice and siRNA knockdown in the same cell type showed reduced secretion and lower Cdc42-dependent exocytic activity (Malacombe et al. 2006; Yu et al. 2008). Once again, details of how Itsn1 coordinates exo-endocytic trafficking remain to be determined.

Additional Functions for Itsn

Intersectins are also expressed in non-neuronal tissues and are therefore expected to have non-neuronal functions (Sengar et al. 1999). In support of this, Itsn1S has been implicated in endocytosis of the renal K^+ channel ROMK1 and Itsn2L in T-cell antigen receptor endocytosis (McGavin et al. 2001; He et al. 2007). Itsn1 and 2 have also been reported to regulate caveolar-based endocytosis. In endothelial cells, Itsn1S associates with Dynamin and Snap23 patches to help facilitate caveolae-mediated internalization (Predescu et al. 2003, Klein et al. 2009). In contrast, knockdown of Itsn2L by siRNA increased endocytosis through caveolae, whereas overexpression of the Cdc42-activating GEF domain of Itsn2L blocked it, suggesting that Itsn2L-dependent actin polymerization may inhibit caveolae-mediated endocytosis (Klein et al. 2009).

Itsn1 is also thought to control cell survival, polarity and mitosis. For example, the Itsn1 mouse knockout has decreased levels of NGF in the septal region of the brain, suggesting a role for Itsn1 in NGFR endocytosis or trafficking (Yu et al. 2008). Indeed, Itsn1 regulates survival signaling (Loeb et al. 2006; Das et al. 2007; Predescu et al. 2007). siRNA knockdown of Itsn1 in differentiated neurons causes cell death, which may be associated with loss of an Itsn1/PI3K-C2β/Akt survival signal (Das et al. 2007). siRNA knockdown of Itsn1 in endothelial cells also causes cell death (Predescu et al. 2007). Itsn1 can activate Epidermal Growth Factor Receptor (EGFR) internalization and signaling to the mitogenic transcription factor, Elk-1. Regulation of this pathway may involve Sos, Ras, and Jnk signaling but not Erk1/2 (Mohney et al. 2003). Expression of a dominant active form of Itsn1L in NIH3T3 cells was strongly transforming as a result of Cdc42 and Ras activation (Wang et al. 2005). In *Drosophila*, Dap160 directly binds and activates aPKC in an endocytosis-independent pathway to modulate neuroblast polarity and cell cycle progression (Chabu and Doe 2008). In mammalian cells, Itsn2 is thought to control activation of Cdc42 near centrosomes in order to control spindle orientation and lumen formation. In this context, Itsn2L SH3 domains bound to p150Glued, a subunit of the Dynactin complex (Rodriguez-Fraticelli et al. 2010). Interestingly, a Dynactin complex protein was also identified in association with *C. elegans* ITSN1 (Wang et al. 2008).

Summary

Thus, a series of elegant studies in organisms ranging from flies to worms to mice have revealed a conserved role for Itsn proteins in coordinating endocytosis and signaling in neurons. For example, Itsn functions in the periactive zone of neuromuscular junctions to regulate BMP-mediated synaptic growth (O'Connor-Giles et al. 2008). It also functions in distinct complexes to coordinate synaptic vesicle recycling at this location. Many non-neuronal Itsn functions have been identified on the basis of in vitro or ex vivo experiments. Genetic studies in *Drosophila*, *C. elegans* and mice have yet to support such roles (Koh et al. 2004; Marie et al. 2004; Rose et al. 2007; Wang et al. 2008; Yu et al. 2008). This may be due to redundancy between Itsn and other scaffolding or signaling proteins, and also to an early focus of genetic studies on synaptic transmission. Since an Itsn2 knockout mouse has yet to be described, it is difficult to interpret the rather limited effect of deleting Itsn1 in mice (Yu et al. 2008). Indeed, Itsn2 deletion may cause major problems in endocytosis and/or trafficking of channel proteins, antigen receptors, caveolae-based internalization, or lumen formation. Of course, Itsn1 and Itsn2 may function redundantly in a number of processes. Double mutant mice will be required to test this possibility. Beyond expected redundancies based on the homology of Itsn1 and 2 with each other, or homologies between Itsn proteins and other proteins with EH domains, SH3 domains, or DBL/PH domains that hint at shared functions, a small screen for synthetic lethal or synthetic sick interactions in *C. elegans* has revealed that Itsn proteins can function redundantly with unrelated proteins like Disabled, Dab-1 (Wang et al. 2008). This redundancy may be based on parallel Itsn- and Dab-mediated pathways for endocytosis and/or signal transduction downstream of common cargo proteins.

Future genetic studies are likely to yield insights into novel functions for Itsn proteins in neurons and many other cell types.

References

Chabu C, Doe CQ. Dap160/intersectin binds and activates aPKC to regulate cell polarity and cell cycle progression. Development. 2008;135(16):2739–46.

Das M, Scappini E, Martin NP, Wong KA, Dunn S, Chen YJ, et al. Regulation of neuron survival through an intersectin-phosphoinositide 3′-kinase C2beta-AKT pathway. Mol Cell Biol. 2007;27(22):7906–17.

Evergren E, Gad H, Walther K, Sundborger A, Tomilin N, Shupliakov O. Intersectin is a negative regulator of dynamin recruitment to the synaptic endocytic zone in the central synapse. J Neurosci. 2007;27(2):379–90.

Ferguson SM, Raimondi A, Paradise S, Shen H, Mesaki K, Ferguson A, et al. Coordinated actions of actin and BAR proteins upstream of dynamin at endocytic clathrin-coated pits. Dev Cell. 2009;17(6):811–22.

Guipponi M, Scott HS, Hattori M, Ishii K, Sakaki Y, Antonarakis SE. Genomic structure, sequence, and refined mapping of the human intersectin gene (ITSN), which encompasses 250 kb on chromosome 21q22.1– > q22.2. Cytogenet Cell Genet. 1998;83(3–4):218–20.

He G, Wang HR, Huang SK, Huang CL. Intersectin links WNK kinases to endocytosis of ROMK1. J Clin Invest. 2007;117(4):1078–87.

Henne WM, Boucrot E, Meinecke M, Evergren E, Vallis Y, Mittal R, et al. FCHo proteins are nucleators of clathrin-mediated endocytosis. Science. 2010;328:1281–4.

Hussain NK, Yamabhai M, Ramjaun AR, Guy AM, Baranes D, O'Bryan JP, et al. Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells. J Biol Chem. 1999;274(22):15671–7.

Hussain NK, Jenna S, Glogauer M, Quinn CC, Wasiak S, Guipponi M, et al. Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP. Nat Cell Biol. 2001;3(10):927–32.

Irie F, Yamaguchi Y. EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. Nat Neurosci. 2002;5(11):1117–8.

Khanna R, Li Q, Stanley EF. 'Fractional recovery' analysis of a presynaptic synaptotagmin 1-anchored endocytic protein complex. PLoS One. 2006;1:e67.

Khanna R, Li Q, Schlichter LC, Stanley EF. The transmitter release-site CaV2.2 channel cluster is linked to an endocytosis coat protein complex. Eur J Neurosci. 2007;26(3):560–74.

Khodosh R, Augsburger A, Schwarz TL, Garrity PA. Bchs, a BEACH domain protein, antagonizes Rab11 in synapse morphogenesis and other developmental events. Development. 2006;133(23):4655–65.

Klein I, Predescu D, Sharma T, Knezevic I, Malik A, Predescu S. Intersectin-2 L regulates caveolae endocytosis secondary to Cdc42-mediated actin polymerization. J Biol Chem. 2009;21:2009.

Koh TW, Verstreken P, Bellen HJ. Dap160/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. Neuron. 2004;43(2):193–205.

Koh TW, Korolchuk VI, Wairkar YP, Jiao W, Evergren E, Pan H, et al. Eps15 and Dap160 control synaptic vesicle membrane retrieval and synapse development. J Cell Biol. 2007;178(2):309–22.

Loeb CR, Harris JL, Craik CS. Granzyme B proteolyzes receptors important to proliferation and survival, tipping the balance toward apoptosis. J Biol Chem. 2006;281(38):28326–35.

Majumdar A, Ramagiri S, Rikhy R. Drosophila homologue of Eps15 is essential for synaptic vesicle recycling. Exp Cell Res. 2006;312(12):2288–98.

Malacombe M, Ceridono M, Calco V, Chasserot-Golaz S, McPherson PS, Bader MF, et al. Intersectin-1 L nucleotide exchange factor regulates secretory granule exocytosis by activating Cdc42. EMBO J. 2006;25(15):3494–503.

Marie B, Sweeney ST, Poskanzer KE, Roos J, Kelly RB, Davis GW. Dap160/intersectin scaffolds the periactive zone to achieve high-fidelity endocytosis and normal synaptic growth. Neuron. 2004;43(2):207–19.

Maritzen T, Podufall J, Haucke V. Stonins–specialized adaptors for synaptic vesicle recycling and beyond? Traffic. 2010;11(1):8–15.

McCabe BD, Marques G, Haghighi AP, Fetter RD, Crotty ML, Haerry TE, et al. The BMP homolog Gbb provides a retrograde signal that regulates synaptic growth at the Drosophila neuromuscular junction. Neuron. 2003;39(2):241–54.

McGavin MK, Badour K, Hardy LA, Kubiseski TJ, Zhang J, Siminovitch KA. The intersectin 2 adaptor links Wiskott Aldrich Syndrome protein (WASp)-mediated actin polymerization to T cell antigen receptor endocytosis. J Exp Med. 2001;194(12):1777–87.

Mohney RP, Das M, Bivona TG, Hanes R, Adams AG, Philips MR, et al. Intersectin activates Ras but stimulates transcription through an independent pathway involving JNK. J Biol Chem. 2003;278(47):47038–45.

Nahm M, Kim S, Paik SK, Lee M, Lee S, Lee ZH, et al. dCIP4 (Drosophila Cdc42-interacting protein 4) restrains synaptic growth by inhibiting the secretion of the retrograde glass bottom boat signal. J Neurosci. 2010;30(24):8138–50.

Nakatsu F, Perera RM, Lucast L, Zoncu R, Domin J, Gertler FB, et al. The inositol 5-phosphatase SHIP2 regulates endocytic clathrin-coated pit dynamics. J Cell Biol. 2010;190(3):307–15.

Nishimura T, Yamaguchi T, Tokunaga A, Hara A, Hamaguchi T, Kato K, et al. Role of numb in dendritic spine development with a Cdc42 GEF intersectin and EphB2. Mol Biol Cell. 2006;17(3):1273–85.

O'Connor-Giles KM, Ho LL, Ganetzky B. Nervous wreck interacts with thickveins and the endocytic machinery to attenuate retrograde BMP signaling during synaptic growth. Neuron. 2008;58(4):507–18.

Okamoto M, Schoch S, Sudhof TC. EHSH1/intersectin, a protein that contains EH and SH3 domains and binds to dynamin and SNAP-25. A protein connection between exocytosis and endocytosis? J Biol Chem. 1999;274(26):18446–54.

Pechstein A, Bacetic J, Vahedi-Faridi A, Gromova K, Sundborger A, Tomlin N, et al. Regulation of synaptic vesicle recycling by complex formation between intersectin 1

and the clathrin adaptor complex AP2. Proc Natl Acad Sci USA. 2010a;16:2010.

Pechstein A, Shupliakov O, Haucke V. Intersectin 1: a versatile actor in the synaptic vesicle cycle. Biochem Soc Trans. 2010b;038(1):181–6.

Predescu SA, Predescu DN, Timblin BK, Stan RV, Malik AB. Intersectin regulates fission and internalization of caveolae in endothelial cells. Mol Biol Cell. 2003;14(12):4997–5010.

Predescu SA, Predescu DN, Knezevic I, Klein IK, Malik AB. Intersectin-1 s regulates the mitochondrial apoptotic pathway in endothelial cells. J Biol Chem. 2007;282(23):17166–78.

Pucharcos C, Estivill X, de la Luna S. Intersectin 2, a new multimodular protein involved in clathrin-mediated endocytosis. FEBS Lett. 2000;478(1–2):43–51.

Quinn CC, Chen E, Kinjo TG, Kelly G, Bell AW, Elliott RC, et al. TUC-4b, a novel TUC family variant, regulates neurite outgrowth and associates with vesicles in the growth cone. J Neurosci. 2003;23(7):2815–23.

Rikhy R, Kumar V, Mittal R, Krishnan KS. Endophilin is critically required for synapse formation and function in Drosophila melanogaster. J Neurosci. 2002;22(17):7478–84.

Rodal AA, Motola-Barnes RN, Littleton JT. Nervous wreck and Cdc42 cooperate to regulate endocytic actin assembly during synaptic growth. J Neurosci. 2008;28(33):8316–25.

Rodriguez-Fraticelli AE, Vergarajauregui S, Eastburn DJ, Datta A, Alonso MA, Mostov K, et al. The Cdc42 GEF Intersectin 2 controls mitotic spindle orientation to form the lumen during epithelial morphogenesis. J Cell Biol. 2010; 189(4):725–38.

Roos J, Kelly RB. Dap160, a neural-specific Eps15 homology and multiple SH3 domain-containing protein that interacts with Drosophila dynamin. J Biol Chem. 1998;273(30): 19108–19.

Rose S, Malabarba MG, Krag C, Schultz A, Tsushima H, Di Fiore PP, et al. Caenorhabditis elegans intersectin: a synaptic protein regulating neurotransmission. Mol Biol Cell. 2007;18(12):5091–9.

Sengar AS, Wang W, Bishay J, Cohen S, Egan SE. The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. EMBO J. 1999;18(5):1159–71.

Smith SM, Renden R, von Gersdorff H. Synaptic vesicle endocytosis: fast and slow modes of membrane retrieval. Trends Neurosci. 2008;31(11):559–68.

Sweeney ST, Davis GW. Unrestricted synaptic growth in spinster-a late endosomal protein implicated in TGF-beta-mediated synaptic growth regulation. Neuron. 2002;36(3): 403–16.

Thomas S, Ritter B, Verbich D, Sanson C, Bourbonniere L, McKinney RA, et al. Intersectin regulates dendritic spine development and somatodendritic endocytosis but not synaptic vesicle recycling in hippocampal neurons. J Biol Chem. 2009;284(18):12410–9.

Verstreken P, Koh TW, Schulze KL, Zhai RG, Hiesinger PR, Zhou Y, et al. Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating. Neuron. 2003;40(4): 733–48.

Wang JB, Wu WJ, Cerione RA. Cdc42 and Ras cooperate to mediate cellular transformation by intersectin-L. J Biol Chem. 2005;280(24):22883–91.

Wang W, Bouhours M, Gracheva EO, Liao EH, Xu K, Sengar AS, et al. ITSN-1 controls vesicle recycling at the neuromuscular junction and functions in parallel with DAB-1. Traffic. 2008;9(5):742–54.

Xie J, Vandenbroere I, Pirson I. SHIP2 associates with intersectin and recruits it to the plasma membrane in response to EGF. FEBS Lett. 2008;582(20):3011–7.

Yamabhai M, Hoffman NG, Hardison NL, McPherson PS, Castagnoli L, Cesareni G, et al. Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. J Biol Chem. 1998;273(47):31401–7.

Yu Y, Chu PY, Bowser DN, Keating DJ, Dubach D, Harper I, et al. Mice deficient for the chromosome 21 ortholog Itsn1 exhibit vesicle-trafficking abnormalities. Hum Mol Genet. 2008;17(21):3281–90.

J

JAB (JANUS Kinase Binding Protein)

► SOCS

JAW1-Related Protein Isoform A

► Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

JBP (c-Jun-Binding Protein)

► CKIP-1

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

K

K-Acetyltransferase 2B (KAT2B)

► PCAF Lysine Acetyltransferase

KAT6A

► MOZ and MORF Lysine Acetyltransferases

KAT6B

► MOZ and MORF Lysine Acetyltransferases

K-FGF

► FGF (Fibroblast Growth Factor)

KGF

► FGF (Fibroblast Growth Factor)

KIAA0001

► $P2Y_{14}$ Receptor

KIAA0209

► DOCK2; Dedicator of Cytokinesis 2

KIAA1415

► P-Rex

KIAA1795

► LCoR

Killer Cell Lectin-Like Receptor Subfamily K

► NKG2D

Killer Immunoglobulin-Like Receptors (KIR)

► NK Receptor

Killer Lectin-Like Receptors (KLR)

► NK Receptor

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

Kinin B_1

► Bradykinin Receptors

Kinin B_2

► Bradykinin Receptors

Ki-Ras

► RAS (H-, K-, N-RAS)

Kiz

► LIMK

Kss1 (*Saccaromyces cerevisiae*)

► ERK1/ERK2

L

LAD

► SH2D2A

Laforin: Function and Action of a Glucan Phosphatase

Amanda R. Sherwood, Vikas V. Dukhande and Matthew S. Gentry
Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Synonyms

EPM2A; LAF-PTPase; MELF

Historical Background

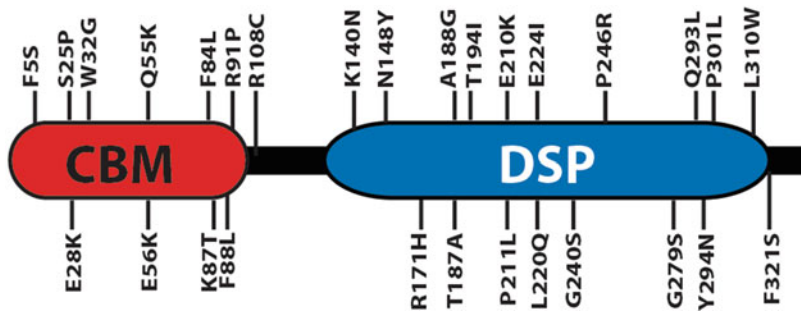
Two groups searching for genes mutated in Lafora disease patients simultaneously discovered the *EPM2A* gene (*epilepsy, progressive myoclonic type 2A*) that encodes the protein laforin (Minassian et al. 1998; Serratosa et al. 1995). Laforin is a bimodular protein containing a dual specificity phosphatase (DSP) domain and a carbohydrate-binding module (CBM) (Fig. 1). Dual specificity phosphatases (DSPs) are a diverse group of phosphatases whose members dephosphorylate phosphoserine/phosphothreonine, phosphotyrosine, phosphoinositols, ribo/deoxyribonucleotide 5′-triphosphates, pyrophosphate/triphosphate, or phospho-glucans. DSPs are members of the larger protein tyrosine phosphatase (PTP) superfamily that all utilize a cysteine-dependent mechanism to hydrolyze phosphoester bonds. This mechanism is dependent on the conserved CX_5R active site motif common to all protein tyrosine phosphatases. Laforin is the founding member of a small group within the DSPs called the glucan phosphatases, that is, laforin liberates phosphate from glucans such as glycogen. CBMs are divided into 63 families based on primary, secondary, and, when available, tertiary structure. In addition to binding and dephosphorylating glucans, laforin also appears to act as a scaffolding protein. The gene encoding laforin is conserved in all vertebrates and in a subset of protists and invertebrates (Gentry et al. 2007). Loss of function recessive mutations in the gene encoding laforin results in a fatal disease called Lafora disease (LD).

Lafora Disease

Lafora disease (LD) is a fatal neurodegenerative epilepsy that is autosomal recessive and is characterized by declining neurological functions over time. LD is one of four diseases that represent the inherited progressive myoclonus epilepsy (PME) family; the PMEs collectively constitute ≈ 1% of all epilepsies. Due to the progressive nature of neurodegeneration, the frequency of myoclonic seizures, and the variety of seizure types that can present, PMEs are often difficult to diagnose at onset (reviewed in Minassian 2001).

Lafora disease is characterized by myoclonic, tonic-clonic, and focal-occipital seizures among other

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



Laforin: Function and Action of a Glucan Phosphatase,
Fig. 1 A schematic of laforin. Laforin contains an amino-terminal carbohydrate-binding module (CBM) followed by a dual specificity phosphatase (DSP) domain. Lafora disease missense mutations are shown

seizure types that increase in frequency and severity with age, with the first tonic-clonic epileptic presentation occurring in late childhood or early adolescence. Other symptoms include dementia, visual hallucinations, ataxia, absence and grand mal seizures along with epilepsy triggered by stimulus (reviewed in Minassian 2001). Death occurs within 10 years following progressive neurodegeneration due to complications such as status epilepticus, aspiration pneumonia, or respiratory failure. There is significant neuronal loss across the CNS upon death with no signs of inflammation (reviewed in Ganesh et al. 2006; Minassian 2001).

Approximately 50% of LD cases result from recessive inactivating mutations in the *EPM2A* gene that encodes laforin. Mutations in laforin disrupt activity, substrate binding, interaction partner binding, or localization, and are evenly distributed across the protein (Fig. 1) (reviewed in Dukhande et al. 2010). LD also results from recessive mutations in the *EPM2B* (*epilepsy, progressive myoclonic type 2B*) gene encoding the laforin-binding partner and E3 ubiquitin ligase malin (Chan et al. 2003).

A hallmark of LD is the presence of insoluble polyglucosan bodies termed Lafora bodies (LBs) in the cytoplasm of cells from most tissues. These tissues, including neurons, cardiac muscle, and liver, also display increased laforin expression in normal individuals (reviewed in Ganesh et al. 2006). The spherical LB inclusions are 3–40 μm in size and can grow large enough to occupy the entire cytoplasm. The inclusions found in LD contain only a small amount of ubiquitinated protein and are instead composed of improperly branched and hyperphosphorylated glucose moieties. Despite the presence of LBs in a variety of tissues throughout the body, non-neurological symptoms are uncommon (reviewed in Minassian 2001).

Laforin Expression, Isoforms, and Localization

Laforin mRNA is widely expressed in the human body. All tissues examined contain laforin mRNA, including brain, heart, skeletal muscle, liver, lung, placenta, kidney, testis, spleen, thymus, prostate, ovary, small intestine, and pancreas (Minassian et al. 1998). Various brain regions such as cerebellum, cortex, medulla, putamen, and spinal cord also express laforin transcript at similar levels, demonstrating the ubiquitous expression of laforin in brain.

There are at least two laforin isoforms. One isoform is a 331 amino acid protein (laforin-331) and the other is a 317 amino acid product (laforin-317) (Ganesh et al. 2002b). Laforin-317 lacks 22 residues from the carboxy-terminus of laforin-331 and has a unique 8 amino acid extension. Laforin-331 is the dominant species and unless otherwise noted is the one discussed throughout this review.

Overexpressed laforin is largely found in the cytosol with some signs of endoplasmic reticulum (ER) localization, and lower levels are found in the nucleus (reviewed in Minassian 2001). Overexpressed laforin also localizes at polyribosomes on rough ER and at the inner surface of cell membranes. The ER-localized laforin forms aggregates at centrosomes following treatment with proteasomal inhibitor. Conversely, laforin-317 localizes to both the ER and nucleus when overexpressed (Ganesh et al. 2002a).

Due to its CBM, laforin has the inherent ability to bind glucans and this domain leads to co-localization of laforin with glycogen synthase and glycogen granules. Mutations in the CBM disrupt this co-localization. A study employing mice overexpressing catalytically inactive phosphatase domain (C266S) of laforin showed that laforin preferentially binds LBs

over glycogen. While multiple studies have investigated laforin localization when overexpressed in mammalian cell culture, no study has described the localization of endogenous laforin in a vertebrate. Endogenous laforin localization was reported for the red algae *Cyanidioschyzon merolae*. In *C. merolae*, laforin co-localized with the outer surface of starch granules as demonstrated by immunogold electron microscopy (Gentry et al. 2007).

There is no study describing transcriptional or translational regulation of laforin concentration. However, laforin protein levels are regulated by the ubiquitin-proteasome system and a direct correlation has been described between laforin protein levels and muscle glycogen content. Both of these mechanisms are discussed in detail below in "Regulation of Laforin Activity and Concentration".

Laforin Activity

Consistent with its designation as a dual specificity phosphatase, laforin exhibits in vitro phosphatase activity against phospho-serine, threonine, and tyrosine residues of various exogenous substrates (reviewed in Ganesh et al. 2006). In addition to this activity, laforin can utilize the artificial substrates 3-O-methylfluorescein phosphate (OMFP) and *para*-nitrophenyl phosphate (*p*-NPP) (reviewed in Dukhande et al. 2010; Gentry et al. 2007). While none of these substrates are physiologically relevant, they do establish that laforin is an active phosphatase and allow one to compare the activity of laforin to other phosphatases.

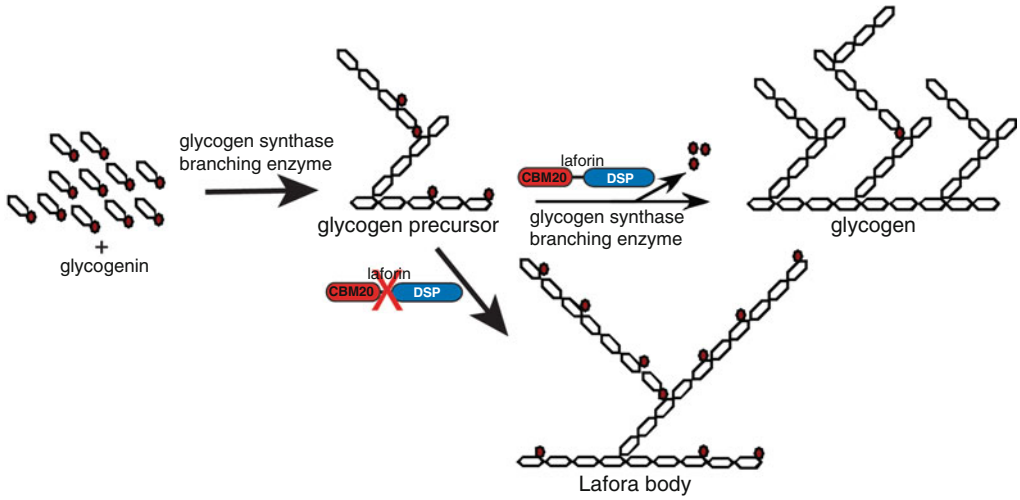
Dixon and colleagues first identified that laforin contains a CBM and demonstrated that this domain allows laforin to bind glucans (Wang et al. 2002). LD patient mutations in the CBM of laforin disrupt glucan binding both in vitro and in vivo. Out of 68 dual specificity phosphatases, laforin is the only DSP with a CBM. This fact coupled with a finding from the 1960s that Lafora bodies have increased phosphate prompted Gentry and colleagues to test whether laforin could liberate phosphate from glucans. Laforin does indeed dephosphorylate glucans, while other DSPs and phosphatases from other families lack this activity (Worby et al. 2006). Subsequently, multiple labs have demonstrated that laforin utilizes its two domains to bind and dephosphorylate glycogen. In addition, loss of function mutations in laforin leads to increased phosphate content in glycogen (Sakai et al. 1970; Tagliabracci et al. 2007). Furthermore, LD patient mutations in the CBM or DSP of laforin abolish its glucan phosphatase activity (Gentry et al. 2007). Recent work from the Roach lab demonstrated that as phosphate levels increase in glycogen the glycogen becomes insoluble (Tagliabracci et al. 2007, 2008). These inclusions eventually form LBs and more closely resemble plant starch than glycogen.

Cumulatively, these results strongly suggest that laforin regulates glycogen phosphate levels to inhibit LB formation. The targeting of laforin to sites of glycogen synthesis by the CBM in order to regulate phosphate is critical for normal glycogen formation, with covalent phosphate occurring as an error of glycogen synthesis (reviewed in Gentry et al. 2007, 2009; Tagliabracci et al. 2011) (Fig. 2).

Regulation of Laforin Activity and Concentration

One mechanism that appears to modulate laforin protein levels is fluctuations in glycogen stores. Investigation into mouse models that accumulate higher or lower levels of glycogen revealed that laforin protein expression is upregulated as glycogen stores increase (Wang et al. 2006a). This result suggests that glycogen and laforin levels are closely linked. In addition, laforin levels in the brains of adult mice have also been seen to increase with age, indicating that laforin may have a vital role in the maturation of the CNS. While these links have been described, exact mechanisms regulating these fluctuations in laforin protein levels are unknown.

The ubiquitin-proteasome degradation pathway carries out a second mechanism regulating laforin protein levels. The single-subunit RING-type E3 ubiquitin ligase malin binds, polyubiquitinates, and promotes the proteasomal degradation of laforin in cell culture (Gentry et al. 2005). The interaction between laforin and malin is mediated by the NHL (NCL-1, HT2A, and LIN-41 proteins) domains of malin (Gentry et al. 2005). Inhibition of proteasome activity by MG-132 results in increased laforin levels, indicating that laforin levels are regulated by the proteasome. In addition, malin ubiquitination of laforin was recapitulated using purified components in vitro (Gentry et al. 2005). In support of this finding, inactivating mutations in malin leads to increased levels of laforin in both humans and mice



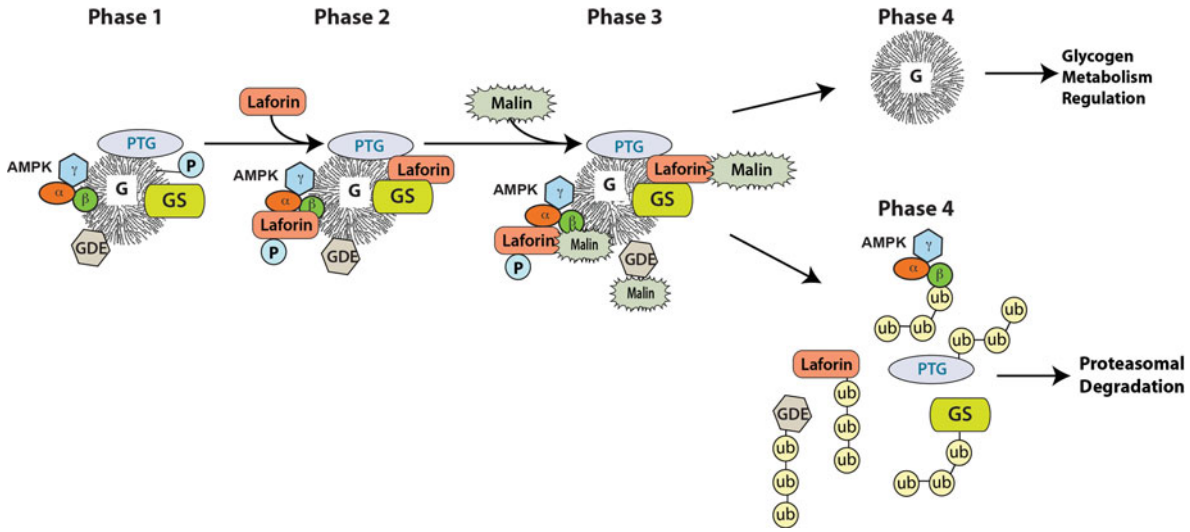
Laforin: Function and Action of a Glucan Phosphatase, Fig. 2 Model of Lafora body formation caused by loss of laforin activity. Glycogen synthesis involves the concerted efforts of glycogen synthase and branching enzyme covalently attaching glucose moieties, initially in the form of UDP-glucose, to glycogenin. UDP-glucose are depicted on the far *left* as hexagons with phosphate (*red circles*). Glycogen is composed of α-1,4-linked glucose moieties with branches occurring via α-1,6-glycosidic linkages. Glycogen contains a small amount of covalently linked phosphate that is present as both a phosphomonoester and phosphodiester. *Red circles* represent phosphate with monoester linkage depicted adjacent to hexagons, and diester linkage depicted between two glucose hexagons. Glycogen synthase incorporates ≈ 1 phosphate per 10,000 glucose residues as a phosphomonoester during glycogen synthesis. Laforin dephosphorylates glycogen phosphomonoesters so that glycogen remains water-soluble. In the absence of laforin, glycogen phosphomonoesters accumulate and glycogen becomes less branched; phosphomonoesters inhibit glycogen solubility and eventually result in Lafora body formation

(Chan et al. 2003; DePaoli-Roach et al. 2010). Currently, it is unknown what triggers malin ubiquitination of laforin, but in vitro biochemical and in vivo LD patient data both support this finding.

Inactivating recessive mutations in *EPM2B*, the gene encoding malin, also results in Lafora disease. The downregulation of laforin by malin is puzzling with regard to LD pathogenesis, as LD occurs due to loss of function of either malin or laforin. Why then would one protein that inhibits LD (i.e., malin) trigger degradation of the second protein (i.e., laforin)? The answer to this question is still unresolved, but malin-directed ubiquitination of laforin is supported by in vitro biochemical data, in vivo mouse models, and LD patient data (Chan et al. 2003; DePaoli-Roach et al. 2010; Gentry et al. 2005).

A third means of regulating laforin activity is homodimerization and heterodimerization of different laforin splice variants. Ganesh and colleagues characterized the interaction of two laforin isoforms that possess different carboxy termini (Dubey and Ganesh 2008). The major isoform consists of 331 amino acids and the minor isoform consists of 317 amino acids. They found that monomeric laforin-317 and homodimers of laforin-317 lack phosphatase activity and both are unable to bind glucans. In addition, these two isoforms form heterodimers that lack phosphatase activity and are unable to bind glucans. Thus, heterodimer formation is a mechanism to modulate laforin glucan binding and its phosphatase activity.

Recently, insights into the regulation of the laforin-malin interaction were provided by a study reporting that AMP-activated protein kinase (AMPK) phosphorylates laforin (Solaz-Fuster et al. 2008). A subsequent study identified serine 25 within the CBM of laforin as the residue that is phosphorylated by AMPK. Ser25 phosphorylation results in increased interaction between laforin and malin. Thus, the laforin-malin interaction is enhanced in conditions that activate AMPK. These studies identify a direct signaling link responsible for modulating the laforin-malin complex and a means to regulate laforin-directed malin ubiquitination of glycogen metabolism enzymes.



Laforin: Function and Action of a Glucan Phosphatase, Fig. 3 Schematic depicting proposed role of laforin and its interactions in glycogen metabolism. In phase 1, the heterotrimeric AMP-activated protein kinase (AMPK), protein targeting to glycogen (PTG), glycogen synthase (GS), and glycogen debranching enzyme (GDE) bind to glycogen. Laforin binds glycogen via its CBM in phase 2 and dephosphorylates glycogen. In addition, AMPK phosphorylates laforin. In phase 3, malin is targeted to laforin and laforin acts as a targeting subunit to direct malin ubiquitination of PTG, GS, and AMPK. Laforin is also ubiquitinated by malin. In addition, malin ubiquitinates GDE, but the role of laforin in this event is unknown. These ubiquitination events lead to the degradation of laforin, AMPK, GS, GDE, and PTG in phase 4. Despite data from multiple labs utilizing multiple models and techniques, malin-deficient mice do not show increased levels of PTG, GS, or GDE. Therefore, the validity of these findings is currently in question. For simplicity purposes, the spatial-temporal nature of these interactions is not depicted and many of them are unknown

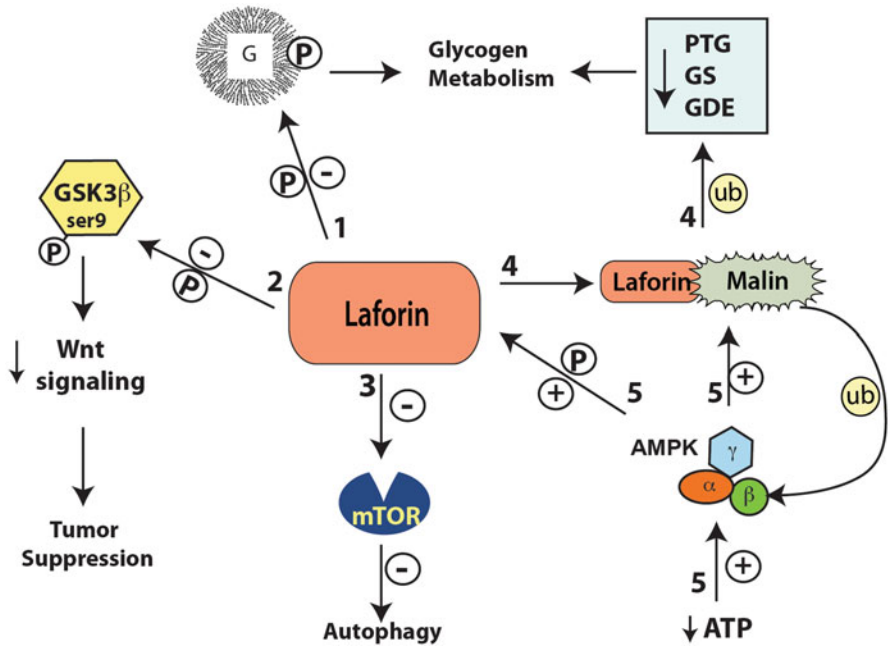
Interactions and Signaling

Laforin interacts with multiple cellular components via its DSP and CBM domains. Laforin interactions can be classified based on the following: (1) binding to glucans, (2) dephosphorylation of substrates, (3) role as a scaffolding protein, and (4) other protein-protein interactions. Many of these interactions are directly involved in glycogen metabolism (Fig. 3).

Laforin is the only phosphatase in vertebrate genomes to have a CBM and the presence of the CBM enables laforin to bind cellular glycogen (Wang et al. 2002). Similarly, the functional homolog of laforin in plant and green algal genomes called as Starch EXcess4 (SEX4) binds and dephosphorylates the glucan starch (Gentry et al. 2007). Mutations in the CBM of SEX4 or laforin disrupt this interaction and abolish their glucan phosphatase activity (Gentry et al. 2007; Tagliabracci et al. 2007). Laforin not only interacts with glucans in vitro, but it also co-localizes with Lafora bodies in mice overexpressing inactivated laforin. Thus, the glucan binding of laforin is supported by data from both in vivo and in vitro systems.

There is now overwhelming evidence that laforin is a glucan phosphatase. First, laforin dephosphorylates glucans such as glycogen and amylopectin in vitro (Tagliabracci et al. 2007; Worby et al. 2006). Conversely, other phosphatases tested are not able to liberate phosphate from glucans (Worby et al. 2006). In addition, laforin homologs in protists and plants also dephosphorylate glucans (Gentry et al. 2007). The glucan phosphatase activity of laforin is also supported by the finding of increased phosphate content of LBs isolated from LD patients compared to phosphate levels in glycogen (reviewed in Gentry et al. 2009; Sakai et al. 1970). Similarly, the phosphate content of LBs isolated from muscle and hepatic tissue of *EPM2A* knockout mice is higher than the phosphate content of glycogen from wildtype mice (Tagliabracci et al. 2007, 2008) (Fig. 4).

In addition to dephosphorylating glycogen, some have reported that laforin also dephosphorylates glycogen synthase kinase3β (GSK3β) at serine-9 and modifies the function of GSK3β in Wnt signaling (Lohi et al. 2005; Wang et al. 2006b). However, this point is currently under debate since other groups have



Laforin: Function and Action of a Glucan Phosphatase, Fig. 4 Model of the proposed roles of laforin in signaling events. (1) Laforin dephosphorylates glycogen to allow normal glycogen metabolism and inhibit Lafora body formation. (2) Laforin is proposed to dephosphorylate pSer9 of GSK3β and decreasing Wnt signaling. Loss of laforin is proposed to result in increased tumor formation. As discussed in the text, data from other labs do not support these findings. (3) Recently, laforin was shown to positively affect autophagosome formation by decreasing mTOR activity via an unknown mechanism. (4) Laforin forms a complex with malin that ubiquitinates protein targeting to glycogen (PTG), glycogen synthase (GS), and AMP-activated protein kinase (AMPK). In addition, malin has been shown to ubiquitinate glycogen debranching enzyme (GDE). As discussed in the text and in Fig. 3, data from the malin knockout mouse do not support these findings. (5) Decreases in ATP activate AMPK to phosphorylate laforin and this increases the interaction between laforin and malin

not observed GSK3β dephosphorylation by laforin (Wang et al. 2007; Worby et al. 2006a). Additionally, laforin has been shown to dephosphorylate the microtubule-stabilizing protein tau (Puri et al. 2009). It is interesting to note that pathological accumulations of misfolded tau, denoted as tangles, are observed in brains of Alzheimer's disease patients, thus uncovering a potential similar mechanism of neurodegeneration between Alzheimer's disease progression and that of LD. Lastly, loss of laforin was recently shown to decrease the activity of the mTOR pathway and inhibit autophagosome formation (Aguado et al. 2010). Thus, it appears that laforin positively influences autophagy (Fig. 4).

In addition to binding and dephosphorylating glucans and perhaps proteins, laforin is also postulated to act as a scaffolding protein for the E3 ubiquitin ligase malin. As discussed above, malin is a single-subunit RING-type E3 ubiquitin ligase that ubiquitinates laforin, and autosomal recessive mutations in the gene encoding malin also cause LD (Chan et al. 2003; Gentry et al. 2005). In addition to ubiquitinating laforin, malin also appears to form a functional complex with laforin (Moreno et al. 2010; Solaz-Fuster et al. 2008; Worby et al. 2008). In this complex, laforin acts as a targeting protein to direct the laforin-malin complex to ubiquitinate and degrade protein targeting to glycogen (PTG), glycogen synthase, and AMP-activated protein kinase (AMPK) (Moreno et al. 2010; Solaz-Fuster et al. 2008; Worby et al. 2008) (Fig. 4). Despite complementary work from multiple labs, the levels of PTG and glycogen synthase did not change in tissues from malin knockout mice compared to wild-type (DePaoli-Roach et al. 2010). Thus, the functional significance of laforin-malin-mediated degradation of these proteins is not completely elucidated.

Lastly, laforin interacts with two other proteins whose relevance to LD is not currently understood

(Fig. 4). Both proteins were identified by yeast two-hybrid screens and verified by immunoprecipitation of overexpressed proteins. The first protein is EPM2A-interacting protein1 (EPM2AIP1). EPM2AIP1 has no reported domains and no known function. In addition to EPM2AIP1, the CBM of laforin interacts with the NifU domain of HIRIP5. NifU is an iron-sulfur cluster assembly domain and HIRIP5 has been implicated in iron-sulfur cluster biosynthesis in yeast. Currently, the functional significance of these interactions is not known.

The Role of Laforin in Lafora Disease

Laforin prevents the accumulation of polyglucosan inclusions by decreasing the amount of phosphate present in glycogen. Failure to remove phosphate covalently attached to glucose moieties disrupts packing within glycogen or glycogen branching (Fig. 2), leading to the accumulation of insoluble LBs. Previously, it was not understood whether LB formation was a result of an overactive polyglucosan biosynthetic pathway or a disrupted degradative metabolic pathway. Recent work determined that phosphate is incorporated into glycogen at a rate of ≈ 1 phosphate per 10,000 glucose residues by glycogen synthase during glycogen metabolism (Tagliabracci et al. 2011). Based on these results, the role of laforin is to negate the errors of glycogen synthase and remove phosphate from glycogen that is incorporated by glycogen synthase (Tagliabracci et al. 2011). The lack of this function in patients with mutations in the *EPM2A* gene leads to hyperphosphorylated, insoluble glycogen accumulations that eventually form LBs.

The accumulation of LBs appear to be particularly detrimental in neurons, possibly disrupting cellular trafficking, disturbing neuronal membrane excitability, or perturbing energy homeostasis that results in neuronal cell death. However, cell death has been observed in the absence of LBs, indicating that other factors may be influencing neuron death (Ganesh et al. 2002b).

In addition to LBs, cells overexpressing mutant laforin also exhibit impaired clearance of misfolded proteins with the appearance of ubiquitin-positive perinuclear aggresomes (Ganesh et al. 2002b). These aggregates of proteins marked for proteasomal degradation are also present in neurons of both LD patients as well as laforin-deficient mice. Impaired protein clearance may also be involved in neuronal cell death in LD patients. Upregulation of the unfolded protein response due to endoplasmic reticulum stress is seen when laforin is mutated or missing. The fact that malin, an E3-ubiquitin ligase, is mutated in LD also provides support that LD is a disease of impaired protein clearance as the ubiquitination pathway is involved in protein turnover by the proteasome. Furthermore, disruption of exon 4 of the *EPM2A* gene in mice leads to cell death often prior to the formation of LBs, and neurons containing LBs often did not deteriorate in these mice (Ganesh et al. 2002b). Therefore, neuronal cell death might be driven by a combination of impaired protein clearance and LBs.

Summary

Laforin is a bimodular protein that contains an amino-terminal CBM followed by a DSP domain and is encoded by the *EPM2A* gene. Recessive, loss of function mutations in *EPM2A* results in Lafora disease (LD). Laforin participates in a number of interactions, and these interactions can be classified based on the following: (1) binding to glucans, (2) dephosphorylation of substrates, (3) role as a scaffolding protein, and (4) other protein-protein interactions. While there is currently debate regarding the role of laforin in many of its proposed functions, it is clear that laforin activity impacts glycogen metabolism. The central theme of Lafora disease revolves around glycogen metabolism: LBs are a "foreign" type of "glycogen," laforin binds glucans, and malin regulates the level of proteins involved in glycogen metabolism. Multiple labs have now demonstrated that laforin binds and dephosphorylates glycogen, making it a glucan phosphatase. Designation of laforin as a glucan phosphatase is supported by in vitro biochemical data, data from mouse models, and LD patient data. While the other proposed functions of laforin are intriguing and supported by some lines of evidence, they require more research to determine which are biologically correct and relevant to Lafora disease.

References

Aguado C, Sarkar S, Korolchuk VI, Criado O, Vernia S, Boya P, et al. Laforin, the most common protein mutated in Lafora disease, regulates autophagy. Hum Mol Genet. 2010; 19:2867–76.

Chan EM, Young EJ, Ianzano L, Munteanu I, Zhao X, Christopoulos CC, et al. Mutations in NHLRC1 cause progressive myoclonus epilepsy. Nat Genet. 2003;35:125–7.

DePaoli-Roach AA, Tagliabracci VS, Segvich DM, Meyer CM, Irimia JM, Roach PJ. Genetic depletion of the malin E3 ubiquitin ligase in mice leads to lafora bodies and the accumulation of insoluble laforin. J Biol Chem. 2010;285:25372–81.

Dubey D, Ganesh S. Modulation of functional properties of laforin phosphatase by alternative splicing reveals a novel mechanism for the EPM2A gene in Lafora progressive myoclonus epilepsy. Hum Mol Genet. 2008;17:3010–20.

Dukhande VV, Sherwood AR, Gentry MS. Laforin- nature molecule page. Nature molecule pages. 2010. doi: 10.1038/mp.a000032.01

Ganesh S, Delgado-Escueta AV, Sakamoto T, Avila MR, Machado-Salas J, Hoshii Y, et al. Targeted disruption of the Epm2a gene causes formation of Lafora inclusion bodies, neurodegeneration, ataxia, myoclonus epilepsy and impaired behavioral response in mice. Hum Mol Genet. 2002b;11:1251–62.

Ganesh S, Suzuki T, Yamakawa K. Alternative splicing modulates subcellular localization of laforin. Biochem Biophys Res Commun. 2002a;291:1134–7.

Ganesh S, Puri R, Singh S, Mittal S, Dubey D. Recent advances in the molecular basis of Lafora's progressive myoclonus epilepsy. J Hum Genet. 2006;51:1–8.

Gentry MS, Worby CA, Dixon JE. Insights into Lafora disease: malin is an E3 ubiquitin ligase that ubiquitinates and promotes the degradation of laforin. Proc Natl Acad Sci USA. 2005;102:8501–6.

Gentry MS, Dowen 3 RH, Worby CA, Mattoo S, Ecker JR, Dixon JE. The phosphatase laforin crosses evolutionary boundaries and links carbohydrate metabolism to neuronal disease. J Cell Biol. 2007;178:477–88.

Gentry MS, Dixon JE, Worby CA. Lafora disease: insights into neurodegeneration from plant metabolism. Trends Biochem Sci. 2009;34:628–39.

Lohi H, Ianzano L, Zhao XC, Chan EM, Turnbull J, Scherer SW, et al. Novel glycogen synthase kinase 3 and ubiquitination pathways in progressive myoclonus epilepsy. Hum Mol Genet. 2005;14:2727–36.

Minassian BA. Lafora's disease: towards a clinical, pathologic, and molecular synthesis. Pediatr Neurol. 2001;25:21–9.

Minassian BA, Lee JR, Herbrick JA, Huizenga J, Soder S, Mungall AJ, et al. Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. Nat Genet. 1998;20:171–4.

Moreno D, Towler MC, Hardie DG, Knecht E, Sanz P. The laforin-malin complex, involved in Lafora disease, promotes the incorporation of K63-linked ubiquitin chains into AMP-activated protein kinase beta subunits. Mol Biol Cell. 2010;21:2578–88.

Puri R, Suzuki T, Yamakawa K, Ganesh S. Hyperphosphorylation and aggregation of Tau in laforin-deficient mice, an animal model for Lafora disease. J Biol Chem. 2009;284:22657–63.

Sakai M, Austin J, Witmer F, Trueb L. Studies in myoclonus epilepsy (Lafora body form). II. Polyglucosans in the systemic deposits of myoclonus epilepsy and in corpora amylacea. Neurology. 1970;20:160–76.

Serratosa JM, Delgado-Escueta AV, Posada I, Shih S, Drury I, Berciano J, et al. The gene for progressive myoclonus epilepsy of the Lafora type maps to chromosome 6q. Hum Mol Genet. 1995;4:1657–63.

Solaz-Fuster MC, Gimeno-Alcaniz JV, Ros S, Fernandez-Sanchez ME, Garcia-Fojeda B, Criado Garcia O, et al. Regulation of glycogen synthesis by the laforin-malin complex is modulated by the AMP-activated protein kinase pathway. Hum Mol Genet. 2008;17:667–78.

Tagliabracci VS, Turnbull J, Wang W, Girard JM, Zhao X, Skurat AV, et al. Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. Proc Natl Acad Sci USA. 2007;104:19262–6.

Tagliabracci VS, Girard JM, Segvich D, Meyer C, Turnbull J, Zhao X, et al. Abnormal metabolism of glycogen phosphate as a cause for Lafora disease. J Biol Chem. 2008;283:33816–25.

Tagliabracci VS, Heiss C, Karthik C, Contreras CJ, Glushka J, Ishihara M, et al. Phosphate incorporation during glycogen synthesis and Lafora disease. Cell Metab. 2011;13:274–82.

Wang J, Stuckey JA, Wishart MJ, Dixon JE. A unique carbohydrate binding domain targets the lafora disease phosphatase to glycogen. J Biol Chem. 2002;277:2377–80.

Wang W, Parker GE, Skurat AV, Raben N, DePaoli-Roach AA, Roach PJ. Relationship between glycogen accumulation and the laforin dual specificity phosphatase. Biochem Biophys Res Commun. 2006a;350:588–92.

Wang Y, Liu Y, Wu C, Zhang H, Zheng X, Zheng Z, et al. Epm2a suppresses tumor growth in an immunocompromised host by inhibiting Wnt signaling. Cancer Cell. 2006b;10:179–90.

Wang W, Lohi H, Skurat AV, DePaoli-Roach AA, Minassian BA, Roach PJ. Glycogen metabolism in tissues from a mouse model of Lafora disease. Arch Biochem Biophys. 2007;457:264–9.

Worby CA, Gentry MS, Dixon JE. Laforin, a dual specificity phosphatase that dephosphorylates complex carbohydrates. J Biol Chem. 2006;281:30412–8.

Worby CA, Gentry MS, Dixon JE. Malin decreases glycogen accumulation by promoting the degradation of protein targeting to glycogen (PTG). J Biol Chem. 2008;283:4069–76.

LAF-PTPase

▶ Laforin: Function and Action of a Glucan Phosphatase

LAMMER Kinases

▶ CLK

LAT

Sarah Sullivan and Weiguo Zhang
Department of Immunology, Duke University Medical Center, Durham, NC, USA

Historical Background

Engagement of the T cell receptor (TCR) with MHC-peptide complexes leads to the activation of protein tyrosine kinases (PTK), ultimately leading to the activation of downstream signaling events, such as calcium flux and mitogen-activated protein kinase (MAPK) activation (Chan et al. 1994). For many years, it was observed that a 36–38 kDa protein, localized to the plasma membrane, was highly tyrosine phosphorylated upon ligation of the TCR and associated with Grb2, PLC-γ1, and the p85 subunit of PI3K. It was hypothesized that this protein served as a critical link connecting TCR engagement at the membrane to activation of signaling events in the cytosol. In 1998, the gene encoding this protein was identified after microsequencing phosphorylated proteins purified from the membrane fractions of activated Jurkat T cells and was named LAT, *l*inker for *a*ctivation of *T* cells. Sequencing of cDNA clones revealed that LAT is a type III transmembrane protein that contains a short extracellular domain, a transmembrane domain, and a long cytoplasmic tail. While LAT has no apparent structural domains, it has multiple tyrosine motifs for binding Grb2. Further biochemical analysis confirmed that it does interact with Grb2, PLC-γ1, and p85 (Zhang 1998a). The indispensible role of LAT in TCR-mediated signaling and thymocyte development was subsequently demonstrated by studies using LAT-deficient Jurkat T cell lines and knockout mice. In 2003, the other member of the LAT protein family, linker for activation of B cells (LAB)/non-T cell activation linker (NTAL), was discovered (Janssen et al. 2003; Brdicka et al. 2002).

LAT Palmitoylation

Biochemical analyses indicate that LAT is constitutively localized to lipid rafts, similar to Lck and Fyn. In the juxtamembrane region of LAT, there are two conserved cysteine residues, which are covalently modified by palmitate, a 16-carbon fatty acid. Studies using LAT cysteine mutants clearly demonstrate that palmitoylation is required for LAT phosphorylation and function in TCR-mediated signaling (Zhang 1998b). Moreover, LAT palmitoylation is impaired in anergic T cells (Hundt et al. 2006). As of yet, the role of LAT palmitoylation remains unclear. While palmitoylation targets LAT to lipid rafts, raft localization is not essential for LAT function (Zhu et al. 2005). It is possible that palmitoylation is required for trafficking of LAT from the Golgi to the plasma membrane (Hundt et al. 2009) or simply for stably tethering LAT to the plasma membrane.

The Positive Role of LAT in TCR-Mediated Signaling

Upon T cell receptor ligation with an MHC-peptide complex, Src family kinases, specifically Lck and Fyn, are activated and phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the TCRζ and ▶ CD3 chains. Phosphorylation of ITAMs creates binding sites for ▶ ZAP-70, which is subsequently activated by Lck and Fyn. ZAP-70 phosphorylates LAT and other signaling proteins (Samelson 2002). There are nine conserved tyrosine residues between murine and human LAT. Among these tyrosine residues, the four distal tyrosines of LAT mediate the binding of Grb2, Gads, and PLC-γ1 and are vital for T cell activation. Grb2 recruits the Ras guanine nucleotide exchange factor, Son of sevenless (Sos), which activates the Ras-Raf-MAP kinase pathway and results in the activation of the transcription factor AP-1. In addition, Grb2 also stabilizes the interactions between LAT and its other binding partners, Gads and PLCγ1 (Zhu et al. 2003). By binding to LAT, Gads mediates the interaction between LAT and ▶ SLP-76, another important adaptor protein. SLP-76 regulates actin polymerization and cytoskeleton rearrangement via recruitment of ▶ Vav, the guanine nucleotide exchange factor for Rac/Rho. Additionally, SLP-76 stabilizes PLC-γ1 binding to LAT and recruits the Tec kinase, Itk, which phosphorylates and activates PLC-γ1 (Jordan et al. 2003). PLC-γ1 is responsible for the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is important for

TCR-mediated calcium mobilization and subsequent activation of the transcription factor ▶ NFAT. DAG has dual functions in that it activates protein kinase C (PKC), triggering downstream ▶ NF-κB nuclear translocation, as well as serving to activate the Ras-Erk pathway through the Ras guanine nucleotide exchange factor, ▶ RasGRP1. Since all of these pathways depend on LAT for their activation, the absence of LAT has severe ramifications on TCR signaling. In LAT-deficient Jurkat cell lines, TCR-mediated calcium flux, Erk activation, and IL-2 production are greatly impaired compared to normal Jurkat cells. Phosphorylation of PLC-γ1 and SLP-76 in these cells is also reduced. These signaling defects can be corrected by the reconstitution of LAT expression in these cells (Finco et al. 1998).

In addition to its vital role in the TCR-mediated signaling pathway, LAT is also essential for T cell development in the thymus. LAT-deficient mice show an early block at the DN3 ($CD44^-CD25^+$) stage, indicating its importance in pre-TCR signaling. Consequently, LAT-deficient mice lack mature αβ T cells. Moreover, γδ T cells also need LAT signaling during development to enter the periphery (Zhang et al. 1999). Recent data indicate that LAT is necessary to transmit signals through a functional rearranged TCRαβ receptor during the transition from DP ($CD4^+CD8^+$) to SP ($CD4^+$ or $CD8^+$) (Shen et al. 2009).

In addition to its well-defined role as a positive regulator of TCR-mediated signaling, LAT is also critical for FcεRI-mediated signaling in mast cells. LAT-deficient mast cells have impaired MAPK activation, cytokine production, and degranulation. $LAT^{-/-}$ mice are resistant to IgE-mediated passive systemic anaphylaxis, indicating the essential role of LAT in FcεRI-mediated signaling in mast cells (Saitoh et al. 2000).

The Negative Role of LAT in TCR-Mediated Signaling

While LAT mainly serves to positively regulate TCR-mediated calcium mobilization and MAPK activation through binding Grb2, Gads, and PLC-γ1, it also negatively impacts TCR signaling by recruiting other proteins, such as ▶ Gab2 and SHIP-1. After TCR activation, Grb2 associated binding protein 2 (Gab2), which is constitutively associated with Gads and Grb2, is recruited to LAT and is then phosphorylated by Zap-70. Gab2, in turn, recruits SH2 domain-containing tyrosine phosphatase-2 (SHP2) to dephosphorylate key signaling molecules, such as CD3ζ (Yamasaki et al. 2003).

LAT can also recruit Src homology 2 domain–containing inositol polyphosphate 5-phosphatase-1 (SHIP-1) to the TCR complex through its interaction with Grb2. SHIP-1 is required to anchor downstream of kinase-2 (Dok-2) to the LAT complex as well as for Dok-2 tyrosine phosphorylation. Together with Dok-1, Dok-2 then negatively regulates Zap-70 and Akt kinase activation (Dong et al. 2006). Through its association with negative regulators, LAT plays a critical role in dampening the signals which emanate from the TCR.

LAT in Autoimmunity

Further evidence indicating that LAT plays a negative role in T cell activation comes from studies examining the interaction between LAT with PLC-γ1. LAT-deficient Jurkat T cells reconstituted with the LATY136F mutant, which fails to bind PLC-γ1, have impaired calcium flux and Erk activation (Zhang et al. 2000). To test the role of this interaction in vivo, two groups independently generated LATY136F knock-in mice. Analyses of these mice show that there is a partial block in thymocyte development at the DN3 stage, demonstrating the positive role of the LAT and PLC-γ1 interaction in thymocyte development. However, a small population of thymocytes is able to progress to the DP and SP stages. Mutant T cells that enter the periphery then undergo uncontrolled expansion, revealing the importance of the LAT and PLC-γ1 interaction in the control of T cell homeostasis. LATY136F mice display lymphadenopathy, splenomegaly, as well as lymphocyte infiltration in the lungs, liver, and kidneys. These mice have very few $CD8^+$ T cells in the periphery and the $CD4^+$ T cells are Th2-skewed, producing high levels of IL-4. They have low levels of surface TCR expression and have an activated/effector memory phenotype, characterized by high surface expression of CD44 and low surface expression of CD62L. As a result of the large population of Th2 T cells, B cells are hyperactivated, producing autoantibodies, mainly IgE and IgG_1 (Sommers et al. 2002; Aguado et al. 2002).

Further analysis of LATY136F mice demonstrates that they do not contain natural T regulatory cells, which are $CD4^+CD25^+Foxp3^+$. Adoptive transfer of normal T regulatory cells into neonatal LATY136F mice can prevent development of the autoimmune syndrome (Koonpaew et al. 2006). In addition, transfer experiments show that LATY136F $CD4^+$ T cells can also expand in MHC Class II-deficient mice, indicating that the uncontrolled expansion of these mutant T cells is independent of the TCR-MHC-peptide interaction (Wang et al. 2008). Interestingly, while mice expressing the LATY136F mutant develop a severe lymphoproliferative syndrome, mice with LAT inducibly deleted in mature T cells also develop a similar, albeit less severe, autoimmune syndrome. LAT-deficient $CD4^+$ T cells expand and secrete large amounts of cytokines despite a severe defect in TCR-mediated signaling (Shen et al. 2010).

Additionally, LAT3YF mice, in which LAT fails to bind Gads and Grb2 due to mutations at Y175, Y195, and Y235, exhibit uncontrolled T cell expansion in the periphery. These mice show a block in thymic development at the DN stage, with a complete block in αβ T cell development. Interestingly, γδ T cell development does not seem to be affected, and these cells exit the thymus and populate the periphery. In aged mice, these γδ T cells have undergone significant expansion and, unexpectedly, produce Th2 cytokines. Thus, B cell populations also expand, causing high serum levels of IgG1 and IgE (Nunez-Cruz, Aguado et al. 2003). These data suggest a role for LAT in the negative regulation of γδ T cell expansion and indicate that αβ and γδ T cells have different requirements for LAT during thymic development. Together, these data clearly indicate the critical role of LAT in T cell homeostasis, but the mechanism by which LAT controls T cell homeostasis remains to be further investigated.

Summary

LAT, a transmembrane adaptor protein that is exclusively expressed in hematopoietic cells, is required for thymocyte development and LAT deficiency leads to a complete lack of mature T cells. LAT is also vital for T cell activation downstream of the TCR. LAT is constitutively localized in lipid rafts and contains two cysteine residues that can be palmitoylated. LAT palmitoylation is essential for its function in T cells. Upon T cell activation, LAT is phosphorylated on multiple tyrosine residues by ZAP-70 tyrosine kinase and interacts with Grb2, Gads, and PLC-γ1 directly, as well as Cbl, SLP-76, Vav, and other proteins indirectly. The ability of LAT to interact with these signaling proteins is required for TCR-mediated MAPK activation, calcium mobilization, and cytokine production. Recent studies indicate that LAT also controls T cell homeostasis. The deletion or mutation of LAT causes the development of a lymphoproliferative autoimmune syndrome due to uncontrolled T cell expansion and cytokine production. In summary, LAT is a key signaling molecule that functions in T cell development, activation, and homeostasis. LAT or LAT-mediated signaling pathways could serve as effective targets for the design of improved therapies to enhance or inhibit T cell function during immune responses.

References

Aguado E, Richelme S, Nunez-Cruz S, Miazek A, Mura AM, Richelme M, et al. Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. Science. 2002;296:2036–40.

Brdicka T, Imrich M, Angelisova P, Brdickova N, Horvath O, Spicka J, et al. Non-T cell activation linker (NTAL): a transmembrane adaptor protein involved in immunoreceptor signaling. J Exp Med. 2002;196:1617–26.

Chan AC, Desai DM, Weiss A. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. Annu Rev Immunol. 1994;12:555–92.

Dong S, Corre B, Foulon E, Dufour E, Veillette A, Acuto O, et al. T cell receptor for antigen induces linker for activation of T cell-dependent activation of a negative signaling complex involving Dok-2, SHIP-1, and Grb-2. J Exp Med. 2006;203:2509–18.

Finco TS, Kadlecek T, Zhang W, Samelson LE, Weiss A. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. Immunity. 1998;9:617–26.

Hundt M, Tabata H, Jeon MS, Hayashi K, Tanaka Y, Krishna R, et al. Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. Immunity. 2006;24:513–22.

Hundt M, Harada Y, De Giorgio L, Tanimura N, Zhang W, Altman A. Palmitoylation-dependent plasma membrane transport but lipid raft-independent signaling by linker for activation of T cells. J Immunol. 2009;183:1685–94.

Janssen E, Zhu M, Zhang W, Koonpaew S. LAB: a new membrane-associated adaptor molecule in B cell activation. Nat Immunol. 2003;4:117–23.

Jordan MS, Singer AL, Koretzky GA. Adaptors as central mediators of signal transduction in immune cells. Nat Immunol. 2003;4:110–16.
Koonpaew S, Shen S, Flowers L, Zhang W. LAT-mediated signaling in CD4+CD25+ regulatory T cell development. J Exp Med. 2006;203:119–29.
Nunez-Cruz S, Aguado E, Richelme S, Chetaille B, Mura AM, Richelme M, et al. LAT regulates gammadelta T cell homeostasis and differentiation. Nat Immunol. 2003; 4(10):999–1008.
Saitoh S, Arudchandran R, Manetz TS, Zhang W, Sommers CL, Love PE, et al. LAT is essential for Fc(epsilon)RI-mediated mast cell activation. Immunity. 2000;12:525–35.
Samelson LE. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. Annu Rev Immunol. 2002;20:371–94.
Shen S, Zhu M, Lau J, Chuck M, Zhang W. The essential role of LAT in thymocyte development during transition from the double-positive to single-positive stage. J Immunol. 2009;182:5596–604.
Shen S, Chuck MI, Zhu M, Fuller DM, Yang CW, Zhang W. The importance of LAT in the activation, homeostasis, and regulatory function of T cells. J Biol Chem. 2010;285:35393–405.
Sommers CL, Park CS, Lee J, Feng C, Fuller CL, Grinberg A, et al. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science. 2002;296:2040–3.
Wang Y, Kissenpfennig A, Mingueneau M, Richelme S, Perrin P, Chevrier S, et al. Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3+ regulatory T cells. J Immunol. 2008;180:1565–75.
Yamasaki S, Nishida K, Sakuma M, Berry D, McGlade CJ, Hirano T, et al. Gads/Grb2-mediated association with LAT is critical for the inhibitory function of Gab2 in T cells. Mol Cell Biol. 2003;23:2515–29.
Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell. 1998a;92:83–92.
Zhang W, Trible RP, Samelson LE. LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity. 1998b;9:239–46.
Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP, et al. Essential role of LAT in T cell development. Immunity. 1999;10:323–32.
Zhang W, Trible RP, Zhu M, Liu SK, McGlade CJ, Samelson LE. Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell angigen receptor-mediated signaling. J Biol Chem. 2000;275:23355–61.
Zhu M, Janssen E, Zhang W. Minimal requirement of tyrosine residues of linker for activation of T cells in TCR signaling and thymocyte development. J Immunol. 2003; 170:325–33.
Zhu M, Shen S, Liu Y, Granillo O, Zhang W. Cutting edge: localization of linker for activation of T cells to lipid rafts is not essential in T cell activation and development. J Immunol. 2005;174:31–5.

LCoR

John H. White and Mario R. Calderon
Department of Physiology, McGill University
McIntyre Medical Sciences Building, Montréal, Québec, Canada

Synonyms

FLJ38026; KIAA1795; Ligand-dependent nuclear receptor corepressor (LCoR); Mblk1-related protein 2 (MLR2); RP11-175O19.1

Historical Background

Gene expression is a fundamental part of cell biology as it initially produces an mRNA transcript that ultimately results in a functional protein. Within this process, gene transcription is a tightly controlled mechanism in cell nuclei through the action of specific DNA-binding transcription factors and an array of ancillary proteins known as co-activators or corepressors, depending on whether they act to stimulate or repress transcription. In general, most co-activators have histone acetyltransferase (HAT) activity, which acts in part to loosen the association of positively charged histones with DNA, facilitating chromatin remodeling and recruitment of the transcriptional machinery. On the other hand, corepressors often recruit histone deacetylases (HDACs) or methyltransferases, which reinforce the structural integrity of the histone–DNA association, effectively denying DNA access to the transcriptional machinery. However, evidence has accumulated showing that the precise transcriptional roles of cofactors can be promoter-specific, as many examples exist in the literature of proteins that can function as a co-activator or corepressor in different contexts. As such, it is perhaps better to globally refer to these proteins as transcriptional co-regulators.

Ligand-dependent nuclear receptor corepressor (LCoR) was originally identified by two independent laboratories through the use of cDNA library screens. Both laboratories identified LCoR as a transcriptional co-regulator. However, Fernandes et al. (2003) extensively characterized it as a ligand-dependent transcriptional corepressor of nuclear receptor–mediated transactivation, whereas Kunieda et al. (2003) showed

preliminary evidence that suggested that it is a transcriptional activator through sequence similarity to mushroom body large-type Kenyon cell-specific protein 1 (Mblk-1). Future studies should provide more definitive evidence confirming that LCoR also regulates gene expression in a context-specific manner.

Domain Structure and Function

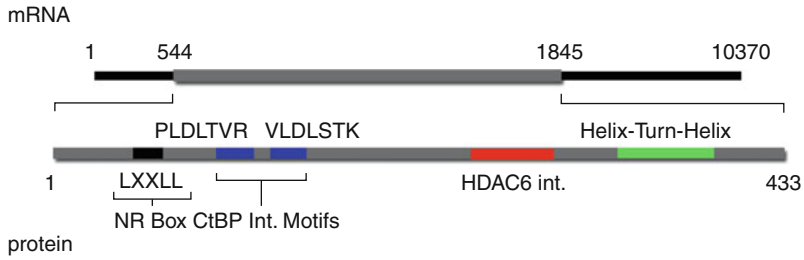
Currently, there exists no direct experimental evidence for different LCoR splice variants, or if these variants display any differences in terms of tissue/cellular localization or function. However, the National Center for Biotechnology Information (NCBI)-curated genomic Reference Sequences lists two potential isoforms of LCoR. Isoform 2 differs from isoform 1 in its 5′ UTR, 3′ UTR, and coding region, while maintaining the open reading frame. This results in similar proteins, with isoform 2 lacking 27 C-terminal amino acids. The region truncated in isoform 2 does not appear to contain any functional domain, yet there is a putative sumoylation motif. Accumulating evidence suggests that sumoylation is important for general repressor function (Geiss-Friedlander and Melchior 2007), but no experimental data exists that suggests differential LCoR isoform function. For the remainder of this entry, when referencing LCoR, it is the longer 433 (isoform 1) amino acid variant that is referenced.

Domain characterization of LCoR (Fig. 1) revealed that it contains a nuclear receptor (NR) box or LXXLL motif in its N-terminal region through which it interacts with class I and II agonist-bound nuclear receptors (Fernandes et al. 2003). The human nuclear receptor superfamily comprises 48 transcription factors that function in a ligand-dependent manner. Each nuclear receptor responds to a particular ligand that can range from steroids and related compounds, retinoic acids, and thyroid hormones to fatty acids, thereby creating classes depending on which ligand they respond to: steroidal (I), nonsteroidal (II), and orphan (III). They regulate many biologically important processes from development to homeostasis (Perissi and Rosenfeld 2005). Specifically, LCoR was shown to associate directly in a ligand-dependent manner through its NR box with estrogen receptor α (ERα, NR3A1), estrogen receptor β (ERβ, NR3A2), progesterone receptor (PR, NR3C3), Vitamin D receptor (VDR, NR1I1), retinoic acid receptor (RAR)-α (NR1B1), RARβ (NR1B2), and RARγ (NR1B3), and retinoid X receptor α (RXRα; NR2B1) (Fernandes et al. 2003). LCoR suppressed transactivation by ERα, PR, glucocorticoid receptor (GR, NR3C1), VDR, and thyroid receptor α (TRα, NR1A1) (Fernandes et al. 2003; Wang et al. 2007). This suggests that LCoR plays an important physiological role through regulation of nuclear-receptor-mediated transcription.

LCoR also contains tandem extended N-terminal PXDLS motifs that recruit C-terminal Binding Proteins 1 (CtBP1) and 2 (Fig. 1) (Fernandes et al. 2003; Palijan et al. 2009b). CtBP1 and CtBP2 function predominanlty as transcriptional corepressors whose activity is modulated by the nuclear ratio of NADH/NAD+ (Chinnadurai 2003). Mutation of both CtBP-binding motifs is required in order to abolish CtBP binding, and attenuation of LCoR corepressor function occurs upon individual mutations at the binding sites in transcription regulated by PR and TRα, with the greatest effect observed when both sites are mutated (Palijan et al. 2009b; Wang et al. 2007). A negligible effect occurred in the transcription regulated by ERα or GR, when individual or combined mutations of the binding sites was carried out, which suggests a minimal role for CtBPs in LCoR-mediated repression of these receptors (Fernandes et al. 2003).

The helix-turn-helix motif located near the C-terminal tail (Fig. 1) also appears to be critical for LCoR function, as deletion of this motif attenuates corepression of estrogen-regulated gene expression and abolishes corepression of progesterone-regulated transcription in T47D human breast cancer cells (Palijan et al. 2009b). How this motif functions in repression, however, remains a topic for future research.

LCoR also recruits HDAC3 and 6 through central domains. HDAC6 recruitment occurs through a region that is delineated by amino acid 203–319 in LCoR (Fernandes et al. 2003; Palijan et al. 2009a). The interaction between HDAC6 and LCoR is somewhat surprising, given that LCoR is nuclear, but HDAC6, unlike HDAC3, is cytoplasmic in many malignant cells (Yang and Seto 2003). However, studies in normal breast epithelial cells show that HDAC6 is nuclear, and that responsiveness to endocrine therapy correlates with HDAC6 localization in breast cancer (Saji et al. 2005). Palijan et al. (2009a) also found that HDAC6 was partially nuclear in ERα-positive MCF-7 breast cancer cells. This suggests that the LCoR–HDAC6 interaction



LCoR, Fig.1 Domain structure of LCoR

is lost in malignant cells in which HDAC6 is cytoplasmic resulting in unknown effects in tumorigenesis.

Cyclical recruitment of LCoR to the promoters of several ERα and PR target genes was demonstrated with a peak occurring 30–45 min after hormone treatment (Palijan et al. 2009a; Palijan et al. 2009b). Although no direct interaction was found between LCoR and CtBP-interacting protein (CtIP), they were found to interact indirectly, and colocalize on the promoters of several ERα and PR target genes (Palijan et al. 2009b). Hence, it is possible that they function as a complex on specific nuclear receptor target genes. Additionally, LCoR was shown to bind specific target gene promoters together with the PR, ERα, CtBP1, or HDAC6, substantiating previous experiments that indicate that these proteins are cofactors (Palijan et al. 2009a; Palijan et al. 2009b).

Furthermore, LCoR was found in a multi-subunit CtBP corepressor complex, which represses the tumor invasion suppressor E-cadherin through specific transcription factor promoter targeting and coordinated histone modifications (Shi et al. 2003). LCoR was also found to interact with lysine (K)-specific demethylase 1 (LSD1), a pivotal member of the Krüppel-like zinc finger E-box binding homeobox 1 (ZEB1)-LSD1-repressor element 1 silencing transcription factor corepressor (CoREST)–CtBP repressive complex (Wang et al. 2007). This large complex binds to the promoters of several genes such as growth hormone 1 (*GH1*) in developing pituitary lactotropes, and is thought to direct a precise transcriptional program that includes both activation and repression (Wang et al. 2007).

Small interfering RNA (siRNA)-mediated knockdown of LCoR revealed an increase in endogenous expression of progesterone-regulated target genes, which is consistent with reporter gene assays that show a role as a corepressor in T47D breast cancer cells (Palijan et al. 2009b). Surprisingly, results from the same type of experiment on estrogen-regulated transcription were conflicting. An increase in estrogen induced expression of an ectopic reporter gene after LCoR knockdown confirmed its role as a repressor of estrogen-mediated transcription (Palijan et al. 2009a). However, analysis of endogenous estrogen target gene expression revealed that abrogation of LCoR expression had either no effect or was required for optimal expression of specific target genes such as insulin-like growth factor binding protein 4 (IGFBP4) and cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1) (Kaipparettu et al. 2008; Palijan et al. 2009b). Future studies might reveal the mechanism through which LCoR functions as a co-activator/-repressor with precise tissue and gene specificity such as another member of the ligand-dependent corepressor family, RIP140 (Cavailles et al. 1995; Lee et al. 1998). The specificity of the effect on transcriptional regulation might depend on the constituents of the transcriptional regulating complex, developmental stage, and tissue. The most compelling evidence for this was presented in a study which found opposing, activator or repressor, functions for a multi-protein complex containing LCoR during mouse development (Wang et al. 2007). Which signals direct the assembly of this function-specific multifactor complex, and how this regulates transcription remain topics of future research.

Regulation of LCoR

Currently, there is very little experimental data that explains how LCoR mRNA or protein expression is regulated. It is not known if different signals direct differential isoform expression, or if this translates into altered functionality. Nevertheless, Wang and colleagues demonstrated that LCoR mRNA expression is increased by estrogen-treatment of human osteosarcoma U2OS cells (Wang et al. 2007). However, widespread and early LCoR expression suggests that there

must be other signals that direct its expression, and clarifying these mechanisms might shed new light on its role in development and potentially disease.

Expression

Tissue Localization

LCoR mRNA is expressed as early as the two-cell stage during development, and is ubiquitously expressed in a variety of fetal and adult human tissues. Highest expression is observed in the placenta, the cerebellum and corpus callosum of the brain, the adult kidney, and a number of fetal tissues (Fernandes et al. 2003). Analysis of placental tissue revealed that LCoR predominantly localizes to the syncytiotrophoblast layer of terminally differentiated cells, which is critical for controlling maternal hormonal signals that regulate fetal metabolism and development (Pepe and Albrecht 1995).

Curiously, generation of a mouse LCoR knockout (*Lcor* $^{-}/^{-}$) model revealed that even though LCoR is expressed early during development it is not essential for it as most *Lcor* $^{-}/^{-}$ animals survived to birth, but failed to suckle and died within hours. Additionally, a growth difference was noticeable starting at the 18th day of gestation (E18.5) as *Lcor* $^{-}/^{-}$ mouse embryos were slightly (~15%), but significantly, smaller than wild-type littermates (Dr. Yaacov Barak, unpublished results). Furthermore, immunohistochemical analysis of neonatal mouse pituitary tissue showed that LCoR expression increased postpartum potentially through signaling by estrogen, which is consistent with the mouse knockout model, which suggests a critical role for LCoR postpartum (Wang et al. 2007). Similarly, CtBP1 knockout mice were viable, but were small and died early (Chinnadurai 2003). This suggests that LCoR and CtBP1 might be co-regulating the same genes during development. These results coupled with the observation that LCoR localizes to and represses the *GH1* promoter in a mouse pituitary cell line (Wang et al. 2007) indicate that although its role in early development is not critical, signal-dependent postpartum induction of LCoR and its subsequent regulation of genes is essential in later development.

Subcellular Localization

Immunohistochemical analysis has shown that LCoR localizes to discrete nuclear bodies in the cell. The same type of analysis has also shown that these bodies contain CtBP1, CtBP2, CtIP, and polycomb ring finger oncogene (BMI1) (Fernandes et al. 2003; Palijan et al. 2009a; Palijan et al. 2009b). CtIP and BMI1, integral components of polycomb group repressor (PcG) complexes (Chinnadurai 2006; Fasano et al. 2007; Sewalt et al. 1999), associate indirectly with LCoR (Palijan et al. 2009b), and as such these observations suggest a possible role for LCoR in PcG complexes, which play an important role in cellular identity during development (Schuettengruber and Cavalli 2009).

Disease

Little is known concerning LCoR and its expression in disease states. In bladder cancer, a study found that LCoR mRNA expression was significantly (more than 0.5 fold) lower in more aggressive human cell lines compared with a less aggressive cell line (Abedin et al. 2009). A similar analysis of LCoR expression in a panel of breast cancer cell lines did not establish a correlation between LCoR and expression of ERα, or of cofactors CtBP1 or histone deacetylase 6 (HDAC6), nor did there appear to be a correlation with cancer aggresiveness (Dr. Sylvie Mader, unpublished results). Nevertheless, caution must be exercised when drawing conclusions from mRNA data as it may not correlate well with protein expression. Indeed, early and ubiquitous expression of LCoR, its critical role postpartum, and the possibility of various pleiotropic effects through interactions with different nuclear receptors, suggest that LCoR might be an important player in disease, particularly cancer development. However, determination of whether it acts in a tumor suppressor or oncogene fashion requires more experimental data.

Summary

A survey of the literature on LCoR reveals that it has been characterized extensively as a ligand-dependent nuclear receptor corepressor, which is expressed early in development and ubiquitously. Additionally, its developmental role becomes crucial postpartum. However, no information exists on the specific mechanisms controlling LCoR expression and turnover neither in the embryo nor the adult.

LCoR functions by recruiting the NAD(H)-dependent repressors, CtBPs, and histone deacetylases. The fact

that CtBP activity is dependent on the NADH/NAD+ ratio suggests that LCoR repression could be affected by metabolic activity. Furthermore, it is not known how LCoR recruits cofactors, and if it is dependent on other signals. Accumulating evidence shows that LCoR may also have co-activator roles. Differential co-activator/corepressor roles enacted by transcriptional regulating complexes must depend at least in part in endogenous signals. These signals could result in posttranslational modifications that direct the assembly of complexes with transcriptional roles specific to different tissues. How LCoR is modified by these signals and participates in these multiprotein complexes remains poorly understood and highlights the fact that much remains to be accomplished in order to elucidate the mechanisms surrounding LCoR function.

References

Abedin SA, Thorne JL, Battaglia S, Maguire O, Hornung LB, Doherty AP, et al. Elevated NCOR1 disrupts a network of dietary-sensing nuclear receptors in bladder cancer cells. Carcinogenesis. 2009;30:449–56.

Cavailles V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, et al. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J. 1995;14:3741–51.

Chinnadurai G. CtBP family proteins: more than transcriptional corepressors. Bioessays. 2003;25:9–12.

Chinnadurai G. CtIP, a candidate tumor susceptibility gene is a team player with luminaries. Biochim Biophys Acta. 2006;1765:67–73.

Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. Cell Stem Cell. 2007;1:87–99.

Fernandes I, Bastien Y, Wai T, Nygard K, Lin R, Cormier O, et al. Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. Mol Cell. 2003;11:139–50.

Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol. 2007;8:947–56.

Kaipparettu BA, Malik S, Konduri SD, Liu W, Rokavec M, van der Kuip H, et al. Estrogen-mediated downregulation of CD24 in breast cancer cells. Int J Cancer. 2008;123:66–72.

Kunieda T, Park JM, Takeuchi H, Kubo T. Identification and characterization of Mlr1,2: two mouse homologues of Mblk-1, a transcription factor from the honeybee brain(1). FEBS Lett. 2003;535:61–5.

Lee CH, Chinpaisal C, Wei LN. Cloning and characterization of mouse RIP140, a corepressor for nuclear orphan receptor TR2. Mol Cell Biol. 1998;18:6745–55.

Palijan A, Fernandes I, Bastien Y, Tang L, Verway M, Kourelis M, et al. Function of Histone deacetylase 6 as a cofactor of nuclear receptor coregulator LCoR. J Biol Chem. 2009a;284(44):30264–74.

Palijan A, Fernandes I, Verway M, Kourelis M, Bastien Y, Tavera-Mendoza LE, et al. Ligand-dependent corepressor LCoR is an attenuator of progesterone-regulated gene expression. J Biol Chem. 2009b;284(44):30275–87.

Pepe GJ, Albrecht ED. Actions of placental and fetal adrenal steroid hormones in primate pregnancy. Endocr Rev. 1995;16:608–48.

Perissi V, Rosenfeld MG. Controlling nuclear receptors: the circular logic of cofactor cycles. Nat Rev Mol Cell Biol. 2005;6:542–54.

Saji S, Kawakami M, Hayashi S, Yoshida N, Hirose M, Horiguchi S, et al. Significance of HDAC6 regulation via estrogen signaling for cell motility and prognosis in estrogen receptor-positive breast cancer. Oncogene. 2005;24:4531–9.

Schuettengruber B, Cavalli G. Recruitment of polycomb group complexes and their role in the dynamic regulation of cell fate choice. Development. 2009;136:3531–42.

Sewalt RG, Gunster MJ, van der Vlag J, Satijn DP, Otte AP. C-Terminal binding protein is a transcriptional repressor that interacts with a specific class of vertebrate Polycomb proteins. Mol Cell Biol. 1999;19:777–87.

Shi Y, Sawada J, Sui G, El Affar B, Whetstine JR, Lan F, et al. Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature. 2003;422:735–8.

Wang J, Scully K, Zhu X, Cai L, Zhang J, Prefontaine GG, et al. Opposing LSD1 complexes function in developmental gene activation and repression programmes. Nature. 2007;446:882–7.

Yang XJ, Seto E. Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. Curr Opin Genet Dev. 2003;13:143–53.

LCP2

► SLP-76

LD78 Receptor

► Chemokine Receptor CCR1

Leukocyte Surface Antigen CD53

► CD53

Leukosialin

► CD43

Leukotriene

▶ Phospholipase A_2

LFS1

▶ p53

Licorne (lic)

▶ Mek3

Ligand-Dependent Nuclear Receptor Corepressor (LCoR)

▶ LCoR

LIM Kinase

▶ LIMK

LIMK

Ora Bernard
Cytoskeleton and Cancer Unit, St Vincent's Institute of Medical Research, Fitzroy, VIC, Australia

Synonyms

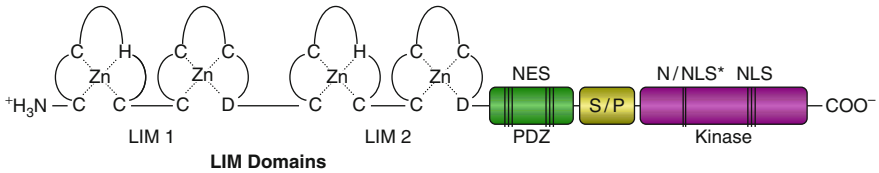
Isl 1; Kiz; LIM kinase; Lin 11; Mec 3 (LIM) kinase

Historical Background

The two members of the LIM kinase (LIMK) family of proteins include LIMK1 and LIMK2. LIMK1, the first of these unique serine kinases to be characterized, was also identified as the murine Kiz-1 protein (Bernard et al. 1994). The second highly related family member, LIMK2, was cloned using a LIMK1 cDNA probe (Okano et al. 1995). These two LIMK family members have identical genomic structures with 16 exons and identical intron/exon boundaries (Bernard et al. 1996; Ikebe et al. 1997). They share a total of ~50% amino acid sequence identity with ~70% identity in their kinase domain (Okano et al. 1995). The structure of the LIMK proteins is unique as they contain two different protein–protein interaction domains, namely, LIM (Lin 11, Isl 1, Mec 3) and PDZ (PSD-95, Dlg, ZO-1) domains. Two tandem LIM domains lie at the N-terminal followed by a central PDZ domain. These two domains are separated from the kinase domain by a serine- and proline-rich region (S/P) with an unknown function (Stanyon and Bernard 1999). The LIMK kinase domain contains putative tyrosine kinase sequences as well as serine/threonine kinase motifs. However, to date no cellular tyrosine kinase activity has been identified as well as cellular substrate phosphorylation on threonine residues, therefore implicating the LIMK proteins as serine kinases. The kinase domain also contains a putative nuclear localization signal and only LIMK2 has a specific nuclear and nucleolar localization signal (Fig. 1).

The LIMK Isoforms

Several isoforms resulting from alternative splicing were identified for LIMK1 and LIMK2. There are three LIMK1 isoforms: full length LIMK1, LIMK1 short (LIMK1-s) lacking 20 amino acids in the kinase domain rendering it inactive because of modifications to the ATP binding site (Stanyon and Bernard 1999), and a truncated protein dLIMK1, which is devoid of the kinase domain (Edwards and Gill 1999). LIMK2 has two major isoforms that are transcribed from two independent sites, resulting in the full-length LIMK2a and the N-terminal truncated LIMK2b that lacks the first zinc finger of the LIM domain but contains 22 new amino acids acquired from the adjacent 5′ intron (Ikebe et al. 1997). Functional differences between the two LIMK2 isoforms have recently been determined and will be discussed below. Another less studied LIMK2 isoform is tLIMK2, a testis-specific isoform that lacks LIM domains and part of the PDZ domain, suggesting a role of this protein in spermatogenesis (Takahashi et al. 2002).



LIMK, Fig. 1 *LIMK protein domain organization*. The LIMK proteins consist of an N-terminal double LIM domain region, a central PDZ domain followed by a serine and proline rich region (S/P), and a C-terminal protein kinase domain. NLS is the site of a putative nuclear localization signal, and NES represents the two nuclear export signals. N/NLS* is the LIMK2 specific nuclear and nucleolar localization signal

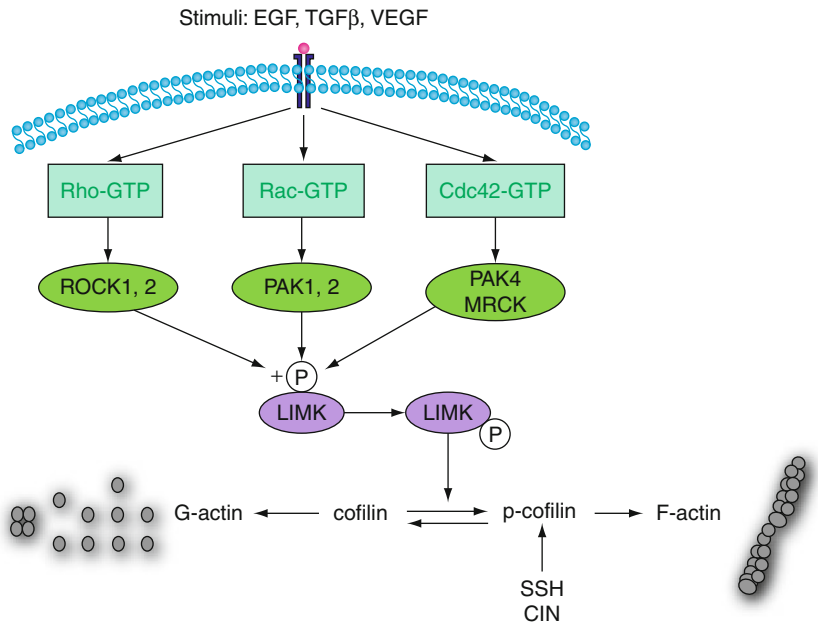
LIMK Substrates: Function and Regulation

LIMK substrates and general function: The most extensively characterized substrates of LIMK are cofilin 1 (non-muscle cofilin), cofilin 2 (muscle cofilin), and destrin (actin depolymerizing factor or ADF). The ADF/cofilin family of actin depolymerizing factors are central players in the regulation of actin-driven cellular processes such as cytokinesis, membrane ruffling, phagocytosis, cell motility, fluid phase endocytosis, and neurite outgrowth (Bamburg 1999). These small molecular weight (15–20 kDa) proteins (referred to herein as cofilin) are essential for the high turnover rates of actin filaments seen in cells. Cofilin binds to both filamentous actin (F-actin) and globular actin (G-actin). Upon binding to F-actin, it induces a conformational change that leads to a twist in the filament and increases the off rate of actin subunits at the pointed end. These conformational changes increase the thermodynamic instability of the actin polymer which is also responsible for cofilin's severing activity (Bamburg 1999). Cofilin is also able to form a complex with G-actin; however, it has a greater binding affinity to actin-ADP than actin-ATP. Binding of cofilin to actin-ADP inhibits nucleotide exchange and thus acts as a sequestering protein by reducing the pool of actin-ATP available for polymerization. The combined functions of cofilin (depolymerizing, severing, and sequestering) increase actin turnover by increasing the number of free-barbed ends as well as actin monomers necessary for the assembly of a new filament (Bamburg 1999). Cofilin's activity is primarily regulated by phosphorylation at the highly conserved serine 3 residue. Phosphorylation at this site inhibits cofilin's ability to bind to F-actin and to induce its depolymerization, resulting in the accumulation of actin filaments (Moriyama et al. 1996; Nebl et al. 1996). The important discovery that LIMK phosphorylates cofilin and inhibits its activity, resulting in the accumulation of F-actin, identified the members of the LIMK family as important regulators of actin dynamics (Arber et al. 1998; Yang et al. 1998).

Other less studied LIMK substrates are the transcription factors ▶ CREB and Nurr1, suggesting a link through LIMK to transcriptional regulation. LIMK activation by FGF leads to increased CREB phosphorylation and CREB-responsive promoter activity (Yang et al. 2004). Nurr1 is an orphan receptor belonging to the nuclear receptor family that regulates gene transcription via hormone response elements in promoter sequences. LIMK1 phosphorylated Nurr1, leading to diminished transcriptional activity from a promoter/reporter construct (Sacchetti et al. 2006).

Another LIMK1 substrate is p25, also known as tubulin polymerization promoting protein (TPPP). When phosphorylated by LIMK1, p25/TPPP loses its ability to promote microtubule (MT) assembly resulting in MT disassembly in vitro and in cells (Acevedo et al. 2007).

LIMK regulation: In the initial studies leading to the discovery that LIMK1 regulates actin dynamics via phosphorylation of cofilin, it was demonstrated that LIMK1 was activated through the Rho-GTPases (Arber et al. 1998; Yang et al. 1998). This identified a novel pathway by which the Rho-GTPases regulate the actin cytoskeletal changes in response to the cellular environment via LIMK1. Furthermore, it was shown that the activity of the LIMK proteins is regulated by the phosphorylation of a threonine residue in the activation loop by the kinases activated by the Rho-GTPases. Both LIMK1 and LIMK2 are phosphorylated by the Rho effectors ▶ Rho-associated kinase 1 and 2 (ROCK1 and ROCK2) on the conserved threonine residues, Thr-508 (LIMK1) (Ohashi et al. 2000) and Thr-505 (LIMK2) (Amano et al. 2001; Sumi et al. 2001a). p21-activated kinase (PAK) 1, 2, and 4 were



LIMK, Fig. 2 *Regulation of LIMK activity*. LIMK activity is regulated by the Rho family of small GTPases via their downstream serine/threonine kinases. ROCK 1 and 2 and PAK 1, 2, and 4. These kinases phosphorylate LIMK on a threonine residue in the activation loop of their kinase domains (LIMK1 Thr508 and LIMK2 Thr505). Activated phospho-LIMK phosphorylates cofilin at Ser3. Phospho-cofilin (p-cofilin) loses its ability to bind to actin filaments (F-actin) and sequester actin monomers (G-actin) resulting in accumulation of F-actin. The cofilin phosphatases SSH and CIN reactivate cofilin and increase its activity

also shown to activate LIMK1 (Edwards et al. 1999; Misra et al. 2005; Dan et al. 2001). PAK 4 activates LIMK1 not only through phosphorylation of Thr-508 but also through phosphorylation and inactivation of the LIMK phosphatase slingshot (see below) (Soosairajah et al. 2005). The myotonic dystrophy kinase-related Cdc42-binding kinase (MRCKα) has been reported to phosphorylate and activate both LIMK1 and LIMK2 (Sumi et al. 2001b). The most studied LIMK activators are ROCK1 and 2. Only limited information on the role of the other kinases mentioned above in LIMK activation is available.

LIMK activity is also regulated by auto- and trans-phosphorylation. Unphosphorylated LIMK1 has a short half-life of ~4 h, in comparison the phosphorylated protein has a half-life of ~24 h. LIMK transphosphorylation is mediated by its association with Hsp90 which promotes homodimerization and transphosphorylation, leading to the formation of stable LIMK dimers with increased specific activity (Li et al. 2006).

Other less studied LIMK1 activators are summarized below:

- MAPK/MK2 phosphorylates both Hsp27 and Ser323 of LIMK1, via VEGF-A regulation.
- Semaphorin upregulates LIMK2 activity in the presence of plexin C1, a receptor for semaphorin 7A.
- Neuregulin interacts with and activates LIMK1.
- The interaction between 14-3-3 and LIMK1 increases cofilin phosphorylation by an unknown mechanism.
- PKA activates LIMK1 by phosphorylation at serine 323 and 596.
- PKC activates LIMK1 by direct interaction.
- SDF-1a activates LIMK1.
- Nogo-66 activates LIMK1 by a ROCK-dependent phosphorylation.
- Ionomycin/CaMKIV activate LIMK1 by direct phosphorylation at Thr508.

Phosphorylation and activation of proteins can be reversed by dephosphorylation. The slingshot 1 (SSH1) phosphatase, previously identified as a cofilin phosphatase (Niwa et al. 2002), was also found to dephosphorylate and inactivate LIMK1 (Soosairajah et al. 2005). Another less studied cofilin phosphatase is chronophin (CIN) (Gohla et al. 2005) (Fig. 2).

The association of the LIM and PDZ domains with the kinase domain inhibits LIMK activity, while deletion of both the LIM and the PDZ domains as well as mutations in the second LIM and PDZ domains lead to a significant increase in LIMK activity, suggesting that the N-terminal region controls the activity of LIMK. LIMK activity can be negatively regulated by unknown, mechanism

through its interaction with several proteins as summarizes below:

- The ubiquitin ligases Rnf6 and parkin induce the proteasomal degradation of LIMK.
- Nischarin inhibits the Rac/PAK activation of both LIMK1 and LIMK2 by direct association with LIMK.
- LATS1 interacts with and reduces LIMK1 activity.
- Par-3 interacts with and inhibits LIMK2 activity in vitro and its overexpression suppresses cofilin phosphorylation that is induced by lysophosphatidic acid.
- The cytoplasmic tail of BMPR-II interacts with and inhibits the activity of LIMK.

In addition, miR-134 inhibits LIMK1 mRNA translation in neurons.

Specific regulation of the LIMK2 isoforms: Recently, it was reported that ▶ p53 induces the expression of LIMK2b, but not that of LIMK2a, after DNA damage by binding to a p53-binding consensus motif in intron 1 of the LIMK2b gene (intron 2 of LIMK2a) (Hsu et al. 2010). The transcription factor p53 is an important cell cycle regulator. After DNA damage, p53 activation induces cell cycle arrest and the activation of pro-survival pathways allowing DNA damage repair. However, with excessive DNA damage, p53 activation may lead to apoptotic cell death. Ectopic p53 expression increases LIMK2b expression and cofilin phosphorylation (Hsu et al. 2010), suggesting that the LIMK2b-mediated actin regulatory function is required for cell cycle arrest. While these results demonstrate that LIMK2b has a role in G2/M cell cycle arrest, the reason for specific p53-mediated LIMK2b induction remains elusive. It is possible that LIMK2b has a distinct role in the cell.

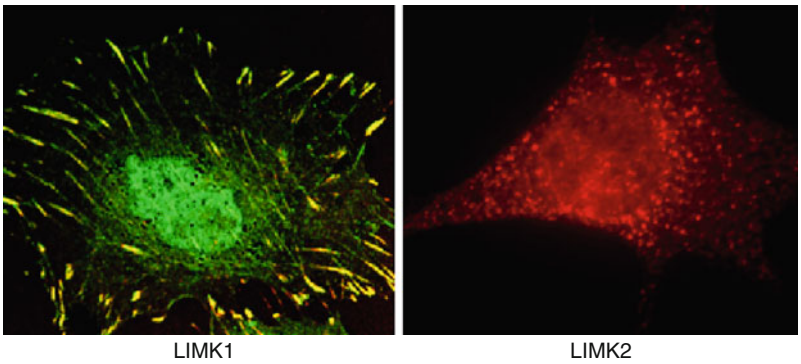
Apart from specific LIMK2b induction following increased p53 levels after DNA damage caused by radiation or drugs such as doxorubicin, no other functional difference between the LIMK2 isoforms have been established to date. Overexpression of LIMK2a or LIMK2b reduced both cell proliferation and viability, and resulted in multi-nucleated cells. These cellular changes were more pronounced with enforced LIMK2b expression, suggesting that the additional 22 amino acids replacing the first zinc-finger may increase LIMK2 activity. Finally, analysis of a large cohort of cell lines by RT-PCR demonstrated that LIMK2a and LIMK2b were differentially expressed in a number of cell lines. Interestingly, the levels of LIMK2a and LIMK2b are downregulated in thyroid cancers while in esophageal cancers LIMK2b is downregulated but LIMK2a levels are increased in comparison to normal tissues (Hsu et al. 2010).

Expression and Cellular Localization of LIMK

LIMK1: Early studies on the expression of LIMK1 mRNA identified it mainly as a brain specific protein (Bernard et al. 1994; Mizuno et al. 1994). However, with the development of good anti-LIMK1 antibodies it was demonstrated that LIMK1 is expressed in all the cells of mouse tissues but at different levels (Foletta et al. 2004). LIMK1 gained special attention due to its high level of expression in cell lines generated from metastatic human breast and prostate tumors, with the notion that LIMK1 may be involved in cancer metastasis (see below).

LIMK1 has a distinct cellular localization. It resides mainly in the cytoplasm with lower levels in the nuclei. In the cytoplasm it localizes with F-actin in stress fibers and at focal adhesions. At focal adhesions it colocalizes with paxillin (Fig. 3) (Foletta et al. 2004). In Human umbilical vein endothelial cells (HUVEC) LIMK1 is localized to microtubules (MT) (Gorovoy et al. 2005). Similarly, LIMK1 colocalizes with MTs during mitosis, in particular during prophase, metaphase, and anaphase, while it accumulates at the contractile ring during telophase. However, Sumi et al. demonstrated that during cell division LIMK1 dynamically localizes to the mitotic apparatus. At metaphase it localizes to the spindle poles, while at late anaphase it is distributed exclusively along the lateral cytoplasmic membrane before transitioning to the contractile ring during telophase (Sumi et al. 2006). The activation and dynamic localization of LIMK1 during mitosis suggests the involvement of LIMK1 in cell division (Kaji et al. 2008).

LIMK2: As for LIMK1, early data on LIMK2 expression in mouse tissues relied on mRNA expression due to the lack of suitable anti-LIMK2 Abs. LIMK2, like LIMK1, is expressed in all mouse tissues (Acevedo et al. 2006). Although both proteins are expressed in both the nuclei and the cytoplasm the subcellular localization of LIMK2 is distinct from that of LIMK1. LIMK2 clearly localizes to punctae resembling endosomes in several cell lines and mouse embryonic fibroblasts (Fig. 3) (Acevedo et al. 2006). In NIH3T3 cells LIMK2 protein is also associated with GM130, a protein associated with the *cis*-compartment of the Golgi apparatus (Acevedo et al. 2006).



LIMK, Fig. 3 *Cellular localization of LIMK1 and LIMK2*. In the cytoplasm, LIMK1 localizes with F-actin in stress fibers (*green*) and at focal adhesions. At focal adhesions it colocalizes with paxillin (*yellow*). LIMK2 clearly localizes to punctae resembling endosome. Both proteins are also found in the nucleus

Immunofluorescence staining showed that during mitosis the localization of LIMK2 is similar to that of LIMK1 (Gamell-Fulla and Bernard). LIMK2 is associated with the mitotic spindle-like structure during mitosis and accumulates at the centrosomes in prometaphase. During the metaphase to anaphase transition, LIMK2 redistributes to the mitotic spindle and finally to the spindle midzone in telophase (Sumi et al. 2006). The distinct subcellular localization of LIMK2 suggests that it may have different substrates and cellular functions to that of LIMK1.

LIMK1 and Cancer Metastasis

Consistent with the role of LIMK1 in the regulation of the actin cytoskeleton there is growing evidence to indicate that LIMK1 plays an important role in tumor cell invasion and metastasis. High LIMK1 expression was found in metastatic melanoma cells (Wang et al. 2004), breast and prostate tumors (Wang et al. 2004; Davila et al. 2003), and tumor cell lines (Davila et al. 2003; Yoshioka et al. 2003). Several studies have implicated the involvement of LIMK1 in cell invasion and cancer metastasis. Downregulation of LIMK1 activity by expression of dominant-negative LIMK1 in the invasive human breast cancer cell line MDA-MB-231 reduced their invasion in vitro and in mice while overexpression of LIMK1 in the non-invasive MCF7 human breast cancer cells increased their invasion (Yoshioka et al. 2003). Similarly, expression of LIMK1 in MDA-MB-435 human breast cancer cells enhanced their proliferation, invasiveness, and promoted angiogenesis in vitro. When injected to immunocompromised nude mice these cells grew faster, promoted tumor angiogenesis, and induced liver and lung metastasis. (Bagheri-Yarmand et al. 2006). Benign prostate epithelial cells overexpressing LIMK1 exhibit increased expression of Membrane type matrix metalloproteinase 1 (MT1-MMP), a critical modulator of extracellular matrix (ECM) turnover through pericellular proteolysis that plays crucial roles in neoplastic cell invasion and metastasis. MT1-MMP and LIMK1 are both highly expressed in prostate tumor tissues (Tapia et al. 2011).

A recent study demonstrated that not only LIMK1 but also LIMK2 promote the metastasis of pancreatic cancer cells using a cell-based in vitro migration assay, as well as two zebra fish xenograft assays. The double knock down of LIMK1 and LIMK2 completely blocked invasion and formation of micrometastasis in vivo suggesting that both LIMK proteins have an important role in tumor progression and metastasis formation (Vlecken and Bagowski 2009). Scott et al. demonstrated that inhibition of LIMK activity by the pharmacological agent BMS3 (LIMKi) or by siRNA mediated knockdown blocks the collective invasion of MDA-MB-231 breast carcinoma cells growing in three-dimensional (3D) matrices (Scott et al. 2010). LIMK activity was also required for the collective invasion of squamous carcinoma cells (SCCs) in a 3D organotypic skin model, but it was not required for cell motility in two dimensions (2D). However, as described previously (Bagheri-Yarmand et al. 2006), LIMK was responsible for extracellular matrix (ECM) degradation in 3D cultures but not for path finding in MDA-MB-231 or SCCs (Scott et al. 2010).

Summary

The members of the LIM kinase family, LIMK1 and LIMK2 are ubiquitously expressed serine kinases that share identical genomic structure and ~50% overall identity. The most studied substrates of LIMK1 and

LIMK2 are the actin depolymerizing factor ADF/cofilin family of proteins. These actin binding proteins bind to and severe actin filaments (F-actin) and sequester actin monomers resulting in actin depolymerization. Phosphorylation of cofilin by LIMK inhibits its actin binding activity resulting in the accumulation of F-actin. The activity of the LIMK proteins is regulated by the Rho-GTPases: Rho, Rac, and Cdc42 via their downstream effector kinases ROCK 1 and 2, PAK 1, 2, and 4, which phosphorylate LIMK on a threonine residue. Although the LIMK proteins show significant structural similarity, their expression pattern, subcellular localization, regulation, and functions are different. They are involved in many cellular functions, such as cell migration, cell cycle, and neuronal differentiation and also have a role in cancer cell invasion and metastasis. The actin cytoskeleton plays a pivotal role in the motility of normal cells and in the invasive capacity of tumor cells. Both polymerization and depolymerization of actin are required for cell motility and invasion. LIMK1 levels are high in metastatic breast, prostate, and melanoma tumors and in a variety of invasive cell lines. Overexpression of LIMK1 in breast and prostate cancers increased their invasion in vitro and in mice while downregulation of its activity reduced their invasiveness, suggesting that inhibition of LIMK activity with pharmacological agents may be used to inhibit the metastatic spread of cancer cells.

References

Acevedo K, Moussi N, Li R, Soo P, Bernard O. LIM kinase 2 is widely expressed in all tissues. J Histochem Cytochem. 2006; 54:487–501.

Acevedo K, Li R, Soo P, Suryadinata R, Sarcevic B, Valova VA, Graham ME, Robinson PJ, Bernard O. The phosphorylation of p25/TPPP by LIM kinase 1 inhibits its ability to assemble microtubules. Exp Cell Res. 2007;313:4091–106.

Amano T, Tanabe K, Eto T, Narumiya S, Mizuno K. LIM-kinase 2 induces formation of stress fibres, focal adhesions and membrane blebs, dependent on its activation by Rho-associated kinase- catalysed phosphorylation at threonine-505. Biochem J. 2001;354:149–59.

Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O, Caroni P. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature. 1998;393:805–9.

Bagheri-Yarmand R, Mazumdar A, Sahin AA, Kumar R. LIM kinase 1 increases tumor metastasis of human breast cancer cells via regulation of the urokinase-type plasminogen activator system. Int J Cancer. 2006;118:2703–10.

Bamburg JR. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Annu Rev Cell Dev Biol. 1999; 15:185–230.

Bernard O, Ganiatsas S, Kannourakis G, Dringen R. Kiz-1, a protein with LIM zinc finger and kinase domains, is expressed mainly in neurons. Cell Growth Differ. 1994;5:1159–71.

Bernard O, Burkitt V, Webb GC, Bottema CD, Nicholl J, Sutherland GR, Matthew P. Structure and chromosomal localization of the genomic locus encoding the Kiz1 LIM-kinase gene. Genomics. 1996;35:593–6.

Dan C, Kelly A, Bernard O, Minden A. Cytoskeletal changes regulated by the PAK4 serine/threonine kinase are mediated by LIM kinase 1 and cofilin. J Biol Chem. 2001; 276:32115–21.

Davila M, Frost AR, Grizzle WE, Chakrabarti R. LIM kinase 1 is essential for the invasive growth of prostate epithelial cells: implications in prostate cancer. J Biol Chem. 2003; 278:36868–75.

Edwards DC, Gill GN. Structural features of LIM kinase that control effects on the actin cytoskeleton. J Biol Chem. 1999; 274:11352–61.

Edwards DC, Sanders LC, Bokoch GM, Gill GN. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol. 1999; 1:253–9.

Foletta VC, Moussi N, Sarmiere PD, Bamburg JR, Bernard O. LIM kinase 1, a key regulator of actin dynamics, is widely expressed in embryonic and adult tissues. Exp Cell Res. 2004; 294:392–405.

Gohla A, Birkenfeld J, Bokoch GM. Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. Nat Cell Biol. 2005;7:21–9.

Gorovoy M, Niu J, Bernard O, Profirovic J, Minshall R, Neamu R, Voyno-Yasenetskaya T. LIM kinase 1 coordinates microtubule stability and actin polymerization in human endothelial cells. J Biol Chem. 2005;280:26533–42.

Hsu FF, Lin TY, Chen JY, Shieh SY. p53-Mediated transactivation of LIMK2b links actin dynamics to cell cycle checkpoint control. Oncogene. 2010;29:2864–76.

Ikebe C, Ohashi K, Fujimori T, Bernard O, Noda T, Robertson EJ, Mizuno K. Mouse LIM-kinase 2 gene – cDNA cloning, genomic organization, and tissue-specific expression of two alternatively initiated transcripts. Genomics. 1997;46:504–8.

Kaji N, Muramoto A, Mizuno K. LIM kinase-mediated cofilin phosphorylation during mitosis is required for precise spindle positioning. J Biol Chem. 2008;283:4983–92.

Li R, Soosairajah J, Harari D, Citri A, Price J, Ng HL, Morton CJ, Parker MW, Yarden Y, Bernard O. Hsp90 increases LIM kinase activity by promoting its homo-dimerization. FASEB J. 2006;20:1218–20.

Misra UK, Deedwania R, Pizzo SV. Binding of activated alpha2-macroglobulin to its cell surface receptor GRP78 in 1-LN prostate cancer cells regulates PAK-2-dependent activation of LIMK. J Biol Chem. 2005;280:26278–86.

Mizuno K, Okano I, Ohashi K, Nunoue K, Kuma K, Miyata T, Nakamura T. Identification of a human cDNA encoding a novel protein kinase with two repeats of the LIM/double zinc finger motif. Oncogene. 1994;9:1605–12.
Moriyama K, Iida K, Yahara I. Phosphorylation of Ser-3 of cofilin regulates its essential function on actin. Genes Cells. 1996; 1:73–86.
Nebl G, Meuer SC, Samstag Y. Dephosphorylation of serine 3 regulates nuclear translocation of cofilin. J Biol Chem. 1996; 271:26276–80.
Niwa R, Nagata-Ohashi K, Takeichi M, Mizuno K, Uemura T. Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell. 2002; 108:233–46.
Ohashi K, Nagata K, Maekawa M, Ishizaki T, Narumiya S, Mizuno K. Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. J Biol Chem. 2000;275:3577–82.
Okano I, Hiraoka J, Otera H, Nunoue K, Ohashi K, Iwashita S, Hirai M, Mizuno K. Identification and characterization of a novel family of serine/threonine kinases containing two N-terminal LIM motifs. J Biol Chem. 1995;270:31321–30.
Sacchetti P, Carpentier R, Segard P, Olive-Cren C, Lefebvre P. Multiple signaling pathways regulate the transcriptional activity of the orphan nuclear receptor NURR1. Nucleic Acids Res. 2006;34:5515–27.
Scott RW, Hooper S, Crighton D, Li A, Konig I, Munro J, Trivier E, Wickman G, Morin P, Croft DR, Dawson J, Machesky L, Anderson KI, Sahai EA, Olson MF. LIM kinases are required for invasive path generation by tumor and tumor-associated stromal cells. J Cell Biol. 2010;191:169–85.
Soosairajah J, Maiti S, Wiggan O, Sarmiere P, Moussi N, Sarcevic B, Sampath R, Bamburg JR, Bernard O. Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin. EMBO J. 2005; 24:473–86.
Stanyon CA, Bernard O. LIM-kinase1. Int J Biochem Cell Biol. 1999;31:389–94.
Sumi T, Matsumoto K, Nakamura T. Specific activation of LIM kinase 2 via phosphorylation of threonine 505 by ROCK, a Rho-dependent protein kinase. J Biol Chem. 2001a; 276:670–6.
Sumi T, Matsumoto K, Shibuya A, Nakamura T. Activation of LIM kinases by myotonic dystrophy kinase-related Cdc42-binding kinase alpha. J Biol Chem. 2001b;276:23092–6.
Sumi T, Hashigasako A, Matsumoto K, Nakamura T. Different activity regulation and subcellular localization of LIMK1 and LIMK2 during cell cycle transition. Exp Cell Res. 2006; 312:1021–30.
Takahashi H, Koshimizu U, Miyazaki J, Nakamura T. Impaired spermatogenic ability of testicular germ cells in mice deficient in the LIM-kinase 2 gene. Dev Biol. 2002; 241:259–72.
Tapia T, Ottman R, Chakrabarti R. LIM kinase1 modulates function of membrane type matrix metalloproteinase 1: implication in invasion of prostate cancer cells. Mol Cancer. 2011;10:6.
Vlecken DH, Bagowski CP. LIMK1 and LIMK2 are important for metastatic behavior and tumor cell-induced angiogenesis of pancreatic cancer cells. Zebrafish. 2009;6:433–9.
Wang W, Goswami S, Lapidus K, Wells AL, Wyckoff JB, Sahai E, Singer RH, Segall JE, Condeelis JS. Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. Cancer Res. 2004;64:8585–94.
Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishidi E, Mizuno K. Cofilin phosphorylation by LIM-kinase1 and its role in Rac-mediated actin reorganization. Nature. 1998;393:809–12.
Yang EJ, Yoon JH, Min DS, Chung KC. LIM kinase 1 activates cAMP-responsive element-binding protein during the neuronal differentiation of immortalized hippocampal progenitor cells. J Biol Chem. 2004;279:8903–10.
Yoshioka K, Foletta V, Bernard O, Itoh K. A role for LIM kinase in cancer invasion. Proc Natl Acad Sci USA. 2003; 100:7247–52.

Lin 11

► LIMK

LNB-TM7 Receptors

► Adhesion GPCRs

LOC9372

► SARA

Location Chromosome 11p12

► TRAF6

Long-Chain Base Phosphate

► Sphingosine-1-Phosphate

Low Density Lipoprotein Receptor-Related Protein 1 (LRP1)

► CD91

LOX

Rozalia Laczko, Kornelia Molnarne Szauter, Charles D. Boyd and Katalin Csiszar
John A. Burns School of Medicine, University of Hawaii, Honolulu, HI, USA

Synonyms

Lysyl oxidase

Background

The extracellular matrix (ECM) is a dynamic milieu that provides a structural and signaling environment necessary for cell proliferation, differentiation, and migration in normal and pathological processes including embryonic development, tissue homeostasis and repair, inflammation, tumor invasion, and metastasis. While the composition of the ECM is unique in each organ, its main components consistently include various types of collagens, noncollagenous glycoproteins, and a range of enzymes essential for ECM assembly and maintenance. Lysyl oxidase (LOX) is one of these enzymes in the ECM. LOX is a copper-dependent amine oxidase that oxidatively deaminates specific peptidyl lysine and hydroxylysine residues within its substrates of which best known are fibrillar collagens and elastin. The resulting reactive aldehyde residues then spontaneously condense to form covalent intra- and intermolecular cross-linkages leading to the development of insoluble ECM matrices. Besides the cross-linking activity of LOX in the ECM of skin, lung, cardiovascular, and other systems, novel roles have also been recognized for LOX including significant ECM and cellular signaling. In mammals, the LOX family has five members, including LOX and four LOX-like proteins (LOXL1-4). The C-terminal region is highly conserved among all five members and includes a copper-binding site, a cytokine receptor-like (CRL) domain, and residues that form the lysine tyrosylquinone (LTQ) cofactor (Csiszar 2001). The N-terminal regions of the LOX-like proteins are more variable and different from LOX and include a proline-rich region in LOXL1 and four scavenger receptor cysteine-rich (SRCR) domains within LOXL2, LOXL3, and LOXL4. Emerging evidence indicates that the LOX-like proteins are also catalytically active but have different temporal and tissue specific expression, different substrates, and different functions.

Regulation of LOX Expression

The mRNA transcribed from the human LOX gene (chromosome 5q23.3–31.2) that encodes a 417 amino acid polypeptide, varies in size from 2.0 to 2.4, 4.3, and 5.5 Kb due to the use of alternate polyadenylation and multiple transcription initiation sites for which the regulating mechanisms and functions are poorly defined. During development and tissue remodeling, LOX expression is prominently associated with the assembly of collagen and elastic fibers in tissues including skin, aorta, placenta, and lung. A large number of cell types have been shown to express LOX and these include fibroblasts, smooth muscle cells from the aorta and lung, osteoblasts, osteosarcoma cells, myofibroblasts, chondrocytes, epithelial and endothelial cells, basal cells, liver parenchymal cells, spleen reticulum cells, kidney glomeruli, medulla, and other renal cell lines. LOX mRNA expression is highly responsive to a variety of cytokines and growth factors. Basic fibroblast growth factor (b-FGF) downregulates LOX in fibroblastic, epithelial, and osteoblastic cell lineages. Similarly, interferon-γ (IF-γ) downregulates LOX in aortic smooth muscle cells and prostaglandin E2 (PGE2) in embryonic smooth muscle cells and neonatal lung fibroblasts. Among the positive LOX regulators, transforming growth factor-β1 (TGF-β1) upregulates LOX mRNA, protein levels and activity in fibroblasts, osteoblasts, and aortic smooth muscle cells. Platelet-derived growth factor (PDGF) upregulates LOX mRNA in retinal pigment epithelial cells and vascular smooth muscle cells. Insulin-like growth factor-I (IGF-I) increases LOX protein level in oral tissue. Among hormonal regulators, follicle stimulating hormone (FSH) decreases LOX mRNA expression and activity in granulosa cells, while testosterone increases both LOX mRNA level and activity. Stem cell factor (SCF), brain-derived neurotropic factor (BDNF), and C-type natriuretic peptide (CNP) stimuli induce LOX transcription as noted during chondrocyte differentiation. Bone Morphogenetic Protein-2 and Bone Morphogenetic Protein-4 (BMP-2, -4) also upregulate LOX protein expression during adipocyte stem cell differentiation, while blocking the BMP-2

pathway by knockdown of Sma and Mad Related Protein 4 (SMAD4) results in reduced LOX expression and abolished acquisition of the adipocyte phenotype that is critically dependent on LOX expression.

Regulation of LOX Activity

The human LOX protein is synthesized as a 48 kDa preproenzyme with a 21 amino acid signal sequence at the N-terminus. Following N-terminal glycosylation, the signal peptide is removed and the copper cofactor is incorporated into the protein within the Golgi apparatus resulting in an intermediary, catalytically inactive 50 kDa proenzyme that is subsequently secreted to the extracellular space. In the ECM, the pro-LOX is activated by proteolytic cleavage by procollagen C-proteinase/BMP-1. The proteolytic activation of the intermediary proenzyme yields a mature ECM enzyme of 30 kDa and an 18 kDa N-terminal propeptide fragment. Fibronectin and the microfibrillar network play a role in regulating LOX activation and localization in the ECM.

LOX in Fibrotic Disorders and TGF-β Signaling

In fibrotic diseases, increased collagen expression and enhanced LOX expression and activity result in increased cross-linking and accumulation of insoluble collagen fibers. Strong association has been reported between organ fibrosis and increased LOX activity in experimental fibrosis, lung, arterial, dermal, kidney fibrosis, and pathological conditions leading to fibrosis.

During the fibrotic process, changes in the expression level of type III collagen were noted to parallel changes both in the level and activity of LOX (Kim et al. 1999). Subsequently, the active LOX was reported to translocate into the nuclei and enhance collagen-3A1 (*COL3A1*) gene promoter activity in monkey renal (COS-7) and human primary skin fibroblasts. In addition to the association between LOX and type III collagen expression, increases in LOX expression were also linked to type I collagen expression (Hong and Trackman 2002) and it was proposed that the LOX and the *COL1A1* and *COL1A2* genes may be co-regulated by their similar TGF-β response elements within their promoters. Consistent with this mechanism, upregulated TGF-β/p38 mitogen-activated protein kinase (p38MAPK) signaling increased both LOX levels and collagen synthesis in maternal obesity-associated fetal muscle fibrogenesis.

Notably, a direct interaction between the LOX protein and TGF-β1 has also been reported by which interaction LOX was able to suppress TGF-β1 induced SMAD3 phosphorylation. Significantly, this effect proved to be due to the BAPN-sensitive amine oxidase activity of LOX, and not to its peroxide production that is a dominant LOX-associated signaling mechanism in multiple tumor types. In interactions with TGF-β1, LOX may be involved in oxidative deamination of lysine residues within the lysine-rich terminus in TGF-β1, a process that could change charges or covalently stabilize the conformation of TGF-β1. Such change may interfere with binding of TGF-β1 to its receptor leading to diminished signaling via SMAD3 in a cross-talk with Phosphoinositide 3-kinase (PI3K) and Akt serine-threonine protein kinase (Atsawasuwan et al. 2008).

Interestingly, in a novel microfibrillar network-associated fibrotic skin disorder, LOX overexpression was not accompanied by either increased TGF-β1 expression or signaling (Szauter et al. 2010). However, genes of the microfibrillar network including fibrillin-2 (FBN2), microfibril-associated glycoprotein 2, microfibril-associated protein 4, latent TGFβ-binding protein 1 (LTBP1), fibulins 1 and 3 (FBLN1, 3), and decorin were found downregulated leading to compromised synthesis and assembly of the microfibrillar scaffold reflected in the disorderly assembly of elastic fibers. Interactions between LOX and microfibrillar proteins FBLN-1, FBLN-4, and fibrillin 1 (FBN1) have been established and in this case of dermal fibrosis the disorderly microfibrillar network clearly contributed to deregulated LOX expression and activity. The microfibrillar network is also known to regulate TGF-β1 activity; however, in this case, there was no increase in SMAD2 phosphorylation further supporting the conclusion that TGF-β1 did not play a major role in LOX regulation in the pathomechanism of this particular fibrotic disease.

LOX in Tumor Development and Invasion

LOX, in addition to its function in the ECM, has also been noted for significant intracellular and intranuclear

activities, a tumor suppressor role (Csiszar 2001), and contrasting essential roles during tumor invasion and metastasis (Payne et al. 2005; Baker et al. 2011). Studies of LOX signal transduction suggested that LOX is able to affect signaling pathways downstream of extracellular signal-regulated kinase (ERK2) and influence oncogenic RAS-dependent meiotic maturation. A RAS-related tumor suppressor activity for LOX (RAS-recision gene or *RRG*) was reported in RAS-transformed mouse NIH3T3 (Kenyon et al. 1991) and other Ha-ras transformed fibroblasts. Some of the complex signaling pathways associated with the tumor suppressor activity of LOX have been since localized to the LOX propeptide (LOX-PP) and are summarized in the subsequent section.

In the hypertrophic stroma surrounding the in situ tumors in human breast and bronchopulmonary carcinomas increased LOX expression likely participates in the formation of a peritumor barrier, however, the significance of LOX functions in the tumor stroma remained largely unexplored. Importantly, a recent study identified a role for tissue stiffness linked to increased LOX cross-linking of collagen I in the tumor ECM resulting in increased focal adhesion and integrin β1 signaling, and elevated phosphorylation of focal adhesion kinase (FAK)^{Y397}and CRISPR-associated protein ^{p130}CAS. Modified ECM-induced integrin β1 signaling additionally activates PI3K and EGF-mediated phosporylation of Akt^{S473} resulting in increased tumor cell adhesion and invasion (Levental et al. 2009).

In breast tumor epithelial cells, increased LOX expression and enzyme activity proved to be strongly associated with invasive properties. As noted earlier in monocytes and smooth muscle cells, in tumor epithelial cells, cell migration depended on H_2O_2 generated during the catalytic reaction of the active LOX with its substrates. LOX-generated H_2O_2 mediated breast tumor cell migration and invasion by activating FAK/SRC signaling (Payne et al. 2005) that was also confirmed in colorectal cancer cells (Baker et al. 2011). Furthermore, LOX expression was associated with increased metastasis of breast tumors (Erler et al. 2006; Payne et al. 2006). Astrocytoma and glioma cell lines and tissues also demonstrated high levels of LOX expression that correlated with increased tumor grade and increased activation of FAK/paxillin signaling (Laczko et al. 2007). In tumor cells, the LOX-associated invasive mechanism further involved increased lamellipodia formation by affecting signaling downstream of FAK, involving the CAS/CRK/DOCK signaling complex, activation of RAC GTPase, and a direct activation of the PI3K/Akt pathway as demonstrated in human colorectal carcinoma cell lines (Pez et al. 2011).

Within tumors, hypoxic conditions are known to upregulate LOX involving the hypoxia inducible factor (HIF) demonstrated in breast (Erler et al. 2006) and colorectal carcinoma cells where the HIF-1α subunit induced LOX expression (Pez et al. 2011). In turn, LOX is able to reciprocally enhance HIF-1α expression through its catalytic activity and the subsequent production of H_2O_2 through PI3K/Akt signaling, thus the mutually regulated HIF and LOX synergistically promote tumor cell growth and proliferation.

The LOX Propeptide in RAS, NF-κB, and FAK Signaling

The 18 kDa LOX propeptide (LOX-PP) is required for optimal LOX secretion and is proteolytically removed in the ECM as part of the activation process by BMP-1. LOX-PP due to its high isoelectric point can reenter the cell and interact with intracellular binding partners in addition to ECM molecules. Moreover, LOX-PP has a putative nuclear localization signal, and has been detected in the nucleus where it was proposed to function as a signaling molecule (Li et al. 2010).

LOX-PP inhibits several distinct signal transduction pathways that oncogenic RAS activates, including the PI3K/Akt and RAF/MAPK/ERK and the Nuclear Factor-κB (NF-κB) pathways. In lung cancer and pancreatic cancer cells ectopic expression of LOX-PP reduces activation of the ERK cascade inhibiting growth and invasive colony formation (Wu et al. 2007). LOX-PP also inhibits Akt-dependent induction of RAS in c-Ha-RAS-transformed NIH3T3 cells resulting in decreased cell proliferation and migration. Signaling of FGF-2 that induces ERK/MAPK phosphorylation was also inhibited by LOX-PP in MC3T3 and osteoblast cultures by reducing FGF-2 binding to its receptors. In androgen-independent prostate cancer cell lines with high levels of RAS, LOX-PP inhibited serum-stimulated DNA synthesis in a dose-dependent manner: in DU145 cells through the MEK/ERK and PI3K/Akt pathways, and in PC-3 cells through a different mechanism, demonstrating that

LOX-PP has more than one molecular target (Palamakumbura et al. 2009). In smooth muscle cells (SMC), LOX-PP effectively reduced TNF-α stimulated ERK/MAP kinases, cell proliferation, and matrix metalloproteinase-9 (MMP-9), mechanisms that are relevant to the development of atherosclerosis (Hurtado et al. 2008).

NF-κB directly induces expression of genes that promote tumor cell survival and proliferation, resistance to chemotherapy, neoplastic transformation, and maintenance of an invasive phenotype. In pancreatic and lung cancer cell lines LOX-PP substantially reduced levels of NF-κB and its tissue specific target BCL-2 and decreased the ability of these cells to migrate (Wu et al. 2007). LOX-PP also inhibited transformation of murine breast cancer cells driven by Her-2/neu, an upstream activator of RAS via NF-κB activation. In these cells LOX-PP reversed Her-2/neu-induced migration and epithelial to mesenchymal transition (EMT) as measured by reduced levels of Snail and vimentin, upregulation of E-cadherin, and inhibited tumor formation (Min et al. 2007). In mice a mutation within neuronal nuclear protein induced by axotomy (Nna1) was noted to increase expression and intranuclear localization of LOX-PP in Purkinje cell nuclei. This excess LOX-PP inhibited NF-κB/RelA signaling and decreased microtubule-associated proteins MAP1B and MAP2, regulators of microtubule stability leading to underdevelopment of Purkinje cell dendrites (Li et al. 2010).

The FAK/SRC/CAS signaling pathway that is crucial for cell migration and invasion is also affected by LOX-PP that was found to inhibit fibronectin-mediated signaling via FAK and CAS^{p130} and fibronectin-stimulated migration of breast cancer cells (Zhao et al. 2009). In murine breast cancer cells, LOX-PP decreased the phosphorylation of FAK Y397 and Y576 that resulted in diminished recruitment of its substrate CAS to focal adhesion sites. Recombinant LOX-PP protein also inhibited the migration of the invasive human MDA-MB-231 and Hs578T breast cancer cells. Recently, a G473A polymorphism resulting in an Arg-to-Gln substitution has been found to impair the ability of LOX-PP to inhibit the invasive phenotype and tumor formation of breast cancer cells (Min et al. 2009). This polymorphism was associated with an increased risk of estrogen receptor (ER)-negative breast cancer, further supporting a role for impaired LOX-PP signaling in breast cancer.

Summary

LOX is a copper-dependent amine oxidase essential for the development and maintenance of the ECM that provides a structural and signaling environment necessary for cellular and tissue functions during normal and pathological processes. LOX is one of five members of the LOX family that have variable N-terminal domains and highly conserved C-terminal regions that include the Cu-binding site and residues that form the LTQ cofactor both critical for catalytic activity, and a CRL domain. Following multiple processing steps, the 48 kDa LOX preproprotein is secreted and activated in the ECM by BMP-1 cleavage that produces an 18 kDa LOX-PP and a 30 kDa active LOX, both with distinct ECM, cellular and intranuclear functions. LOX expression is regulated by various cytokines and growth factors including b-FGF, IF-γ, PGE2, PDGF, TGF-β1, IGF-I, and hormones including FSH and testosterone. While TGF-β1 is a prominent LOX regulator in fibrotic disorders, the active LOX also directly interacts with and likely modifies lysine residues within TGF-β1 leading to reduced binding of TGF-β1 to its receptors and reduced TGF-β1/SMAD signaling. Furthermore, analysis of the pathomechanism of a novel fibrotic disorder also revealed a prominent LOX regulatory function for the microfibrillar network that appears independent of TGF-β1. The active LOX proved essential in promoting tumor development and invasion both through ECM-cell signaling and peroxide-mediated cellular signaling. The LOX-modulated ECM increases integrin β1 signaling through FAK, CAS, and PI3K/Akt signaling resulting in increased tumor cell adhesion and invasion. While peroxide generated by the catalytic reaction of LOX activates FAK/SRC/paxillin and CAS/CRK/DOC signaling and directly upregulates the PI3K/Akt pathway further promoting tumor cell migration, invasion, and metastasis. In contrast, the LOX-PP appears to function as an active inhibitor of the transformed cell phenotype through inhibiting RAS signaling and RAS-activated PI3K/Akt, RAF/MAPK pathways, signaling of FGF-2 and subsequent induction of ERK/MAPK phosphorylation, and NF-κB. Through these mechanisms LOX-PP reduces cell proliferation, migration, EMT, and tumor formation. LOX-PP can also inhibit fibronectin-mediated FAK signaling and recruitment of CAS to focal adhesion sites. Thus, the active LOX and the LOX-PP provide a dynamic balance in contrasting

functions that depend on the expression, ECM processing, and activation of LOX and turnover of the different LOX forms.

References

Atsawasuwan P, Mochida Y, Katafuchi M, Kaku M, Fong KS, Csiszar K, et al. Lysyl oxidase binds transforming growth factor-beta and regulates its signaling via amine oxidase activity. J Biol Chem. 2008;283(49):34229–40.

Baker AM, Cox TR, Bird D, Lang G, Murray GI, Sun XF, et al. The role of lysyl oxidase in SRC-dependent proliferation and metastasis of colorectal cancer. J Natl Cancer Inst. 2011;103(5):407–24.

Csiszar K. Lysyl oxidases: a novel multifunctional amine oxidase family. Prog Nucleic Acid Res Mol Biol. 2001;70:1–32.

Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature. 2006;440(7088):1222–6.

Hong HH, Trackman PC. Cytokine regulation of gingival fibroblast lysyl oxidase, collagen, and elastin. J Periodontol. 2002;73(2):145–52.

Hurtado PA, Vora S, Sume SS, Yang D, St Hilaire D, Guo Y, et al. Lysyl oxidase propeptide inhibits smooth muscle cell signaling and proliferation. Biochem Biophys Res Commun. 2008;366(1):156–61.

Kenyon K, Contente S, Trackman PC, Tang J, Kagan HM, Friedman RM. Lysyl oxidase and rrg messenger RNA. Science. 1991;253(5021):802.

Kim Y, Peyrol S, So CK, Boyd CD, Csiszar K. Coexpression of the lysyl oxidase-like gene (LOXL) and the gene encoding type III procollagen in induced liver fibrosis. J Cell Biochem. 1999;72(2):181–8.

Laczko R, Szauter KM, Jansen MK, Hollosi P, Muranyi M, Molnar J, et al. Active lysyl oxidase (LOX) correlates with focal adhesion kinase (FAK)/paxillin activation and migration in invasive astrocytes. Neuropathol Appl Neurobiol. 2007;33(6):631–43.

Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009;139(5):891–906.

Li J, Gu X, Ma Y, Calicchio ML, Kong D, Teng YD, et al. Nna1 mediates purkinje cell dendritic development via lysyl oxidase propeptide and NF-kappaB signali. Neuron. 2010;68(1):45–60.

Min C, Kirsch KH, Zhao Y, Jeay S, Palamakumbura AH, Trackman PC, et al. The tumor suppressor activity of the lysyl oxidase propeptide reverses the invasive phenotype of Her-2/neu-driven breast cancer. Cancer Res. 2007;67(3):1105–12.

Min C, Yu Z, Kirsc KH, Zhao Y, Vora SR, Trackman PC, et al. A loss-of-function polymorphism in the propeptide domain of the LOX gene and breast cancer. Cancer Res. 2009;69(16):6685–93.

Palamakumbura AH, Vora SR, Nugent MA, Kirsch KH, Sonenshein GE, Trackman PC. Lysyl oxidase propeptide inhibits prostate cancer cell growth by mechanisms that target FGF-2-cell binding and signaling. Oncogene. 2009;28(38):3390–400.

Payne SL, Fogelgren B, Hess AR, Seftor EA, Wiley EL, Fong SF, et al. Lysyl oxidase regulates breast cancer cell migration and adhesion through a hydrogen peroxide-mediated mechanism. Cancer Res. 2005;65(24):11429–36.

Payne SL, Hendrix MJ, Kirschmann DA. Lysyl oxidase regulates actin filament formation through the p130(Cas)/Crk/DOCK180 signaling complex. J Cell Biochem. 2006;98(4):827–37.

Pez F, Dayan F, Durivault J, Kaniewski B, Aimond G, Le Provost GS, et al. The HIF-1-inducible lysyl oxidase activates HIF-1 via the Akt pathway in a positive regulation loop and synergizes with HIF-1 in promoting tumor cell growth. Cancer Res. 2011;71(5):1647–57.

Szauter KM, Ordas A, Laxer RM, Pope E, Wherrett D, Alman B, et al. A novel fibrotic disorder associated with increased dermal fibroblast proliferation and downregulation of genes of the microfibrillar network. Br J Dermatol. 2010;163(5):1102–15.

Wu M, Min C, Wang X, Yu Z, Kirsch KH, Trackman PC, et al. Repression of BCL2 by the tumor suppressor activity of the lysyl oxidase propeptide inhibits transformed phenotype of lung and pancreatic cancer cells. Cancer Res. 2007;67(13):6278–85.

Zhao Y, Min C, Vora SR, Trackman PC, Sonenshein GE, Kirsch KH. The lysyl oxidase pro-peptide attenuates fibronectin-mediated activation of focal adhesion kinase and p130Cas in breast cancer cells. J Biol Chem. 2009;284(3):1385–93.

LpMYO3

► Myosin III

Lps

► TLR4, Toll-Like Receptor 4

LRF-1

► ATF3 Activating Transcription Factor 3

LRG-21

► ATF3 Activating Transcription Factor 3

Lsk

► CSK-Homologous Kinase

L-Type

► Voltage-Gated Calcium Channels: Structure and Function (CACNA)

LXA4R

► FPR2/ALX

Ly 5.2

► CD45 (PTPRC)

Ly100

► CLEC5A

Ly-19

► CD72

Ly-32

► CD72

Ly5.1

► CD45 (PTPRC)

Ly87

► TLR4, Toll-Like Receptor 4

Lyb-2

► CD72

Ly-m19

► CD72

Lymphocyte Cytosolic Protein 2

► SLP-76

Lysine (K) Acetyltransferase 6A

► MOZ and MORF Lysine Acetyltransferases

Lysine (K) Acetyltransferase 6B

► MOZ and MORF Lysine Acetyltransferases

Lysine Acetyltransferase 2B

► PCAF Lysine Acetyltransferase

Lysyl Oxidase

► LOX

M

Macrophage Inflammatory Protein-1 Alpha

► CCL3

Macrophage Inflammatory Protein-1 Beta

► CCL4

Macrophage Mannose Receptor 2

► MRC2

Macrophage-Inducible C-Type Lectin

► CLEC4E

MADHIP, MAD Homolog Interacting Protein

► SARA

Mail

► IkBz

Mal

► Toll-like Receptor Adaptor Protein Family Members

MALT1

► MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1)

MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1)

Andreas Gewies[1] and Jürgen Ruland[1,2]
[1]Laboratory for Signaling in the Immune System, Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany
[2]Third Medical Department, Institute for Molecular Immunology, Technical University of Munich, Klinikum rechts der Isar, Munich, Germany

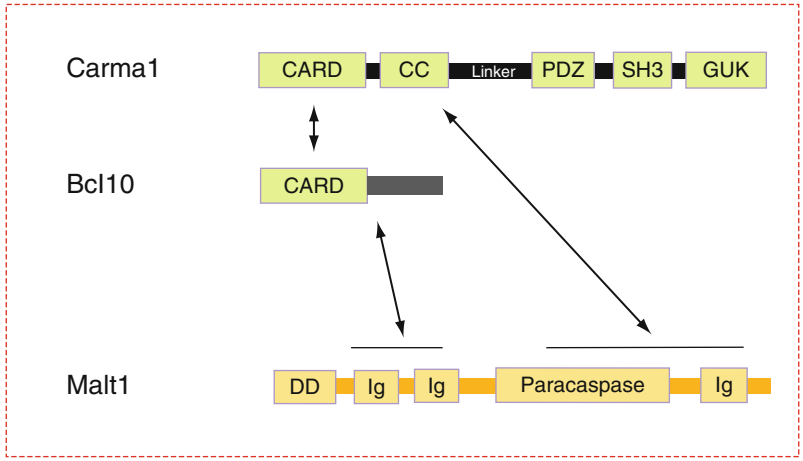
Synonyms

MALT1; MLT; Mucosa-Associated Lymphoid Tissue translocation gene 1; Paracaspase

Historical Background

Around the beginnings of the 1990s several cytogenetic studies reported the occurrence of t(11;18)(q21;q21)

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

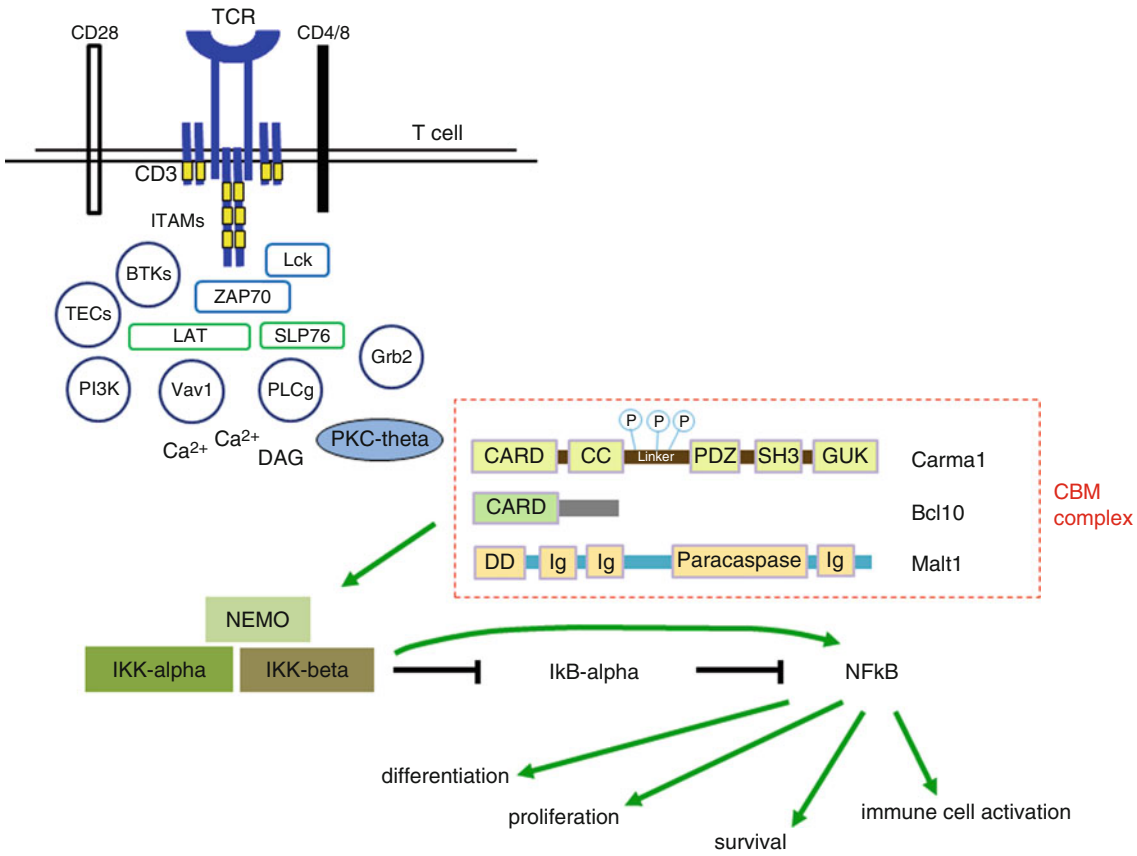


MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1), Fig. 1 *The CBM complex*. The CBM complex consists of the three proteins Carma1 (Card11), Bcl10, and Malt1. Bcl10 and Malt1 interact via a sequence immediately downstream of the Bcl10 CARD domain and the Ig-like domains of Malt1. While the Bcl10-Malt1 interaction is assumed to be constitutive, Bcl10-Malt1 is recruited to Carma1 in response to antigen-receptor signaling. Bcl10 and Carma1 associate via CARD-CARD interaction, and the Carma1 coiled-coil domain can bind to the C-terminal part of Malt1 that contains the paracaspase domain. The CBM complex is believed to form the core of a molecular scaffold that upon activation recruits various further signaling components to activate NF-κB and other signaling pathways

chromosomal translocations in several cases of low-grade Mucosa-Associated Lymphoid Tissue (MALT) lymphoma. Later, Akagi and colleagues identified a novel gene on chromosome 18 to be involved in the t (11;18)(q21;q21) translocation which they named *MALT1* as a candidate gene involved in the pathogenesis of MALT lymphoma (Akagi et al. 1999). Already a few months earlier, Dierlamm and colleagues also reported *MALT1* (which they named *MLT*) as part of the t(11;18)(q21;q21) translocation as well its translocation partner on chromosome 11, the *API2* gene, resulting in the expression of a *5′-API2-MALT1-3′* fusion transcript (Dierlamm et al. 1999). While the function of the *MALT1* gene product was completely unclear at that time, the *API2* gene had already been known to encode for a member of the family of inhibitor of apoptosis (IAP) proteins. Breakpoint analyses showed that the *API2* gene contributed its three baculovirus IAP repeat (BIR) motifs to the 5′ part of the *API2-MALT1* fusion transcript. Since the BIR region was known to be sufficient for the inhibition of caspases and, therefore, for the suppression of apoptosis, it was suggested that the API2-MALT1 fusion might be involved in an inappropriate inhibition of apoptosis and thereby might confer a survival advantage to MALT lymphomas. However, subsequent biochemical and genetic characterization did not support a direct role for API2-MALT1 or MALT1 in apoptosis inhibition but revealed that MALT1 (and the API2-MALT1 fusion) promotes the activation of the ▶ NF-κB signaling pathway.

MALT1 as Part of the CBM Complex

In silico analyses revealed that a C-terminal part of the MALT1 amino acid sequence has similarity to the proteolytic domain of caspases, a family of cysteine proteases centrally involved in the induction of apoptosis. Importantly, the essential catalytic histidine and cysteine diad is conserved in the MALT1 sequence. Uren and colleagues therefore proposed that human MALT1 and its orthologs in, for example, mouse, zebrafish, and *Caenorhabditis elegans* should be classified as a novel family of caspase-related proteins, the paracaspases (Uren et al. 2000). The caspase-like catalytic domain of MALT1 was called the paracaspase domain which is preceded by an N-terminal death domain (DD) and two immunoglobulin (Ig)-like domains (Fig. 1). MALT1 is known to be constitutively associated with the BCL10 protein: the interaction occurs between the MALT1 Ig-like domains and a sequence stretch (aa 107–119) immediately downstream of the Bcl10 CARD domain. BCL10 is encoded



MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1), Fig. 2 *TCR signaling via the CBM complex to NFkB*. The signaling events emanating from the activated T-cell receptor (TCR) complex have been studied in detail. Receptor proximal signaling leads to the activation of various kinases such as Scr and Syk family kinases and to the recruitment of adaptor molecules such as LAT and SLP76. The adaptors assemble and activate a plethora of signaling molecules, among others phospholipase C-gamma (PLCg) and protein kinase C theta (PKC-theta). PKC-theta and other kinases are thought to activate Carma1 by phosphorylation leading to the assembly of the CBM complex for activation of the IKK complex and the NF-κB transcriptional program which affects various biological processes

by a gene that had been previously shown to be involved in t(1;14)(p22;q32) chromosomal translocations associated with cases of MALT lymphomas and which upon overexpression induces NF-κB. BCL10 also interacts with the CARD-containing protein ▶ CARMA1 which also is able to activate NF-κB upon overexpression. The NF-κB-activating potential of CARMA1 and BCL10 is dependent on homotypic interaction between the CARD domains of BCL10 and CARMA1 for the formation of oligomeric protein complexes (Fig. 1). The ectopic overexpression of *Bcl10* or *CARMA1* by itself induces NF-κB, whereas overexpression of *MALT1* alone is not sufficient for NF-κB activation. However, MALT1 was shown to enhance Bcl10-mediated activation of NF-κB and it was proposed that BCL10 mediates the oligomerization of MALT1 molecules for the enhanced activation of NF-κB. This hypothesis was supported by the observation that the oligomerization of the MALT1 paracaspase domain or oligomerization of API2-MALT1 via the N-terminal BIR domains resulted in NF-κB activation. Mutation of the active-site cysteine residue of the proteolytic paracaspase domain reduced the capacity of API2-MALT1 for induction of NF-κB, however, not completely. Taken together, MALT1 was identified to be part of a complex with BCL10 and CARMA1 – the so-called CBM (CARMA1-BCL10-MALT1) complex (Thome et al. 2010) – which is involved in NF-κB activation (Fig. 2). How MALT1 is thought to be activated within the CBM complex and

how MALT1 participates in the regulation of NF-κB under physiological settings will be described within the subsequent paragraphs.

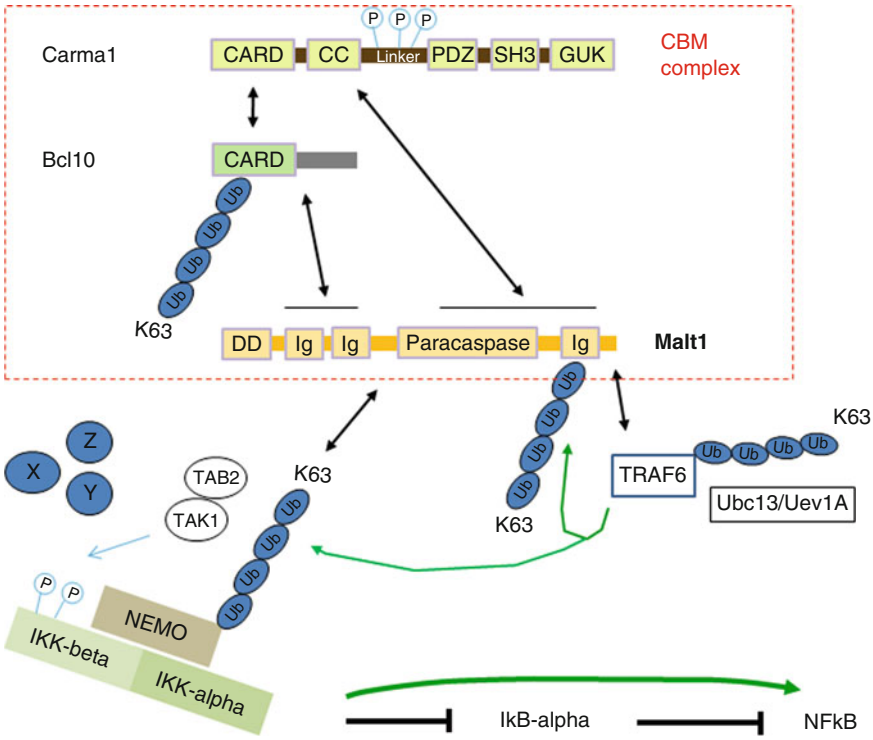
MALT1 in Antigen-Receptor

Phenotype of MALT1 Knockout Mice

A role for the CBM complex in cells of the immune system could be expected from the expression patterns of its components: in the mouse, *Malt1* and *Carma1* are predominantly expressed in lymphoid organs whereas *Bcl10* is quite ubiquitously expressed in various tissues including the lymphoid compartments. Analysis of *Bcl10*-deficient mice revealed severe immunodeficiency with strong defects in antigen receptor (AgR)-mediated activation, proliferation, and NF-κB signaling of B and T cells (Ruland et al. 2001), which is comparable to the phenotype of mice deficient in Carma1. The importance of the CBM complex in the immune system is further supported by the phenotype of *Malt1*-deficient mice (Ruefli-Brasse et al. 2003; Ruland et al. 2003). *Malt1*-deficient mice proved to be viable and fertile, and are born at the expected Mendelian ratio. Of note, while a fraction of *Bcl10*-deficient mice die during embryonic development due to a neural tube closure defect, *Malt1*-deficient mice as well as *Carma1*-deficient mice are not affected by a developmental defect, indicating that Bcl10 can exert functions independent of Malt1 and Carma1. *Malt1*-deficient mice are severely immunodeficient with markedly reduced basal immunoglobulin serum levels and a defective antibody response after immunizations. While Bcl10 is essential for the development of all main subtypes of B cells, follicular B2 cells, marginal zone B (MZB) cells and B1 cells (Xue et al. 2003), Malt1-deficiency revealed impairment of MZB and B1 cell development but showed normal follicular B2 cell development. *Malt1*-deficient mice display normal numbers of peripheral T lymphocytes despite some irregularity during thymocyte development due to a premature maturation of double-negative thymocytes. However, it was demonstrated that Bcl10/Malt1 signaling is dispensable for negative and positive selection of thymocytes (Jost et al. 2007). T cells isolated from *Malt1*-deficient mice reveal severe impairment of proliferation, IL-2 production, and NF-κB activation in response to stimulation with phorbol-12-myristate-13-acetate (PMA) plus ionomycin or with anti-► CD3 plus anti-CD28 antibodies. While ERK1/2 activation, calcium release, and JNK1 activation proves to be normal, the activation of JNK2 is impaired. Ruefli-Brasse and colleagues reported that B lymphocytes isolated from their *Malt1* knockout mouse strain also showed severe defects in the proliferative response and in NF-κB activation (Ruefli-Brasse et al. 2003). However, the *Malt1*-deficient mouse strain of Ruland and colleagues revealed only a moderate effect of Malt1 on B-cell receptor (BCR) signaling, since conventional B2 cells from those mice were still able to activate NF-κB and showed only a partial reduction of proliferation after stimulation (Ruland et al. 2003). More detailed analysis of this *Malt1* knockout mouse strain revealed that Malt1-deficiency in B cells does not affect the activation of the NF-κB subunit RelA but selectively impairs the formation of cRel-containing complexes (Ferch et al. 2007). The same study demonstrated that Malt1, therefore, seems to control only certain aspects of BCR-mediated NF-κB signaling such as activation of survival signals but does not control cell proliferation. This is in contrast to the role of Bcl10 which is essential for all aspects of BCR-mediated NF-κB activation (Ruland et al. 2001). Taken together, analysis of *Malt1*-deficient mice clearly demonstrated its central importance for a regular adaptive immune response and its essential involvement in AgR-mediated NF-κB signaling downstream of Bcl10.

MALT1 as an Adaptor Protein in AgR-Mediated CBM Signaling

During recent years, the mechanisms by which MALT1, as part of the CBM complex, contributes to AgR-mediated NF-κB activation were intensely studied on the molecular level. The early T-cell receptor (TCR)-proximal events had already been studied quite in detail: ligation of the TCR and its costimulatory CD28 receptor at the immunological synapse first results in the recruitment and activation of the Src protein tyrosine kinase Lck. Lck phosphorylates the immunoreceptor tyrosine based activation motives (ITAMs) of ► CD3 subunits to which the Syk family protein tyrosine kinase ► ZAP-70, and subsequently the adaptors ► LAT and ► SLP-76 are recruited. Then several additional molecules, such as Grb2, Vav1, and Tec kinases, associate with the activated receptor complex by which multiple signaling events are initiated, including the activation of the MAP kinases, phospholipase C (PLC)-gamma, and phosphatidylinositole-3-kinase (PI3-K). Those events again trigger a cascade



MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1), Fig. 3 *Malt1 as a scaffold protein in IKK activation*.As part of the activated CBM complex, Malt1 serves as a scaffold protein that recruits further signaling components such as the ubiquitin ligase complex consisting of TRAF6 and Ubc13/Uev1A. This results in the K63-type ubiquitination of several molecules, among others Malt1 and TRAF6 themselves but also of Bcl10 and the regulatory IKK subunit NEMO. Those ubiquitination events contribute to the association of NEMO and the IKK complex to the CBM complex with subsequent activation of the IKK complex and NF-κB

of downstream events such as the formation of diacylglycerol(DAG), the release of intracellular calcium, and activation of the serine/threonine protein kinases Akt/PKB and PKC-theta (Fig. 2). Upon activation, PKC-theta translocates into specialized membrane microdomains, the so-called lipid rafts, where it is essential for TCR-mediated NF-κB and AP-1 activation. Importantly, in unstimulated T cells CARMA1 was identified to be constitutively present in lipid rafts, whereas BCL10 was not. However, upon TCR stimulation, BCL10 was shown to be recruited from the cytoplasm to the membrane lipid raft fraction in a CARMA1-dependent manner. Upon activation of the AgR signaling pathways, PKC-theta in T cells or PKC-beta in B cells as well as other kinases such as IKK-beta and CK1-alpha were described to phosphorylate CARMA1 within its linker region. This might result in a conformational change of CARMA1, enabling its CARD-dependent interaction with BCL10 which on the other hand is constitutively associated with MALT1 (Thome et al. 2010). Once recruited to the immunological synapse by CARMA1 and BCL10, MALT1 contributes to the activation of the inhibitor of NF-κB kinase (IKK) complex for NF-κB activation. Up to now, the mechanisms by which MALT1 (in synergy and/or in parallel with BCL10) activates and regulates IKK activity are not completely understood. It seems to be clear, however, that MALT1 acts as an adaptor protein that recruits several binding partners for the induction of downstream signaling events. For example, the association of MALT1 with ▶ TRAF6-containing ubiquitin ligase complexes results in the addition of K63-linked ubiquitin chains to a multitude of proteins, among others TRAF6, Bcl10, and MALT1 but also to the regulatory domain of the IKK complex, NEMO (Fig. 3). The modification with K63-linked ubiquitin chains is essential for the recruitment of the IKK complex and other signaling molecules to the CBM complex at the immunological synapse and subsequent NF-κB activation (Duwel et al. 2010). First evidence for the participation of a TRAF6-dependent ubiquitin ligase complex in CBM-mediated NF-κB activation was obtained from studying a cell-free system (Sun et al. 2004). Sun and colleagues were able to reconstitute BCL10- and MALT1-dependent IKK activation in vitro in the presence of recombinant TRAF6, Ubc13/Uev1A, and TAK1/TAB1/TAB2. This study also suggests that MALT1 oligomers must be formed via its interaction with BCL10. Those MALT1 oligomers supposedly bind to the RING domain-containing ubiquitin ligase TRAF6 which in concert with the

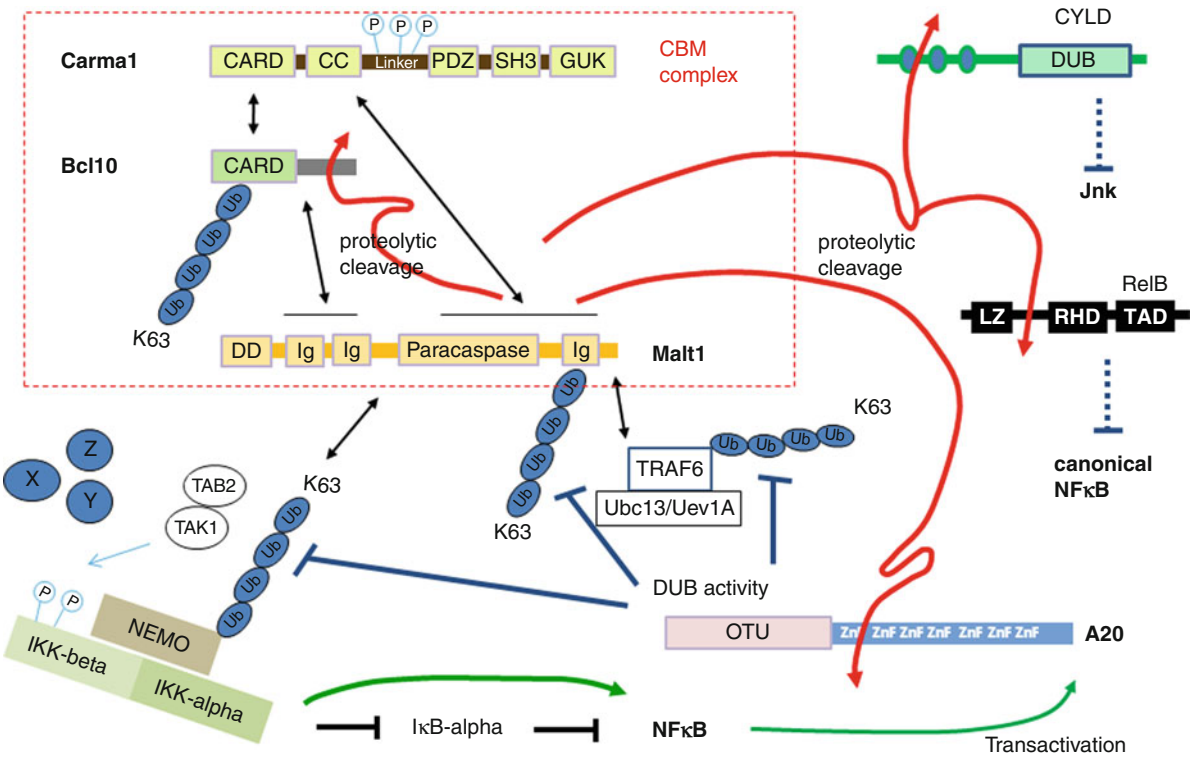
ubiquitin-conjugating enzyme complex Ubc13/Uev1A is activated as a ubiquitin ligase. TRAF6 was shown to directly bind and K63-ubiquitylate MALT1. Subsequently, the IKK complex can be recruited to the CBM complex since NEMO binds to K63-polyubiquitylated MALT1 via its ubiquitin-binding domain (UBD). Besides NEMO, also the TAK1/TAB2 kinase complex was shown to be associated with ubiquitylated MALT1 (Oeckinghaus et al. 2007) and might contribute to the activation of the IKK complex by phosphorylating two serine residues within the activation loop of IKK-β. However, the exact role and mode of function of TAK1 in IKK activation is not yet resolved.

Evidence accumulated for a surprising contribution of caspase-8 to TCR signaling including NF-κB activation. In this setting, caspase-8 does not signal for apoptosis but associates with various NF-κB signaling molecules in lipid rafts. Kawadler and colleagues demonstrated that MALT1 associates with procaspase-8 and promotes a partial proteolytical processing and activation of procaspase-8, which was shown to contribute to the activation of NF-κB via cleavage of $cFlip_L$ (Kawadler et al. 2008). The interaction between MALT1 and procaspase-8 proved to be dependent on the MALT1 paracaspase domain but independent of paracaspase activity since inactivation of the paracaspase active site did not interfere with procaspase-8 binding and processing.

In conclusion, the role of MALT1 as an adapter protein that recruits several signaling molecules to the CBM complex for the activation of NF-κB is well established.

MALT1 as a Protease in the Regulation of AgR-Mediated NF-κB Signaling

Uren and colleagues recognized that MALT1 belongs to a family of proteins that contain a common domain with sequence homology to the large protease subunit of caspases. Members of this protein family were identified in mammals, fish, nematodes, and amoeba and were collectively named paracaspases (Uren et al. 2000). Like caspases, the paracaspases possess an active-site cysteine together with a catalytic histidine and belong to the CD clan of cysteine-dependent proteases. In contrast to caspases, which specifically cleave behind aspartatic acid residues in the substrates P1 position, paracaspases were predicted to have different substrate specificities. Indeed, upon stimulation of human lymphocytes, MALT1 was shown to cleave BCL10 behind an arginine residue at its very C-terminal end (Rebeaud et al. 2008). The tetrapeptide inhibitor z-VRPR-fmk was able to block MALT1-mediated cleavage of BCL10 and thereby was validated as an inhibitor of MALT1 paracaspase proteolytic activity. Z-VRPR-fmk treatment of stimulated human T cells also reduced NF-κB signaling. However, BCL10 cleavage was not required for NF-κB signaling but instead might be important for integrin-mediated adhesion of activated lymphocytes to the extracellular matrix. Those findings suggest that other MALT1 paracaspase substrates should exist. In fact, Coornaert and colleagues recognized that the zinc-finger protein A20 is also cleaved by MALT1 paracaspase activity in stimulated human T and B cells (Coornaert et al. 2008). The cleavage site was identified behind an arginine residue between the first and second zinc finger of human A20. The MALT1 cleavage site in human A20 is different from that in BCL10 and is not conserved in the murine A20 protein which instead is presumably cleaved by MALT1 at another more C-terminal site. MALT1-dependent cleavage of A20 might indeed represent a mechanism that contributes to the regulation of CBM-mediated NF-κB signaling. A20 is a transcriptional target of NF-κB and, implementing a negative feedback loop, downregulates NF-κB signaling by removing K63-linked ubiquitin chains from various signaling molecules such as TRAF6, NEMO, and MALT1. MALT1-mediated A20 cleavage inactivates A20 as a negative regulator of NF-κB and, therefore, might contribute to a more sustained or amplified NF-κB signal (Fig. 4). This view is supported by the findings of Düwel and colleagues upon reconstitution of *Malt1*-deficient murine T cells with either wild-type *MALT1* or the active-site mutant *MALT1* C464A. *MALT1* C464A was able to restore early events in NF-κB activation such as IκBα degradation but displayed a reduced long-term response in terms of IL2 production compared to reconstituted wild-type MALT1 (Duwel et al. 2009). Very recently, MALT1 paracaspase was reported to cleave the deubiquitinating enzyme CYLD and thereby positively regulate JNK signaling (Staal et al. 2012) and to cleave RelB leading to RelB degradation with concomitant increase in canonical NF-κB signaling (Hailfinger et al. 2012). It appears likely that there are even more MALT1 paracaspase substrates which contribute to the regulation of CBM complex-mediated NF-κB signaling and beyond.



MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1), Fig. 4 *MALT1 as a paracaspase for the regulation of CBM signaling*. As a paracaspase Malt1 possesses proteolytic activity which is induced upon CBM activation. It has been reported that Malt1 as a paracaspase cleaves Bcl10, A20, CYLD, and RelB. A20 as a deubiquitinatin enzyme removes polyubiquitin chains from several proteins such as TRAF6, Malt1, and NEMO and, therefore, acts as a negative regulator of NF-κB signaling which transcriptionally upregulates A20 in a negative autoregulatory manner. A20 cleavage by Malt1 paracaspase therefore is believed to enhance CBM-mediated NF-κB signaling. CYLD is also a deubiquitinating enzyme and has been shown to be a negative regulator of Jnk activation. Inactivating cleavage of CYLD by Malt1 paracaspase therefore has been proposed to enhance Jnk signaling. Malt1-mediated cleavage of RelB leads to the degradation of RelB resulting in enhanced canonical NF-κB signaling

MALT1 Functions Beyond AgR Signaling

After AgR ligation, the most immediate sequence of events is the phosphorylation of ITAM motifs within coreceptor transmembrane proteins leading to the recruitment and activation of Syk kinase family members. Activation of ITAMs and Syk kinases is absolutely essential for the activation of most signaling pathways downstream of the AgRs including the formation of the CBM complex for NF-κB activation. Many other immunoreceptors also signal via ITAM or ITAM-like motifs and via activation of Syk kinases (Mocsai et al. 2010). And indeed, Malt1 as part of the CBM complex was found to be centrally involved in the activation of NF-κB upon stimulation of a variety of ITAM-coupled immunoreceptors other than AgRs. As a first example, Klemm and colleagues reported the essential contribution of Bcl10 and Malt1 to FcεRI-mediated NF-κB activation in mast cells, suggesting an important role for the CBM complex in the regulation of allergic inflammatory responses (Klemm et al. 2006; Klemm and Ruland 2006). Of special interest was the finding that Malt1 together with Bcl10 and the Carma1 homolog Card9 mediates NF-κB signaling triggered by innate C-type lectin receptors such as Dectin-1 for the immune response to fungal infections (Gross et al. 2006). This demonstrated for the first time that Bcl10 and Malt1 not only are central for adaptive immunity provided by AgR signaling on T and B lymphocytes but also for innate immunity provided by so-called pattern recognition receptors (PRRs) on dendritic cells (DCs) and macrophages (Hara and

Saito 2009). Another example for the involvement of Card9/Bcl10/Malt1 in the response of PRRs to pathogen-associated molecular patterns (PAMPs) is the activation of DCs and macrophages by the mycobacterial cell wall component TDM via recognition of the ITAM-coupled C-type lectin receptor Mincle (Schoenen et al. 2010). Furthermore, also activating NK cell receptors such as NK1.1 and ▶NKG2D couple to ITAM-containing signaling chains and signal to NF-κB in a Carma1/Bcl10/Malt1-dependent manner (Gross et al. 2008). The CBM complex has been described to also mediate NF-κB activation independently of upstream ITAM-Syk signaling. Tusche and colleagues reported that BAFF-induced alternative NF-κB signaling is dependent on Malt1 in B lymphocytes and that this BAFF pathway is essential for the survival and immune function of MZB but not of follicular B cells (Tusche et al. 2009). In nonimmune cells such as MEFs, Bcl10 and Malt1 were shown to be specifically required for NF-κB signaling upon activation of G protein-coupled receptors (GPCRs) by the phospholipid lysophosphatidic acid, LPA, which might be of relevance for tumorigenesis (Klemm et al. 2007).

MALT1 as a Potential Drug Target in Lymphoma Treatment

As mentioned above, *MALT1* had been initially identified as a gene that is involved in the t(11;18)(q21;q21) translocation in cases of MALT lymphoma. This translocation results in the expression of the API2-MALT1 fusion protein that independently of Bcl10 can auto-oligomerize and mediate ubiquitin ligase activity for deregulated NF-κB signaling in the process of lymphomagenesis. API2-MALT1 is known to activate the classical NF-κB pathway but Rosebeck and colleagues recently observed that also the alternative pathway is involved (Rosebeck et al. 2012). According to this report, the paracaspase activity of API2-MALT1 is directly responsible for the cleavage of the NF-κB-inducing kinase (NIK) behind its arginine residue R325, leading to the stabilization of NIK due to its rescue from proteasomal degradation. Cleavage-stabilized NIK was shown to result in enhanced processing of p100 to p52 and activation of the alternative NF-κB transcriptional program in t(11;18)-positive MALT lymphoma samples. Those data suggest that the inhibition of MALT1 paracaspase activity might offer a potential approach for the treatment of t(11;18)-positive MALT lymphoma. In contrast to the t(11;18)(q21;q21) translocation which generates an API2-MALT1 fusion protein, the recurrent translocation t(14;18)(q23;q21) brings *MALT1* into proximity of the immunoglobulin heavy-chain (*IGH*) locus and leads to high-level expression of MALT1 in the affected lymphoma cells. The mechanisms by which aberrant expression of *MALT1* contributes to NF-κB-dependent lymphomagenesis are not yet clear; nucleocytoplasmic shuttling of MALT1 and associated BCL10 might play a role. MALT1, BCL10, and CARMA1 were demonstrated to be indispensable for the survival of NF-κB-dependent diffuse large B-cell lymphoma (DLBCL) cell lines of the activated B cell (ABC) type and, therefore, might represent "Achilles' heels" that might be targeted for therapeutic intervention. Indeed, ABC-type DLBCL cell lines were shown to be selectively sensitive to treatment with the MALT1 paracaspase tetrapeptide inhibitor z-VRPR-fmk (Ferch et al. 2009). Thus, MALT1 as a component of the CBM complex with enzymatic paracaspase activity may be an attractive drug target for the treatment of lymphomas because inhibition of MALT1 paracaspase would not generally block NF-κB signaling but would only affect certain aspects of NF-κB signaling within a few cell types suggesting low side effects of such a therapeutic approach. However, the importance of MALT1 paracaspase activity in the physiological context of an organism is not known, and therefore, the consequences of MALT1 paracaspase inhibition remain to be investigated.

Summary

MALT1 was initially identified as a gene that is involved in chromosomal translocations in cases of MALT lymphoma. Soon it was recognized that MALT1 interacts and collaborates with BCL10, which also is translocated in MALT lymphoma, for the induction of NF-κB signaling. In response to the activation of adaptive and innate immune receptors MALT1 and BCL10 are recruited by CARD-containing proteins such as CARMA1 and CARD9 to form so-called CBM complexes. CBM complexes mediate the activation of the IKK complex and subsequent NF-κB signaling. Within the CBM complex, MALT1 functions as an adaptor protein that recruits further signaling molecules but also regulates CBM signaling via its paracaspase

protease activity. Regular CBM-mediated NF-κB signaling is essential for intact adaptive and innate immune functions. In contrast, aberrant CBM-mediated NF-κB signaling can contribute to and even might be essential for the development and/or maintenance of lymphomas. MALT1 as a component of the CBM complex with enzymatic paracaspase activity is an attractive drug target and first in vitro data indicate that inhibition of MALT1 paracaspase might indeed impair survival of specific subtypes of lymphoma cells. The physiological functions of MALT1 paracaspase activity, however, remain to be elucidated.

Acknowledgments We thank Frank Oliver Gorka for thoughtfully reading and discussing the manuscript.

References

Akagi T, Motegi M, Tamura A, Suzuki R, Hosokawa Y, Suzuki H, Ota H, Nakamura S, Morishima Y, Taniwaki M, Seto M. A novel gene, MALT1 at 18q21, is involved in t(11;18)(q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. Oncogene. 1999;18(42):5785–94.

Coornaert B, Baens M, Heyninck K, Bekaert T, Haegman M, Staal J, Sun L, Chen ZJ, Marynen P, Beyaert R. T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. Nat Immunol. 2008;9(3):263–71.

Dierlamm J, Baens M, Wlodarska I, Stefanova-Ouzounova M, Hernandez JM, Hossfeld DK, De Wolf-Peeters C, Hagemeijer A, Van den Berghe H, Marynen P. The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. Blood. 1999;93(11):3601–9.

Duwel M, Welteke V, Oeckinghaus A, Baens M, Kloo B, Ferch U, Darnay BG, Ruland J, Marynen P, Krappmann D. A20 negatively regulates T cell receptor signaling to NF-kappaB by cleaving Malt1 ubiquitin chains. J Immunol. 2009;182(12):7718–28.

Duwel M, Hadian K, Krappmann D. Ubiquitin conjugation and deconjugation in NF-kappaB signaling. Subcell Biochem. 2010;54:88–99.

Ferch U, ZumBuschenfelde CM, Gewies A, Wegener E, Rauser S, Peschel C, Krappmann D, Ruland J. MALT1 directs B cell receptor-induced canonical nuclear factor-kappaB signaling selectively to the c-Rel subunit. Nat Immunol. 2007;8(9):984–91.

Ferch U, Kloo B, Gewies A, Pfander V, Duwel M, Peschel C, Krappmann D, Ruland J. Inhibition of MALT1 protease activity is selectively toxic for activated B cell-like diffuse large B cell lymphoma cells. J Exp Med. 2009;206(11):2313–20.

Gross O, Gewies A, Finger K, Schafer M, Sparwasser T, Peschel C, Forster I, Ruland J. Card9 controls a non-TLR signaling pathway for innate anti-fungal immunity. Nature. 2006;442(7103):651–6.

Gross O, Grupp C, Steinberg C, Zimmermann S, Strasser D, Hannesschlager N, Reindl W, Jonsson H, Huo H, Littman DR, Peschel C, Yokoyama WM, Krug A, Ruland J. Multiple ITAM-coupled NK-cell receptors engage the Bcl10/Malt1 complex via Carma1 for NF-kappaB and MAPK activation to selectively control cytokine production. Blood. 2008;112(6):2421–8.

Hailfinger S, Nogai H, Pelzer C, Jaworski M, Cabalzar K, Charton JE, Guzzardi M, Decaillet C, Grau M, Dorken B, Lenz P, Lenz G, Thome M. Malt1-dependent RelB cleavage promotes canonical NF-{kappa}B activation in lymphocytes and lymphoma cell lines. Proc Natl Acad Sci USA. 2012;108(35):14596–601.

Hara H, Saito T. CARD9 versus CARMA1 in innate and adaptive immunity. Trends Immunol. 2009;30(5):234–42.

Jost PJ, Weiss S, Ferch U, Gross O, Mak TW, Peschel C, Ruland J. Bcl10/Malt1 signaling is essential for TCR-induced NF-kappaB activation in thymocytes but dispensable for positive or negative selection. J Immunol. 2007;178(2):953–60.

Kawadler H, Gantz MA, Riley JL, Yang X. The paracaspase MALT1 controls caspase-8 activation during lymphocyte proliferation. Mol Cell. 2008;31(3):415–21.

Klemm S, Gutermuth J, Hultner L, Sparwasser T, Behrendt H, Peschel C, Mak TW, Jakob T, Ruland J. The Bcl10-Malt1 complex segregates Fc epsilon RI-mediated nuclear factor kappa B activation and cytokine production from mast cell degranulation. J Exp Med. 2006;203(2):337–47.

Klemm S, Ruland J. Inflammatory signal transduction from the Fc epsilon RI to NF-kappa B. Immunobiology. 2006;10:815–20.

Klemm S, Zimmermann S, Peschel C, Mak TW, Ruland J. Bcl10 and Malt1 control lysophosphatidic acid-induced NF-kappaB activation and cytokine production. Proc Natl Acad Sci U S A. 2007;104(1):134–8.

Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol. 2010;10(6):387–402.

Oeckinghaus A, Wegener E, Welteke V, Ferch U, Arslan SC, Ruland J, Scheidereit C, Krappmann D. Malt1 ubiquitination triggers NF-kappaB signaling upon T-cell activation. EMBO J. 2007;26(22):4634–45.

Rebeaud F, Hailfinger S, Posevitz-Fejfar A, Tapernoux M, Moser R, Rueda D, Gaide O, Guzzardi M, Iancu EM, Rufer N, Fasel N, Thome M. The proteolytic activity of the paracaspase MALT1 is key in T cell activation. Nat Immunol. 2008;9(3):272–81.

Rosebeck S, Madden L, Jin X, Gu S, Apel IJ, Appert A, Hamoudi RA, Noels H, Sagaert X, Van Loo P, Baens M, Du MQ, Lucas PC, McAllister-Lucas LM. Cleavage of NIK by the API2-MALT1 fusion oncoprotein leads to noncanonical NF-kappaB activation. Science. 2012;331(6016):468–72.

Ruefli-Brasse AA, French DM, Dixit VM. Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. Science. 2003;302(5650):1581–4.

Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS, Mak TW. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell. 2001;104(1):33–42.

Ruland J, Duncan GS, Wakeham A, Mak TW. Differential requirement for Malt1 in T and B cell antigen receptor signaling. Immunity. 2003;19(5):749–58.

Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, Agger EM, Stenger S, Andersen P, Ruland J, Brown GD, Wells C, Lang R. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol. 2010; 184(6):2756–60.

Staal J, Driege Y, Bekaert T, Demeyer A, Muyllaert D, Van Damme P, Gevaert K, Beyaert R. T-cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1. EMBO J. 2012;30(9):1742–52.

Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ. The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol Cell. 2004;14(3): 289–301.

Thome M, Charton JE, Pelzer C, Hailfinger S. Antigen receptor signaling to NF-kappaB via CARMA1, BCL10, and MALT1. Cold Spring Harb Perspect Biol. 2010;2(9):a003004.

Tusche MW, Ward LA, Vu F, McCarthy D, Quintela-Fandino M, Ruland J, Gommerman JL, Mak TW. Differential requirement of MALT1 for BAFF-induced outcomes in B cell subsets. J Exp Med. 2009;206(12):2671–83.

Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell. 2000;6(4):961–7.

Xue L, Morris SW, Orihuela C, Tuomanen E, Cui X, Wen R, Wang D. Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells. Nat Immunol. 2003;4(9):857–65.

Mammalian Target of Rapamycin (mTOR)

▶ mTOR

Mannose Receptor, C-type 2

▶ MRC2

MAP Kinase Kinase 3

▶ Mek3

MAP Kinase Upstream Kinase

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

Map Kinase-Activated Protein Kinase 5 (MK5)

Alexey Kotlyarov
Institute of Biochemistry, Hannover Medical School, Hannover, Germany

Synonyms

MAPKAP kinase 5; MAPKAPK 5; PRAK

Historical Background

Murine and human MK5 were initially isolated in 1998 in two independent screenings for proteins with sequence homology to the MK2 (New et al. 1998; Ni et al. 1998). This novel 54 kDa kinase displays 45% amino acid identity to MK2 and is ubiquitously expressed in all tissues. Both groups showed that MK5 could be phosphorylated and activated in vitro by the p38 MAP kinase, detected by 32P incorporation into peptide substrate (KKRPQRATSNVFS) or Hsp25 (Hspb1). New et al. named this kinase as p38-regulated and activated kinase or PRAK to emphasize its integration in p38 pathway. More recently, MK5 has also been shown to interact with the atypical MAP kinases ERK3 and ERK4 and this also results in phosphorylation and activation of MK5. The amino acid sequence of MK5 is most closely related to MK2 52% amino acid identity within kinase domains and MK3 50%, but MK5 is more distantly related to these kinases than they are to each other (77%). An MK5 gene does not appear to be present in either *C. elegans* or Drosophila, but orthologues are found in most vertebrates.

Structure, Activation, and Expression

According to the structure of the kinase domain, MK5 belong to the family of calcium/calmodulin-dependent protein kinases. The human MK5 mRNA could be spliced on the two slightly different ways, resulting in two isoforms differing in two additional amino acids within the C-terminal extension of MK5 of transcript coding for 473 aa (variant 2) compared to transcript variant 1. In mice, five MK5 isoforms have been

detected, representing combinations of the two amino acid changes found in humans with deletions of the N-terminal portion of the MK5 catalytic domain as well as truncated variant due to frame shift.

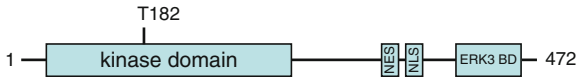
The functional significance of these different splice variants is not known. Similar to the other MKs MK5 contains a conserved LXTP site, where X is threonine for MK2, glutamine for MK3, and methionine for MK5, in the T-loop with the threonine as a phospho-acceptor site. The C-terminal region of MK5 contains a functional nuclear export sequence (NES) and a nuclear localization sequence (NLS). The NLS overlaps with a putative MAPK docking site (D motif) mediating interaction with p38 (Seternes et al. 2002). In contrast to MK2 and MK3, in MK5 NES and NLS are located in close proximity to each other. In addition, MK5 possesses a 100 amino acid extension C-terminal to the NLS, which is not present in other MKs. This extension is indispensible and sufficient for the interaction with phosphorylated MAPKs ERK3 and ERK4 and enables alternative way of MK5 activation. This activation mechanism via atypical MAPKs in addition to p38 is the unique feature of MK5, integrating it in two separate signaling pathways (Fig. 1).

MK5 is ubiquitously expressed in most tissues and cell lines that have been analyzed.

MK5 carries the phospho-acceptor site in the T-loop (T182), conserved of all other MKs (New et al. 1998) which is the only regulatory phosphorylation site in contrast to MK2 and MK3, requiring additional phosphorylation sites for full activation. Comparison of the MK5 sequence with that of MK2 and MK3 reveals that these additional regulatory MAPK phosphorylation sites are absent in MK5.

MK5 can be activated by p38 similar to MK2 (New et al. 1998, 2003; Seternes et al. 2002) at least in vitro and in the case of overexpression. Additionally, overexpressed p38 can bind MK5 leading to the cytoplasmic localization of resulting complex. However endogenous MK5 is not significantly activated by classical p38 stimuli, such as arsenite and sorbitol (Shi et al. 2003). Moreover, the phenotype of MK5-deficient mice does not resemble one of MK2/3-deficient animals, displaying a normal profile of cytokine production and no increased resistance to LPS challenge (Shi et al. 2003).

Recently, MK5 was shown to interact with atypical MAPKs ERK3 and ERK4. These kinases form a tight complex with MK5 resulting in mutual stabilization and phosphorylation. Moreover the expression of either ERK3 or ERK4 does only lead to phosphorylation and activation of MK5 but also cause the translocation of ERK3–MK5 from the nucleus to the cytoplasm (Seternes et al. 2004; Schumacher et al. 2004). However, the physiological conditions, leading to the activation of MK5 by ERK3 and ERK4 remain unknown.



Map Kinase-Activated Protein Kinase 5 (MK5), Fig. 1 Schematic structure of MK5. The phospho-acceptor site T182 in the T-loop nuclear export signal (NES), the D-domain/nuclear localization signal (NLS) and the ERK3 and ERK4 binding domain are shown

Protein kinase A (PKA) signaling also been postulated to influence the cellular distribution of MK5 (Gerits et al. 2007a). Treatment of cells with forskolin or overexpression of a nuclear-targeted PKA C alpha catalytic subunit was shown to induce the transient redistribution of MK5 from nucleus to cytoplasm in rat PC 12 cells.

Substrates

The optimal phosphorylation site motif for MK5 is identical to MK2/3 where it has been defined as (L,F,I)-X-R-(Q,S,T)-L-(pS,pT)-hydrophobic. MK5 is able to phosphorylate HSPB1, glycogen synthetase, tyrosine hydroxylase (preferentially on S19), and myosin heavy chain in vitro (New et al. 1998; Ni et al. 1998; Gaestel 2006; Toska et al. 2002) at the same sites as MK2.

It was shown that MK5 interact with Hspb1 and is responsible for the cAMP/cAMP-dependent protein kinase induced Hspb1 phosphorylation in PC12 cells (Kostenko et al. 2009).

However in MK5 knockout mice, stress-dependent HSPB1 phosphorylation under the stress conditions is not impaired (Shi et al. 2003). Instead, MK5 phosphorylates its interacting partners, atypical MAPKs ERK3 (Seternes et al. 2004; Schumacher et al. 2004) as well as homologous ERK4 (Kant et al. 2006; Aberg et al. 2006) and can also phosphorylate the transcription factor p53 on S37 (Sun et al. 2007).

Physiological Roles

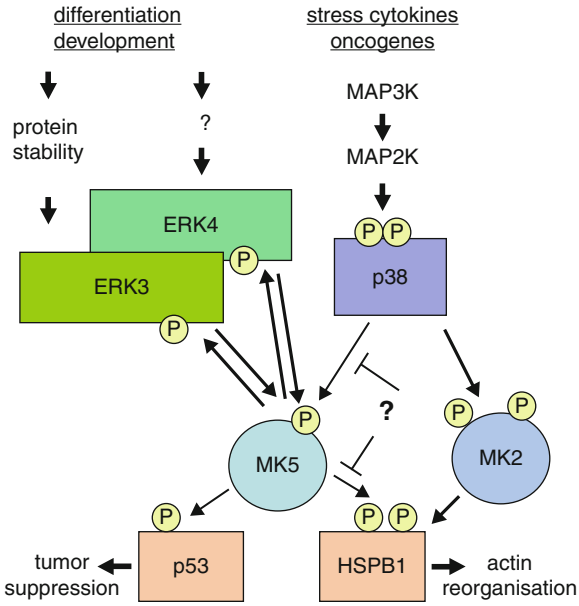
The exact biological function of MK5 is unknown. Originally, due to the high structural similarity and substrate consensus, MK5 was ascribed the same functions as MK2, which is involved in stress response and inflammation.

Generation of MK5-deficient mice challenged this assumption. Indeed, MK5-deficient mice do not display any of the phenotypic changes characteristic for MK2-deficient animals. Disruption of the MK5 gene in mice on mixed genetic background does not manifest in any phenotypic features. MK5 knockout backcrossed to C57Bl/6 genetic background resulted in lethality at E11.5 with incomplete penetrance, indicating the role of MK5 during embryogenesis.

In studies exploring the Ras-induced senescence in primary mouse and human fibroblasts, MK5 was shown to be indispensable for oncogene-induced senescence and hence can act as a tumor suppressor. Consistently, MK5-deficient mice were found to be more susceptible to skin carcinogenesis induced by the mutagen dimethylbenzanthracene. In the same study, the authors demonstrated that MK5 phosphorylates p53 at Ser37, a residue located in transactivation domain. Since this phosphorylation site is not within a perfect consensus for MAPKAP kinases, it could not be excluded that MK5-associated MAPK is responsible for this phosphorylarion.

Overexpression of MK5 in HeLa cells leads to an increase in both F-actin production and cell migration (Tak et al. 2007). Interestingly, MK5 was shown to interact with 14-3-3 epsilon resulting in inhibition of MK5 kinase activity. In another study, treatment of PC12 cells with forskolin caused a transient increase in F-actin levels and this was blocked in PC12 cells transfected with an siRNA against MK5 (Gerits et al. 2007a). How MK5 mediates cytoskeletal rearrangement is still unclear, and further studies are needed to address this.

Finally, a possible link between MK5 and neurological/cognitive function has been proposed based on behavioral analyses of a transgenic mouse that expresses a constitutively active mutant of MK5. This study revealed complex sex-specific changes in both anxiety-related traits and locomotor activity in MK5 expressing mice relative to WT controls (Gerits et al. 2007b) (Fig. 2).



Map Kinase-Activated Protein Kinase 5 (MK5), Fig. 2 ERK3 is an intrinsically instable protein and can be stabilized by differentiation or in development. Mechanism of ERK4 regulation is not revealed until now. ERK3 and ERK3 bind to MK5 and activate later through T182 phosphorylation. As a result of interaction, ERK3 and ERK4 become phosphorylated by MK5 either. P38 is able to phosphorylate MK5; however, MK5 phosphorylation is not essential result of p38 signaling as in the case of MK2/3 and this fact is indicated with inhibition symbol supplied with question mark. However, p53 phosphorylation on S37 stimulated by oncogenic Ras seems to proceed via p38–MK5 axis. Involvement of MK5 in the regulation of actin reorganization is described and could be mediated by MK5-dependent phosphorylation of F-actin capping proteins, shown here exemplary as small heat shock protein HSPB1, although HSPB1 phosphorylation is abrogated in MK2/3 but not MK5-deficient cell lines. The fact, that despite similarity to MK2, MK5 usually is not involved in MK2 substrate phosphorylation is indicated

Summary

MK5 is a MAPK-activated kinase integrated in p38 and ERK3/ERK4 signaling module. P38 was initially described as a MK5 activator, resulting in the name PRAK, which is acronym for p38 activating regulating kinase. However, p38 activation is not usually cause MK5 phosphorylation and activation. Moreover, MK5 – p38 interaction is rather weak, the significance of p38 in MK5 signaling is restricted and needs to be directly addressed in future studies. In the contrast, the involvement of ERK3 and ERK4 in MK5 functions is clearly documented in in vivo studies and supported by strong

interaction between these proteins, mutual stabilization and similar pattern of expression in embryogenesis. However, ERK3/ERK4 – MK5 remains "orphan" signaling module until now, since any physiological stimuli as well as regulatory mechanisms were revealed for it.

Future studies of available MK5 knockout animals and dissection of signaling events using genetic approach by crossing MK5-deficient animals with p38, ERK3/4, and MK2/3 as well as usage of emerging MK5 inhibitors.

References

Aberg E, Perander M, Johansen B, Julien C, Meloche S, Keyse SM, Seternes OM. Regulation of MAPK-activated protein kinase 5 activity and subcellular localization by the atypical MAPK ERK4/MAPK4. J Biol Chem. 2006;281(46):35499–510.

Gaestel M. MAPKAP kinases - MKs - two's company, three's a crowd. Nat Rev Mol Cell Biol. 2006;7(2):120–30.

Gerits N, Mikalsen T, Kostenko S, Shiryaev A, Johannessen M, Moens U. Modulation of F-actin rearrangement by the cyclic AMP/cAMP-dependent protein kinase (PKA) pathway is mediated by MAPK-activated protein kinase 5 and requires PKA-induced nuclear export of MK5. J Biol Chem. 2007a;282(51):37232–43.

Gerits N, Van Belle W, Moens U. Transgenic mice expressing constitutive active MAPKAPK5 display gender-dependent differences in exploration and activity. Behav Brain Funct. 2007b;3(1):58.

Kant S, Schumacher S, Singh MK, Kispert A, Kotlyarov A, Gaestel M. Characterization of the atypical MAPK ERK4 and its activation of the MAPK-activated protein kinase MK5. J Biol Chem. 2006;281(46):35511–9.

Kostenko S, Johannessen M, Moens U. PKA-induced F-actin rearrangement requires phosphorylation of Hsp27 by the MAPKAP kinase MK5. Cell Signal. 2009;21(5):712–8.

New L, Jiang Y, Zhao M, Liu K, Zhu W, Flood LJ, Kato Y, Parry GC, Han J. PRAK, a novel protein kinase regulated by the p38 MAP kinase. EMBO J. 1998;17(12):3372–84.

New L, Jiang Y, Han J. Regulation of PRAK subcellular location by p38 MAP kinases. Mol Biol Cell. 2003;14(6):2603–16.

Ni H, Wang XS, Diener K, Yao Z. MAPKAPK5, a novel mitogen-activated protein kinase (MAPK)-activated protein kinase, is a substrate of the extracellular-regulated kinase (ERK) and p38 kinase. Biochem Biophys Res Commun. 1998;243(2):492–6.

Schumacher S, Laass K, Kant S, Shi Y, Visel A, Gruber AD, Kotlyarov A, Gaestel M. Scaffolding by ERK3 regulates MK5 in development. EMBO J. 2004;23(24):4770–9.

Seternes OM, Johansen B, Hegge B, Johannessen M, Keyse SM, Moens U. Both binding and activation of p38 mitogen-activated protein kinase (MAPK) play essential roles in regulation of the nucleocytoplasmic distribution of MAPK-activated protein kinase 5 by cellular stress. Mol Cell Biol. 2002;22(20):6931–45.

Seternes OM, Mikalsen T, Johansen B, Michaelsen E, Armstrong CG, Morrice NA, Turgeon B, Meloche S, Moens U, Keyse SM. Activation of MK5/PRAK by the atypical MAP kinase ERK3 defines a novel signal transduction pathway. EMBO J. 2004;23(24):4780–91.

Shi Y, Kotlyarov A, Laabeta K, Gruber AD, Butt E, Marcus K, Meyer HE, Friedrich A, Volk HD, Gaestel M. Elimination of protein kinase MK5/PRAK activity by targeted homologous recombination. Mol Cell Biol. 2003;23(21):7732–41.

Sun P, Yoshizuka N, New L, Moser BA, Li Y, Liao R, Xie C, Chen J, Deng Q, Yamout M, Dong MQ, Frangou CG, Yates JR III, Wright PE, Han J. PRAK is essential for ras-induced senescence and tumor suppression. Cell. 2007;128(2):295–308.

Tak H, Jang E, Kim SB, Park J, Suk J, Yoon YS, Ahn JK, Lee JH, Joe CO. 14-3-3epsilon inhibits MK5-mediated cell migration by disrupting F-actin polymerization. Cell Signal. 2007;19(11):2379–87.

Toska K, Kleppe R, Armstrong CG, Morrice NA, Cohen P, Haavik J. Regulation of tyrosine hydroxylase by stress-activated protein kinases. J Neurochem. 2002;83(4):775–83.

MAP Kinases

► Mitogen-Activated Protein Kinases

MAP2K1/2

► Mek

Map2k3

► Mek3

MAP2K6

► MKK6

MAP3K11

► MLK3

Map3k12

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

Map3k8

▶ TPL2

Map4k1

▶ HPK1

MAPK (Mitogen Activating Protein Kinase)

▶ p38 MAPK Family of Signal Transduction Proteins

MAPK Erk Kinase 3

▶ Mek3

MAPK Kinase 1/2 (MAPKK1/2)

▶ Mek

Mapk4 (Erk4)

▶ Erk3 and Erk4

Mapk6 (Erk3)

▶ Erk3 and Erk4

MAPKAP Kinase 2/3

▶ Mapkap Kinase 2/3 (MK2/3)

Mapkap Kinase 2/3 (MK2/3)

Matthias Gaestel
Hannover Medical School, Institute of Biochemistry, Hannover, Germany

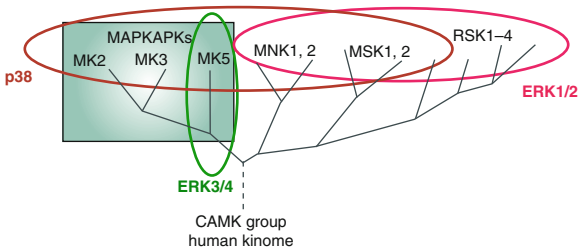
Synonyms

Hsp27-kinase; Hsp25-kinase; Hspb1-kinase; sHsp-kinase; MAPKAP kinase 2/3; MAPKAPK 2/3

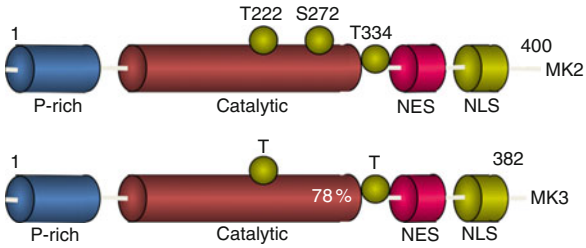
Historical Background

Formally, all protein kinases downstream to ▶ MAP kinases should be regarded as MAPK-activated protein kinases (MKs). This would include ribosomal S6-kinases (RSKs), mitogen- and stress-activated protein kinases (MSKs), MAP kinase-interacting kinases (MNKs), as well as MK2, MK3, and MK5/PRAK (Roux and Blenis 2004). However, due to the specificity of activation, this group of protein kinases is further subdivided (Fig. 1): RSKs are activated by classical ERKs (▶ Erk1/Erk2) and MSKs and MNKs are phosphorylated by both ERKs and p38 MAPK. MK2 and MK3 are the only MKs which are exclusively activated by p38 MAPK. MK5/PRAK has been described as a "p38-regulated and -activated kinase (PRAK)," but its activation by p38 MAPK is challenged and alternative activation by atypical MAPKs (Erk3/Erk4) is discussed (Gaestel 2006). For historical reasons RSK was designated MAPKAPK 1 (MK1) and the name MK4 was given to MK2 of sea urchin.

A stress-induced small heat shock protein-kinase activity was described as early as in 1983 (Kim et al. 1983), but identification of this activity as the protein kinases MK2 (Stokoe et al. 1992) and MK3 (McLaughlin et al. 1996) took more than 10 years. Characterization of the physiological roles of these protein kinases is still ongoing.



Mapkap Kinase 2/3 (MK2/3), Fig. 1 Classification of protein kinases downstream to MAPKs. For details, see text. Adapted from (Gaestel 2006) with modifications



Mapkap Kinase 2/3 (MK2/3), Fig. 2 Primary structure of MK2 and MK3. MK2 and MK3 display 78% identity in their amino acid sequence and contain N-terminal proline-rich domains (P-rich), central protein-kinase domains (catalytic), and C-terminal regulatory tails which contain a nuclear export signal (NES) and a bi-partide nuclear localization motif (NLS). The NLS overlaps with the p38 MAPK-docking site. At least two regulatory phosphorylation sites are present in both enzymes: One threonine within the catalytic domain at the activation loop (T222) and the other threonine in the hinge region between kinase domain and C-terminal tail

Structure, Activation, and Expression

According to their primary structure, MK2/3 belong to the family of calcium/calmodulin-dependent protein kinases (Camk). However, their activity does not depend on calcium, but on the phosphorylation of regulatory sites within the catalytic domain and in a hinge region between the catalytic domain and the C-terminus (Ben-Levy et al. 1995, Engel et al. 1995) (see Fig. 2). In human MK2, two phosphorylation sites are located within the catalytic domain, T222 and S272, and a further phosphorylation site is T334 in the hinge region. Similar sites were also identified in MK3. These are proline-directed sites which are phosphorylated by p38 MAPKα,β (Freshney et al. 1994, Rouse et al. 1994). The N-terminus of MK2/3 contains a proline-rich region which is able to bind to SH3 domain-containing proteins in vitro (Plath et al. 1994). The C-terminus of MK2/3 is a very interesting part, since it contains different signals for regulation of subcellular localization (nuclear export signal – NES; nuclear import signal – NLS). Furthermore, the C-terminus may have inhibitory effects on the catalytic domain (Engel et al. 1995) and tightly binds to the activator kinase p38 MAPKα (White et al. 2007). The binding specificity between MK2 and p38 MAPKα depends on two regions of p38, the common docking (CD) domain and the ED motif, and on the NLS of MK2 (Gum and Young 1999, Tanoue et al. 2001).

A variant of the cDNA of human MK2 that codes for an alternative C-terminus without NES and NLS has been described (Zu et al. 1994). MK2 also migrates as two (mouse, 46 and 54 kDa; human, 53 and 60 kDa) distinct bands in SDS–PAGE (Stokoe et al. 1992, Cano et al. 1996), which are both absent from MK2-knockout fibroblasts (Kotlyarov et al. 1999), and has two biochemically distinct forms (p43 and p49) in cardiac myocytes (Chevalier and Allen 2000). So far, it is not completely clear whether both bands detected for MK2 correspond to different proteins based on an alternatively spliced transcript or to posttranslational modification/processing of MK2.

MK2 and MK3 are highly expressed in heart and skeletal muscle, but their activity can also be detected in most other tissues and cell lines. Compared to expression of MK2, expression of MK3 is much lower, making it a "minor isoenzyme" of MK2. This explains the clear phenotype of the MK2-deletion (Kotlyarov et al. 1999), which cannot be compensated by MK3 because of its low expression (see below).

The regulation of MK2/3's subcellular localization is an interesting issue. GFP-tagged, overexpressed MK2/3 is mainly localized in the nucleus and translocated to the cytoplasm after activation (Engel et al. 1998). Whether this mechanism of coupled activation and translocation is of importance to the endogenous enzymes and whether this mechanism is modulated by complex formation of MK2/3 with p38 MAPK is so far not clear.

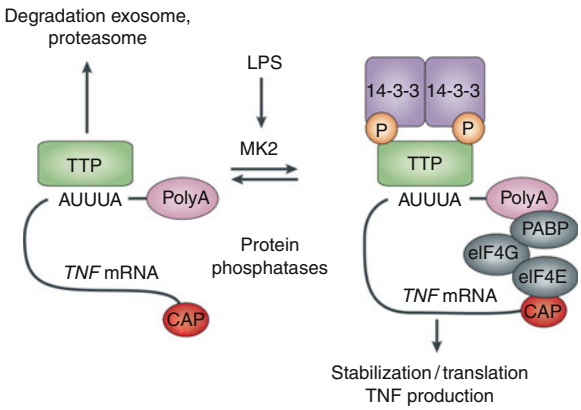
Substrates

The human kinome contains more than 500 protein kinases and the human proteome is thought to comprise more than 10,000 different phosphorylation sites.

Hence, an average protein kinase should phosphorylate more than 20 amino acid residues of various substrate proteins. Knowing the complete substrate spectrum of MK2/3 could be very helpful for understanding its downstream signaling in different cell types and physiological situations. The optimal phosphorylation site motif for MK2/3 has been defined as (L,F,I)-X-R-(Q,S,T)-L-(pS,pT)-hydrophobic. So far, no difference in substrate-specificity between MK2 and MK3 has been detected (Clifton et al. 1996). Several substrates of MK2/3 have been described. Besides the major substrate Hspb1, the following substrates have been identified: myosin II regulatory light chain, lymphocyte-specific protein 1, tyrosine hydroxylase, αB-crystallin, vimentin, serum response factor, transcription factors E47 and ER81, 5-lipoxygenase, poly (A)-binding protein 1, tuberin, hnRNP A0, p16-Arc, LIM-kinase 1 (▶ LIMK), 14-3-3ζ, tristetraproline (TTP), p66-Shc, Bcl-2-associated athanogene 2, polycomb-group protein Bmi 1, DNA-damage response protein phosphatase ▶ Cdc25B/C, and the p53 E3 ubiquitin ligase HDM2 (for references see supplementary table of (Gaestel 2006)). However, at the moment, only a few in vivo substrates of MK2/3, such as Hspb1, SRF, TTP, and keratin 20 (Menon et al. 2010), have been verified. The physiological function of phosphorylation of these substrates is far from being completely understood.

Physiological Roles

The physiological role of MK2 became mainly evident from the MK2-knockout mouse, which is viable and fertile but displays resistance against endotoxic shock (Kotlyarov et al. 1999) and collagen-induced arthritis (Hegen et al. 2006) as well as increased susceptibility in a *Listeria* infection model (Lehner et al. 2002). These phenotypes can be explained by MK2/3-dependent regulation of expression of the inflammatory master cytokine TNF at the posttranscriptional level of mRNA stability and translation (Kotlyarov et al. 1999, Neininger et al. 2002). Mechanistically, this regulation proceeds for AU-rich element (ARE)-containing mRNAs, such as TNF mRNA, via phosphorylation of ARE-binding proteins, such as hnRNP A0 (Rousseau et al. 2002) and TTP (Stoecklin et al. 2004) (Fig. 3). In a MK2-free genetic background deletion of MK3 leads to a further slight, but significant



Mapkap Kinase 2/3 (MK2/3), Fig. 3 Proposed mechanism of molecular action of MK2/3 in cytokine biosynthesis. MK2 phosphorylates AU-rich element (ARE – "AUUUA")-binding proteins such as hnRNP A0 or TTP. In the case of TTP, phosphorylation leads to 14-3-3 binding of the TNF mRNA-TTP complex, stabilization, and/or translation of the mRNA and increased TNF production. (From Gaestel 2006)

reduction of TNF production, indicating cooperative action of both enzymes (Ronkina et al. 2007). Apart from displaying catalytic activity, MK2/3 bind to p38 MAPK and mutually stabilize each other by protein complex formation. In MK2 knockout and MK2/3-double knockout mice, a significantly reduced p38 MAPK level is detected (Ronkina et al. 2007). Further physiological roles for MK2/3 include cell cycle checkpoint control, cell migration, and general stress response.

Because of its contribution to the production of inflammatory cytokines and because of the toxicity of p38 MAPK-inhibitors, MK2 becomes increasingly of interest as a target for anti-inflammatory therapy. First small molecule inhibitors of MK2 and/or MK3 have been reported. Of these, the orally available small molecule MK2 inhibitor of the benzothiophene type, PF-3644022, was demonstrated to be effective in a chronic streptococcal cell-wall-induced arthritis model in rats (Mourey et al. 2010).

Summary

MK2/3 are p38 MAPK-activated kinases that are stimulated by different stresses such as heat shock, hypo- and hyperosmolarity, and treatment with anisomycin or arsenite as well as by bacterial lipopolysaccharide (LPS) and chemotaxis-inducing formyl peptides. MK2 and MK3 show different levels of expression and

activity, making MK2 the major and MK3 the minor "isoform." MK2/3 are involved in stress and immune response by the modulation of cytokine production, mainly at the posttranscriptional level, regulating cytokine messenger RNA stability and translation. mRNA-binding substrates such as tristetraproline (TTP), hnRNP A0, and possibly also poly(A)-binding protein 1 are involved in this regulation. The existence of a wide variety of further substrates identified for MK2/3 indicates various other physiological functions for these protein kinases in vivo. One of the major substrates of MK2/3, the small heat shock protein Hspb1, contributes to stabilization of the actin cytoskeleton and acts as a molecular chaperone.

References

Ben-Levy R, Leighton IA, Doza YN, Attwood P, Morrice N, Marshall CJ, et al. Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. EMBO J. 1995;14:5920–30.

Cano E, Doza YN, Ben-Levy R, Cohen P, Mahadevan LC. Identification of anisomycin-activated kinases p45 and p55 in murine cells as MAPKAP kinase-2. Oncogene. 1996;12:805–12.

Chevalier D, Allen BG. Two distinct forms of MAPKAP kinase-2 in adult cardiac ventricular myocytes. Biochemistry. 2000;39:6145–56.

Clifton AD, Young PR, Cohen P. A comparison of the substrate specificity of MAPKAP kinase-2 and MAPKAP kinase-3 and their activation by cytokines and cellular stress. FEBS Lett. 1996;392:209–14.

Engel K, Schultz H, Martin F, Kotlyarov A, Plath K, Hahn M, et al. Constitutive activation of mitogen-activated protein kinase-activated protein kinase 2 by mutation of phosphorylation sites and an A-helix motif. J Biol Chem. 1995;270:27213–21.

Engel K, Kotlyarov A, Gaestel M. Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. EMBO J. 1998;17:3363–71.

Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, et al. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell. 1994;78:1039–49.

Gaestel M. MAPKAP kinases – MKs – two's company, three's a crowd. Nat Rev Mol Cell Biol. 2006;7:120–30.

Gum RJ, Young PR. Identification of two distinct regions of p38 MAPK required for substrate binding and phosphorylation. Biochem Biophys Res Commun. 1999;266:284–9.

Hegen M, Gaestel M, Nickerson-Nutter CL, Lin LL, Telliez JB. MAPKAP kinase 2-deficient mice are resistant to collagen-induced arthritis. J Immunol. 2006;177:1913–7.

Kim YJ, Shuman J, Sette M, Przybyla A. Phosphorylation pattern of a 25 Kdalton stress protein from rat myoblasts. Biochem Biophys Res Commun. 1983;117:682–7.

Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD, et al. MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. Nat Cell Biol. 1999;1:94–7.

Lehner MD, Schwoebel F, Kotlyarov A, Leist M, Gaestel M, Hartung T. Mitogen-activated protein kinase-activated protein kinase 2-deficient mice show increased susceptibility to listeria monocytogenes infection. J Immunol. 2002;168:4667–73.

McLaughlin MM, Kumar S, McDonnell PC, Van Horn S, Lee JC, Livi GP, et al. Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. J Biol Chem. 1996;271:8488–92.

Menon MB, Schwermann J, Singh AK, Franz-Wachtel M, Pabst O, Seidler U, et al. p38 MAP kinase and MAPKAP kinases MK2/3 cooperatively phosphorylate epithelial keratins. J Biol Chem. 2010. doi:M110.132357.

Mourey RJ, Burnette BL, Brustkern SJ, Daniels JS, Hirsch JL, Hood WF, et al. A benzothiophene inhibitor of mitogen-activated protein kinase-activated protein kinase 2 inhibits tumor necrosis factor alpha production and has oral anti-inflammatory efficacy in acute and chronic models of inflammation. J Pharmacol Exp Ther. 2010;333:797–807.

Neininger A, Kontoyiannis D, Kotlyarov A, Winzen R, Eckert R, Volk HD, et al. MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. J Biol Chem. 2002;277:3065–8.

Plath K, Engel K, Schwedersky G, Gaestel M. Characterization of the proline-rich region of mouse MAPKAP kinase 2: influence on catalytic properties and binding to the c-abl SH3 domain in vitro. Biochem Biophys Res Commun. 1994;203:1188–94.

Ronkina N, Kotlyarov A, Dittrich-Breiholz O, Kracht M, Hitti E, Milarski K, et al. The mitogen-activated protein kinase (MAPK)-activated protein kinases MK2 and MK3 cooperate in stimulation of tumor necrosis factor biosynthesis and stabilization of p38 MAPK. Mol Cell Biol. 2007;27:170–81.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, et al. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell. 1994;78:1027–37.

Rousseau S, Morrice N, Peggie M, Campbell DG, Gaestel M, Cohen P. Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs. EMBO J. 2002;21:6505–14.

Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 2004;68:320–44.

Stoecklin G, Stubbs T, Kedersha N, Wax S, Rigby WF, Blackwell TK, et al. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. EMBO J. 2004;23:1313–24.

Stokoe D, Engel K, Campbell DG, Cohen P, Gaestel M. Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. FEBS Lett. 1992;313:307–13.

Tanoue T, Maeda R, Adachi M, Nishida E. Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. Embo J. 2001;20:466–79.

White A, Pargellis CA, Studts JM, Werneburg BG, Farmer 2nd BT. Molecular basis of MAPK-activated protein kinase 2: p38 assembly. Proc Natl Acad Sci USA. 2007;104:6353–8.
Zu YL, Wu F, Gilchrist A, Ai Y, Labadia ME, Huang CK. The primary structure of a human MAP kinase activated protein kinase 2. Biochem Biophys Res Commun. 1994;200:1118–24.

MAPKAP Kinase 5

► Map Kinase-Activated Protein Kinase 5 (MK5)

MAPKAPK (MAP Kinase-Activated Kinase)

► p38 MAPK Family of Signal Transduction Proteins

MAPKAPK 2/3

► Mapkap Kinase 2/3 (MK2/3)

MAPKAPK 5

► Map Kinase-Activated Protein Kinase 5 (MK5)

MAPKK3

► Mek3

MAPKK6

► MKK6

Marelle

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

MARK1: EMK3, hPAR-1c, KIAA1477

► Microtubule Affinity Regulating Kinases (MARK)

MARK2: EMK1, hPAR-1b

► Microtubule Affinity Regulating Kinases (MARK)

MARK3: EMK3, hPAR-1a, C-TAK1, KP78

► Microtubule Affinity Regulating Kinases (MARK)

MARK4: MARKL1, hPAR-1d, KIAA1860

► Microtubule Affinity Regulating Kinases (MARK)

MAT1

► Pea15

MAT1H

► Pea15

Matk

► CSK-Homologous Kinase

Mblk1-Related Protein 2 (MLR2)

► LCoR

MBP1

► Fibulins

M-CAT Binding Factor

▶ Tead

MCIP1

▶ Regulator of Calcineurin 1 (RCAN1)

MCL-1 (Myeloid Cell Leukemia-1), BCL2L3 (BCL-2 like 3)

▶ BCL-2 Family

mCLEC-2

▶ CLEC-2

mD52 (Mouse)

▶ TPD52 (Tumor Protein D52)

MDL1

▶ CLEC5A

MDL-1

▶ CLEC5A

Mec 3 (LIM) Kinase

▶ LIMK

Mek

Shiri Procaccia and Rony Seger
Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel

Synonyms

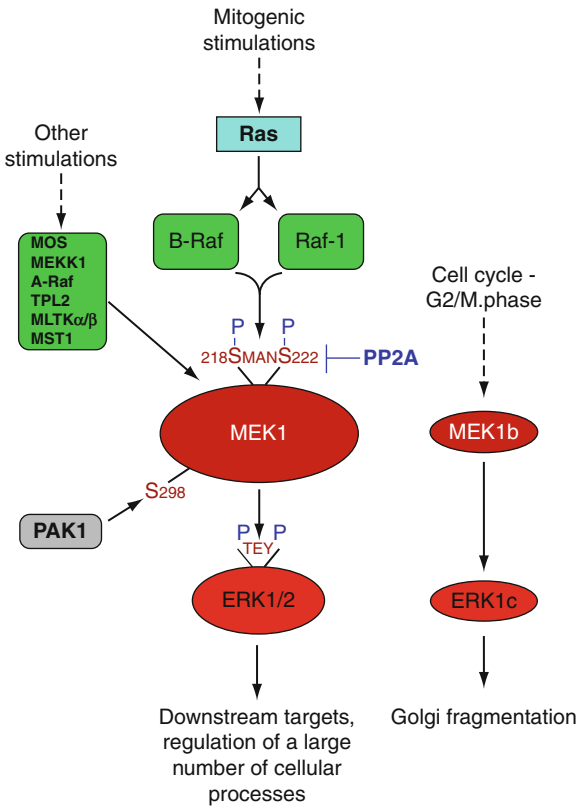
MAP2K1/2; MAPK kinase 1/2 (MAPKK1/2); Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinases 1/2 (MEK1/2); MKK1/2; p45MAPKK; PRKMK1/2

Historical Background

The transmission of extracellular signals to their intracellular targets is mediated by a network of interacting proteins that governs a large number of cellular processes including proliferation, differentiation, stress response, and apoptosis. One of the central components in the transmission network is the ERK cascade, which is composed of sequential phosphorylation and activation of the protein kinases Raf, MEK, ERK and ▶ MAPKAPKs. In turn, the latter two components of the cascade phosphorylate a large number of regulatory proteins, culminating in the induction and regulation of proper downstream cellular processes. This cascade was identified in the late 1980s and early 1990s of the previous century by several research groups that studied growth factor receptor signaling. At that time it was already known that growth factor receptors operate via an integral or associated protein tyrosine kinases that, upon stimulation, rapidly phosphorylate tyrosine residues on many substrates. It was also known that the initial Tyr phosphorylation is replaced by a Ser/Thr phosphorylation, which is found on a large number of Proteins, 15–60 min after stimulation. An important approach to study the role of these phosphorylations, was to establish a cellular event affected by growth factors, and identify components that regulate it up to the receptor (upstream approach; [Seger and Krebs 1995]). One protein that undergoes significant Ser/Thr phosphorylation shortly after growth factor stimulation is ribosomal protein S6, which became a useful readout for this upstream approach. Due to the work of several laboratories in

the 1980s it was found that the stimulated phosphorylation of S6 is mediated, in part, by RSK, which acts downstream of an enzyme that was initially termed microtubule-associated protein 2 (MAP-2) protein kinase, but later renamed mitogen-activated protein kinase (▶ MAP kinase; MAPK) and extracellular signal-regulated kinase (ERK; [Seger and Krebs 1995]).

As a central component in this novel pathway, ERK activation has attracted considerable attention. Soon after the cloning of ERK, it became clear that this protein kinase is activated due to phosphorylation of two residues in its activation loop (Thr183 and Tyr185 of ERK2). The phosphorylation of both these residues was found to be an essential step in ERK activation, as dephosphorylation of each of them completely abolished it. Initially, it was thought that the Tyr phosphorylation can be mediated by tyrosine kinases such as the growth factor receptors, while the Thr phosphorylation is mediated either by autophosphorylation or by another Ser/Thr kinase. However, this thought was rapidly changed due to studies by Ahn et al. (1991) who combined inactive ERK with fractions from growth factor–stimulated cells and looked for enhanced ERK activity. These studies led to the identification of two non-receptor ERK activating factors, which were able to induce phosphorylation of both activatory residues on ERK. Similar activators were then identified in NGF-stimulated PC-12 cells (Gomez and Cohen 1991), and later, in many other systems as well. Subsequently, the proteins were purified and cloned, giving rise to two main proteins and one alternatively spliced isoform that were eventually termed MEK1, MEK2, and MEK1b. These were shown to be protein kinases that catalyze Ser/Thr as well as Tyr phosphorylation, and therefore, belong to the small group of dual specificity protein kinases. These two kinases are also part of a group of MAPK kinases (MAPKKs) that are the specificity determinant of the MAPK signaling cascades in mammals and in other organisms. Finally, at that stage, it was important to elucidate the mechanism of MEK activation upon stimulation. Studies in that direction revealed that MEKs are activated by phosphorylation of two Ser residues in their activation loop. The main Ser/Thr kinase that executes this phosphorylation after stimulation was found to be the proto-oncogene Raf (Kyriakis et al. 1992), but other protein kinases were shown to act as activators (e.g., MOS, MEKK1) as well (see Fig. 1). In view of the above, it is clear today that



Mek, Fig. 1 Schematic representation of the ERK1/2 MAPK pathway. For more details, see text. MEK1 regulations are emphasized

MEK1/2 are the specificity determining component of the ERK cascade, and therefore, are central components in the regulation of proliferation and many other physiological processes (Seger and Krebs 1995).

The MEK1/2 Subfamily of MAPKKs

Key intracellular mediators of extracellular signals are the mitogen-activated protein kinase (MAPK) signaling cascades. They are evolutionarily conserved from yeast to mammals, and are expressed in nematodes, insects, slime molds, and plants as well. Each of the cascades is composed of three core protein kinases (MAP3K, MAPKK, and MAPK), which might be complemented in some cells and conditions by upstream MAP4K and downstream MAPK-activated protein kinase (MAPKAPK), giving rise to either 3, 4, or 5 tiered cascades. The transmission of signals

within the cascades is mediated by a sequential phosphorylation and activation of the components of the cascade, whereby the downstream components transmit the signal further by phosphorylating many regulatory proteins, which further governs an array of intracellular responses. Four such MAPK cascades are currently known in mammals, named according to the components of their MAPK tier: ERK1/2, JNK, p38 and ERK5 cascades. Main specificity determining components of the MAPK cascades are the MAPKK levels proteins, which are MEK1/2 for the ERK1/2 cascade, MKK3, 4, 6, 7 for the JNK and p38 cascades, and MEK5 for the ERK5 cascades. These kinases are unique in having a very stringent specificity toward Thr and Tyr residues in the activation loop of their cognate MAPKs. For that reason, the MAPKK are considered as the main specificity-determining components of their cognate cascades, and thereby are central regulatory components of essentially all stimulated cellular processes including proliferation, differentiation, adhesion, cellular morphology, stress response, and apoptosis.

The first identified and best-studied component of the MAPKK family is MEK1, which was shown to activate specifically ERK1/2 (Bendetz-Nezer and Seger 2005). This protein (45 kDa) is encoded by one gene (MAP2K1) that also encodes an alternatively spliced isoform termed MEK1b (43 kDa). The latter is very specific to the alternatively spliced isoform of ERK1 termed ERK1c (Shaul et al. 2009). Mammals contain another gene termed MAP2K2 that encodes the close homologue MEK2 (46 kDa), which phosphorylates and activates specifically ERK1/2 as well. This is an evolutionary conserved group of protein kinases, having very close orthologues in yeasts, worms, insects, and plants. MEK1/2, like other MAPKKs, are activated by many extracellular and intracellular stimuli. These stimuli usually transmit their signals to MEK1/2 via membranal receptors that recruit adaptor proteins together with nucleotide exchange factors, to consequently activate the small GTPase, Ras. Active Ras, in turn, recruits the protein kinases Raf1 and B-Raf (Rafs) to the plasma membrane, where they are activated via an unknown mechanism. The Rafs are the main MAP3Ks that activate MEK1/2 by phosphorylating them on two Ser residues (Ser218, 222 in human MEK1) in their activation loops. However, the ability to induce this MEK1/2 phosphorylation is not restricted to Rafs, since under different conditions, other kinases can serve as MEK kinases as well. For example, MOS acts as a MEK1/2 kinase in the reproductive system, and MEKK1 acts under stress conditions. A-Raf, ▶ MLTKα/β, ▶ TPL2 and MST1 have been implicated in the activation of MEK1/2 as well, but the conditions under which they operate are not fully understood.

MEK1/2 Activity and Its Regulation

As mentioned above, the main function of MEK1/2 is to phosphorylate and activate ERK1/2. In all species, MEK1/2 isoforms exhibit unique specificity toward the native forms of these two downstream targets, phosphorylating them non-processively on their activatory Thr and Tyr that are separated by Glu residue (Seger and Krebs 1995). This Thr-Glu-Tyr phosphorylation induces a big conformational change in the active pocket of ERK1/2, which consequently, results in the activation of the proteins and the rest of the cascade. Aside from this most important role of MEK1/2 in ERK1/2 activation, these MAPKKs were implicated in additional processes, including: (1) Association with ERK1/2 to induce either cytoplasmic localization of the latter in resting cells or their nuclear export at later time points after stimulation (Fukuda et al. 1997; Rubinfeld et al. 1999). (2) Direct binding to DNA in gene promoters that consequently results in a direct regulation of transcription (Perry et al. 2001). (3) Regulation of the subcellular localization of PPARγ (Burgermeister and Seger 2007). These additional functions, which are not directly dependent on the kinase activity of MEK1/2, indicate that these two MAPKKs may exert their activity in a kinase-independent manner. Furthermore, these additional activities demonstrate ERK-independent functions that seem to complement the main functions of ERK1/2 in the regulation of mitogenic signals.

Being important components of the central ERK cascade and other processes, MEK1/2 activation is well regulated by various mechanisms. Like many other components of signaling cascades, the activation of MEK1/2 is transient, as their activity usually peaks 2–4 min after stimulation and returns to basal levels within 10–90 min, dependent on the stimuli and conditions. The activation is mediated by the MAP3Ks described above, while the removal of the phosphates from the two activatory Ser residues, which

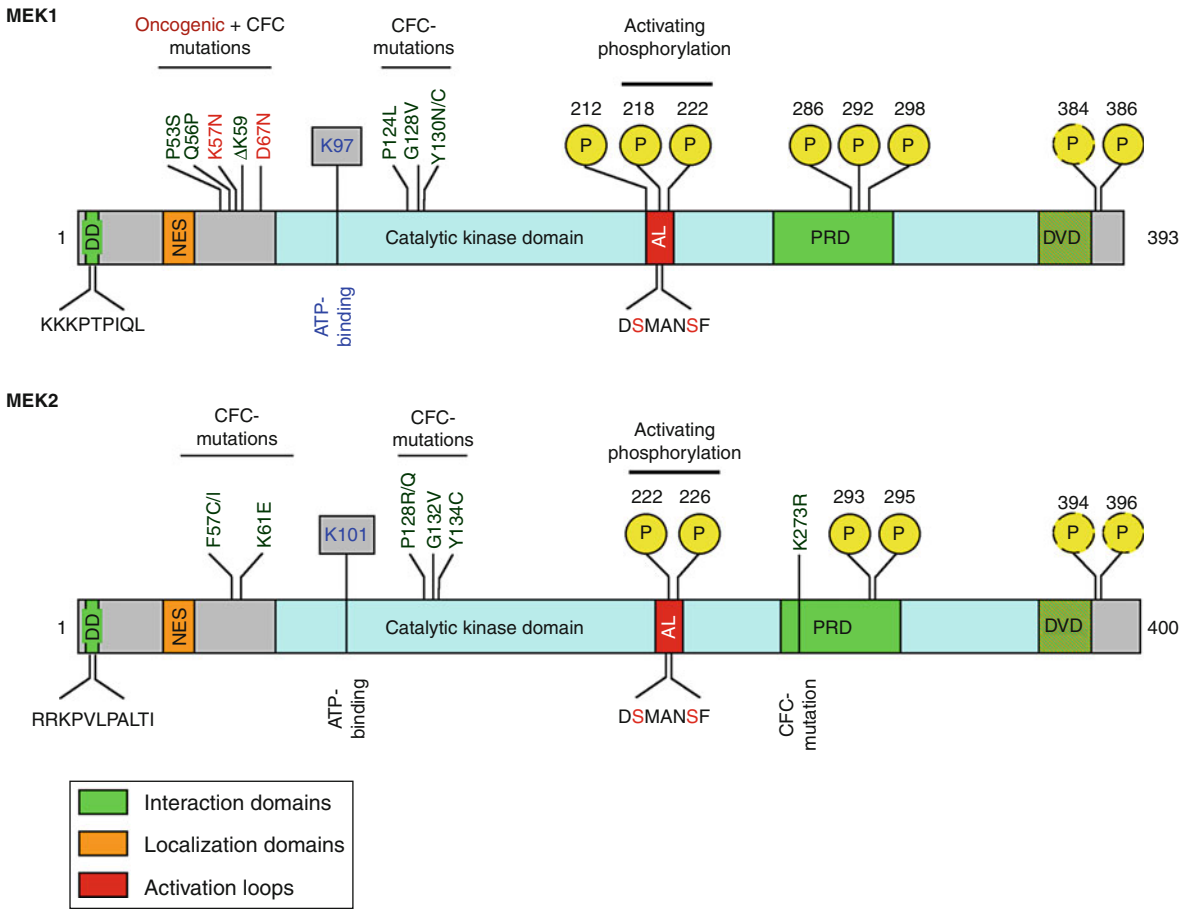
consequently leads to inactivation of MEK1/2, is mainly mediated by the Ser/Thr phosphatase PP2A. However, aside from the dynamic phosphorylation of the activatory Ser residues, MEK1/2 are regulated by additional phosphorylations. One such phosphorylation is that of Ser298 of MEK1 by the protein kinase ▶ Pak1, which acts downstream of the morphology regulator Rho. This phosphorylation does not activate MEK1 by itself, but seems to accelerate the Ser218/222-dependent activation, thereby serving as a convergence point of distinct signals. Other phosphorylations of MEK1 are inhibitory ones, including MEK1's Thr286 and Thr292, which seem to inhibit mainly Ser298 phosphorylation, and MEK1's Ser212 that inhibits the activity of MEK1/2 by an unknown mechanism. These, and other phosphorylations, as well as a few docking domains described below, induce better interactions of MEK1/2 with scaffold proteins such as Paxillin, MP1, and more. These interactions can bring MEK1/2 to close proximity to their upstream activators, to ERK1/2 and to their correct site of action, and therefore, may accelerate properly situated MEK activity. In addition, the activity of MEK1/2 is also regulated by their heterologous dimerization and by varying their subcellular localization. Finally, MEK1/2 are excellent targets for synthetic inhibitors, and many such small molecular weight inhibitors (e.g., PD98059, U0126, PD184352, and AZD6244) are currently being used either in the biochemical studies of the ERK cascade or in the development of anticancer drugs (see below).

Structure–Function Relationships

Most of the structure–function relationships have been conducted on MEK1, but because of their sequence (Seger and Krebs 1995) and conformation (Ohren et al. 2004) similarities, MEK1 and MEK2 probably share many of the features identified. Human MEK1 is a 393 amino acid protein kinase that contains all known kinase subdomains and residues, including ATP binding and catalytic sites. Indeed, this protein exhibits a significant phosphorylation activity that ranges from ~10 nmole/min/mg in basal state to ~1 mmole/min/mg in its active state. The significant change in MEK1 activity (10^5 fold) is mediated by two Ser phosphorylation, as described above. The phosphorylation of both residues is essential for full catalytic MEK1 activity, while phosphorylation of just one of the Ser residues is sufficient to induce only small MEK1 activation (up to ~15% of total activity for p-Ser222). Interestingly, phosphomimetic mutations of the two Ser residues induce a constitutive, but rather low MEK1 activity, which can be used to study MEK1 functions.

As central signaling components, the activity of MEK1/2 is well-regulated by interacting proteins, protein kinases, and protein phosphatases. In order to allow this regulation, MEK1/2 contain several docking domains, as well as phosphorylation sites that are spread all over the molecules (Fig. 2). Thus, the very N-terminus of MEK (residues 3–11) contains a docking domain (D-domain), which interacts specifically with the CRS/CD domain of ERK1/2, and thereby, directly determines ERK1/2 activation (Tanoue et al. 2000). This region is also important for other MEK1 activities, including its effects on PPARγ and ERK1/2 localization. Another important domain in the N-terminus of MEK1 lies within residues 31–68. Deletion of residues 31–51, or substitution of residues within this area, cause high constitutive activation of MEK1, which together with its phosphomimetic mutations, is used for functional MEK studies. In addition, few activatory oncogenic (see below) or Cardio-Facio-Cutaneous (CFC) syndrome–causing mutations were identified C-terminal to residue 51 (Phe53Ser, Gln56Pro, Lys57Asn, ΔLys59, and Asp67Asn). Therefore, it is likely that the 31–68 region is important for maintaining the low basal MEK1 activity. Finally, another important part in this region is its nuclear export signal (NES; residues 32–44), which determines its subcellular localization, as described below.

Based on sequence alignment, the core kinase domain of MEK1 starts at residue 74 with the ATP binding site, and is stretched all the way to residue 361. This catalytic region contains several regulatory parts, including the activation loop (residues 212–225) and a Pro reach domain (PRD; residues 271–307). First, CFC-causing mutations in residues 124–130 (Pro124Leu, Gly128Val and Tyr130Asn/Cys) indicate that similar to residues 31–68, this region participates in maintaining the low basal activity of MEK1. Another important functional region is the activation loop of MEK1 containing three phosphorylation sites, including the inhibitory Ser212, and activatory Ser218 and Ser222. Finally, the third functional region of the kinase domain is PRD, which unlike other domains is



Mek, Fig. 2 Schematic representations of MEK1 and MEK2. The docking domains (*DD*), nuclear export signals (*NES*), activation loops (*AL*), proline rich domain (*PRD*), domain of versatile docking (*DVD*, MAP3K binding), and ATP binding site are marked. MEK1's natural oncogenic (*red*), and CFC-causing (*green*) mutations and phosphorylation sites (*yellow*) are stated. Putative phosphorylation site is dashed

somewhat different between MEK1 and MEK2. This region mediates/regulates MEK1's interactions and activity, but the full scope of its activities still requires clarification.

The C-terminal region of MEK1 is, as yet, another regulatory part of MEK1/2. It contains the domain for versatile docking (DVD; residues 362–381), which is conserved among other MAPKKs, and seems to be important for interaction with upstream kinases (Takekawa et al. 2005). Interestingly, a region close to the DVD was also implicated in the determination of MEK1's localization in the cytoplasm (Bendetz-Nezer and Seger 2005). In addition, Thr386, very close to the C-terminal edge of MEK1, was identified as a robust phosphorylation site that regulates MEK1 interactions. This phosphorylation, together with that of the nearby Thr384, was recently implicated in mediating the short-term nuclear translocation of MEK1 (Chuderland et al. 2008). Finally, Thr23, Ser24/25, Ser218, and Ser299 in MEK1 also serve as phosphorylation/autophosphorylation sites, but their role is not fully understood yet.

Subcellular Localization

The downstream targets of the ERK cascade are localized in various cellular organelles, including the cytoplasm, nucleus, plasma membranes, cytoskeleton, mitochondria, Golgi, and ER. However, in resting cells, the inactive MEK1/2, ERK1/2, and most of their MAPKAPKs are localized primarily in the

cytoplasm (Yao and Seger 2009). This cytoplasmic localization of the various components is maintained mainly by binding to cytoplasmic anchoring proteins. In the case of MEK1/2, the anchoring proteins include KSR, β-arrestin, paxillin, MP1, Grb10, IQGAP1, and others that interact with either the D- or PR-domains of MEK1/2. These interactions also seem to be important for the rapid downstream signaling by the ERK cascade upon activation. Interestingly, MEK1/2 are localized in the cytoplasm not only by docking interactions, but also due to their N-terminal CRM-dependent NES, which rapidly exports any nuclear MEK1/2, making the nucleus completely devoid of MEK1/2 molecules at this stage. Since MEK1/2 are known to interact with inactive ERK1/2 via their D-domain, the cytoplasmic MEK1/2 molecules whose D-domain is not hindered by interacting proteins, can by themselves anchor ERK1/2 in the cytoplasm. Thus, it was proposed that MEK1/2 serve as main cytoplasmic anchoring proteins for ERK1/2 in non-stimulated cells, and therefore are responsible for a significant part of the cytoplasmic distribution of these protein kinases, as well as some of their interacting proteins.

The cytoplasmic distribution of MEK1/2 and the other ERK cascade components is dramatically changed upon cellular stimulations. It was shown that upon stimulation, Rafs rapidly translocate to the plasma membrane, while ERK1/2 and RSKs translocate to the nucleus and other cellular organelles. Unlike the other components, initial studies described MEK1 as a constant cytoplasm-resident protein, both in resting cells and after stimulation. It was even proposed that the nuclear translocation of MEK1/2 may be hazardous to cells, and therefore, the NES serves as a safety system to avoid this unwanted nuclear accumulation. However, it was later shown that MEK1, and presumably MEK2, could rapidly translocate into the nucleus upon cellular stimulation. Thus, initiation of signaling via the cascade causes MEK1/2 to detach from ERK1/2 and from other anchoring molecules, and translocate separately from ERK1/2 into the nucleus. Unlike ERK1/2 that can be retained in the nucleus for minutes to hours, most MEK1/2 molecules are rapidly exported back to the cytoplasm shortly after translocation due to their NES, giving rise to the apparent constant cytoplasmic distribution. The role of this rapid MEK1/2 shuttle in and out of the nucleus has not been fully resolved, but might include phosphorylation of nuclear ERK isoforms such as the alternatively spliced forms ERK1b and, to some extent, ERK1c. Another possibility is that the translocated MEK1/2 are involved in the export of ERK1/2 out of the nucleus at late stages after stimulation (Yao and Seger 2009). Finally, MEK1 can also interact with other nuclear proteins (e.g., PPARγ) via its D-domain and induce their nuclear export upon stimulation. Based on all the data described above, it is clear that the subcellular localization of MEK1/2 plays an important role in the regulation of function of the ERK1/2 cascade and probably additional signaling components upon various cellular stimulations.

MEK in Cancer

As soon as the ERK cascade was elucidated, it became clear that it plays a role in the induction and development of cancer. It was initially shown that the ERK cascade transmits signals of many oncogenes such as growth factors, growth factor receptors, and other signaling components. In particular, B-Raf that is a part of the ERK cascade is known as a potent oncogen, especially in melanoma. Today, activation of ERK1/2 was reported in more than 85% of cancer cases, even those that are transformed by oncogenes that are thought to act downstream of the cascade, indicating that downstream components may utilize a positive feedback loop to activate the ERK cascade. In all these cases, the ERK cascade is implicated mainly in mediating uncontrolled proliferation which is one of the key features that underlie oncogenic transformation. Since MEK1/2 are the sole activators of ERK1/2, it is very likely that these components are activated in cancer as well. Staining of a limited number of cancer samples indeed confirmed the phosphorylation of MEK1/2 in cancer, but a widespread screen for MEK1/2 phosphorylation in various cancer types has not been performed as yet.

Although activating mutations of MEK1/2 that can induce transformation of tissue culture cells have been identified already in the early 1990s, early studies failed to identify oncogenic forms of MEK1/2 in tumors. However, the extensive sequencing efforts of human cancers in the last few years did identify relatively rare oncogenic mutations of MEK1 in a limited number of human cancers. Thus, the activating mutation Lys57Asn was identified in lung cancer and Asp67Asn in ovarian and column cancers. Interestingly, mutations in two inhibitory regions of MEK1

(Gln56Pro and Pro124Leu) were shown to confer resistance to MEK1/2 and B-RAF inhibition, indicating the possible activating properties of these regions in human cancers. Although activating mutations have been identified in CFC, and despite large sequencing efforts, no activating MEK2 mutations were identified in human cancer so far. The reason for this is not fully understood, but may be derived from the fact that MEK1 seems to regulate MEK2 activity mainly by their heterodimerization (Catalanotti et al. 2009).

The involvement of the ERK cascade in cancer, and the ability of dominant negative mutants of Rafs and MEK1/2 to reverse oncogenic transformation, prompted many studies aimed to develop efficient inhibitors for components of this cascade. Indeed, several Raf inhibitors have been developed over the years, and one of them, Sorafinib, is already in clinical use for several cancer types. Efficient small molecular weight inhibitors of MEK1/2 have been developed as well. When applied to animal models, these inhibitors significantly reduced the phosphorylation of ERK1/2 and, as a consequence, inhibited the growth of tumors in these models (Sebolt-Leopold et al. 1999). Therefore, several such noncompetitive inhibitors have been used in clinical trials, and indeed demonstrated impressive inhibition of ERK1/2 activity, as well as a very low toxicity. Unfortunately, at present, the efficacy of the inhibitors examined is not sufficient, even in melanomas that are driven by the oncogenic form of the MEK1/2 upstream activator B-Raf. Although it seems that recent MEK inhibitors are somewhat more successful, and likely to be approved for clinical use, new generation of MEK inhibitors should still be developed to fully exploit the centrality of MEK1/2 activity in various types of cancer.

Summary

MEK1/2 are members of the MAPKK family of signaling protein kinases. They function within the ERK signaling cascade, constituting an evolutionarily conserved group with three mammalian isoforms: MEK1, MEK1b, and MEK2. MEK1/2 are activated via phosphorylation on two Ser residues in their activation loops, and when activated, they phosphorylate ERK1/2 on their regulatory Tyr and Thr residues, thereby causing their activation. Importantly, ERK1/2 are the only known phosphorylation substrates of MEK1/2, and therefore, the latter serve as specificity determinants of the ERK cascade. However, protein and DNA interactions of MEK1 also implicate it in the regulation of the subcellular localization of ERK1/2 and PPARγ and in regulating MyoD transcription. Upon activation MEK1/2 rapidly translocate into the nucleus, and then are rapidly exported back to the cytoplasm in a mechanism involving CRM1. Significantly, the centrality of MEK1/2 and the whole ERK cascade indicate that their dysregulation may result in various diseases. Indeed, activating mutations of MEK1/2 were shown to induce developmental disorders, and additional activating mutations of MEK1 were shown to act as oncogenes in a limited number of cancer. Furthermore, MEK1/2 are sensitive to synthetic inhibitors, and several of them are currently being developed as anticancer drugs mainly for melanoma.

Acknowledgments This work was supported by a grant from the Israel Science Foundation. RS is an incumbent of the Yale S. Lewine and Ella Miller Lewine professorial chair for cancer research.

References

Ahn NG, Seger R, Bratlien RL, Diltz CD, Tonks NK, Krebs EG. Multiple components in an epidermal growth factor-stimulated protein kinase cascade. *In vitro* activation of myelin basic protein/microtubule-associated protein-2 kinase. J Biol Chem. 1991;266:4220–7.

Bendetz-Nezer S, Seger R. Full molecular page of MEK1. AfCS/Nature Signaling Gateway. 2005. doi:10.1038/mp.a001505.01.

Burgermeister E, Seger R. MAPK kinases as nucleo-cytoplasmic shuttles for PPARgamma. Cell Cycle. 2007;6:1539–48.

Catalanotti F, Reyes G, Jesenberger V, Galabova-Kovacs G, et al. A Mek1–Mek2 heterodimer determines the strength and duration of the Erk signal. Nat Struct Mol Biol. 2009;16:294–303.

Chuderland D, Konson A, Seger R. Identification and characterization of a general nuclear translocation signal in signaling proteins. Mol Cell. 2008;31:850–61.

Fukuda M, Gotoh I, Adachi M, Gotoh Y, Nishida E. A novel regulatory mechanism in the mitogen-activated protein (MAP) kinase cascade. Role of nuclear export signal of MAP kinase kinase. J Biol Chem. 1997;272:32642–8.

Gomez N, Cohen P. Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. Nature. 1991;353:170–3.

Kyriakis JM, App H, Zhang FX, Banerjee P, Brautigan DL, et al. Raf-1 activates MAP kinase–kinase. Nature. 1992;358:417–21.

Ohren JF, Chen H, Pavlovsky A, Whitehead C, Zhang E, et al. Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. Nat Struct Mol Biol. 2004;11:1192–7.

Perry RL, Parker MH, Rudnicki MA. Activated MEK1 binds the nuclear MyoD transcriptional complex to repress transactivation. Mol Cell. 2001;8:291–301.
Rubinfeld H, Hanoch T, Seger R. Identification of a cytoplasmic-retention sequence in ERK2. J Biol Chem. 1999;274:30349–52.
Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat Med. 1999;5:810–6.
Seger R, Krebs EG. The MAPK signaling cascade. FASEB J. 1995;9:726–35.
Shaul YD, Gibor G, Plotnikov A, Seger R. Specific phosphorylation and activation of ERK1c by MEK1b: a unique route in the ERK cascade. Genes Dev. 2009;23:1779–90.
Takekawa M, Tatebayashi K, Saito H. Conserved docking site is essential for activation of mammalian MAP kinase kinases by specific MAP kinase kinase kinases. Mol Cell. 2005;18:295–306.
Tanoue T, Adachi M, Moriguchi T, Nishida E. A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat Cell Biol. 2000;2:110–6.
Yao Z, Seger R. The ERK signaling cascade–views from different subcellular compartments. Biofactors. 2009;35:407–16.

Mek3

Shin Yasuda[1], Hiroko Sugiura[1] and Kanato Yamagata[1,2]
[1]Department of Neuropharmacology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan
[2]Department of Pharmacology, Shukutoku University, Chuo–ku, Chiba, Japan

Synonyms

Licorne (lic); MAP kinase kinase 3; Map2k3; MAPK Erk kinase 3; MAPKK3; Mitogen-activated protein kinase kinase 3; MKK3; mMMK3b; Mpk3; p38MAPKK; Prkmk3; Protein kinase, mitogen-activated kinase 3

Historical Background

Polymyxin B sensitivity (PBS) 2, a yeast homologue of mitogen-activated protein kinase kinase 3 (Mek3), was originally cloned as a gene that conferred polymyxin B resistance to yeast cells (Boguslawski and Polazzi 1987). The amino acid sequence of the *PBS2* gene product showed strong homology to the serine/threonine protein kinase family (Boguslawski and Polazzi 1987). In 1993, it was shown that *PBS2* and its downstream *HOG1* genes, which code for a Mek3 homologue and a ▶ p38 mitogen-activated protein kinase (MAPK) homologue, respectively, are necessary for yeast cells to grow at high osmolarity (Brewster et al. 1993). Two years after that study, *Mek3* was first amplified by degenerative PCR as a human homologue of yeast *PBS2*, and overexpression of its gene product was revealed to activate p38 MAPK in response to osmotic stress, UV irradiation, and inflammatory cytokines (interleukin (IL)-1 and ▶ tumor necrosis factor (TNF)) in COS-1 cells (Derijard et al. 1995).

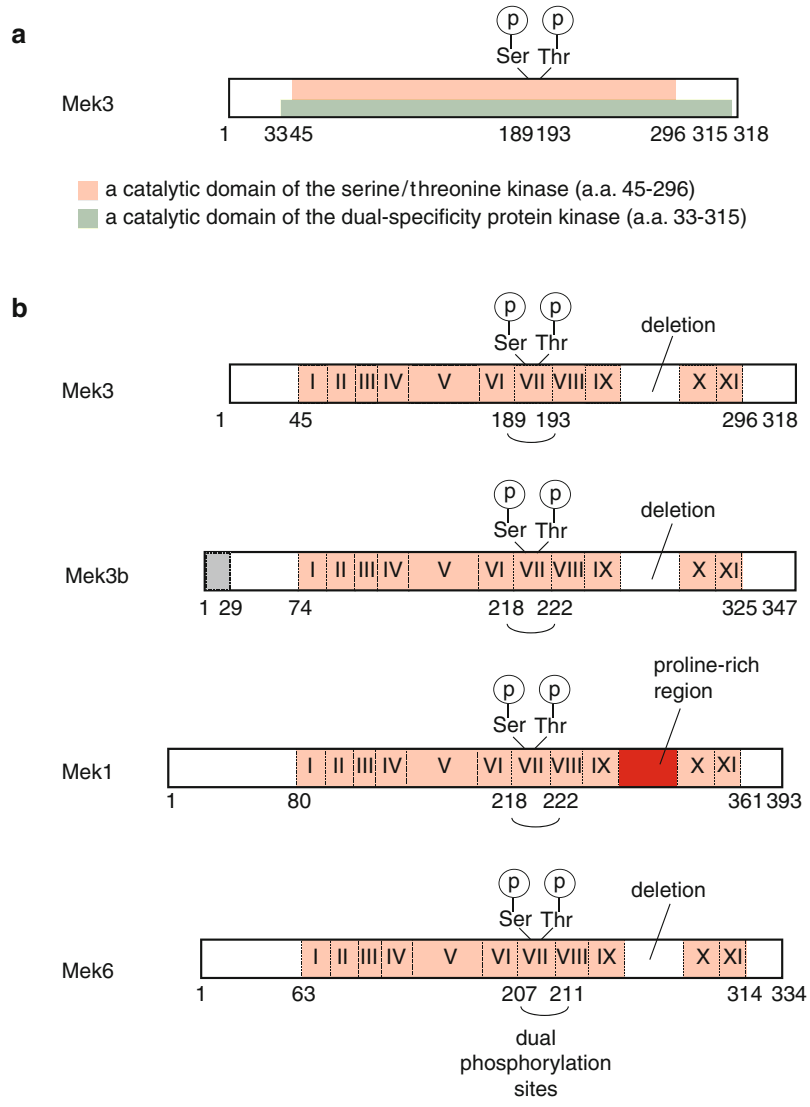
Protein and Gene Structure

The human *Mek3* gene has been mapped to 17q11.2 (Derijard et al. 1995). The *Mek3* gene has 12 exons and 11 introns, and its coding region contains 957 base pairs organized in nine exons (exons 2–10).

The human Mek3 protein consists of 318 amino acids, and its predicted molecular weight and isoelectric point are 36172.7 and 5.87, respectively. Mek3 contains two conserved motifs, a catalytic serine/threonine kinase domain (residues 45–296) and a catalytic dual-specificity protein kinase domain (residues 33–315) (Fig. 1a). Active Mek3 phosphorylates p38α, δ and γ MAPK and acts as a key mediator of various stress-mediated cellular responses (Cuadrado and Nebreda 2010).

The human ▶ Mek protein family contains seven kinases, Mek 1–7. Each member of the Mek family contains the Mek-kinase subdomains I–XI (Hanks et al. 1988) (Fig. 1b). Mek3, Mek4, and Mek6 phosphorylate p38 MAPK, whereas Mek1 and Mek2 phosphorylate extracellular signal-regulated kinase Erk1 and Erk2. Mek4 and Mek7 phosphorylate c-Jun N-terminal kinase (Jnk (1–3)). Mek5 is the only identified kinase upstream of Erk5.

Mek3 is approximately 50% and 80% homologous to Mek4 and Mek6, respectively (Derijard et al. 1995; Stein et al. 1996). In contrast to Mek1 and Mek2, all p38 MAPK stimulators include a large deletion that is located between subdomains IX and X (Fig. 1b). Both Mek1 and Mek2 contain a proline-rich region between these subdomains, which is required for the efficient activation of downstream Erk1 and Erk2 (Dang et al. 1998). Thus, the deletion could define the kinase substrate specificity of Mek3. A Mek3-specific inhibitor



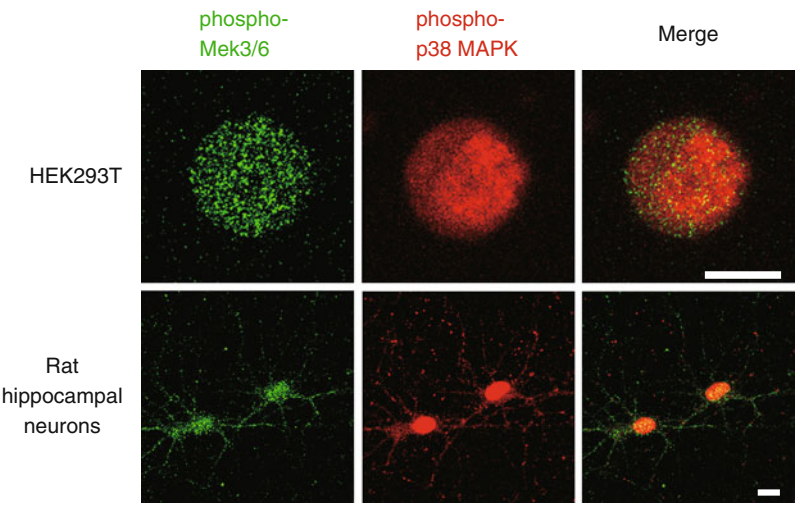
Mek3, Fig. 1 A deletion between subdomains IX and X may determine the kinase substrate specificity of Mek3. (**a**) Mek3 contains two catalytic domains. One is a serine/threonine kinase domain (residues 45–296), and the other is a dual-specificity protein kinase domain (residues 33–315). (**b**) Comparison of human Mek3 subdomains with those of human Mek3b, Mek1, and Mek6. The N-terminal extended region of MKK3b is shown in gray. Note that Mek3, Mek3b, or Mek6 do not contain a proline-rich region inserted between Mek-kinase subdomains IX and X of Mek1

has not yet been developed. UO126 is shown to weakly attenuate Mek3 activity. However, it preferentially suppresses Mek1 and Mek2.

Mek3b is an alternatively spliced form of Mek3. Mek3b is distinct from Mek3 at the 5′-end, encoding 29 extra amino acids at the N-terminus. Reminiscent of Mek3 and Mek6, Mek3b is activated by osmotic shock and phosphorylates p38 when expressed in cultured cells. Both Mek3 and Mek3b activate p38α, whereas only Mek3b phosphorylates p38β2, suggesting a role for the N-terminal region of Mek3 in the determination of substrate specificity (Enslen et al. 2000).

Localization

Mek3 is widely expressed, with its highest expression in skeletal muscle (Derijard et al. 1995). Immunostaining of HEK293T cells with Mek3-specific antibody indicates that this protein is localized to both the cytoplasm and the nucleus. Activated Mek3 and p38 MAPK are diffusely distributed in HEK293T cells and in hippocampal neurons. However, these phosphorylated proteins do not fully colocalize with each other (Fig. 2). There is no evidence for the subcellular translocation of Mek3 in response to cellular stresses.



Mek3, Fig. 2 Subcellular distribution of activated Mek3, Mek6, and p38 MAPK in HEK293T cells. Activated Mek3, Mek6, and p38 MAPK are widely distributed in HEK293T cells (*upper panel*) and rat cultured hippocampal neurons (*lower panel*) (P7). However, the localization of activated Mek3 does not necessarily correspond to that of activated p38 MAPK. The scale bar represents 10 μm

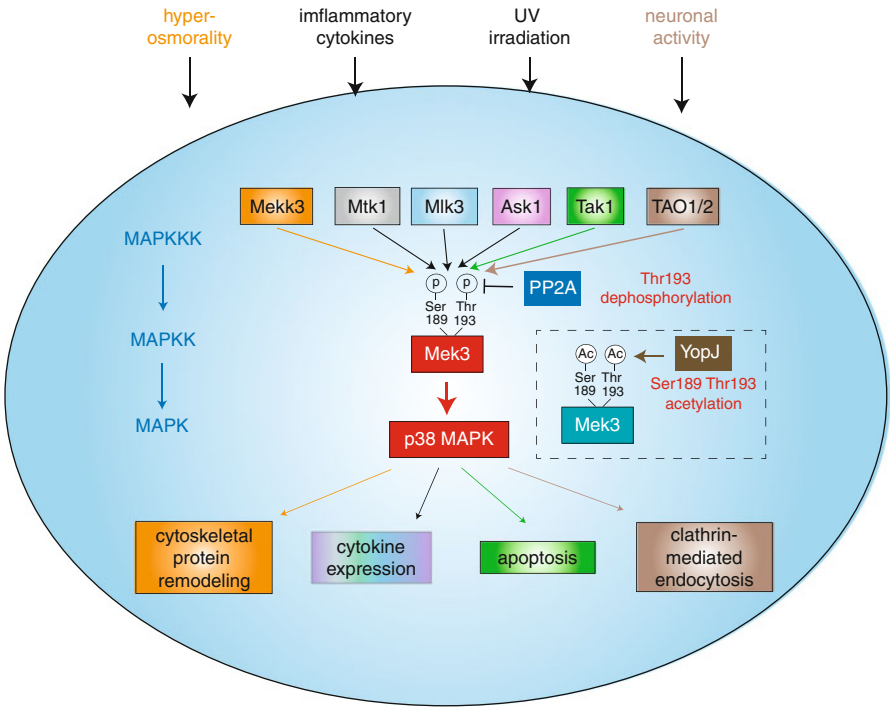
Interaction and Regulation

Mek3 binds to its upstream MAPKKKs, such as mitogen-activated protein/ERK kinase kinase (Mekk)3, Mekk4 (also known as MAP 3 Kinase 1; Mtk1), mixed lineage kinase (Mlk)3, apoptosis signal-regulating kinase (Ask)1, transforming growth factor (TGF)-beta-activated kinase (Tak)1, and thousand and one amino acid kinase (TAO)1/2 (Fig. 3). Through various external stimuli, such as hyperosmolarity, UV irradiation, inflammatory cytokine, and neuronal activity, MAPKKKs are first activated and then phosphorylate Mek3 at Ser189 and Thr193 in its activation loop (a region that lies outside the active-site cleft, residues 178–193 and 195–200) (Fig. 1) (Derijard et al. 1995). However, it is not well understood which MAPKKK is activated by each external stimulation.

Mek3 activity is down-regulated by phosphatases such as type 2 protein phosphatase (PP2A) (Prickett and Brautigan 2007). Phosphorylated Mek3 binds to alpha-4/immunoglobulin-binding protein 1 (Igbp1) and PP2A, which then enhances the site-specific dephosphorylation of Thr193 (but not Ser189) in the activation loop of Mek3 (Fig. 3). Moreover, Ser189 and Thr193 may be modified by an acetyltransferase, Yersinia outer protein (Yop) J (Fig. 3) (Mukherjee et al. 2006). This acetylation could consequently block phosphorylation of these residues, leading to the attenuation of Mek3 activity (Mukherjee et al. 2006). These results indicate that the level of Mek3 kinase activity is dependent on the phosphorylation, dephosphorylation, or acetylation state of its activation loop.

In addition, Mek3 binds to its own downstream proteins. For example, Mek3 is associated with Mirk (Minibrain-related kinase; also called Dyrk1B), which belongs to the Dyrk/minibrain family of dual-specificity tyrosine-regulated, arginine-directed protein kinases. The homophilic interaction of E-cadherin (a cell adhesion molecule) by cell-cell contact triggers the activation of a small G protein Rac and its downstream Mek3 (Jin et al. 2005). Phosphorylated Mek3 associates with and activates Mirk, which causes the phosphorylation and activation of transcriptional regulator hepatocyte nuclear factor (HNF)-1α. Mek3 up-regulates the Mirk kinase activity and the transcriptional activation of HNF1α, inducing gene expression associated with cell proliferation (Fig. 4a). Conversely, p38α and β-MAP kinases suppress Mirk by the dissociation of Mirk from Mek3. Mirk predominantly localizes in some carcinoma cell lines and may regulate cell proliferation by functioning at the junction between the p38 and Mirk signaling cascades (Fig. 4a).

Mek3 also binds to dynactin1 and microtubules. Dynactin1 binds to and cooperates with a microtubule-dependent motor protein, dynein, and this protein complex induces the movement of various cellular cargos, especially membranous vesicles. The disruption of dynactin1 or treatment with microtubule-disrupting drugs suppresses Mek3 and Mek6 phosphorylation in response to hyperosmolar stresses, suggesting that the



Mek3, Fig. 3 The MAPKKKs, Mek3, and p38 MAPK signaling pathways mediate various stress-dependent cellular functions

function of the dynein-dynactin complex may be to partly regulate the Mek3-p38 MAPK signaling cascade (Fig. 4b).

Apart from the interaction mentioned above, Mek3 is known to associate with several proteins including the osmosensing scaffold for Mekk (Osm), JNK-interacting protein 2 (Jip2), Smad7, c-Src, c-Met, phospholipase C (PLC)-β2, and Rho effector protein kinase N1 (Pkn1) (Yasuda et al. 2009). Such Mek3-interacting proteins are involved in various cellular functions. Details of Osm, Smad7, c-Src, and c-Met are described in the section Protein Function.

Protein Function

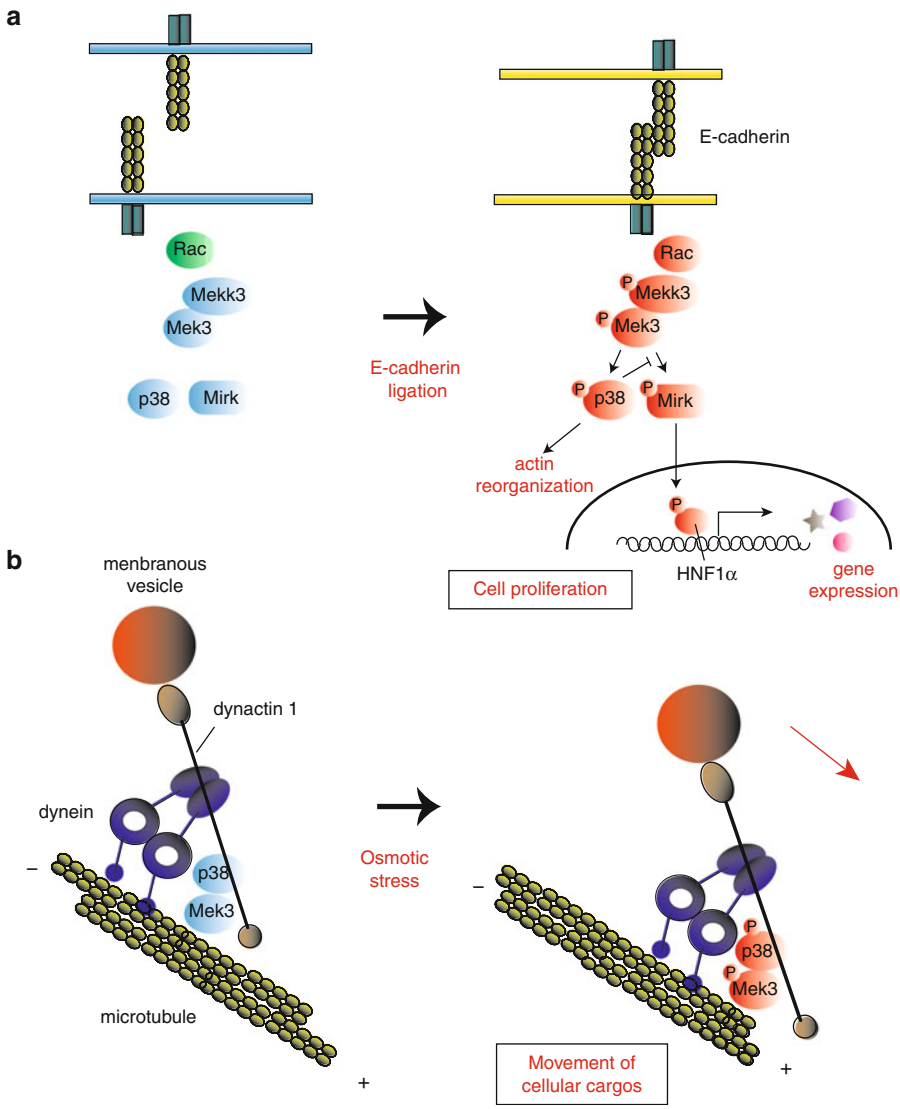
Whereas Mek6 activates all p38 isoforms, Mek3 is somewhat selective in that it preferentially phosphorylates the α, γ and δ subtypes of p38 MAPK at its regulatory Thr and Tyr residues (Cuadrado and Nebreda 2010). The activation of Mek3 and p38 MAPK is associated with several cellular functions, such as cytoskeletal protein remodeling, cytokine expression, apoptosis, and clathrin-mediated endocytosis.

Regulation of Cytoskeletal Proteins

Mek3 activation is involved in the stress-mediated remodeling of cytoskeletal proteins. For example, osmotic shock may activate the Mek3-p38 MAPK signal. Elevated Mek3 and p38 activities cause the recruitment of the scaffold protein Osm with Rac/Mekk3/Mek3/p38 to the actin cytoskeleton. This protein recruitment is likely to enhance the Mek3 and p38 interaction and kinase activity. Activated Mek3/p38 may consequently trigger actin polymerization and the formation of an actin-based ruffle structure (Fig. 5a).

Mek3 also regulates actin polymerization by binding to c-Met (a hepatocyte growth factor/scatter factor (HGF/SF) receptor) and its associated cytoplasmic binding protein kinase c-Src (a member of the cytoplasmic tyrosine kinase family). In this pathway, c-Met stimulates c-Src and its downstream molecule Rac1 that activates the Mek3-p38 MAPK signaling, leading to actin polymerization (Fig. 5a). The active forms of Rac, Mek3, and p38 MAPK are detected in signet-ring carcinoma cell lines (Kobayashi et al. 1999).

How does the activation of the Rac/Mek3/p38 MAPK pathway affect cytoskeletal reorganization? Hitherto, the p38 MAPK substrate, ▶ Mapkap kinase 2 (MK2), has been revealed to be involved in this mechanism. MK2 phosphorylates its downstream heat shock protein HSP27, which acts as an actin-capping protein. Phospho-HSP27 causes the release of free actin filament

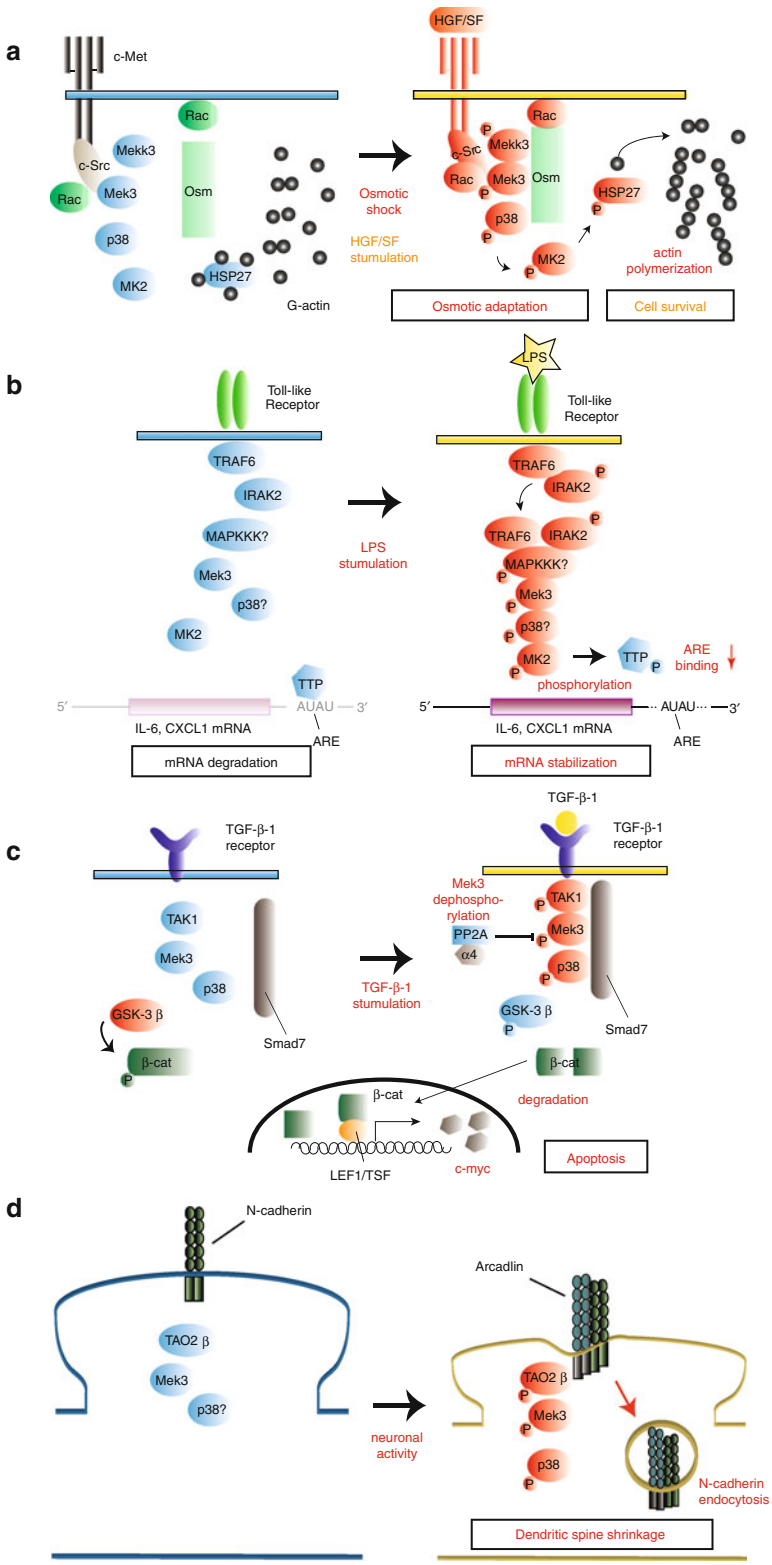


Mek3, Fig. 4 Mek3-interacting proteins. (**a**) Mek3 associates with its downstream protein Mirk. (**b**) Mek3 binds to dynactin 1, a component of the dynactin complex

barbed ends, which may result in actin polymerization (Fig. 5a) (Cuadrado and Nebreda 2010). Thus, Mek3 might control cell migration, cell adhesion, or cell shape by influencing cytoskeletal proteins under pathological conditions.

Cytokine Expression

The stress-mediated activation of Mek3 positively regulates the expression of several cytokines such as interleukin (IL)-1α, IL-1β, IL-6, and IL-12 (Cuadrado and Nebreda 2010). Mek3 knockdown shows selective defects in the response of fibroblasts to tumor necrosis factor (TNF)-α, including reduced p38 MAPK activation and cytokine expression (Cuadrado and Nebreda 2010). In addition, Mek3 mediates lipopolysaccharide (LPS)-triggered posttranscriptional control of cytokine and chemokine expression. LPS stimulation induces the formation of a complex consisting of Irak2 (a member of the IL-1 receptor-associated kinase (Irak) protein family), ▶ Traf6, MK2, and Mek3, and this interaction appears to regulate Toll-like receptor-mediated signaling (Fig. 5b). Elevated Mek3, p38, and MK2 activities cause the phosphorylation of its downstream mRNA destabilizing protein, tristetraprolin (TTP), which binds to the AU-rich element (ARE) of cytokine mRNA in the 3′-noncoding region. The phosphorylation of TTP leads to a reduction in the affinity to ARE and consequently stabilizes cytokine and chemokine mRNAs, such as IL-6



Mek3, Fig. 5 Mek3 plays a pivotal rote in various stress-mediated cellular functions. (**a**) Mek3 activity affects actin polymerization in response to stress. (**b**) LPS stimulation causes the activation of the Mek3-p38-MK2 signal, which may stabilize cytokine mRNAs. (**c**) Activated Mekm3/p38 signal may trigger TGF-β1-induced apoptosis. (**d**) Mek3/p38 activity regulates N-cadherin endocytosis, which causes dendritic spine shrinkage in a neural activity-dependent manner

and chemokine (C-X-C Motif) ligand 1 (CXCL1) (Fig. 5b) (Hitti et al. 2006). These findings indicate that Mek3 might contribute to the stabilization of cytokine mRNAs after exposure to various forms of stress.

Apoptosis

Activation of Mek3 also regulates the induction of apoptosis. That is, the Tak1-Mek3-p38 MAPK signaling complex mediates transforming growth factor (TGF) β1-induced apoptosis. In addition, Smad7 might facilitate this TGF-β-induced apoptosis as a scaffold protein by binding to Tak1, Mek3, and p38, and enhancing each interaction. As a downstream target of p38 MAPK, glycogen synthase kinase (GSK)-3 is phosphorylated and inactivated. The inactive form of GSK-3β causes dephosphorylation and degradation of ▶ β-catenin, which binds to a transcriptional regulator, lymphoid-enhancer binding factor 1 (LEF1)/T-cell-specific factor (TSF), and may increase the expression of some apoptotic inducers, such as ▶ *c-myc* (Fig. 5c) (Edlund et al. 2005). These results suggest that apoptosis may be regulated by Mek3 kinase activity.

Clathrin-Mediated Endocytosis

Activation of Mek3 is related to the mechanism of clathrin-mediated endocytosis. For instance, the endocytosis of N-cadherin is regulated by the Mek3-p38 MAPK activity in neurons. The cytoplasmic region of a neuronal activity-inducible protein, arcadlin, binds to the TAO2β/Mek3/p38 signalosome and initiates N-cadherin endocytosis, causing neuronal activity-dependent dendritic spine retraction in hippocampal neurons (Fig. 5d) (Yasuda et al. 2007). This is the only situation in which a specific external stimulation regulates the activity of Mek3. In addition, the activation of a signaling cascade containing Mek3 and p38 may also cause α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor endocytosis in the dendritic spines of hippocampal neurons, suggesting that Mek3 may play a pivotal role in endocytosis in CNS neurons.

Phenotypes

Mek3-deficient mice appear to be viable and fertile (Cuadrado and Nebreda 2010). By contrast, the *Mek3*/*Mek6*-double knockout (KO) is lethal during midgestation at embryonic day 11.0–11.5 because of major defects during the development of the embryonic vasculature. The double KO mice also show developmental delay (Cuadrado and Nebreda 2010). This phenotype exhibited by *Mek3*/*Mek6* double KO mice is similar to that previously described for *p38α MAPK* KO embryos (Cuadrado and Nebreda 2010). Such a phenotypic difference between single and double KO mice suggests that some compensatory mechanisms can maintain p38 MAPK signaling. In fact, the amount of Mek6 protein is substantially increased in the unilateral ureteric obstruction kidney of *Mek3*-deficient mice (Ma et al. 2007).

Mek3-deficient mice show several phenotypes, including a reduction of cytokine production and impairment of immune responses (Cuadrado and Nebreda 2010). Additionally, *Mek3* KO mice exhibit a reduced progression of arthritis and type 1 diabetes (Fukuda et al. 2008; Inoue et al. 2006). Furthermore, mice with Mek3 deletions show a reduction of bone mass secondary to defective osteoblast differentiation (Greenblatt et al. 2010). Irrespective of the functional significance of Mek3, such minor phenotypes of its deficiency suggest the existence of possible redundant or compensatory mechanisms by Mek6 in vivo.

Summary

Mek3 is phosphorylated by various MAPKKKs, which become activated in response to various physical and chemical stresses, such as oxidative stress, UV irradiation, hypoxia, ischemia, and the presence of various cytokines. Activated Mek3 then specifically phosphorylates p38α, δ and γ MAPK. Conversely, Mek3 kinase activity is down-regulated by dephosphorylation or acetylation of the Ser and Thr residues in its activation loop. Although the crystal structure of Mek3 has not yet been determined, the primary structure indicates that it consists of Mek-kinase subdomains I–XI. An interesting structural characteristic of Mek3 is that it has a deletion between subdomain IX and X that could define the kinase substrate specificity for p38 MAPK. Mek3 converts diverse cellular stimuli into various stress responses, such as cytokine expression, cytoskeletal protein remodeling, apoptosis, and clathrin-mediated endocytosis. Further studies may be required to clarify the regulatory mechanism of Mek3 in response to various external stresses and the p38 MAPK activation machinery. The development of a Mek3-specific inhibitor is also needed to evaluate its pathophysiological significance of Mek3.

Acknowledgments This work was partly supported by KAKENHIs (20591426 to SY, 21500332 to HS, 20300135 and 23110525 to KY) and the Naito Foundation (to KY).

References

Boguslawski G, Polazzi JO. Complete nucleotide sequence of a gene conferring polymyxin B resistance on yeast: similarity of the predicted polypeptide to protein kinases. Proc Natl Acad Sci USA. 1987;84(16):5848–52.

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC. An osmosensing signal transduction pathway in yeast. Science. 1993;259(5102):1760–3.

Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem J. 2010;429(3):403–17.

Dang A, Frost JA, Cobb MH. The MEK1 proline-rich insert is required for efficient activation of the mitogen-activated protein kinases ERK1 and ERK2 in mammalian cells. J Biol Chem. 1998;273(31):19909–13.

Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, et al. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science. 1995;267(5198):682–5.

Edlund S, Lee SY, Grimsby S, Zhang S, Aspenstrom P, Heldin CH, et al. Interaction between Smad7 and beta-catenin: importance for transforming growth factor beta-induced apoptosis. Mol Cell Biol. 2005;25(4):1475–88.

Enslen H, Brancho DM, Davis RJ. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. EMBO J. 2000;19(6):1301–11.

Fukuda K, Tesch GH, Yap FY, Forbes JM, Flavell RA, Davis RJ, et al. MKK3 signalling plays an essential role in leukocyte-mediated pancreatic injury in the multiple low-dose streptozotocin model. Lab Invest. 2008;88(4):398–407.

Greenblatt MB, Shim JH, Zou W, Sitara D, Schweitzer M, Hu D, et al. The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. J Clin Invest. 2010;120(7):2457–73.

Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science. 1988;241(4861):42–52.

Hitti E, Iakovleva T, Brook M, Deppenmeier S, Gruber AD, Radzioch D, et al. Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element. Mol Cell Biol. 2006;26(6):2399–407.

Inoue T, Boyle DL, Corr M, Hammaker D, Davis RJ, Flavell RA, et al. Mitogen-activated protein kinase kinase 3 is a pivotal pathway regulating p38 activation in inflammatory arthritis. Proc Natl Acad Sci USA. 2006;103(14):5484–9.

Jin K, Lim S, Mercer SE, Friedman E. The survival kinase Mirk/dyrk1B is activated through Rac1-MKK3 signaling. J Biol Chem. 2005;280(51):42097–105.

Kobayashi M, Nagata S, Iwasaki T, Yanagihara K, Saitoh I, Karouji Y, et al. Dedifferentiation of adenocarcinomas by activation of phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA. 1999;96(9):4874–9.

Ma FY, Tesch GH, Flavell RA, Davis RJ, Nikolic-Paterson DJ. MKK3-p38 signaling promotes apoptosis and the early inflammatory response in the obstructed mouse kidney. Am J Physiol Renal Physiol. 2007;293(5):F1556–63.

Mukherjee S, Keitany G, Li Y, Wang Y, Ball HL, Goldsmith EJ, et al. Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. Science. 2006;312(5777):1211–4.

Prickett TD, Brautigan DL. Cytokine activation of p38 MAPK and apoptosis is opposed by alpha-4 targeting of PP2A for site-specific dephosphorylation of MEK3. Mol Cell Biol. 2007;27(12):4217–27.

Stein B, Brady H, Yang MX, Young DB, Barbosa MS. Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. J Biol Chem. 1996;271(19):11427–33.

Yasuda S, Tanaka H, Sugiura H, Okamura K, Sakaguchi T, Tran U, et al. Activity-induced protocadherin arcadlin regulates dendritic spine number by triggering N-cadherin endocytosis via TAO2beta and p38 MAP kinases. Neuron. 2007;56(3):456–71.

Yasuda S, Sugiura H, Yamagata K. Mek3. UCSD Nat Mol Pages. 2009. doi:10.1038/mp.a001507.01.

Mek4 (Mouse)

► EphA3, Erythropoietin-Producing Hepatocellular Carcinoma Cell Receptor A3

MEK5/ERK5

Nhat-Tu Le[1], Jay Yang[2] and Jun-ichi Abe[1]
[1]Aab Cardiovascular Research Institute, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA
[2]Department of Anesthesiology, University of Wisconsin, Madison, WI, USA

Synonyms

MEK5/ERK5: Mitogen-activated protein kinase 5/Extracellular signal-regulated kinase 5

Historical Background

► MEK5 is a dual-specificity protein kinase that belongs to the ► MAP kinase kinase family. MEK5 and ERK5 were first identified as the two components of a new protein kinase signaling cascade

(Zhou et al. 1995; English et al. 1995). MEK5 is the only identified immediate upstream MAP kinase kinase of ERK5. Alternative splicing of *mek5* at the 5'-end (NH_2-terminus) results in a 50 kDa (MEK5α) and a 40 kDa (MEK5β) isoforms. MEK5α acquires an extended NH_2-terminus with 89 amino acids longer than that of MEK5β and the longer NH_2-terminus endows MEK5α with unique features. This NH_2-terminus contains a Phox and Bem1 (PB1) protein dimerization/oligomerization domain, which is particular importance in the interaction between this kinase and other molecules. Besides an alternative splicing at 5'-end, both *mek5α* and *mek5β* appear to exhibit an additional alternative splicing at the 3'-end (COOH-terminus) where splicing at this 3'-end causes a deletion of ten amino acids in the catalytic domain. Thus, splicing at both 5'-end and 3'-end comprises four MEK5 alternative splice variants.

Functional difference between MEK5α and MEK5β due to the 5'-end splicing has been extensively examined (Cameron et al. 2004a; Nakamura and Johnson 2003; Nakamura and Johnson 2007; Nakamura et al. 2006; Seyfried et al. 2005). The constitutively active form of MEK5α (CA-MEK5α) but not CA-MEK5β activates ERK5 (Cameron et al. 2004a). Expression of CA-MEK5α but not CA-MEK5β promotes the nuclear translocation of ERK5, demonstrating the critical role of MEK5α NH_2-terminus in ERK5 activation. Deletion of 11 amino acids in the NH_2-terminus produces a mutant protein that is expressed but devoid of any kinase activity (Zhou et al. 1995). MEK5α and MEK5β have distinct tissue-wide distribution and cellular localization. MEK5α is highly expressed in liver and brain whereas MEK5β is ubiquitously present (English et al. 1995).

Null mice

ERK5 is not only a kinase but also a co-activator with a transcriptional domain in its COOH-terminus and this structure makes ERK5 different from ERK1/2. *Erk5*-null mutation results in embryonic lethality due to cardiovascular defects. Conditional *erk5*-knockout in cardiomyocytes, hepatocytes, and neuronal cells develops normally, but the depletion of ERK5 in Mx-1 Cre transgenic mice showed endothelial cell apoptosis and increased vascular permeability (Hayashi et al. 2004). *Mek5*-null mice are in a close phenocopy of *erk5*-null mice (Wang et al. 2005) providing indirect evidence that ERK5 is most likely a unique substrate of MEK5.

Physiological Roles in Different Cell Types

Epithelial Cells

MEK5/ERK5 signaling has been implicated in Epidermal Growth Factor (EGF)-mediated proliferation of epithelial cells (Kato et al. 1998). However, *mek5*-null embryo shows normal cell-cycle progression and the precise role of MEK5/ERK5 in epithelial cell proliferation is inconclusive. Rather, evidence supports an anti-apoptotic role of ERK5 (Squires et al. 2002; Wang et al. 2005) tipping the balance toward increase in viable cell number with an apparent increase in cell proliferation.

Endothelial Cells (ECs)

Steady laminar blood flow (s-flow) generates a frictional dragging force on the endothelium surface (fluid shear stress), which is known to possess anti-apoptotic and anti-atherosclerotic effects (Traub and Berk 1998). ERK5 is strongly activated by s-flow (Yan et al. 1999), and plays a key role in anti-apoptotic effects by increasing Bad phosphorylation, but the detailed mechanism remains unclear (Pi et al. 2004).

Due to its unique structure with a transcriptional domain on COOH-terminus, the activation of ERK5 by various stimuli is distinct from that of ERK1/2 (Abe et al. 1996). TNF-mediated JNK activation is inhibited by s-flow. This inhibition is completely reversed by treatment of ECs with BIX02188, a MEK5-selective inhibitor. However, treatment of ECs with PD184352, at a concentration that blocks ERK1/2 but not ERK5, shows no effect on flow inhibition of TNF-mediated JNK activation (Li et al. 2008). Moreover, s-flow can increase PPARγ1 activation which inhibits the expression of VCAM1 (vascular cellular adhesion molecule 1) and ICAM1 (intercellular adhesion molecule 1) (Pasceri et al. 2000).

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a process that demands highly coordinated migration, proliferation, and differentiation of ECs (Carmeliet 2005). Activation of Epac, a cyclic adenosine 5'-monophosphate-activated guanine nucleotide exchange factor for Rap1, or Rap1 deteriorates angiogenesis via downregulation of ID1 (the inhibitor of differentiation 1). MEK5/ERK5 antagonizes the anti-angiogenic effect of Epac/Rap1 via upregulation of ID1 and increases angiogenesis (Biyashev et al. 2010; Doebele et al. 2009).

Studies on the effects of VEGF-mediated MEK5/ERK5 activation in primary human dermal microvascular

ECs (HDMECs) point out that the activation of MEK5/ERK5 is specifically essential for tubular morphogenesis but not for cellular proliferation. During tubular morphogenesis MEK5/ERK5 appears to be vital in mediating VEGF-stimulated Akt activation with a consequent suppression of caspase-3 activity and facilitation of EC survival (Roberts et al. 2010). However, the mechanism by which MEK5/ERK5 regulates Akt is still obscure.

FAK (focal adhesion kinase) is a central signaling molecule in focal contact turnover (Mitra et al. 2005). Constant hyper-activation of FAK and its downstream target p130Cas augments migration and motility, diminishes matrix-cell interaction, and stress fiber formation (Johnson et al. 2008; Kaneda et al. 2008). Constitutive activation of ERK5 by expressing MEK5DD not only phosphorylates FAK at Ser^{910} but also decreases p130Cas level, and blocks cell migration (Spiering et al. 2009).

Cardiomyocytes

MEK5/ERK5 cascade provides a critical link between extrinsic and intrinsic signals leading to cardiac hypertrophy (Nicol et al. 2001; Suzaki et al. 2002; Cameron et al. 2004b; Nadruz et al. 2003; Takahashi et al. 2005). To examine the impact of ERK5 in hypertrophic remodeling, cardiomyocyte-specific deletion of the *erk5* gene ($ERK5^{cko}$) was generated. Following hypertrophic stress, $ERK5^{cko}$ exhibits decreased hypertrophic growth and fibrosis than the control. However, cardiomyocyte apoptosis is increased in the absence of ERK5 (Kimura et al. 2010). Inhibition of ERK5 using BIX02189 (selective ERK5 inhibitor) or silencing ERK5 using siERK5 shows a reduction of MEF2 (myocyte enhancer factor 2) transcriptional activity and the attenuation of ERK5-mediated hypertrophic response. These observations provide clear evidence of the role of MEK5/ERK5 in hypertrophic remodeling via regulation of MEF2 activity (Nadruz et al. 2003; Kimura et al. 2010).

In addition to hypertrophic remodeling, MEK5/ERK5 can protect cardiomyocyte from apoptosis by regulating PDE3A (phosphodiesterase 3)/ICER (inducible cAMP early repressor) feedback loop (Ding et al. 2005; Yan et al. 2007; Kimura et al. 2010). Inhibition of PDE3 significantly increases cardiomyocyte apoptosis (Ding et al. 2005; Mongillo et al. 2004). Persistent downregulation of PDE3A and concomitant upregulation of ICER (PDE3A/ICER feedback loop) has been shown to play a key role in the pathogenesis of cardiomyocyte apoptosis. (Tomita et al. 2003; Ding et al. 2005). It has been reported that insulin-like growth factor-1 represses cardiomyocyte apoptosis via activation of ERK5 and inhibition of the PDE3A/ICER feedback loop (Yan et al. 2007). The cardio-protective effect of ERK5 activation also involves CHIP (ubiquitin ligase of the C terminus of Hsc-interacting protein). Upon activation, ERK5 binds to CHIP, increases CHIP ubiquitin E3 ligase activity, promotes degradation of ICER, and ultimately leads to apoptosis inhibition (Woo et al. 2010).

Embryonic Progenitor Cells (Myoblast)

Activated by the key fetal growth factor IGF-2, MEK5/ERK5 pathway is required for the pro-myogenic action of IGF-2 in myoblast differentiation (Carter et al. 2009).

Fibroblasts

Studies using fibroblasts derived from *erk5*- or *mek5*-null embryo suggest that MEK5/ERK5 is important for fibroblast cell survival. Bim, a protein of ▶ Bcl-2 family, is known to become hyper-phosphorylated upon apoptotic stress. Under basal condition, ERK5 is required to inhibit Bim phosphorylation, therefore repressing its pro-apoptotic activity (Puthalakath et al. 1999). The loss of ERK5 expression correlates with increased death of mouse embryonic fibroblasts under basal conditions. This phenomenon confirms the role of MEK5/ERK5 in fibroblast survival under basal condition via inhibition of the Bim pro-apoptotic effect. However, under an osmotic stress condition, MEK5/ERK5 uses a different mechanism to protect cells from apoptosis. Although the incubation of fibroblasts with sorbitol causes Bim phosphorylation, there is no significant difference in the ability of sorbitol to phosphorylate Bim between wild type and *erk5*−/− or *mek5*−/− mouse embryonic fibroblasts. Instead, in *erk5*−/− and *mek5*−/− mouse embryonic fibroblasts treated with sorbitol, the expression of death receptor FasL is elevated via upregulation of Foxo3a activity. This increase of FasL is responsible for enhanced cell death. This observation demonstrates that, under osmotic condition, MEK5/ERK5 prevents fibroblast cell death by suppressing the expression of FasL via downregulation of Foxo3a (Wang et al. 2006).

Prostate Cancer (PCa) Cells

Evidence supports the important role of MEK5/ERK5 cascade in PCa (Mehta et al. 2003; McCracken et al. 2008). Reduced ERK5 expression using siRNA, or

reduced EKR5 function using the MEK inhibitor leads to the inhibition of the motility and invasive capacity of human prostate cancer cell line PC3 both in vitro and in vivo (Ramsay et al. 2011).

Breast Cancer Cells

STAT3, a major member of the STAT family, has a role in cell proliferation and differentiation (Darnell et al. 1994; Schaefer et al. 1995; Buettner et al. 2002). STAT3 activation is detected in breast cancer specimens in patients with advanced disease and breast carcinoma cell lines, but not in normal breast epithelial cells (Watson and Miller 1995; Garcia et al. 1997, 2001). Similarly, increased MEK5 expression is noted in breast carcinoma cell lines and breast cancer tissues concomitant hyper- phosphorylation of STAT3 is present. Gene profile examination of apoptosis-resistant and -sensitive MCF-7 breast cancer cell lines identified a 22-fold increase in MEK5/ERK5. Overexpression of a constitutively active STAT3 results in a marked induction of MEK5 expression, whereas blocking of STAT3 activity inhibits MEK5 expression in breast cancer cells. Therefore, the role of STAT3 as an upstream regulator for MEK5 induction was proposed (Weldon et al. 2002; Song et al. 2004). However, details on how STAT3 regulates MEK5 expression are largely unexplored.

Hepatocytes

TGF-β (transforming growth factor β) is a multifunctional cytokine that is involved in the regulation of hepatic growth (Bissell et al. 2001; Zavadil and Bottinger 2005). Snail, a zinc-finger transcription factor, is an early TGF-β-responsive gene (Cicchini et al. 2008), with its activity controlled by various signaling pathways at multiple levels (Barrallo-Gimeno and Nieto, 2005). Previous studies show the role of GSK-3β (glycogen synthase kinase-3β) in inactivation and degradation of Snail through the proteasomal pathway (Bachelder et al. 2005; Zhou et al. 2004). The degradation of Snail mediated by GSK-3β occurs in a MAPK-dependent manner (Zhou et al. 2004). TGFβ-mediated activation of MEK5/ERK5 appears essential for the functional inactivation of GSK-3β, and stabilization of the snail protein expression (Marchetti et al. 2008).

Neuronal Cells

Peripheral target-derived neurotrophins acting on neurotrophin receptors on the nerve terminals results in endocytosis and transport of the neurotrophin-receptor complex through the axon to the cell body. This retrograde signaling by neurotrophins acts via activation of ERK5 in the cell soma (Watson et al. 2001). A recent review indicates that the MEK5/ERK5 pathway mediates the promotion of neuronal survival and differentiation both in vitro and in vivo (Obara and Nakahata 2010).

Regulation of MEK5/ERK5 Activity

PB1 Domain-Containing Molecules

MEK5/ERK5 interacts specifically with one another but does not interact with kinases in the MEK1/ERK1 signaling pathway. This specificity is likely due to MEK5 PB1 domain, which determines the binding partners for this kinase. Molecules contain a PB1 domain such as atypical PKCς (aPKCς), ▶ MEK kinase (MEKK)2/3, p62 (Sequestosome 1), and Par-6 (Partitioning defective 6 homolog a) (Moscat et al. 2006) can associate with MEK5 via the PB1 domain (Lamark et al. 2003).

aPKCζ aPKCς regulates MEK5/ERK5 activity in an enzymatic activity-independent manner. The interaction between aPKCς and MEK5 is needed for EGF-mediated ERK5 stimulation (Diaz-Meco and Moscat 2001). Upon activation of Gq-couple G protein-coupled receptors (GPCR), both aPKCς and MEK5 bind to Gαq thus forming a ternary complex that seems essential for ERK5 activation (Garcia-Hoz et al. 2010). A recent study reported that aPKCς directly associates with and phosphorylates ERK5, and these events are required to increase eNOS protein degradation in ECs (Nigro et al. 2010).

(MEKK)2/3 Unlike aPKCς, (MEKK)2/3 activates MEK5/ERK5 in an enzymatic activity-dependent manner (Diaz-Meco and Moscat 2001). Although both MEKK2 and MEKK3 specifically interact with MEK5 and activate ERK5, the activation of ERK5 by MEKK2 is more potent than that of MEKK3. MEKK2 associates with Lad, an SH2 domain-containing adaptor protein, and activates Src kinase that is necessary for ERK5 activation in response to EGF. Thus, the association between MEKK2 and Lad indicates an important role of Src–Lad in MEKK2–MEK5/ERK5 signaling (Sun et al. 2003).

p62 Depletion of p62 using antisense oligonucleotide inhibits MEK5-mediated MEF2C transactivation

presenting a possible functional role of the p62–MEK5/ERK5 interaction (Lamark et al. 2003).

Others

AMP-activated Protein Pinase (AMPK) AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) is an agonist of AMPK. AICAR has been found to increase MEK5/ERK5 signaling. This increase is impeded by a dominant negative MEK5, thus rendering AMPK as an upstream regulator of MEK5 (Young et al. 2009). The exact regulatory mechanism between AMPK and MEK5 is unclear.

Epac1 (exchange factor for the small GTPase Rap1) The muscle-specific A kinase anchoring protein (mAKAP) signal transduction complex (mAKAP-PKA-PDE4D3) regulates cardiac hypertrophy. PDE3D3 acts as a scaffolding protein to recruit Epac1 to the mAKAP complex. ERK5-mediated PDE4D3 phosphorylation decreases phosphodiesterase activity and maintains a sustained peak of cAMP concentration (Hoffmann et al. 1999; Dodge-Kafka et al. 2005; Dodge-Kafka and Kapiloff 2006).

ERK5 SUMOylation

SUMOylation is a process of covalent attachment of SUMO (small ubiquitin-like modifier) proteins to specific lysine residues of target proteins (Johnson 2004). ERK5 has been reported to be SUMOylated at the NH_2-terminus (Lys-7 and Lys-22) when stimulated by ROS (reactive oxygen species) and AGE (Advanced Glycogen End product). The SUMOylation of ERK5 significantly inhibits ERK5 transcriptional activity (Woo et al. 2008). Inhibition of ERK5 SUMOylation by constitutively active (CA)-MEK5α is independent of ERK5 kinase activity, but dependent on the binding between MEK5-ERK5 (Shishido et al. 2008). Additionally, in diabetic hearts with exacerbation of left ventricular dysfunction after myocardial infarction, ERK5 SUMOylation is increased. Reduction of ERK5 SUMOylation using CA-MEK5α significantly improves cardiac function after myocardial infarction in diabetic mice, but not in nondiabetic mice. This observation implicates the crucial role of ERK5 SUMOylation in ROS-mediated ERK5 transcriptional repression and the contribution of this process to poor cardiac function after myocardial infarction in diabetics (Shishido et al. 2008).

Downstream Targets of MEK5/ERK5

Unlike other MAP kinases, ERK5 possesses not only a kinase domain that phosphorylates its substrates but also a transcriptional domain which is encoded by its long C-terminal region. Consequently, ERK5 transmits signals from MEK5 to downstream molecules either by phosphorylating or trans-activating. In the latter mechanism, ERK5 acts as a co-activator, binding to transcription factors and enhancing their activities (Akaike et al. 2004; Kasler et al. 2000). Furthermore, via direct or indirect protein–protein interaction, MEK5/ERK5 activates downstream signaling by phosphorylation.

Regulation via Phosphorylation

MEF2 (Myocyte Enhancer Factor 2) MEF2 belongs to a super family of transcription factors, consists of four *mef2* genes, referred to as MEF2A, – B,-C, and –D (Yang et al. 1998). MEK5/ERK5 specifically phosphorylate and activate MEF2A, –C, and –D but not MEF2B. MEK5/ERK5-induced MEF2 phosphorylation is necessary for the activation of MEF2 transcriptional activity by both ERK5 and growth factors (Kato et al. 1997; Kato et al. 2000). However, there is an alternative mechanism suggested that views the MEF2D transcriptional activity as predominantly regulated by ERK5 transcriptional activation domain and not by the kinase domain (Kasler et al. 2000). Members of the MEF2 family can bind to the endogenous KLF2 promoter and are crucial component of the transcriptional machinery required for the regulation of KLF2 expression (Parmar et al. 2006).

KLFs (Kruppel-Like Factors) KLF2: A mechano-activated transcription factor that control vasoprotective, anti-thrombotic, and anti-inflammatory responses to laminar flow (Dekker et al. 2002; SenBanerjee et al. 2004; Suzuki et al. 2005). KLF2 enhances endothelial nitric oxide synthase (eNOS) expression and reduces cytokine-mediated adhesion molecule expression (Lin et al. 2005; Parmar et al. 2006; SenBanerjee et al. 2004). The increase in KLF2 activity is triggered by steady laminar flow via the MEK5/ERK5/MEF2 signaling pathway.

KLF4: Likewise, *Klf4* has recently been reported as a shear stress-activated gene and an important regulator of inflammation in ECs. The activation of MEK5/ERK5 mediated by shear stress inhibits inflammatory responses in microvascular ECs, partly via ERK5-dependent induction of KLF4 (Clark et al. 2010). The expression

of KLF4 largely reproduces the protective phenotype in ECs. Overall, these results collectively underscore a major protective role of MEK5/ERK5/MEF2/KLF2 as well as -/KLF4 in ECs in response to pro-inflammatory stimuli (Parmar et al. 2006; Ohnesorge et al. 2010; Clark et al. 2010).

Cx43 (Connexin 43) MEK5/ERK5 activation is critical for EGF-mediated uncoupling of Cx43 gap junction, in which the activated ERK5 directly associates with, selectively phosphorylates Cx43 at Ser255, and regulates Cx43 gap junction uncoupling (Cameron et al. 2003).

Sap1a (TCF/Ets Transcription Factor SRF Accessory Protein-1a) The MEK5/ERK5 cascade activates the Serum Response Element (SRE) via Sap1a but not via the related transcription factor Elk1 and corroborated by the observation that MEK5/ERK5 can phosphorylate Sap1a but not Elk1. In contrast, ERK1/2 can phosphorylate and activate both Sap1a and Elk1. This difference proposes an overlapping but distinct function in regulating immediate early genes expression between the MEK5/ERK5 pathway and the ERK1/2 pathway (Kamakura et al. 1999).

Fos Family *c-Fos:* Activated MEK5/ERK5 augments the expression of oncogenic protein c-Fos (Kamakura et al. 1999), accounts for the phosphorylation and stabilization of c-Fos (Terasawa et al. 2003). Through the phosphorylation of c-Fos at Thr232 in the nuclear extraction signal (residues 221–233), MEK5/ERK5 inhibits c-Fos nuclear export. In addition, ERK5-mediated phosphorylation of c-Fos at Ser32 disrupts the interaction between c-Fos and the E3 ubiquitin ligase enzyme UBR1, and MEK5/ERK5 stabilizes c-Fos via phosphorylation of both Thr232 and Ser32 on c-Fos (Sasaki et al. 2006). ERK1/2 can also cause phosphorylation and stabilization of c-Fos. However, the activation of MEK5/ERK5 increases c-Fos transcriptional activity, but ERK1/2 does not. Both ERK5 kinase domain and transcriptional domain are required for the modulation of the transcriptional activity of c-Fos (Terasawa et al. 2003).

Fra-1: A member of the Fos family proteins, Fra-1 was previously found phosphorylated by ERK1/2. This phosphorylation is important for the activation of Fra-1 transcriptional activity (Young et al. 2002). Later, Terasawa et al. reported that the MEK5/ERK5 also regulates Fra-1 activity (Terasawa et al. 2003).

SGK (Serum- and Glucocorticoid-Inducible Kinase) Attempt to understand the role of MEK5/ERK5 in EGF-induced cell proliferation recognizes SGK as a cellular protein that interacts with ERK5. SGK, a serine/threonine kinase, is crucial for the G1/S cell cycle transition (Buse et al. 1999). Activated ERK5 phosphorylates SGK at Ser78 which is necessary for the activation of SGK and, more importantly, for cell proliferation (Hayashi et al. 2001).

p90RSK MEK5/ERK5 can phosphorylate p90RSK. However, physiological role of the p90RSK activation by ERK5 is controversial and may depend on the stimuli and cell type (Pi et al. 2005; Ranganathan et al. 2006).

Regulation via Interaction: PPARs (Peroxisome Proliferator-Activated Receptors)

PPARγ-1: Although activated MEK5/ERK5 mediates PPARγ-1 activation, a direct phosphorylation of PPARγ1 by ERK5 is not detected. Instead, association between the middle region (arginine-rich) on ERK5 and the hinge-helix region on PPARγ1 has been found (Akaike et al. 2004). The inactive N-terminal ERK5 kinase domain acts as a negative regulator of its middle region, and ERK5 activation induced by MEK5 disrupts this N-terminal inhibitory effect, leading to association of ERK5 with PPARγ1 and transactivation of PPARγ1.

PPARδ: MEK5/ERK5 also increases PPARδ transcriptional activity by a similar protein–protein association mechanism, but the binding site of ERK5 on PPARδ is different from that on PPARγ (Woo et al. 2006).

Nuclear Translocation

When ERK5 is non-phosphorylated the transcriptional activity in COOH-terminus is suppressed (Kondoh et al. 2006). Activation of ERK5 by MEK5 facilitates ERK5 auto-phosphorylation in the COOH-terminus and ERK5 is translocated from the cytoplasm to the nucleus (Mody et al. 2003; Carter et al. 2009).

Pharmacological Inhibitors

The unavailability of selective MEK5/ERK5 inhibitors is one of reasons why the number of studies for MEK5/ERK5 is limited compared to those for ERK1/2. In 2008, two novel selective MEK5 inhibitors, BIX02188 and BIX02189, were identified and these inhibitors were shown to inhibit the catalytic

function of purified MEK5 enzyme (Tatake et al. 2008). Availability of these selective MEK5/ERK5 inhibitors should contribute to further deciphering of the biological role of this signaling pathway.

Summary

Although strongly activated by s-flow which protects ECs from becoming dysfunctional (Ziegler et al. 1998; Gimbrone et al. 2000), the precise role of ERK5 in atherosclerosis is unknown. Depletion of ERK5 in Mx-1-Cre transgenic mice shows EC apoptosis and increased vascular permeability, but the role of ERK5 in other vessel functions such as vessel dilation and inflammation remains to be explored. Exposure of cultured ECs to s-flow potently activates the kinase and transcriptional activities of ERK5, and inhibits both leukocyte binding and adhesion molecule expression (Surapisitchat et al. 2001; Yamawaki et al. 2003). Identifying molecules that can negatively regulate ERK5 activity, and examining the interplay between those molecules and ERK5 might reveal a potential therapeutic venue for protecting ECs from becoming dysfunctional and treating vascular diseases.

References

Abe J, Kusuhara M, Ulevitch RJ, Berk BC, Lee JD. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. J Biol Chem. 1996;271:16586–90.

Akaike M, Che W, Marmarosh NL, Ohta S, Osawa M, Ding B, Berk BC, Yan C, Abe J. The hinge-helix 1 region of peroxisome proliferator-activated receptor gamma1 (PPARgamma1) mediates interaction with extracellular signal-regulated kinase 5 and PPARgamma1 transcriptional activation: involvement in flow-induced PPARgamma activation in endothelial cells. Mol Cell Biol. 2004;24(19):8691–704.

Bachelder RE, Yoon SO, Franci C, de Herreros AG, Mercurio AM. Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. J Cell Biol. 2005;168:29–33.

Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development. 2005;132:3151–61.

Bissell DM, Roulot D, George J. Transforming growth factor beta and the liver. Hepatology. 2001;34:859–67.

Biyashev D, Veliceasa D, Kwiatek A, Sutanto MM, Cohen RN, Volpert OV. Natural angiogenesis inhibitor signals through Erk5 activation of peroxisome proliferator-activated receptor gamma (PPARgamma). J Biol Chem. 2010;285:13517–24.

Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res. 2002;8:945–54.

Buse P, Tran SH, Luther E, Phu PT, Aponte GW, Firestone GL. Cell cycle and hormonal control of nuclear-cytoplasmic localization of the serum- and glucocorticoid-inducible protein kinase, Sgk, in mammary tumor cells. A novel convergence point of anti-proliferative and proliferative cell signaling pathways. J Biol Chem. 1999;274:7253–63.

Cameron SJ, Abe J, Malik S, Che W, Yang J. Differential role of MEK5alpha and MEK5beta in BMK1/ERK5 activation. J Biol Chem. 2004a;279?(2):1506–12.

Cameron SJ, Itoh S, Baines CP, Zhang C, Ohta S, Che W, Glassman M, Lee JD, Yan C, Yang J, Abe J. Activation of big MAP kinase 1 (BMK1/ERK5) inhibits cardiac injury after myocardial ischemia and reperfusion. FEBS Lett. 2004b;566:1–3.

Cameron SJ, Malik S, Akaike M, Lerner-Marmarosh N, Yan C, Lee JD, Abe J, Yang J. Regulation of epidermal growth factor-induced connexin 43 gap junction communication by big mitogen-activated protein kinase1/ERK5 but not ERK1/2 kinase activation. J?Biol Chem. 2003;278?(20):18682–8.

Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005;438:932–6.

Carter EJ, Cosgrove RA, Gonzalez I, Eisemann JH, Lovett FA, Cobb LJ, Pell JM. MEK5 and ERK5 are mediators of the pro-myogenic actions of IGF-2. J Cell Sci. 2009;122:3104–12.

Cicchini C, Laudadio I, Citarella F, Corazzari M, Steindler C, Conigliaro A, Fantoni A, Amicone L, Tripodi M. TGFbeta-induced EMT requires focal adhesion kinase (FAK) signaling. Exp Cell Res. 2008;314:143–52.

Clark PR, Jensen T, Kluger MS, Morelock M, Hanidu A, Qi Z, Tatake RJ, Pober JS. MEK5 is activated by shear stress, activates ERK5 and induces KLF4 to modulate TNF responses in human dermal microvascular endothelial cells. Microcirculation. 2010;18(2):102–17.

Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264:1415–21.

Dekker RJ, van Soest S, Fontijn RD, Salamanca S, de Groot PG, VanBavel E, Pannekoek H, Horrevoets AJ. Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLF2). Blood. 2002;100:1689–98.

Diaz-Meco MT, Moscat J. MEK5, a new target of the atypical protein kinase C isoforms in mitogenic signaling. Mol Cell Biol. 2001;21:1218–27.

Ding B, Abe J, Wei H, Xu H, Che W, Aizawa T, Liu W, Molina CA, Sadoshima J, Blaxall BC, Berk BC, Yan C. A positive feedback loop of phosphodiesterase 3 (PDE3) and inducible cAMP early repressor (ICER) leads to cardiomyocyte apoptosis. Proc Natl Acad Sci USA. 2005;102:14771–6.

Dodge-Kafka KL, Kapiloff MS. The mAKAP signaling complex: integration of cAMP, calcium, and MAP kinase signaling pathways. Eur J Cell Biol. 2006;85:593–602.

Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS, Scott JD. The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. Nature. 2005;437:574–8.

Doebele RC, Schulze-Hoepfner FT, Hong J, Chlenski A, Zeitlin BD, Goel K, Gomes S, Liu Y, Abe MK, Nor JE, Lingen MW, Rosner MR. A novel interplay between

Epac/Rap1 and mitogen-activated protein kinase kinase 5/extracellular signal-regulated kinase 5 (MEK5/ERK5) regulates thrombospondin to control angiogenesis. Blood. 2009;114:4592–600.

English JM, Vanderbilt CA, Xu S, Marcus S. Cobb MHIsolation of MEK5 and differential expression of alternatively spliced forms. J?Biol Chem. 1995;270?(48):28897–902.

Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, Cox CE, Falcone R, Fairclough R, Parsons S, Laudano A, Gazit A, Levitzki A, Kraker A, Jove R. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene. 2001;20:2499–513.

Garcia R, Yu CL, Hudnall A, Catlett R, Nelson KL, Smithgall T, Fujita DJ, Ethier SP, Jove R. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. Cell Growth Differ. 1997;8:1267–76.

Garcia-Hoz C, Sanchez-Fernandez G, Diaz-Meco MT, Moscat J, Mayor F, Ribas C. G alpha(q) acts as an adaptor protein in protein kinase C zeta (PKCzeta)-mediated ERK5 activation by G protein-coupled receptors (GPCR). J Biol Chem. 2010;285:13480–9.

Gimbrone, MA, Jr. Topper, JN, Nagel T, Anderson KR, Garcia-Cardena G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. Ann NY Acad Sci. 2000;902:230-9; discussion 239–40.

Hayashi M, Kim SW, Imanaka-Yoshida K, Yoshida T, Abel ED, Eliceiri B, Yang Y, Ulevitch RJ, Lee JD. Targeted deletion? of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. J Clin Invest. 2004;113 (8): 1138–48.

Hayashi M, Tapping RI, Chao TH, Lo JF, King CC, Yang Y, Lee JD. BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of serum and glucocorticoid-inducible kinase. J Biol Chem. 2001;276:8631–4.

Hoffmann R, Baillie GS, MacKenzie SJ, Yarwood SJ, Houslay MD. The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. EMBO J. 1999;18:893–903.

Johnson ES. Protein modification by SUMO. Annu Rev Biochem. 2004;73:355–82.

Johnson TR, Khandrika L, Kumar B, Venezia S, Koul S, Chandhoke R, Maroni P, Donohue R, Meacham RB, Koul HK. Focal adhesion kinase controls aggressive phenotype of androgen-independent prostate cancer. Mol Cancer Res. 2008;6:1639–48.

Kamakura S, Moriguchi T, Nishida E. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. J Biol Chem. 1999;274:26563–71.

Kaneda T, Sonoda Y, Ando K, Suzuki T, Sasaki Y, Oshio T, Tago M, Kasahara T. Mutation of Y925F in focal adhesion kinase (FAK) suppresses melanoma cell proliferation and metastasis. Cancer Lett. 2008;270:354–61.

Kasler HG, Victoria J, Duramad O, Winoto A. ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. Mol Cell Biol. 2000;20:8382–9.

Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. EMBO J. 1997;16:7054–66.

Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ, Lee JD. Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature. 1998;395:713–6.

Kato Y, Zhao M, Morikawa A, Sugiyama T, Chakravortty D, Koide N, Yoshida T, Tapping RI, Yang Y, Yokochi T, Lee JD. Big mitogen-activated kinase regulates multiple members of the MEF2 protein family. J Biol Chem. 2000;275 (24):18534–40.

Kimura TE, Jin J, Zi M, Prehar S, Liu W, Oceandy D, Abe J, Neyses L, Weston AH, Cartwright EJ, Wang X. Targeted deletion of the extracellular signal-regulated protein kinase 5 attenuates hypertrophic response and promotes pressure overload-induced apoptosis in the heart. Circ Res. 2010;106:961–70.

Kondoh K, Terasawa K, Morimoto H, Nishida E. Regulation of nuclear translocation of extracellular signal-regulated kinase 5 by active nuclear import and export mechanisms. Mol Cell Biol. 2006;26:1679–90.

Lamark T, Perander M, Outzen H, Kristiansen K, Overvatn A, Michaelsen E, Bjorkoy G, Johansen T. Interaction codes within the family of mammalian Phox and Bem1p domain-containing proteins. J Biol Chem. 2003;278:34568–81.

Li L, Tatake RJ, Natarajan K, Taba Y, Garin G, Tai C, Leung E, Surapisitchat J, Yoshizumi M, Yan C, Abe J, Berk BC. Fluid shear stress inhibits TNF-mediated JNK activation via MEK5-BMK1 in endothelial cells. Biochem Biophys Res Commun. 2008;370:159–63.

Lin Z, Kumar A, Senbanerjee S, Staniszewski K, Parmar K, Vaughan DE, Gimbrone Jr MA, Balasubramanian V, Garcia-Cardena G, Jain MK. Kruppel-like factor 2 (KLF2) regulates endothelial thrombotic function. Circ Res. 2005;96 (5):e48–57.

Marchetti A, Colletti M, Cozzolino AM, Steindler C, Lunadei M, Mancone C, Tripodi M. ERK5/MAPK is activated by TGFbeta in hepatocytes and required for the GSK-3beta-mediated Snail protein stabilization. Cell Signal. 2008;20:2113–8.

McCracken SR, Ramsay A, Heer R, Mathers ME, Jenkins BL, Edwards J, Robson CN, Marquez R, Cohen P, Leung HY. Aberrant expression of extracellular signal-regulated kinase 5 in human prostate cancer. Oncogene. 2008;27:2978–88.

Mehta PB, Jenkins BL, McCarthy L, Thilak L, Robson CN, Neal DE, Leung HY. MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion. Oncogene. 2003;22:1381–9.

Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol. 2005;6:56–68.

Mody N, Campbell DG, Morrice N, Peggie M, Cohen P. An analysis of the phosphorylation and activation of extracellular-signal-regulated protein kinase 5 (ERK5) by mitogen-activated protein kinase kinase 5 (MKK5) in vitro. Biochem J. 2003;372:567–75.

Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M. Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ Res. 2004;95:67–75.

Moscat J, Diaz-Meco MT, Albert A, Campuzano S. Cell signaling and function organized by PB1 domain interactions. Mol Cell. 2006;23:631–40.

Nadruz W Jr, Kobarg CB, Constancio SS, Corat PD, Franchini KG. Load-induced transcriptional activation of c-jun in rat myocardium: regulation by myocyte enhancer factor 2. Circ Res. 2003;92:243–51.

Nakamura K, Johnson GL. Noncanonical function of MEKK2 and MEK5 PB1 domains for coordinated extracellular signal-regulated kinase 5 and c-Jun N-terminal kinase signaling. Mol Cell Biol. 2007;27:4566–77.

Nakamura K, Johnson GL. PB1 domains of MEKK2 and MEKK3 interact with the MEK5 PB1 domain for activation of the ERK5 pathway. J Biol Chem. 2003;278:36989–92.

Nakamura K, Uhlik MT, Johnson NL, Hahn KM, Johnson GL. PB1 domain-dependent signaling complex is required for extracellular signal-regulated kinase 5 activation. Mol Cell Biol. 2006;26:2065–79.

Nicol RL, Frey N, Pearson G, Cobb M, Richardson J, Olson EN. Activated MEK5 induces serial assembly of sarcomeres and eccentric cardiac hypertrophy. EMBO J. 2001;20:2757–67.

Nigro P, Abe J, Woo CH, Satoh K, McClain C, O'Dell MR, Lee H, Lim JH, Li JD, Heo KS, Fujiwara K, Berk BC. PKCzeta decreases eNOS protein stability via inhibitory phosphorylation of ERK5. Blood. 2010;116:1971–9.

Obara Y, Nakahata N. The signaling pathway leading to extracellular signal-regulated kinase 5 (ERK5) activation via G-proteins and ERK5-dependent neurotrophic effects. Mol Pharmacol. 2010;77:10–6.

Ohnesorge N, Viemann D, Schmidt N, Czymai T, Spiering D, Schmolke M, Ludwig S, Roth J, Goebeler M, Schmidt M. Erk5 activation elicits a vasoprotective endothelial phenotype via Induction of Krüppel-like Factor 4 (KLF4). J Biol Chem. 2010;285 (34):26199–210.

Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, Kratz JR, Lin Z, Jain MK, Gimbrone Jr MA, Garcia-Cardena G. Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. J Clin Invest. 2006;116:49–58.

Pasceri V, Wu HD, Willerson JT, Yeh ET. Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. Circulation. 2000;101:235–8.

Pi X, Garin G, Xie L, Zheng Q, Wei H, Abe J, Yan C, Berk BC. BMK1/ERK5 is a novel regulator of angiogenesis by destabilizing hypoxia inducible factor 1alpha. Circ Res. 2005;96 (11):1145–51.

Pi X, Yan C, Berk BC. Big mitogen-activated protein kinase (BMK1)/ERK5 protects endothelial cells from apoptosis. Circ Res. 2004;94:362–9.

Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell. 1999;3:287–96.

Ramsay AK, McCracken SR, Soofi M, Fleming J, Yu AX, Ahmad I, Morland R, Machesky L, Nixon C, Edwards DR, Nuttall RK, Seywright M, Marquez R, Keller E, Leung HY. ERK5 signalling in prostate cancer promotes an invasive phenotype. Br J Cancer. 2011;104:664–72.

Ranganathan A, Pearson GW, Chrestensen CA, Sturgill TW, Cobb MH. The MAP kinase ERK5 binds to and phosphorylates p90 RSK. Arch Biochem Biophys. 2006;449:8–16.

Roberts OL, Holmes K, Muller J, Cross DA, Cross MJ. ERK5 is required for VEGF-mediated survival and tubular morphogenesis of primary human microvascular endothelial cells. J Cell Sci. 2010;123:3189–200.

Sasaki T, Kojima H, Kishimoto R, Ikeda A, Kunimoto H, Nakajima K. Spatiotemporal regulation of c-Fos by ERK5 and the E3 ubiquitin ligase UBR1, and its biological role. Mol Cell. 2006;24 (16):63–75.

Schaefer TS, Sanders LK, Nathans D. Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3. Proc Natl Acad Sci USA. 1995;92:9097–101.

SenBanerjee S, Lin Z, Atkins GB, Greif DM, Rao RM, Kumar A, Feinberg MW, Chen Z, Simon DI, Luscinskas FW, Michel TM, Gimbrone Jr MA, Garcia-Cardena G, Jain MK. KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. J Exp Med. 2004;199:1305–15.

Seyfried J, Wang X, Kharebava G, Tournier C. A novel mitogen-activated protein kinase docking site in the N terminus of MEK5alpha organizes the components of the extracellular signal-regulated kinase 5 signaling pathway. Mol Cell Biol. 2005;25:9820–8.

Shishido T, Woo CH, Ding B, McClain C, Molina CA, Yan C, Yang J, Abe J. Effects of MEK5/ERK5 association on small ubiquitin-related modification of ERK5: implications for diabetic ventricular dysfunction after myocardial infarction. Circ Res. 2008;102 (11):1416–25.

Song H, Jin X, Lin J. Stat3 upregulates MEK5 expression in human breast cancer cells. Oncogene. 2004;23:8301–9.

Spiering D, Schmolke M, Ohnesorge N, Schmidt M, Goebeler M, Wegener J, Wixler V, Ludwig S. MEK5/ERK5 signaling modulates endothelial cell migration and focal contact turnover. J Biol Chem. 2009;284:24972–80.

Squires MS, Nixon PM, Cook SJ. Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1. Biochem J. 2002;366: 673–80.

Sun W, Wei X, Kesavan K, Garrington TP, Fan R, Mei J, Anderson SM, Gelfand EW, Johnson GL. MEK kinase 2 and the adaptor protein Lad regulate extracellular signal-regulated kinase 5 activation by epidermal growth factor via Src. Mol Cell Biol. 2003;23 (7):2298–308.

Surapisitchat J, Yoshizumi M, Hoefen R, Yan C, Berk BC. Fluid shear stress inhibits TNF-alpha activation of JNK, but not ERK1/2 or p38 in human umbilical vein endothelial cells: Inhibitory crosstalk among MAPK family members. Proc Natl Acad Sci USA. 2001;98(11):6476–81.

Suzaki Y, Yoshizumi M, Kagami S, Koyama AH, Taketani Y, Houchi H, Tsuchiya K, Takeda E, Tamaki T. Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. J Biol Chem. 2002;277:9614–21.

Suzuki T, Aizawa K, Matsumura T, Nagai R. Vascular implications of the Kruppel-like family of transcription factors. Arterioscler Thromb Vasc Biol. 2005;25:1135–41.

Takahashi N, Saito Y, Kuwahara K, Harada M, Tanimoto K, Nakagawa Y, Kawakami R, Nakanishi M, Yasuno S, Usami S, Yoshimura A, Nakao K. Hypertrophic responses to cardiotrophin-1 are not mediated by STAT3, but via a MEK5-ERK5 pathway in cultured cardiomyocytes. J Mol Cell Cardiol. 2005;38:185–92.

Tatake RJ, O'Neill MM, Kennedy CA, Wayne AL, Jakes S, Wu D, Kugler Jr SZ, Kashem MA, Kaplita P, Snow RJ. Identification of pharmacological inhibitors of the MEK5/ERK5 pathway. Biochem Biophys Res Commun. 2008;377:120–5.

Terasawa K, Okazaki K, Nishida E. Regulation of c-Fos and Fra-1 by the MEK5-ERK5 pathway. Genes Cells. 2003;8:263–73.

Tomita H, Nazmy M, Kajimoto K, Yehia G, Molina CA, Sadoshima J. Inducible cAMP early repressor (ICER) is a negative-feedback regulator of cardiac hypertrophy and an important mediator of cardiac myocyte apoptosis in response to beta-adrenergic receptor stimulation. Circ Res. 2003;93:12–22.

Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler Thromb Vasc Biol. 1998;18:677–85.

Wang X, Finegan KG, Robinson AC, Knowles L, Khosravi-Far R, Hinchliffe KA, Boot-Handford RP, Tournier C. Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. Cell Death Differ. 2006;13:2099–108.

Wang X, Merritt AJ, Seyfried J, Guo C, Papadakis ES, Finegan KG, Kayahara M, Dixon J, Boot-Handford RP, Cartwright EJ, Mayer U, Tournier C. Targeted deletion of mek5 causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. Mol Cell Biol. 2005;25:336–45.

Watson CJ, Miller WR. Elevated levels of members of the STAT family of transcription factors in breast carcinoma nuclear extracts. Br J Cancer. 1995;71:840–4.

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci. 2001;4:981–8.

Weldon CB, Scandurro AB, Rolfe KW, Clayton JL, Elliott S, Butler NN, Melnik LI, Alam J, McLachlan JA, Jaffe BM, Beckman BS, Burow ME. Identification of mitogen-activated protein kinase kinase as a chemoresistant pathway in MCF-7 cells by using gene expression microarray. Surgery. 2002;132:293–301.

Woo CH, Le NT, Shishido T, Chang E, Lee H, Heo KS, Mickelsen DM, Lu Y, McClain C, Spangenberg T, Yan C, Molina CA, Yang J, Patterson C, Abe J. Novel role of C terminus of Hsc70-interacting protein (CHIP) ubiquitin ligase on inhibiting cardiac apoptosis and dysfunction via regulating ERK5-mediated degradation of inducible cAMP early repressor. FASEB J. 2010;24:4917–28.

Woo CH, Massett MP, Shishido T, Itoh S, Ding B, McClain C, Che W, Vulapalli SR, Yan C, Abe J. ERK5 activation inhibits inflammatory responses via peroxisome proliferator-activated receptor delta (PPARdelta) stimulation. J Biol Chem. 2006;281(43):32164–74.

Woo CH, Shishido T, McClain C, Lim JH, Li JD, Yang J, Yan C, Abe J. Extracellular signal-regulated kinase 5 SUMOylation antagonizes shear stress-induced antiinflammatory response and endothelial nitric oxide synthase expression in endothelial cells. Circ Res. 2008;102(5):538–45.

Yamawaki H, Lehoux S, Berk BC. Chronic physiological shear stress inhibits tumor necrosis factor-induced proinflammatory responses in rabbit aorta perfused ex vivo. Circulation. 2003;108:1619–25.

Yan C, Ding B, Shishido T, Woo CH, Itoh S, Jeon KI, Liu W, Xu H, McClain C, Molina CA, Blaxall BC, Abe J. Activation of extracellular signal-regulated kinase 5 reduces cardiac apoptosis and dysfunction via inhibition of a phosphodiesterase 3A/inducible cAMP early repressor feedback loop. Circ Res. 2007;100:510–9.

Yan C, Takahashi M, Okuda M, Lee JD, Berk BC. Fluid shear stress stimulates big mitogen-activated protein kinase 1 (BMK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. J Biol Chem. 1999;274:143–50.

Yang CC, Ornatsky OI, McDermott JC, Cruz TF, Prody CA. Interaction of myocyte enhancer factor 2 (MEF2) with a mitogen- activated protein kinase, ERK5/BMK1. Nucleic Acids Res. 1998;26:4771–7.

Young A, Wu W, Sun W, Larman HB, Wang N, Li YS, Shyy JY, Chien S, Garcia-Cardena G. Flow activation of AMP-activated protein kinase in vascular endothelium leads to Kruppel-like factor 2 expression. Arterioscler Thromb Vasc Biol. 2009;29:1902–8.

Young MR, Nair R, Bucheimer N, Tulsian P, Brown N, Chapp C, Hsu TC, Colburn NH. Transactivation of Fra-1 and consequent activation of AP-1 occur extracellular signal-regulated kinase dependently. Mol Cell Biol. 2002;22:587–98.

Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene. 2005;24:5764–74.

Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol. 2004;6:931–40.

Zhou G, Bao ZQ, Dixon JE. Components of a new human protein kinase signal transduction pathway. J Biol Chem. 1995;270(21):12665–9.

Ziegler, T, Bouzourene K, Harrison VJ, Brunner, HR, Hayoz D. Influence of oscillatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in cultured endothelial cells. Arterioscler Thromb Vasc Biol. 1998;18:686–92.

MEK5/ERK5: Mitogen-Activated Protein Kinase 5/Extracellular Signal-Regulated Kinase 5

► MEK5/ERK5

MEK6

► MKK6

MEL

► Rab8

Mel Transforming Oncogene (RAB8 Homolog)

▶ Rab8

Melastatin-Like TRP Channels, TRPM1-8

▶ TRP (Transient Receptor Potential Cation Channel)

MELF

▶ Laforin: Function and Action of a Glucan Phosphatase

Member 1 (KLRK1)

▶ NKG2D

Membrane Component, Chromosome 17, Surface Marker 1 (M17S1)

▶ Flotillin-2 (FLOT2)

Metabotropic Glutamate Receptors

▶ Glutamate Receptors

Metal-Dependent Protein Phosphatase

▶ PP2C

MGC:3310

▶ TRAF6

MGC124948

▶ Rab8

MGC129998

▶ RasGRP1

MGC129999

▶ RasGRP1

MGC131738

▶ PHLDA1 (Pleckstrin Homology-like Domain, Family A, Member): Alias: PHRIP; TDAG51; DT1P1B11; MGC131738

MGC131975

▶ CDK11

MGC39955

▶ Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

MGC9013

▶ CD40

Microtubule Affinity Regulating Kinases (MARK)

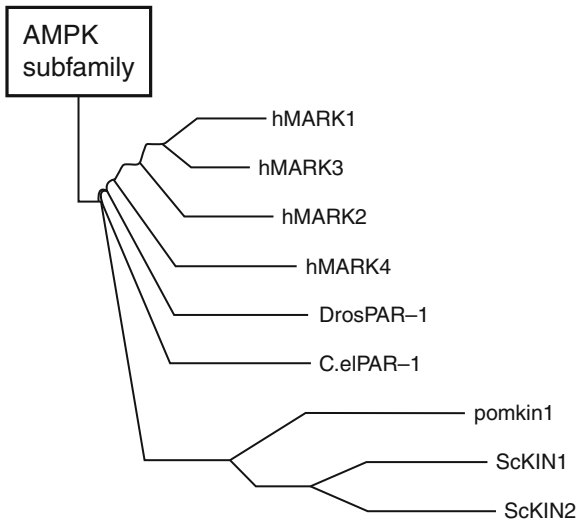
Gerard Drewes
Discovery Research, Cellzome AG, Heidelberg, Germany

Synonyms

MARK1: EMK3, hPAR-1c, KIAA1477; MARK2: EMK1, hPAR-1b; MARK3: EMK3, hPAR-1a, C-TAK1, KP78; MARK4: MARKL1, hPAR-1d, KIAA1860

Historical Background

Microtubule affinity regulating kinase (MARK) was first identified as an activity in brain extract with the ability to phosphorylate the microtubule-associated protein (MAP) tau and inhibit its binding to microtubules (MTs) (Drewes et al. 1995). All four human MARKs phosphorylate tau and the structurally related proteins MAP2 and MAP4 on their MT-binding domains, causing their dissociation from MTs in purified systems and in cells, which in turn leads to destabilization of MTs (Drewes et al. 1997, 1998). Since hyperphosphorylation and mislocalization of tau represent hallmarks of the neurofibrillar pathology of Alzheimer's disease (AD), MARKs have attracted interest as potential therapeutic targets early on (Drewes 2004). Other clues for the function of MARKs in cytoskeletal regulation and cell polarity resulted from studies in epithelial cells (Bohm et al. 1997) and from MARK orthologues in fission yeast (Drewes and Nurse 2003) and the extensive data from the nematode *Caenorhabditis elegans* (Kemphues 2000).

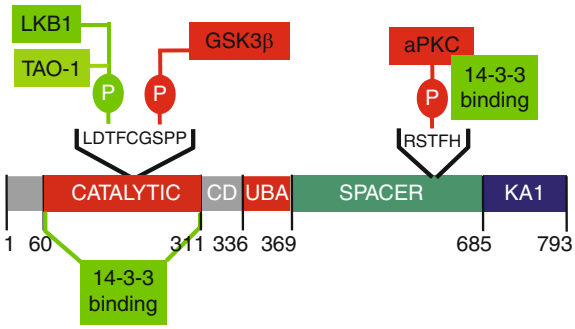


Microtubule Affinity Regulating Kinases (MARK), Fig. 1 MARK kinases are a part of the subfamily of the AMPK (AMP-dependent protein kinase)-related kinases. In humans, there are four MARK genes and 28 pseudogenes. The phylogenetic tree displays the relationship between the four human MARK gene products (GenBank numbers: MARK1; AB040910, MARK2; X97630, MARK3; U64205, MARK4; AY057448) and their orthologues from *Drosophila melanogaster* (PAR-1; AF258462), *Caenorhabditis elegans* (PAR-1; U22183), *Schizosaccharomyces pombe* (kin1; M64999), and *Saccharomyces cerevisiae* (KIN1; M69017, KIN2; M69018)

Phylogeny, Structure, and Regulation

Phylogeny: The MARK kinases represent a subgroup within the AMPK subfamily of the Ca^{2+}-calmodulin-dependent protein kinase branch of the human kinome. They are related to orthologous kinases in flies, worms, and yeast (Fig. 1). Humans express the four paralogous genes MARK1–MARK4 which undergo alternative splicing (Drewes et al. 1998). All paralogues comprise an N-terminal header (N), followed by a conserved catalytic domain and a ubiquitin-associated domain (UBA), which links via a more divergent spacer domain to the C-terminal "kinase associated domain" (KA1) (Fig. 2). The UBA and KA1 domains are only present in the AMPK-related protein kinases. The UBA domain plays a role in MARK activation (Jaleel et al. 2006).

Structure: Several crystal structures have been reported including the catalytic and UBA domains of MARK1, MARK2, and MARK3 (Marx et al. 2010), and the KA domain (Moravcevic et al. 2010). The structures of the catalytic domain are all in the inactive state and indicate a propensity for dimer formation. The overall structure of the kinase domain resembles that of other eukaryotic protein kinases consisting of a smaller N-terminal lobe and a larger C-terminal lobe with the active site pocket with the nucleotide located in between (Marx et al. 2010). The larger lobe contains the activation segment which, depending on its phosphorylation state, inhibits or permits the proper access of the peptidic substrate to the MgATP in the active site (see below). The UBA domain is located at the C-terminal end of the large lobe and may be able to confer autoinhibition by directly binding to the N-terminal lobe. The catalytic and UBA domains are connected via an acidic stretch of 15 amino acids, which bears similarity to the common docking domain in MAPK family kinases where it functions to bind upstream activating kinases and inactivating phosphatases. The C-terminus of MARK kinases folds into a compact domain ("KA1 domain") which is formed by five beta strands and two alpha helices and ends in an ELKL motif. This domain is less conserved in MARK4 which terminates with a DLEL sequence. The KA1 domain is a membrane association domain that binds acidic phospholipids like phosphatidylserine (Moravcevic et al. 2010).



Microtubule Affinity Regulating Kinases (MARK), Fig. 2 The domain structure of the four mammalian MARK/Par-1 kinases is conserved. A short diverse N-terminal sequence is followed by the catalytic domain, followed by a UBA (ubiquitin-associated) domain, which might be involved in the interaction with other proteins in a ubiquitin-dependent fashion. The C-terminal KA1 (kinase-associated) domain functions as a membrane anchor. All four human MARKs are activated by the phosphorylation of a conserved threonine residue by LKB1, and MARK2 can also be activated by TAO-1. The serine-proline motif nearby can be phosphorylated by GSK3b and this appears to confer inhibition. The serine in the spacer domain is phosphorylated by aPKC and regulates membrane localization. MARK/Par-1 can form complexes with 14-3-3 family proteins, which bind to the catalytic domain with a site on 14-3-3 outside the known phospholigand binding pocket

Regulation: MARK kinases are subject to diverse regulatory mechanisms including posttranslational modifications and the association with inhibitory proteins. The posttranslational modifications include phosphorylation and ubiquitination (Al-Hakim et al. 2008; Brajenovic et al. 2004; Lizcano et al. 2004; Timm et al. 2003). MARK2 purified from tissue is phosphorylated on T208 and S212 in the activation loop (Drewes et al. 1997). Phosphorylation of T208 (and the homologous sites on other MARKs) is required for activation and can be induced by LKB1, which constitutes a mammalian orthologue of *Drosophila melanogaster* and *C. elegans* PAR-4, and by TAO1/MAP3K16. Both upstream kinases increase MT dynamics through MARK activation (Kojima et al. 2007; Timm et al. 2003). Phosphorylation of S212 is mediated by GSK3β and leads to inactivation (Timm et al. 2008), although there is a conflicting report (Kosuga et al. 2005). Phosphorylation of S595 on MARK2 (and the homologous sites on other MARK isoforms) by aPKC (atypical PKC, a member of the Par-6 polarity complex) inactivates MARK by inducing its association with 14-3-3 proteins which in turn causes the delocalization from membranous compartments in polarized cells (Hurov and Piwnica-Worms 2007; Suzuki et al. 2004). MARK3 was shown to be inhibited by phosphorylation by ▶ Pim-1 (Bachmann et al. 2006) and MARK2 is inhibited by association with PAK5. It was proposed that the interplay between MARKs and the actin-modulating kinases PAK5 and TESK1 may provide a mechanism to route incoming signals to antagonistic MT network- or actin network-dependent cytoskeletal activity (Matenia et al. 2005). The proteomic analysis of MARK4 by tandem affinity purification revealed co-purification of several additional proteins including 14-3-3 proteins, the ubiquitin-dependent proteases USP7 and USP9x, subunits of protein phosphatase 2, and gamma-tubulin (Brajenovic et al. 2004). MARK4 and possibly other MARKs are subject to inhibitory polyubiquitination in vivo by unusual K29/K33-linked ubiquitin chains, which are regulated by USP9X (Al-Hakim et al. 2008). The interaction with 14-3-3 proteins appears to constitute a major function of MARKs (see below).

MARKs and Cell Morphology

MARKs and its orthologues in lower eukaryotes comprise protein kinases conserved from yeast to *C. elegans* and *Drosophila* to mammals, which are essential for cellular polarity, governing the establishment of the embryonic body axis and maintaining cell differentiation. The MARK/Par-1 kinase regulates the localization of structural PAR-proteins, and tightly regulated phosphorylation events provide a cellular regulatory mechanism governing important developmental decisions (Chen et al. 2006). In epithelial cells MARK2 is asymmetrically localized and knockdown or overexpression of inactive mutants perturbs the polarity in this cell line suggesting a conserved mechanism governing polarization from *C. elegans* embryos to mammalian cells (Cohen et al. 2007). Many of the effects of MARK on cell polarity are mediated by the MT system. In neurons, the phosphorylation of MAPs controls the binding to MTs, and bound MAPs stabilize the tubulin polymer, and their phosphorylation potentially provides a mechanism for regulating stability of MTs in a spatial and temporal fashion. MTs function as structural elements of cell morphology and as "tracks" for intracellular transport of vesicles and organelles (Matenia and Mandelkow 2009). Therefore, kinases that regulate MT stability may not only control cell shape and polarity but also cellular transport mediated

by the interplay of MAPs and motor proteins. In neurons, MARK2 and possibly MARK4 play role in neurite outgrowth and maintenance of neuronal polarity, a process requiring dynamic instability of MTs (Yoshimura et al. 2010; Trinczek et al. 2004). In hippocampal neurons, MARK2 overexpression inhibits axon formation whereas RNAi-mediated MARK2 knockdown induces multiple axons (Chen et al. 2006). MARK4 is absent from neuronal progenitor cells, but upregulated during neuronal differentiation (Moroni et al. 2006). MARK2 is also involved in the control of neuronal migration through the phosphorylation of doublecortin, a MAP present in the leading process of migrating neurons, which has been implied in neuronal migration disorders (Sapir et al. 2008). MARKs also impact body axis specification and morphogenic signaling via modulation of the Wnt signaling pathway by the phosphorylation of dishevelled (Dsh) which acts at a branching point for developmental decisions (Elbert et al. 2006).

Substrates of MARKs

Microtubule-associated proteins: Several of the effects of MARKs on the microtubule system are mediated via the phosphorylation of MAPs on serine and threonine residues. All four MARK isoforms can phosphorylate Tau and the related MAP2, MAP2c, and MARK4 at the repeated Lys-Xaa-Gly-Ser motifs in the MT-binding domain (Drewes et al. 1998). MARKs also phosphorylate other components of the MT system including doublecortins and motor proteins (Sapir et al. 2008; Yoshimura et al. 2010).

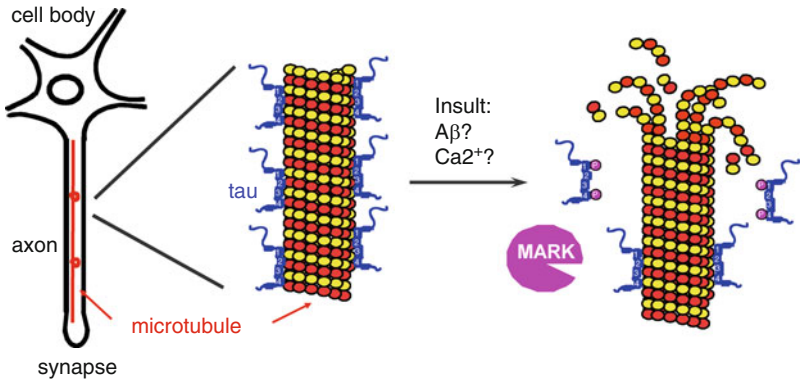
14-3-3 binding proteins: 14-3-3 proteins are phosphoserine adaptor proteins binding to multiple clients with pleiotropic cellular functions, e.g., in protein translocation and vesicle trafficking. MARK3 was shown to phosphorylate 14-3-3 binding motifs on different substrates: the phosphatases PTPH1 and CDC25; the suppressor of Ras/MAPK signaling KSR; the desmosomal protein plakophilin-2, and histone deacetylases. The phosphorylation of these substrates by MARK induces the formation of a complex between the substrate protein and 14-3-3, and changes the cellular localization and thus the function of the substrate (Matenia and Mandelkow 2009). 14-3-3 proteins also interact directly with the MARK catalytic domain, enabling the kinase to target substrates to 14-3-3 and re-phosphorylate sites quickly after dephosphorylation and disruption of 14-3-3 binding (Muller et al. 2003). Alternatively, the spacer domain of MARK2 can be phosphorylated by aPKC to generate a 14-3-3 binding motif leading to relocalization and inactivation (Hurov and Piwnica-Worms 2007).

Role of MARKs in Disease

Helicobacter infection: *Helicobacter pylori* is a pathogen associated with gastritis, ulcerations, and gastric adenocarcinoma. The virulence factor CagA can bind MARK2, inhibit its aPKC-dependent activation, and delocalize it from the membrane, causing junctional and polarity defects in gastric epithelial cells which results in mucosal damage, inflammation, and carcinogenesis (Saadat et al. 2007).

Neurodegenerative disease: The degeneration of neurons in AD and related diseases is characterized by filamentous aggregates of hyperphosphorylated proteins in neurons. Tau protein is the major component of these neurofibrillary tangles which cause neuronal death. Evidence implicating MARK in this process stems from models of tauopathies in the fruit fly *D. melanogaster* where the overexpression of the fly MARK orthologue PAR-1 causes neuronal degeneration dependent on both kinase activity and the presence of endogenous fly tau, and degeneration induced by exogenous expression of human tau was exacerbated by overexpression of PAR-1 (Nishimura et al. 2004). Taking together the biochemical studies on tau phosphorylation and function and the PAR-1 fly model, a cascade of events was proposed which is initiated by phosphorylation of microtubule-bound tau by MARK/Par-1 (Fig. 3) (Drewes 2004; Matenia and Mandelkow 2009). Both MARK4 and its upstream kinase LKB1 are rapidly upregulated in neurons after induced focal ischaemia in the mouse, a model for human stroke (Schneider et al. 2004). A genetic locus close to the MARK4 gene was reported to be associated with late-onset AD in a large genome-wide association study (Seshadri et al. 2010).

Metabolic syndrome: MARK2 knockout mice display growth retardation and dysfunctions in fertility, immune homeostasis, and learning and memory, but are lean, insulin hypersensitive, resistant to high-fat-diet-induced weight gain, and hypermetabolic (Hurov et al. 2007). MARK3 appears to have a related but



Microtubule Affinity Regulating Kinases (MARK), Fig. 3 Destabilization of microtubules by MARK. Axonal microtubules (*red*) function as tracks for synaptic vesicles. Microtubule function is regulated by bound tau protein (*blue*), which binds to the microtubules via the sequence repeats in its microtubule-binding domain. Activation of MARKs, possibly by an external insult to the cell such the disruption of intracellular calcium homeostasis by amyloid peptides, triggers phosphorylation of tau on its microtubule-binding domain. Phosphorylation triggers the dissociation of tau from the microtubule surface, changing the properties of the microtubule surface for transport processes and ultimately destabilizing the entire microtubule

nonredundant function as knockout mice exhibit increased energy expenditure, reduced adiposity with unaltered glucose handling, but normal insulin sensitivity. These mice were protected against diet-induced obesity and showed slow weight gain with resistance to fatty liver, with improved glucose handling and decreased insulin secretion. The crossing of MARK2 null mice with MARK3 null mice revealed that at least one allele is necessary for embryonic survival (Lennerz et al. 2010). The animal model data indicate that MARKs are important regulators of glucose and lipid metabolism and may be valuable drug target for the treatment of metabolic syndrome.

Summary

MARK genes are conserved in lower eukaryotic organisms like yeast, nematodes, and fruit flies where their function has been extensively studied by genetic means. From these studies MARKs have emerged as key regulators of cell polarity. Humans express four paralogous MARK genes encoding structurally related but functionally nonredundant protein serine/threonine kinases. MARKs are regulated by activating and inactivating phosphorylation events on distinct residues located in the activation loop and outside the catalytic domain. Key regulators of these pathways are the kinases LKB1 and aPKC which themselves constitute major regulators of cell polarity. MARK activity is also regulated by a tightly regulated interplay of 14-3-3 protein binding and membrane localization mediated by the C-terminal KA1-domain. Biochemical studies have proposed different classes of microtubule-binding proteins as substrates which are likely to transduce the effect of MARKs on cytoskeletal rearrangements during cellular transport processes and cell morphology. In disease, a deregulation of MARK activity may contribute to the loss of cell polarity in the affected highly polarized cell types including neurons (in Alzheimer's disease and other forms of dementia), gastric epithelial cells (in *H. pylori*-induced gastritis and in gastric cancer), and possibly in immune cell homeostasis.

References

Al-Hakim AK, Zagorska A, Chapman L, Deak M, Peggie M, Alessi DR. Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains. Biochem J. 2008;411:249–60.

Bachmann M, Kosan C, Xing PX, Montenarh M, Hoffmann I, Moroy T. The oncogenic serine/threonine kinase Pim-1 directly phosphorylates and activates the G2/M specific phosphatase Cdc25C. Int J Biochem Cell Biol. 2006;38:430–43.

Bohm H, Brinkmann V, Drab M, Henske A, Kurzchalia TV. Mammalian homologues of C. elegans PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. Curr Biol. 1997;7:603–6.

Brajenovic M, Joberty G, Kuster B, Bouwmeester T, Drewes G. Comprehensive proteomic analysis of human Par protein

complexes reveals an interconnected protein network. J Biol Chem. 2004;279:12804–11.

Chen YM, Wang QJ, Hu HS, Yu PC, Zhu J, Drewes G, Piwnica-Worms H, Luo ZG. Microtubule affinity-regulating kinase 2 functions downstream of the PAR-3/PAR-6/atypical PKC complex in regulating hippocampal neuronal polarity. Proc Natl Acad Sci USA. 2006;103:8534–9.

Cohen D, Tian Y, Musch A. Par1b promotes hepatic-type lumen polarity in Madin Darby canine kidney cells via myosin II- and E-cadherin-dependent signaling. Mol Biol Cell. 2007;18:2203–15.

Drewes G, Ebneth A, Mandelkow EM. MAPs, MARKs and microtubule dynamics. Trends Biochem Sci. 1998;23: 307–11.

Drewes G, Ebneth A, Preuss U, Mandelkow EM, Mandelkow E. MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. Cell. 1997;89:297–308.

Drewes G, Nurse P. The protein kinase kin1, the fission yeast orthologue of mammalian MARK/PAR-1, localises to new cell ends after mitosis and is important for bipolar growth. FEBS Lett. 2003;554:45–9.

Drewes G, Trinczek B, Illenberger S, Biernat J, Schmitt-Ulms G, Meyer HE, Mandelkow EM, Mandelkow E. Microtubule-associated protein/microtubule affinity-regulating kinase (p110mark). A novel protein kinase that regulates tau-microtubule interactions and dynamic instability by phosphorylation at the Alzheimer-specific site serine 262. J Biol Chem. 1995;270:7679–88.

Drewes G. MARKing tau for tangles and toxicity. Trends Biochem Sci. 2004;29:548–55.

Elbert M, Cohen D, Musch A. PAR1b promotes cell-cell adhesion and inhibits dishevelled-mediated transformation of Madin-Darby canine kidney cells. Mol Biol Cell. 2006;17:3345–55.

Hurov J, Piwnica-Worms H. The Par-1/MARK family of protein kinases: from polarity to metabolism. Cell Cycle. 2007;6:1966–9.

Hurov JB, Huang M, White LS, Lennerz J, Choi CS, Cho YR, Kim HJ, Prior JL, Piwnica-Worms D, Cantley LC, Kim JK, Shulman GI, Piwnica-Worms H. Loss of the Par-1b/MARK2 polarity kinase leads to increased metabolic rate, decreased adiposity, and insulin hypersensitivity in vivo. Proc Natl Acad Sci USA. 2007;104:5680–5.

Jaleel M, Villa F, Deak M, Toth R, Prescott AR, Van Aalten DM, Alessi DR. The ubiquitin-associated domain of AMPK-related kinases regulates conformation and LKB1-mediated phosphorylation and activation. Biochem J. 2006;394: 545–55.

Kemphues K. PARsing embryonic polarity. Cell. 2000;101: 345–8.

Kojima Y, Miyoshi H, Clevers HC, Oshima M, Aoki M, Taketo MM. Suppression of tubulin polymerization by the LKB1-microtubule-associated protein/microtubule affinity-regulating kinase signaling. J Biol Chem. 2007;282:23532–40.

Kosuga S, Tashiro E, Kajioka T, Ueki M, Shimizu Y, Imoto M. GSK-3beta directly phosphorylates and activates MARK2/PAR-1. J Biol Chem. 2005; 280:42715–22.

Lennerz JK, Hurov JB, White LS, Lewandowski KT, Prior JL, Planer GJ, Gereau RW, Piwnica-Worms D, Schmidt RE, Piwnica-Worms H. Loss of Par-1a/MARK3/C-TAK1 kinase leads to reduced adiposity, resistance to hepatic steatosis, and defective gluconeogenesis. Mol Cell Biol. 2010;30:5043–56.

Lizcano JM, Goransson O, Toth R, Deak M, Morrice NA, Boudeau J, Hawley SA, Udd L, Makela TP, Hardie DG, Alessi DR. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J. 2004;23:833–43.

Marx A, Nugoor C, Panneerselvam S, Mandelkow E. Structure and function of polarity-inducing kinase family MARK/Par-1 within the branch of AMPK/Snf1-related kinases. FASEB J. 2010;24:1637–48.

Matenia D, Mandelkow EM. The tau of MARK: a polarized view of the cytoskeleton. Trends Biochem Sci. 2009;34:332–42.

Matenia D, Griesshaber B, Li XY, Thiessen A, Johne C, Jiao J, Mandelkow E, Mandelkow EM. PAK5 kinase is an inhibitor of MARK/Par-1, which leads to stable microtubules and dynamic actin. Mol Biol Cell. 2005;16:4410–22.

Moravcevic K, Mendrola JM, Schmitz KR, Wang YH, Slochower D, Janmey PA, Lemmon MA. Kinase associated-1 domains drive MARK/PAR1 kinases to membrane targets by binding acidic phospholipids. Cell. 2010;143:966–77.

Moroni RF, De BS, Colapietro P, Larizza L, Beghini A. Distinct expression pattern of microtubule-associated protein/microtubule affinity-regulating kinase 4 in differentiated neurons. Neuroscience. 2006;143:83–94.

Muller J, Ritt DA, Copeland TD, Morrison DK. Functional analysis of C-TAK1 substrate binding and identification of PKP2 as a new C-TAK1 substrate. EMBO J. 2003;22: 4431–42.

Nishimura I, Yang Y, Lu B. PAR-1 kinase plays an initiator role in a temporally ordered phosphorylation process that confers tau toxicity in Drosophila. Cell. 2004;116:671–82.

Saadat I, Higashi H, Obuse C, Umeda M, Murata-Kamiya N, Saito Y, Lu H, Ohnishi N, Azuma T, Suzuki A, Ohno S, Hatakeyama M. Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. Nature. 2007;447:330–3.

Sapir T, Shmueli A, Levy T, Timm T, Elbaum M, Mandelkow EM, Reiner O. Antagonistic effects of doublecortin and MARK2/Par-1 in the developing cerebral cortex. J Neurosci. 2008;28:13008–13.

Schneider A, Laage R, von Ahsen O, Fischer A, Rossner M, Scheek S, Grunewald S, Kuner R, Weber D, Kruger C, Klaussner B, Gotz B, Hiemisch H, Newrzella D, Martin-Villalba A, Bach A, Schwaninger M. Identification of regulated genes during permanent focal cerebral ischaemia: characterization of the protein kinase 9b5/MARKL1/MARK4. J Neurochem. 2004;88:1114–26.

Seshadri S, Fitzpatrick AL, Ikram MA, DeStefano AL, Gudnason V, Boada M, Bis JC, Smith AV, Carassquillo MM, Lambert JC, Harold D, Schrijvers EM, Ramirez-Lorca R, Debette S, Longstreth Jr WT, Janssens AC, Pankratz VS, Dartigues JF, Hollingworth P, Aspelund T, Hernandez I, Beiser A, Kuller LH, Koudstaal PJ, Dickson DW, Tzourio C, Abraham R, Antunez C, Du Y, Rotter JI, Aulchenko YS, Harris TB, Petersen RC, Berr C, Owen MJ, Lopez-Arrieta J, Varadarajan BN, Becker JT, Rivadeneira F, Nalls MA, Graff-Radford NR, Campion D, Auerbach S, Rice K, Hofman A, Jonsson PV, Schmidt H, Lathrop M, Mosley TH, Au R, Psaty BM, Uitterlinden AG, Farrer LA, Lumley T, Ruiz A,

Williams J, Amouyel P, Younkin SG, Wolf PA, Launer LJ, Lopez OL, van Duijn CM, Breteler MM. Genome-wide analysis of genetic loci associated with Alzheimer disease. JAMA. 2010;303:1832–40.
Suzuki A, Hirata M, Kamimura K, Maniwa R, Yamanaka T, Mizuno K, Kishikawa M, Hirose H, Amano Y, Izumi N, Miwa Y, Ohno S. aPKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity. Curr Biol. 2004;14:1425–35.
Timm T, Balusamy K, Li X, Biernat J, Mandelkow E, Mandelkow EM. Glycogen synthase kinase (GSK) 3beta directly phosphorylates Serine 212 in the regulatory loop and inhibits microtubule affinity-regulating kinase (MARK) 2. J Biol Chem. 2008; 283:18873–82.
Timm T, Li XY, Biernat J, Jiao J, Mandelkow E, Vandekerckhove J, Mandelkow EM. MARKK, a Ste20-like kinase, activates the polarity-inducing kinase MARK/PAR-1. EMBO J. 2003;22: 5090–101.
Trinczek B, Brajenovic M, Ebneth A, Drewes G. MARK4 is a novel microtubule-associated proteins/microtubule affinity-regulating kinase that binds to the cellular microtubule network and to centrosomes. J Biol Chem. 2004;279: 5915–23.
Yoshimura Y, Terabayashi T, Miki H. Par1b/MARK2 phosphorylates kinesin-like motor protein GAKIN/KIF13B to regulate axon formation. Mol Cell Biol. 2010;30:2206–19.

Microtubule-Associated Protein Kinases

► Mitogen-Activated Protein Kinases

MINCLE

► CLEC4E

MIP-1α/R

► Chemokine Receptor CCR1

MIP-1α/RANTES

► Chemokine Receptor CCR1

MITF/Microphthalmia-Associated Transcription Factor (AGS13)

► Activators of G-Protein Signaling (AGS)

Mitogen- and Stress-Activated Protein Kinase 1

► MSK1

Mitogen-Activated Protein Kinase (MAPK)/Extracellular Signal-Regulated Kinase (ERK) Kinases 1/2 (MEK1/2)

► Mek

Mitogen-Activated Protein Kinase 1 and 2

► ERK1/ERK2

Mitogen-Activated Protein Kinase Kinase 3

► Mek3

Mitogen-Activated Protein Kinase Kinase 6

► MKK6

Mitogen-Activated Protein Kinase Kinase Kinase 11

► MLK3

Mitogen-Activated Protein Kinase Kinase Kinase 12

► DLK (Dual Leucine Zipper-Bearing Kinase)

Mitogen-Activated Protein Kinase Kinase Kinase 8

► TPL2

Mitogen-Activated Protein Kinase Kinase Kinase Kinase 1

▶ HPK1

Mitogen-Activated Protein Kinases

Sylvain Meloche
Department of Pharmacology, Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada
Program of Molecular Biology, Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada

Synonyms

MAP kinases; Microtubule-associated protein kinases

Historical Background

Mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases that play a key role in transducing chemical and physical extracellular signals into a variety of intracellular responses. These protein kinases are among the most highly studied signaling molecules, as reflected by the more than 65,000 papers on "MAP kinase" listed in PubMed as of July 2011. This chapter provides a broad overview of the different subfamilies of MAP kinases. Each subfamily will then be treated in more detail in individual entries of this encyclopedia.

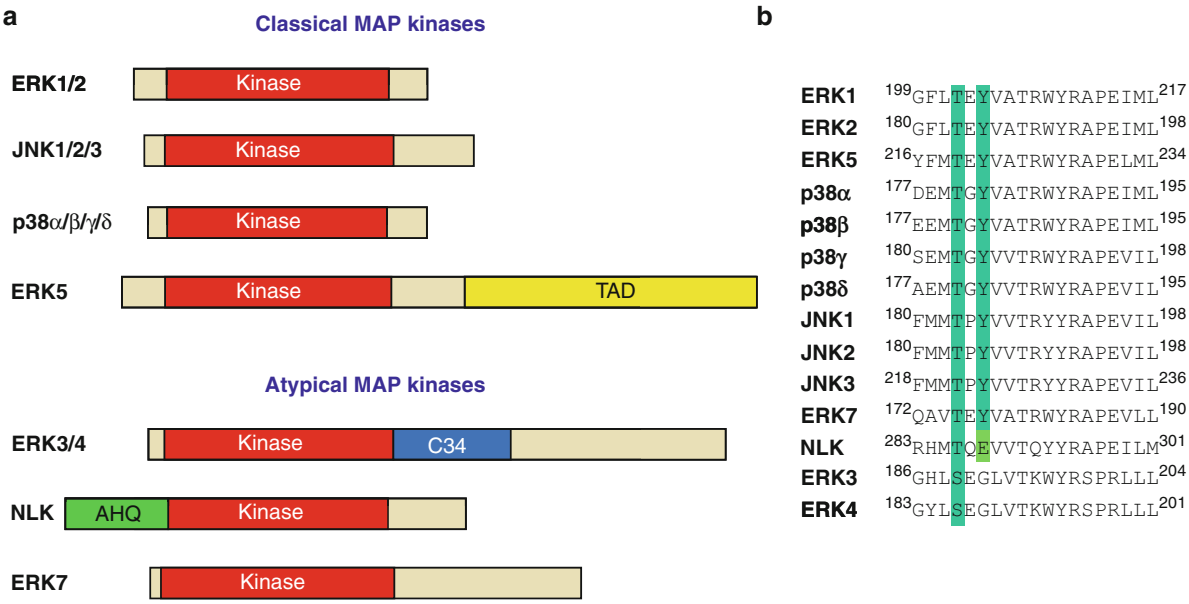
The discovery of MAP kinases goes back to 1987 when Ray and Sturgill reported the identification of a novel insulin-stimulated serine/threonine kinase activity from extracts of 3T3-L1 adipocytes that was capable of phosphorylating microtubule-associated protein-2 in vitro (Ray and Sturgill 1987). As a result, the enzyme was called microtubule-associated protein (MAP) kinase (Ray and Sturgill 1988). Partial purification of the novel kinase revealed that its activation was accompanied by phosphorylation on threonine and tyrosine residues, suggesting that it may be a direct substrate of the insulin receptor or another insulin-regulated tyrosine kinase (Ray and Sturgill 1988). Subsequent work indicated that the insulin-activated MAP kinase was identical to phosphoproteins of 41–43 kDa, previously shown to be phosphorylated on tyrosine in response to a variety of mitogens and growth factors (Rossomando et al. 1989). This led to the redesignation of the MAP acronym from microtubule-associated protein to mitogen-activated protein.

The first sequence of a mammalian MAP kinase, the insulin-stimulated MAP kinase named extracellular signal-regulated kinase 1 (Erk1), was reported in 1990 by the group of Melanie Cobb (Boulton et al. 1990). Interestingly, this protein kinase was found to be homologous to two recently identified budding yeast protein kinases, Kss1p and Fus3p, involved in cell cycle arrest in response to mating pheromones. This finding provided the first evidence for the conservation of MAP kinase functions across eukaryotic evolution. Later on, the discovery of additional MAP kinase pathways, first in yeast and then in mammalian cells, led to the concept of multiple MAP kinase subfamilies (Avruch 2007; Pearson et al. 2001; Widmann et al. 1999).

The initial observation that MAP kinase contains both phosphothreonine and phosphotyrosine, followed by the demonstration that dephosphorylation of either residue leads to inactivation of the kinase (Anderson et al. 1990) suggested that MAP kinase could serve to integrate information from two different signaling pathways. An intense effort was then made to identify these upstream MAP kinase kinase(s). Surprisingly, purification of a MAP kinase activator revealed that the threonine and tyrosine phosphorylation activities reside in the same polypeptide (Ahn et al. 1992). Shortly thereafter, a series of studies established that the MAP kinase kinases are themselves activated by phosphorylation by upstream MAP kinase kinase kinases, defining the basic architecture of MAP kinase pathways.

The Subfamilies of MAP Kinases

MAP kinases belong to the CMGC group of eukaryotic protein kinases, which also includes cyclin-dependent kinases (Cdks), glycogen synthase kinases, and Cdk-like kinases. They are proline-directed serine/threonine kinases, meaning that they phosphorylate substrates on Ser-Pro or Thr-Pro consensus motifs. In human, the MAP kinase family is encoded by 14 genes and is classified into 7 distinct subfamilies (Fig. 1)



Mitogen-Activated Protein Kinases, Fig. 1 Structure of MAP kinases. (**a**) Schematic representation of the structure of human MAP kinase subfamilies. Red, kinase domain; *TAD*, transactivation domain; *C34*, conserved in Erk3 and Erk4; *AHQ*, rich in alanine, histidine, glutamine. (**b**) Amino acid sequence of the activation loop of MAP kinase family members

(Coulombe and Meloche 2007). Many of the MAP kinase genes also encode alternatively spliced variants, further expanding the repertoire of these signaling enzymes.

Structure and Regulation of MAP Kinases

At the structural level, MAP kinases are composed of a catalytic kinase domain flanked by amino- and carboxy-terminal extensions of varying lengths (Fig. 1). All the family members display more than 40% identity to the founding member Erk1 in the kinase domain. One feature that distinguishes the different MAP kinase subfamilies is the sequence of the activation loop, which is the site of activating phosphorylation by upstream protein kinases. Several MAP kinases possess the motif Thr-Xxx-Tyr in the activation loop and are phosphorylated by a family of dual-specificity protein kinases known as MKKs (MAP kinase kinases) or ▶ MEKs (MAP kinase or ERK kinases). However, the Thr-Xxx-Tyr motif is absent in the MAP kinases Erk3, Erk4, and Nlk. Erk3 and Erk4 bear the motif Ser-Glu-Gly instead, whereas Nlk has a glutamic acid residue at the position of the tyrosine. The MAP kinase Erk7 has the Thr-Xxx-Tyr motif in its loop but is activated by a different mechanism. Based on these structural and regulatory features, it has been proposed to divide MAP kinases into classical and atypical enzymes (Coulombe and Meloche 2007). Classical MAP kinases, which are phosphorylated and activated by MKK family members, include the four subfamilies Erk1/2, Jnk1/2/3, p38α/β/γ/δ, and Erk5. Atypical MAP kinases, which are not substrates of MKKs, include the subfamilies Erk3/4, Erk7, and Nlk (Fig. 1).

Classical MAP kinases are organized into modules of three sequentially acting protein kinases (Pearson et al. 2001; Widmann et al. 1999). Typically, exposure to an extracellular stimulus induces the oligomerization and/or activation of a cell-surface receptor, often leading to the activation of a small GTPase of the Ras/Rho family, to a MAP kinase kinase kinase kinase, or to the binding of specialized adaptor proteins. These events ultimately lead to the activation of the upstream MAP kinase kinase kinase by a complex mechanism involving phosphorylation events and protein–protein interactions. The MAP kinase kinase kinase then phosphorylates and activates the MAP kinase kinase, which in turn activates the effector MAP kinase by dual phosphorylation of Thr and Tyr residues. Each MAP kinase kinase kinase confers responsiveness to

a distinct set of stimuli. Once activated, classical MAP kinases can phosphorylate a vast array of substrates present in all cellular compartments. The regulation of atypical MAP kinases is much less well understood (Coulombe and Meloche 2007).

MAP Kinases in Physiology and Disease

MAP kinases are activated in response to a variety of extracellular stimuli and cellular perturbations to regulate numerous cellular responses such as gene expression, cell proliferation and differentiation, cell survival, metabolism, motility, and adaptation (Coulombe and Meloche 2007; Cuadrado and Nebreda 2010; Hayashi and Lee 2004; Pearson et al. 2001; Weston and Davis 2007). Pharmacological and genetic studies have revealed that each of the MAP kinase subfamilies has distinct, but sometimes overlapping cellular functions. The best-characterized MAP kinase subfamilies are the Erk1/2, Jnk1/2/3, and p38α/β/γ/δ. The Erk1/2 MAP kinase pathway plays a major role in the regulation of cell proliferation in response to mitogenic factors. The Jnk pathway is involved in cell death signaling and metabolism, while the p38 pathway has been implicated in cytokine production and inflammatory responses.

Deregulated activity of MAP kinase pathways has been linked to many diseases, including cancer, inflammatory disorders, diabetes, and neurodegenerative diseases (Fremin and Meloche 2010; Kim and Choi 2010; Lawrence et al. 2008). For example, hyperactivation of the Ras-dependent Raf/▶ Mek/Erk1/2 pathway is frequently observed in human cancer as a result of aberrant activation of receptor tyrosine kinases or gain-of-function mutations in *RAS* or *RAF* genes (Schubbert et al. 2007). This has led to the clinical evaluation of small-molecule inhibitors of Mek1/2, the upstream activators of Erk1/2 MAP kinases, for the targeted therapy of cancer (Fremin and Meloche 2010). Inhibitors of p38 MAP kinase have also entered clinical trials to evaluate their efficacy in the management of rheumatoid arthritis. More recently, the p38δ MAP kinase inhibitor pirfenidone has been approved in Europe for the treatment of the fatal lung disease idiopathic pulmonary fibrosis. This represents the first small-molecule inhibitor of a MAP kinase to enter the market.

MAP kinases also play a causative role in certain congenital disorders. Indeed, germline mutations within components of the Erk1/2 MAP kinase signaling cascade have been associated with a number of clinical syndromes that are collectively termed "neuro-cardio-facial-cutaneous syndromes" (Bentires-Alj et al. 2006; Tidyman and Rauen 2009). Also, individuals with microdeletions encompassing the *Erk2* gene (*MAPK1*) in distal chromosome 22q11 exhibit a spectrum of craniofacial abnormalities, cardiac defects, and neurodevelopmental deficits (Ben-Shachar et al. 2008).

Summary

MAP kinases are components of evolutionarily conserved signaling modules that evolved to control a multitude of physiological responses required to maintain normal cellular and tissue homeostasis. Aberrant activity of MAP kinases has been observed in many human diseases, fostering the development of small-molecule inhibitors targeting these pathways. Considerable progress has been made over the last 20 years in identifying the core components of classical MAP kinase pathways and clarifying their mechanisms of regulation by extracellular stimuli. The physiological functions of classical MAP kinases have been extensively studied by the use of pharmacological inhibitors and genetic approaches. On the other hand, much less is known about the regulation and functions of atypical MAP kinases. Future challenges in the field include defining the individual contributions of MAP kinase isoforms using gene targeting and chemical genetic approaches, systematically characterizing the substrate profile of these enzymes by global proteomic strategies, further our understanding of the spatiotemporal regulation of MAP kinase pathways using mathematical modeling and synthetic biology, and exploiting the therapeutic potential of these enzymes.

References

Ahn NG, Seger R, Krebs EG. The mitogen-activated protein kinase activator. Curr Opin Cell Biol. 1992;4:992–9.

Anderson NG, Maller JL, Tonks NK, Sturgill TW. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature. 1990;343:651–3.

Avruch J. MAP kinase pathways: the first twenty years. Biochim Biophys Acta. 2007;1773:1150–60.

Ben-Shachar S, Ou Z, Shaw CA, Belmont JW, Patel MS, Hummel M, Amato S, Tartaglia N, Berg J, Sutton VR,

Lalani SR, Chinault AC, Cheung SW, Lupski JR, Patel A. 22q11.2 distal deletion: a recurrent genomic disorder distinct from DiGeorge syndrome and velocardiofacial syndrome. Am J Hum Genet. 2008;82:214–21.
Bentires-Alj M, Kontaridis MI, Neel BG. Stops along the RAS pathway in human genetic disease. Nat Med. 2006;12:283–5.
Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science. 1990;249:64–7.
Coulombe P, Meloche S. Atypical mitogen-activated protein kinases: structure, regulation and functions. Biochim Biophys Acta. 2007;1773:1376–87.
Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem J. 2010;429:403–17.
Fremin C, Meloche S. From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. J Hematol Oncol. 2010;3:8.
Hayashi M, Lee JD. Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice. J Mol Med. 2004;82:800–8.
Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta. 2010;1802:396–405.
Lawrence MC, Jivan A, Shao C, Duan L, Goad D, Zaganjor E, Osborne J, McGlynn K, Stippec S, Earnest S, Chen W, Cobb MH. The roles of MAPKs in disease. Cell Res. 2008;18:436–42.
Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001;22:153–83.
Ray LB, Sturgill TW. Rapid stimulation by insulin of a serine/threonine kinase in 3 T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc Natl Acad Sci USA. 1987;84:1502–6.
Ray LB, Sturgill TW. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. Proc Natl Acad Sci USA. 1988;85:3753–7.
Rossomando AJ, Payne DM, Weber MJ, Sturgill TW. Evidence that pp 42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc Natl Acad Sci USA. 1989;86:6940–3.
Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer. 2007;7:295–308.
Tidyman WE, Rauen KA. The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation. Curr Opin Genet Dev. 2009;19:230–6.
Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol. 2007;19:142–9.
Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999;79:143–80.

Mixed Lineage Kinase 7 (MLK7)

► MLTK

Mixed-Lineage Kinase 3

► MLK3

mKIAA1415

► P-Rex

MKK (MAP Kinase Kinase)

► p38 MAPK Family of Signal Transduction Proteins

MKK1/2

► Mek

MKK3

► Mek3

MKK6

Sonia-Vanina Forcales
Institute of Predictive and Personalized Medicine of Cancer (IMPPC), Badalona, Barcelona, Spain

Synonyms

MAP2K6; MAPKK6; MEK6; Mitogen-activated protein kinase kinase 6; PRKMK6; SAPKK3

Historical Background

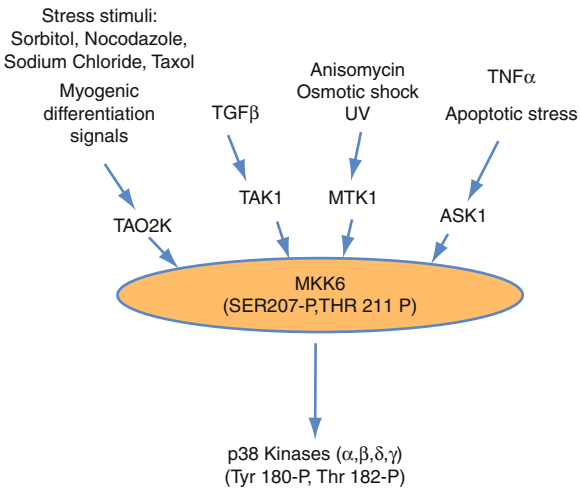
MKK6 is a dual specificity mitogen-activated protein kinase that was first cloned by PCR using degenerate primers for the conserved kinase domain of MKKs

(Han et al. 1996). MKK6 amino-acid sequence shares about 80% of similarity with MKK3 and 40% with MKK4 (Stein et al. 1996). Two MKK6 isoforms have been described in mouse and human (Cuenda et al. 1996; Han et al. 1996). The bigger form contains 334 amino acids and is highly expressed in heart, skeletal muscle, pancreas, and liver, while the smaller isoform of 278 amino acids has been detected only in skeletal muscle (Han et al. 1996).

Regulation of MKK6 Activity

Several extracellular stimuli initiate a cascade of upstream kinases that activate MKK6 by phosphorylation of two residues located in its activation loop domain (serine 207 and threonine 211) (Fig. 1). For instance, osmotic stress, ultraviolet light, and anisomycin activate MAP three kinase-1 (MTK1) which in turn phosphorylates MKK6 in a dose-dependent manner in COS and HeLa cells (Takekawa et al. 1997). Transforming growth factor beta–activated protein kinase 1 (TAK1) has been shown to phosphorylate and activate MKK6 in vitro and in vivo (Moriguchi et al. 1996) and to be required to activate MKK6-p38 pathway during myogenic differentiation (Bhatnagar et al. 2010). Thousand and one amino acid kinase-2 (TAO2) coimmunoprecipitates and phosphorylates MKK6 during myogenic differentiation in the presence of stress stimuli such as sorbitol, sodium chloride, taxol, and nocodazole (Chen and Cobb, 2001). Apoptosis signal-regulating kinase 1 (ASK1) activates MKK6-p38 in response to pro-inflammatory cytokines and apoptotic stress (Ichijo et al. 1997). PKNα (a fatty-acid-activated and rho-activated serine/threonine protein kinase) has been shown to play an activating role in the MKK6-p38 pathway as an upstream activator of MTK1 and as a scaffold protein, associating with each member of the p38γ MAPK signaling pathway (p38γ, MKK6 and MTK1) (Takahashi et al. 2003). MKK6 activation has also been shown to be mediated by microtubules and Dctn1, a component of the dynein-dynactin complex involved in organelle transportation along the microtubules (Cheung et al. 2004).

Upon activation, MKK6 phosphorylates ▶ p38α, p38β, p38γ, and p38δ on their regulatory threonine and tyrosine residues within a tripeptide motif (Thr–Gly–Tyr) located in the activation loop of p38 (Raingeaud et al. 1995). The specific recognition of



MKK6, Fig. 1 Activation of MKK6 by upstream kinases. Several extracellular stimuli activate MAPKKKs (MTK1, ASK1, TAK1, TAO2K) that phosphorylate MKK6, thereby activating it. Active MKK6 phosphorylates and activates p38 kinases alpha, beta, delta, and gamma

MKK6-p38 binding is mediated by a consensus docking domain (Lys/Arg–Xaa3–Leu/Ile–Xaa–Leu/Ile) present in the N-terminal region of MKK6 (Enslen et al. 2000).

MKK3 and MKK4, who share highly similar catalytic domains to MKK6, can also phosphorylate and activate p38 mitogen-activated protein kinase (MAPK) family members (Derijard et al. 1995). Genetic and biochemical evidence demonstrates nonredundant and selective functions for these MAPKKs in regulating the activity of p38 kinases in response to specific stimuli. For instance, p38α is activated by MKK6 and MKK3 in response to TNFα stimuli, while in response to UV light, p38α is phosphorylated and activated by MKK6, MKK3, and also MKK4. Environmental stress activates MKK6 and ▶ MKK3, which in turn activate p38β and p38γ. However, MKK6 activation by TNFα results in activation of p38γ, while MKK3 activation by TNFα, UV light, osmotic shock, and anisomycin results in activation of p38δ (Remy et al. 2010). The complexity of MAPKs' activation may reflect the ability to respond to multiple stimuli.

While phosphorylation of MKK6 in serine 207 and threonine 211 is an activatory event, MKK6 acetylation of these same residues by YopJ (Yersinia Outer protein J) has been shown to inhibit MKK6 activity. Therefore, MKK6 acetylation may be the mechanism by which YopJ blocks the innate immune response (Mukherjee et al. 2006).

MKK6 has major roles in several biological processes. The specificity of its action depends on the diversity of its activators, the selectivity of the available substrates, and the concomitant signaling pathways being activated or repressed in a particular situation. Because of its prominent signaling role in differentiation, inflammatory diseases, and cancer, the exploration of drugs targeting MKK6 activity is a challenging field to develop new therapeutic approaches.

Functional Roles of MKK6 Activity

Role of MKK6 in Differentiation

MKK6 plays a crucial role in promoting myogenesis through the activation of p38 pathway (Zetser et al. 1999). Myogenic differentiation stimuli such as cell-to-cell contact activate the CDO-JLP immunoglobulin-scaffold protein complex which in turn activates the MKK6-p38-α/β pathway (Takaesu et al. 2006). Other ligands, such as TNFα and amphoterin/RAGE, have been shown to induce myogenic differentiation through MKK6-p38 activation (Sorci et al. 2004). MKK6 exerts its pro-myogenic functions mainly through the p38 alpha kinase isoform (Perdiguero et al. 2007), which mediates the assembly of an active myogenic transcriptional complex by direct phosphorylation of its components: MEF2 myogenic transcriptional factors (Zhao et al. 1999; Rampalli et al. 2007), ▶ SWI/SNF (SWItch/Sucrose NonFermentable) chromatin remodeling subunits (Simone et al. 2004), and E47 (Lluís et al. 2005). p38 beta and gamma have been reported to promote and inhibit muscle gene expression by different groups (Wang et al. 2008; Gillespie et al. 2009).

The MKK6-p38 pathway has been shown to play a role in the survival of differentiated neurons. In response to calcium influx, the activation of MKK6-p38 pathway induces MEF2C phosphorylation in serine 387 and this event may activate the expression of survival genes and repress the expression of apoptotic genes (Mao et al. 1999).

Role of MKK6 in Heart Physiology

MKK6-p38 pathway plays an important cardioprotective role in the heart. In an in vivo model of infarction, transgenic mouse hearts overexpressing MKK6 show a better functional recovery and less injury than non-transgenic mice (Martindale et al. 2005). One possible mechanism could be that MKK6-p38 activation in these MKK6-transgenic mice induced the expression of the heat shock protein αB-crystallin and the antiapoptotic Bcl-2 protein, which have been shown to play important cardioprotective roles.

MKK6 Roles in the Immune System

MKK6 knockout (KO) mice are viable, fertile, and they do not show developmental or tissue abnormalities. However, these mice show impairment in double positive thymocyte apoptosis; therefore, MKK6 plays a role in thymocyte fate (Tanaka et al. 2002).

In macrophages, LPS induces MKK6 phosphorylation and its binding with IRAK2. This event is required for the posttranscriptional control of cytokine and chemokine expression involved in the innate immune response (Wan et al. 2009).

MKK6 Role in Cancer

A tumor suppressor role has been described by different groups for the MKK6-p38 pathway. Fibroblasts isolated from MKK6$^{-/-}$ mice show an increased proliferation rate in serum-free medium compared to WT fibroblasts, correlating with the maintained expression of D-cyclins and the presence of phosphorylated Rb. Subcutaneous injection of MKK6$^{-/-}$ fibroblasts into nude mice induces larger tumors compared to the WT fibroblasts (Brancho et al. 2003). A deficient MKK6-p38 signaling has been shown in certain types of rhabdomyosarcomas, where restoring a persistent activation of p38 MAPK pathway by a constitutively active mutant of MKK6 (MKK6EE) leads to tumor cell growth arrest and terminal differentiation (Puri et al. 2000). Activation of the MKK6-p38 pathway is sufficient to suppress in vivo tumorigenesis. Nude mice injected with HeLa (human cervical carcinoma) cells expressing a constitutively active MKK6 show a reduced tumor formation (Timofeev et al. 2005). In addition, an anti-metastatic effect has been attributed to the MKK6-p38 pathway. Ectopic expression of MKK6 in an ovarian carcinoma cell line prevented the metastatic process when these cells were injected intraperitoneally (Hickson et al. 2006). All these works show an important role of MKK6-p38 signaling pathway in the control of neoplastic growth.

MKK6 Role in Inflammation

MKK6 activation occurs in inflammatory diseases such as rheumatoid arthritis (RA) and osteoarthritis

(OA) (Chabaud-Riou and Firestein 2004). Blocking MKK6-p38 activity has been shown to attenuate these inflammatory diseases. For instance, a dominant negative MKK6 impairs the production of inflammatory cytokines (IL-6, IL-8) and the protease MMP-3 in IL-1 stimulated fibroblasts-like synoviocytes (Inoue et al. 2005). Furthermore, in a passive K/B × N mouse serum transfer model of arthritis, MKK6$^{-/-}$ mice show attenuation of arthritis, cartilage destruction, and bone erosion (Yoshizawa et al. 2009). In this study, MKK6 would be contributing to the colocalization of p38 with its substrate MK2 (MAPKAPK2).

Summary

Mitogen-activated protein kinase kinase-6 (MKK6) belongs to the MAPK kinase (MAPKK) family of enzymes, which specifically activates p38 MAPK. MKK6 has a similar sequence to MKK3 (80%) and to MKK4 (40%). Cellular stresses such as osmotic shock, UV irradiation, hypoxia, cytokine stimulation, and cell-to-cell contact activate the MKK6-p38 pathway by activating upstream kinases (MAPKKKs) including Ask1 (apoptosis signal-regulating kinase 1), Tak1 (transforming growth factor beta–activated kinase 1), Tao2 (1001 amino acids kinases 2), and Mtk1 (Mekk4/MAP three kinase 1). All of these protein kinases activate MKK6 by phosphorylating it on serine 207 and threonine 211 in its activation loop. Acetylation of these sites by acetyltransferase YopJ (Yersinia outer protein J) inhibits MKK6 activity, indicating that the phosphorylation and acetylation states of the activation loop are crucial to regulate MKK6 kinase activity. Active MKK6 phosphorylates p38α, β, δ, and γ MAPKs, on their regulatory threonine and tyrosine residues, activating p38 MAPK signaling. MKK6 is widely expressed in mammalian tissues with higher accumulation in skeletal muscle. MKK6 is a key mediator of many stress-responsive signaling cascades that are crucial for differentiation, inflammation, and tumorigenic processes.

References

Bhatnagar S, Kumar A, Makonchuk DY, Li H, Kumar A. Transforming growth factor-beta-activated kinase 1 is an essential regulator of myogenic differentiation. J Biol Chem. 2010;285(9):6401–11.

Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ. Mechanism of p38 MAP kinase activation in vivo. Genes Dev. 2003;17(16):1969–78.

Chabaud-Riou M, Firestein GS. Expression and activation of mitogen-activated protein kinase kinases-3 and -6 in rheumatoid arthritis. Am J Pathol. 2004;164(1):177–84.

Chen Z, Cobb MH. Regulation of stress-responsive mitogen-activated protein (MAP) kinase pathways by TAO2. J Biol Chem. 2001;276(19):16070–5.

Cheung PY, Zhang Y, Long J, Lin S, Zhang M, Jiang Y, Wu Z. p150(Glued), Dynein, and microtubules are specifically required for activation of MKK3/6 and p38 MAPKs. J Biol Chem. 2004;279(44):45308–11.

Cuenda A, Alonso G, Morrice N, Jones M, Meier R, Cohen P, Nebreda AR. Purification and cDNA cloning of SAPKK3, the major activator of RK/p38 in stress-and cytokine-stimulated monocytes and epithelial cells. EMBO J. 1996;15(16):4156–64.

Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ. Independent human MAP- kinase signal transduction pathways defined by MEK and MKK isoforms. Science. 1995;267(5198):682–5.

Enslen H, Brancho DM, Davis RJ. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. EMBO J. 2000;19(6):1301–11.

Gillespie MA, Le Grand F, Scimè A, Kuang S, von Maltzahn J, Seale V, Cuenda A, Ranish JA, Rudnicki MA. p38-{gamma}-dependent gene silencing restricts entry into the myogenic differentiation program. J Cell Biol. 2009;187(7):991–1005.

Han J, Lee JD, Jiang Y, Li Z, Feng L, Ulevitch RJ. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). J Biol Chem. 1996;271(6):2886–91.

Hickson JA, Huo D, Vander Griend DJ, Lin A, Rinker-Schaeffer CW, Yamada SD. The p38 kinases MKK4 and MKK6 suppress metastatic colonization in human ovarian carcinoma. Cancer Res. 2006;66(4):2264–70.

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science. 1997;275(5296):90–4.

Inoue T, Hammaker D, Boyle DL, Firestein GS. Regulation of p38 MAPK by MAPK kinases 3 and 6 in fibroblast-like synoviocytes. J Immunol. 2005;174(7):4301–6.

Lluís F, Ballestar E, Suelves M, Esteller M, Muñoz-Cànoves P. E47 phosphorylation by p38 MAPK promotes MyoD/E47 association and muscle-specific gene transcription. EMBO J. 2005;24(5):974–84.

Mao Z, Bonni A, Xia F, Nadal-Vicens M, Greenber ME. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science. 1999;286(5440):785–90.

Martindale JJ, Wall JA, Martinez-Longoria DM, Aryal P, Rockman HA, Guo Y, Bolli R, Glembotski CC. Overexpression of mitogen-activated protein kinase kinase 6 in the heart improves functional recovery from ischemia in vitro and protects against myocardial infarction in vivo. J Biol Chem. 2005;280(1):669–76.

Moriguchi T, Toyoshima F, Gotoh Y, Iwamatsu A, Irie K, Mori E, Kuroyanagi N, Hagiwara M, Matsumoto K, Nishida E. Purification and identification of a major activator for p38

from osmotically shocked cells. Activation of mitogen-activated protein kinase kinase 6 by osmotic shock, tumor necrosis factor-alpha, and H_2O_2. J Biol Chem. 1996;271(43):26981–8.
Mukherjee S, Keitany G, Li Y, Wang Y, Ball HL, Goldsmith EJ, Orth K. Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. Science. 2006;312(5777):1211–4.
Perdiguero E, Ruiz-Bonilla V, Serrano AL, Muñoz-Cánoves P. Genetic deficiency of p38alpha reveals its critical role in myoblast cell cycle exit: the p38alpha-JNK connection. Cell Cycle. 2007;6(11):1298–303.
Puri PL, Wu Z, Zhang P, Wood LD, Bhakta KS, Han J, Feramisco JR, Karin M, Wang JY. Induction of terminal differentiation by constitutive activation of p38 MAP kinase in human rhabdomyosarcoma cells. Genes Dev. 2000;14(5):574–84.
Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem. 1995;270(13):7420–6.
Rampalli S, Li L, Mak E, Ge K, Brand M, Tapscott SJ, Dilworth FJ. p38 MAPK signaling regulates recruitment of Ash2L-containing methyltransferase complexes to specific genes during differentiation. Nat Struct Mol Biol. 2007;12:1150–6.
Remy G, Risco AM, Iñesta-Vaquera FA, González-Terán B, Sabio G, Davis RJ, Cuenda A. Differential activation of p38MAPK isoforms by MKK6 and MKK3. Cell Signal. 2010;22(4):660–7.
Simone C, Forcales SV, Hill DA, Imbalzano AN, Latella L, Puri PL. p38 pathway targets SWI-SNF chromatin-remodeling complex to muscle-specific loci. Nat Genet. 2004;36(7):738–43.
Sorci G, Riuzzi F, Arcuri C, Giambanco I, Donato R. Amphoterin stimulates myogenesis and counteracts the antimyogenic factors basic fibroblast growth factor and S100B via RAGE binding. Mol Cell Biol. 2004;24(11):4880–94.
Stein B, Brady H, Yang MX, Young DB, Barbosa MS. Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. J Biol Chem. 1996;271(19):11427–33.
Takaesu G, Kang JS, Bae GU, Yi MJ, Lee CM, Reddy EP, Krauss RS. Activation of p38alpha/beta MAPK in myogenesis via binding of the scaffold protein JLP to the cell surface protein Cdo. J Cell Biol. 2006;175(3):383–8.
Takahashi M, Gotoh Y, Isagawa T, Nishimura T, Goyama E, Kim HS, Mukai H, Ono Y. Regulation of a mitogen-activated protein kinase kinase kinase, MLTK by PKN. J Biochem. 2003;133(2):181–7.
Takekawa M, Posas F, Saito H. A human homolog of the yeast Ssk2/Ssk22 MAPkinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. EMBO J. 1997;16(16):4973–82.
Tanaka N, Kamanaka M, Enslen H, Dong C, Wysk M, Davis RJ, Flavell RA. Differential involvement of p38 mitogen-activated protein kinases MKK3 and MKK6 in T-cell apoptosis. EMBO Rep. 2002;3(8):785–91.
Timofeev O, Lee TY, Bulavin DV. A subtle change in p38 MAPK activity is sufficient to suppress in vivo tumorigenesis. Cell Cycle. 2005;4(1):118–20.
Wan Y, Xiao H, Affolter J, Kim TW, Bulek K, Chaudhuri S, Carlson D, Hamilton T, Mazumder B, Stark GR, Thomas J, Li X. Interleukin-1 receptor-associated kinase 2 is critical for lipopolysaccharide-mediated post-transcriptional control. J Biol Chem. 2009;284(16):10367–75.
Wang H, Xu Q, Xiao F, Jiang Y, Wu Z. Involvement of the p38 mitogen-activated protein kinase alpha, beta, and gamma isoforms in myogenic differentiation. Mol Biol Cell. 2008;19(4):1519–28.
Yoshizawa T, Hammaker D, Boyle DL, Corr M, Flavell R, Davis R, Schett G, Firestein GS. Role of MAPK kinase 6 in arthritis: distinct mechanism of action inflammation and cytokine expression. J Immunol. 2009;183(2):1360–7.
Zetser A, Gredinger E, Bengal E. p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. J Biol Chem. 1999;274(8):5193–200.
Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di Padova F, Olson EN, Ulevitch RJ, Han J. Regulation of the MEF2 family of transcription factors by p38. Mol Cell Biol. 1999;1:21–30.

MKKEE (Activated form of MKK6)

► p38 MAPK Family of Signal Transduction Proteins

MKKK (MAP Kinase Kinase Kinase)

► p38 MAPK Family of Signal Transduction Proteins

MKP (MAPK Phosphatase)

► p38 MAPK Family of Signal Transduction Proteins

MK-STYX

Natalie M. Niemi[1,2] and Jeffrey P. MacKeigan[2]
[1]Van Andel Institute Graduate School, Van Andel Research Institute, Grand Rapids, MI, USA
[2]Center for Cancer Genomics and Quantitative Biology, Van Andel Research Institute, Grand Rapids, MI, USA

Synonyms

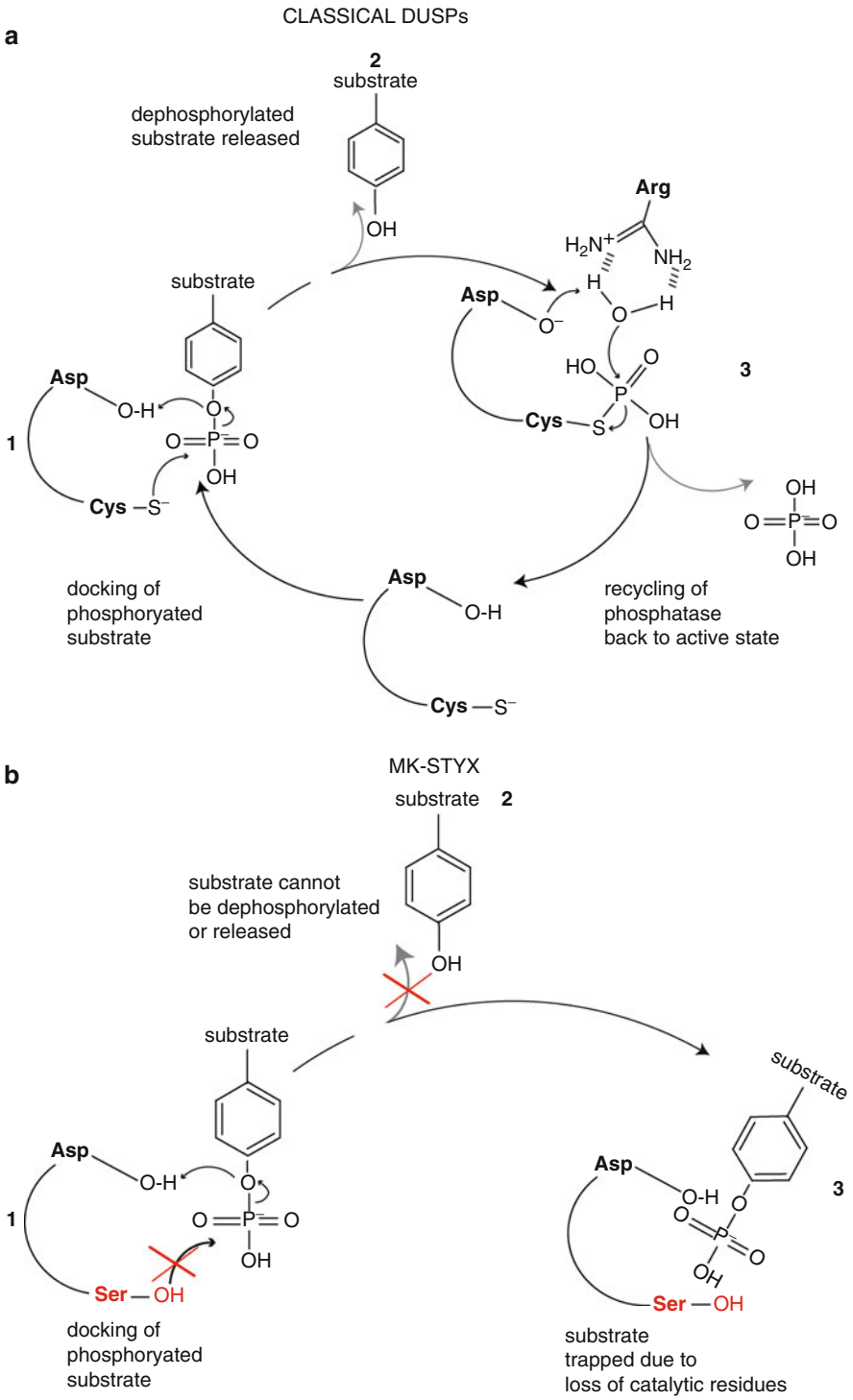
DUSP24; Styxl1

Historical Background

MK-STYX was first identified bioinformatically due to its homology with the dual specificity phosphatases (DUSPs), a family of enzymes known for their ability to dephosphorylate not only tyrosine residues but also serine and threonine residues (Wishart and Dixon 1998). Specifically, MK-STYX resembles a MAP kinase phosphatase (MKP), which contains a rhodenase or ▶ Cdc25 homology (CH2) domain in its N-terminus, while containing a DUSP domain in its C-terminus. The name of the protein reflects this similarity, as MK-STYX was coined from the phrase *M*AP *K*inase Phosphatase-like *S*erine *T*hreonine T*y*rosine inter*act*ion domain (Wishart and Dixon 1998). The Ser/Thr/Tyr interaction (STYX) domain was so named based on the observation that, though this domain shares significant homology to a DUSP domain, it lacks a critical cysteine residue necessary for catalysis; the conservative substitution of a serine in place of this residue renders this enzyme completely inactive. Mechanistically, this is explained though examination of the enzymatic reaction necessary for DUSPs to carry out the dephosphorylation reaction. After binding a phosphorylated substrate, a classical DUSP will utilize a conserved cysteine residue to nucleophilically attack the phosphate group which is to be removed from its substrate (Fig. 1a). This cysteine contains a thiolate intermediate, kept in its ionic state by an environment promoting an extremely low pKa within the pocket of the phosphatase. The substitution of a cysteine to a serine, however, abolishes this thiolate intermediate, and even the very low pKa of the phosphatase pocket is not sufficient to render the hydroxyl group on this serine as a potent nucleophile (Fig. 1b). Thus, the serine mutation does not affect the binding of the substrate, but rather the initiaton of the first step of catalysis. This Cys → Ser substitution has been exploited by researchers in the field of phosphatase biology to create what are known as "substrate-trapping mutants" (Flint et al. 1997). These mutants are utilized as a tool in which phosphatases bind their substrates in a more stable fashion, allowing for easier identification and characterization of binding partners and downstream effector molecules. Thus, MK-STYX is an endogenous "substrate-trapping mutant," although whether this molecule uses its STYX domain as a tool to bind phosphorylated residues on other molecules has yet to be determined.

MK-STYX is part of a family of two known proteins in humans with a STYX domain. The other protein, simply named STYX, is also catalytically inactive, due to a Cys → Gly mutation within its active site (Wishart and Dixon 1998). Reversion of the glycine back to a cysteine is sufficient to restore catalytic activity, demonstrating the preservation of the phosphatase domain and binding pocket in this protein (Wishart et al. 1995). Due to this structural preservation, it has been speculated that the STYX domain could bind to phosphorylated proteins, analogous to an SH2 domain binding phosphotyrosine residues. This would be a particularly interesting possibility, as DUSPs are able dephosphorylate both tyrosine residues, as well as serine/threonine residues due to a particularly shallow pocket which accommodates the active site. Indeed, a recent study demonstrated that the mutation of two residues within the active site, the serine → cysteine along with the −1 position, is sufficient to promote phosphatase activity of MK-STYX (Hinton et al. 2010). These data suggest that while the enzyme is catalytically inactive, it retains a phosphatase binding pocket that could be important in its cellular functions.

Another interesting possibility of MK-STYX function is to regulate the localization or enzymatic activity of an active phosphatase, presumably a DUSP. Surprisingly, there are numerous phosphatases present in the human genome which are rendered catalytically inactive due to mutations at various points within their active sites (Tonks 2006). This includes the D2 domains of many receptor protein tyrosine phosphatases (R-PTPs), which are implicated in controlling enzymatic activity. Interestingly, a family of lipid phosphatases, the myotubularins, consists of 14 different members, 6 of which are catalytically inactive (Begley and Dixon 2005). Importantly, these catalytically inactive myotubularins are critical for modulating enzymatic activity and/or cellular sublocalization of their active counterparts, creating an additional regulatory layer into lipid phosphatase biology (Begley et al. 2006; Robinson et al. 2008). While a phosphatase interactor for STYX and/or MK-STYX has yet to be found, the possibility that these proteins could be similar to a regulatory module for active phosphatases is an attractive model with clear precedence within the cell.



MK-STYX, Fig. 1 Catalytic mechanism of DUSPs and MK-STYX. All dual specificity phosphatases contain a consensus motif, DX_nCX_5R, which defines their phosphatase domain. The cysteine is critical for the initial nucleophilic attack of the phosphoryl group to be removed from the phosphorylated substrate (**a**, step 1). The phosphorylated residue is released from the enzyme, and the aspartate and arginine are involved in recycling the enzyme back to its active state (**a**, steps 2 and 3). In MK-STYX, the initial catalytic cysteine is substituted with a serine group, which is not a potent nucleophile (**b**, step 1). Due to the loss of this cysteine, the enzyme cannot instigate dephosphorylation of the substrate (**b**, steps 1 and 2). Instead, the phosphatase pocket creates a stable intermediate, with the inactive phosphatase "trapping" its substrate through docking in this binding pocket (**b**, step 3)

MK-STYX and STYX Domains Throughout Evolution

MK-STYX seems to be a relatively recent molecular addition in the evolution of animals, as definitive homologues of the protein can only be found within the phylum Chordata, which includes deuterosomes such as the sea cucumber as well as zebrafish, mice, and humans. It is not conserved, however, in insects, C. *elegans*, or yeast. It is interesting to note, however, that "STYX-domain" containing proteins do exist in these organisms (Wishart and Dixon 1998). While they are probably not direct homologues of MK-STYX, it is notable that catalytically inactive phosphatases have been utilized in even early life forms, though little is known about the functionality of these genes.

MK-STYX Regulation

As MK-STYX is relatively uncharacterized, it comes as no surprise that little is known about its regulation at a transcriptional or posttranscriptional level. There are multiple studies, however, that have demonstrated that MK-STYX transcript levels increase in the context of the Ewing's Sarcoma fusion product, EWS-FLI (Guillon et al. 2009; Siligan et al. 2005). EWS-FLI is a transcription factor with aberrant-binding capacities which is known to be sufficient in causing Ewing's Sarcoma. While MK-STYX transcript levels have been shown to increase to this aberrant fusion protein, presumably through transcriptional upregulation, little is known of the physiological significance of this observation, as MK-STYX has also been hypothesized to function as a tumor suppressor (see RNAi phenotypes, below).

MK-STYX has also been identified as being transcriptionally upregulated by the induction of the p21 protein (Chang et al. 2000), though this would have to be an indirect form of regulation, as p21 itself is not a direct transcriptional regulator. It is interesting, however, in light of the potential tumor suppressive functions of MK-STYX uncovered by MacKeigan et al., that p21 could upregulate MK-STYX to halt cell cycle progression or promote an apoptotic phenotype. The broad applicability p21-mediated control of MK-STYX expression will have to be confirmed and explored in much more detail before a clear understanding of these implications is uncovered.

RNAi-Mediated Phenotypes of MK-STYX Knockdown

Interestingly, MK-STYX has been identified as having numerous phenotypes in multiple RNAi screens designed to study very different cellular processes. The first study aimed to identify novel kinases and phosphatases involved in cellular survival or apoptotic potential (MacKeigan et al. 2005). The authors used siRNA sequences to all known and putative human kinases and phosphatases and transfected them into cancer cells. RNAi-mediated loss of MK-STYX promoted the most highly chemoresistant phenotype of all enzymes assayed. Importantly, this chemoresistance was shown in response to multiple drugs with different mechanisms of action, implicating a general cellular mechanism of chemoresistance. As chemoresistance is a highly significant clinical problem for patients with advanced and recurrent cancers, studies on the significance of this gene in treatment response and/or prediction of response rate could be an important future direction in oncology research.

A second RNAi-screening paper identified MK-STYX as potentially tumor suppressive in the context of breast cancer. This paper demonstrated that the RNAi-mediated loss of MK-STYX promoted a highly aberrant migratory phenotype in MCF-10A cells, a nontransformed mammary epithelial cell line (Simpson et al. 2008). This seemed to be coupled with a striking loss in cell polarity, which is a typical feature of cancer cells.

An additional independent study identified loss of MK-STYX within a set of genes whose downregulation is associated with breast cancer metastasis to the brain (Bos et al. 2009). In the study, two cell lines were passaged in vivo to create a daughter cell line which was highly metastatic to the brain. Importantly, both cell models had statistically significant downregulation of MK-STYX in the metastatic cell lines relative to the parental lines. This data, coupled with the RNAi-screening data, suggests that MK-STYX could be a potent tumor and/or metastasis suppressor in breast cancers.

While no molecular mechanism was worked out for either of these phenotypes, it is interesting to note that the loss of MK-STYX seems to promote both resistance to therapy, as well as a prometastatic phenotype. These data suggest that the loss of MK-STYX could be an important event in the later stages of cancer progression and could be a valuable therapeutic target if these observations validate in follow-up studies.

MK-STYX and Stress Granule Formation

Currently, the only known protein identified as an interaction partner of MK-STYX is the RNA-binding protein G3BP1. A recent study has shown that MK-STYX can bind to endogenous G3BP, a protein intimately involved in the formation and maintenance of stress granules within the cell (Hinton et al. 2010). Importantly, MK-STYX overexpression alleviated stress granule formation within cells, implicating a functional role to this interaction. Interestingly, the authors noted that the interaction between MK-STYX and G3BP is abrogated when MK-STYX is reverted to an active enzyme through the mutation of two key residues within its active site, suggesting that the MK-STYX-G3BP1 interaction axis is mediated through its STYX domain, potentially through a substrate-trapping mechanism. It will be interesting in the future to understand whether G3BP is phosphorylated and whether this phosphorylation is what mediates the interaction of these two proteins.

Summary and Future Directions

MK-STYX, while relatively uncharacterized, is suggested to have numerous independent and interesting cellular phenotypes. Many studies remain to be done to elucidate how a single gene could have such pleiotropic effects; subcellular localization, regulation, and turnover, as well as identification of interaction partners will be critical for these analyses. Although many facts remain to be uncovered about this gene, the few studies that have been done on this protein suggest that it could play a very interesting role in the etiology of diseases, such as cancer. As such, studies in the future should take note of this interesting gene, and efforts should be made to uncover its cellular function and what role it plays in the etiology of disease.

References

Begley MJ, Dixon JE. The structure and regulation of myotubularin phosphatases. Curr Opin Struct Biol. 2005;15:614–20.

Begley MJ, Taylor GS, Brock MA, Ghosh P, Woods VL, Dixon JE. Molecular basis for substrate recognition by MTMR2, a myotubularin family phosphoinositide phosphatase. Proc Natl Acad Sci USA. 2006;103:927–32.

Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA, Massague J. Genes that mediate breast cancer metastasis to the brain. Nature. 2009;459:1005–9.

Chang BD, Watanabe K, Broude EV, Fang J, Poole JC, Kalinichenko TV, Roninson IB. Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. Proc Natl Acad Sci USA. 2000;97:4291–6.

Flint AJ, Tiganis T, Barford D, Tonks NK. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc Natl Acad Sci USA. 1997;94:1680–5.

Guillon N, Tirode F, Boeva V, Zynovyev A, Barillot E, Delattre O. The oncogenic EWS-FLI1 protein binds in vivo GGAA microsatellite sequences with potential transcriptional activation function. PLoS One. 2009;4:e4932.

Hinton SD, Myers MP, Roggero VR, Allison LA, Tonks NK. The pseudophosphatase MK-STYX interacts with G3BP and decreases stress granule formation. Biochem J. 2010;427:349–57.

MacKeigan JP, Murphy LO, Blenis J. Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. Nat Cell Biol. 2005;7:591–600.

Robinson FL, Niesman IR, Beiswenger KK, Dixon JE. Loss of the inactive myotubularin-related phosphatase Mtmr13 leads to a charcot-marie-tooth 4B2-like peripheral neuropathy in mice. Proc Natl Acad Sci U S A. 2008;105:4916–21.

Siligan C, Ban J, Bachmaier R, Spahn L, Kreppel M, Schaefer KL, Poremba C, Aryee DN, Kovar H. EWS-FLI1 target genes recovered from Ewing's sarcoma chromatin. Oncogene. 2005;24:2512–24.

Simpson KJ, Selfors LM, Bui J, Reynolds A, Leake D, Khvorova A, Brugge JS. Identification of genes that regulate epithelial cell migration using an siRNA screening approach. Nat Cell Biol. 2008;10:1027–38.

Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. Nat Rev Mol Cell Biol. 2006;7:833–46.

Wishart MJ, Dixon JE. Gathering STYX: phosphatase-like form predicts functions for unique protein-interaction domains. Trends Biochem Sci. 1998;23:301–6.

Wishart MJ, Denu JM, Williams JA, Dixon JE. A single mutation converts a novel phosphotyrosine binding domain into a dual-specificity phosphatase. J Biol Chem. 1995;270:26782–5.

MLCK

► MYLK (Myosin Light Chain Kinase)

MLCK108

► MYLK (Myosin Light Chain Kinase)

MLCK210

▶ MYLK (Myosin Light Chain Kinase)

MLK3

Jian Chen[1], Chotirat Rattanasinchai[3] and Kathleen A. Gallo[2,3]
[1]Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI, USA
[2]Department of Physiology, Michigan State University, East Lansing, MI, USA
[3]Cell & Molecular Biology Program, Michigan State University, East Lansing, MI, USA

Synonyms

MAP3K11; Mitogen-activated protein kinase kinase kinase 11; Mixed-lineage kinase 3; SH3 domain–containing proline-rich kinase; SPRK

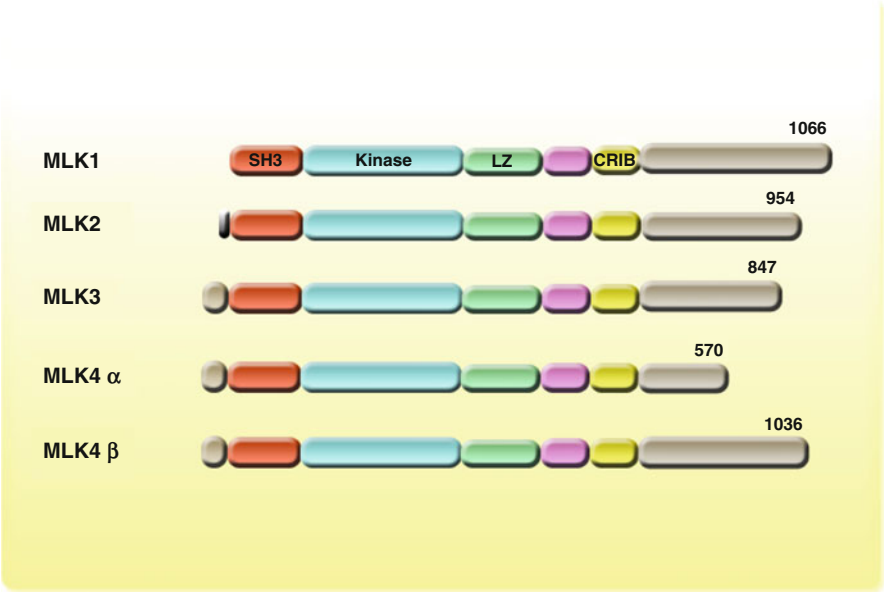
Historical Background

The mixed-lineage kinases are so named for their sequence similarity to both tyrosine kinases and serine/threonine kinases. However, based on biochemical assays, only serine/threonine kinase activity has been demonstrated for the MLKs. The three subfamilies of MLKs reside within the "tyrosine kinase-like" branch of the human kinome. Members of the MLK subfamily, comprised of MLK1-4, share a conserved domain arrangement and 75% identity within their catalytic domains (Fig. 1). The dual leucine zipper–bearing kinase (▶ DLK) subgroup of MLKs, which includes DLK/ZPK/MUK and LZK, is characterized by a kinase catalytic domain followed by two leucine zipper motifs. A third subgroup of MLKs represented by ZAK/▶ MLTK contains both a leucine zipper motif and a sterile-alpha motif. MLK3 has emerged as the paradigm for the MLK subfamily. There is no ortholog for MLK3 in yeast but in Drosophila, the MLK1-4 ortholog, Slipper, is critical for the cell sheet movement during dorsal closure in the fly embryo, which involves activation of the JNK pathway. MLK3-deficient mice are fully viable but have a reduced thickness of the dorsal epidermal tissue, which parallels the effects of disruption of Slipper in *Drosophila* (reviewed in Gallo and Johnson 2002; Schachter et al. 2006).

Regulation of MLK3 Activity

MLK3 contains several protein interaction domains that are important in regulating its activity. A short glycine-rich region is followed sequentially by a Src homology 3 (SH3) domain, a kinase catalytic domain, a leucine zipper region, and a Cdc42/Rac Interactive Binding (CRIB) motif (Fig. 1). The carboxyl terminal region of MLK3 is rich in proline, serine, and threonine residues (Gallo and Johnson 2002). Like many protein kinases, MLK3 activation involves phosphorylation within the activation loop of the kinase domain. Based on site-directed mutagenesis studies, Thr 277 and Ser 281 in the activation loop act as positive regulatory phosphorylation sites; and phospho-specific antibodies directed against these sites are widely used to monitor MLK3 activity. Notably these potential phosphorylation sites are conserved within the activation loops of MLK1-4, suggesting that they may serve a similar function. Leucine zippers form coiled coil dimers that are stabilized by the interaction of leucine or other nonaromatic aliphatic residues at the interface of the helices. Deletion of the entire zipper or introduction of a helix disrupting Pro residue for one of the conserved Leu residues prevents dimerization and activation loop phosphorylation of MLK3. Thus, leucine zipper–mediated dimerization can lead to MLK3 activation. These findings are consistent with the high MLK3 activity observed upon overexpression in many cultured cell lines. Presumably when MLK3 is expressed at high levels, a portion of MLK3 is dimerized in the absence of a physiologically appropriate stimulus, leading to high basal MLK3 activity.

MLK3 contains a CRIB motif, a short conserved sequence required for binding the Rho family GTPases, Cdc42 and Rac. Subsequent work demonstrated that the small GTPases, Cdc42 and Rac, are indeed capable of activating MLK3. Like other GTPase effector proteins, MLK3 interacts with the active, GTP-bound GTPase, but not the inactive, GDP-bound form. Binding of activated Cdc42 (or Rac) promotes MLK3 dimerization, activation loop phosphorylation, increases MLK3 catalytic activity, and translocates MLK3 to the cell



MLK3, Fig. 1 Conserved domains in the MLK subfamily. The domain arrangement within the MLK subfamily, depicting the relative positions of the Src-homology-3 (SH3) kinase, leucine-zipper (LZ), and Cdc42/Rac1 interactive binding (CRIB) motifs. The number of amino acids in each kinase is shown. See text for details

periphery (Du et al. 2005). Posttranslational COOH-terminal prenylation (geranylgeranylation) of Cdc42 and Rac allows for membrane targeting. A prenylation-defective site-directed mutant of activated Cdc42 retains the ability to bind MLK3 and promote activation loop phosphorylation of MLK3, but fails to translocate MLK3 to membranes. Thus the physical interaction between the activated GTPase and MLK3 is the key mechanism by which it activates MLK3. However, membrane targeting by activated, prenylated Cdc42 is accompanied by additional phosphorylation events on MLK3 and further enhances MLK3 in vitro kinase and cellular signaling activities.

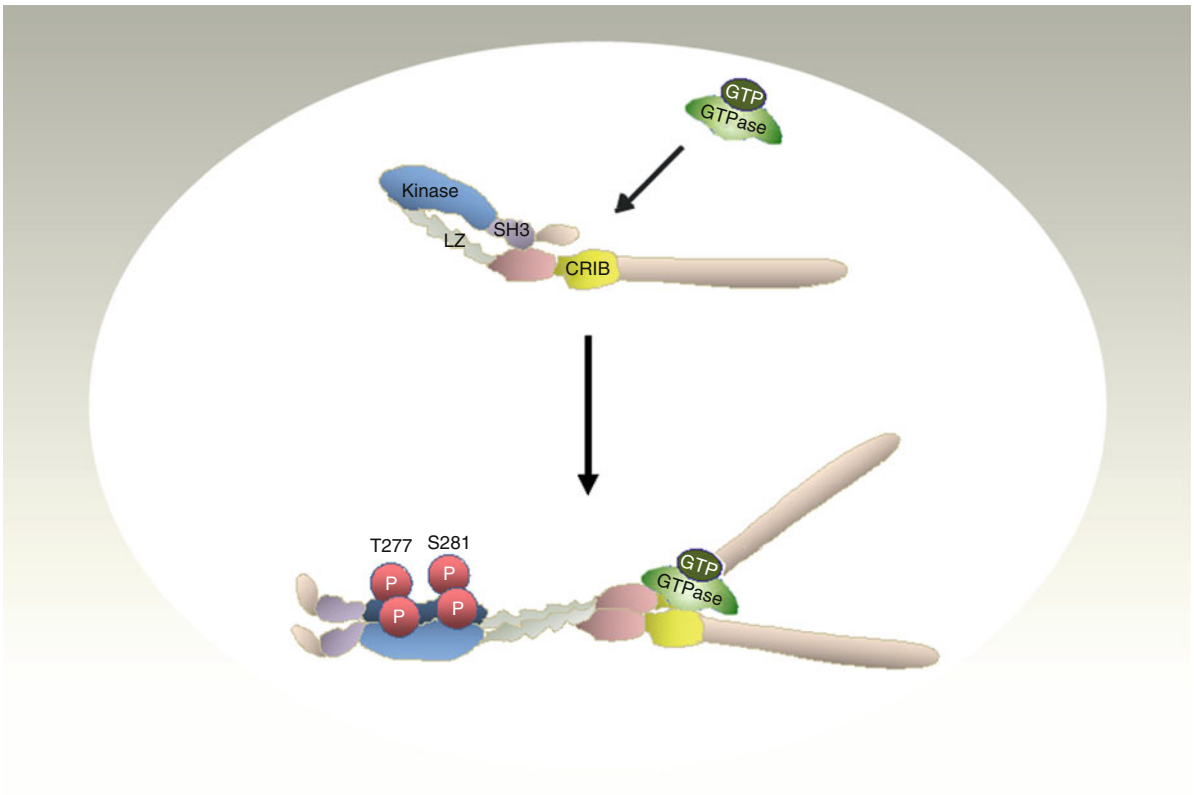
SH3 domains are modular domains of about 60 amino acids that typically bind proline-rich sequences to interact with intracellular signaling partners. The N-terminal SH3 domain of MLK3 functions as an autoinhibitory domain. A single Pro residue located between the leucine zipper and CRIB motif is required for the interaction with its SH3 domain. Though not formally shown, this autoinhibitory interaction is presumed to be intramolecular. Since this Pro residue is conserved in MLK1-4, it is likely that these MLKs are also regulated by SH3-mediated autoinhibition.

An integrated model for MLK3 activation by Cdc42 and Rac consistent with available data is shown in Fig. 2. Binding of the active, GTP-bound GTPase to MLK3 in a region containing the CRIB motif disrupts the SH3 autoinhibitory interaction, promoting leucine zipper–mediated dimerization and trans-autophosphorylation within the activation loop, yielding the active kinase at cell membranes (reviewed in Gallo and Johnson 2002; Schachter et al. 2006).

In addition to phosphorylation within the activation loop, numerous MLK3 phosphorylation sites have been identified through mass spectrometry, the majority of which are Ser or Thr residues followed immediately by Pro residues, conforming to the consensus sequence for proline-directed kinases. The downstream MAPK, JNK, has been shown to phosphorylate MLK3 at multiple proline-directed kinase sites within the COOH terminal region in a positive feedback loop that stabilizes MLK3 and/or redistributes MLK3 into Triton-soluble cellular fractions. Ser 674 has been identified as an Akt(PKB)-mediated phosphorylation site on MLK3 that inhibits MLK3-mediated apoptosis (reviewed in Handley et al. 2007; Schachter et al. 2006). In contrast, Ser 789 and Ser 793 have been identified as sites for GSK-3 beta-mediated phosphorylation on MLK3 that promote neuronal cell death (Mishra et al. 2007).

MLK3 Signaling Triggered by Cell-Surface Receptors

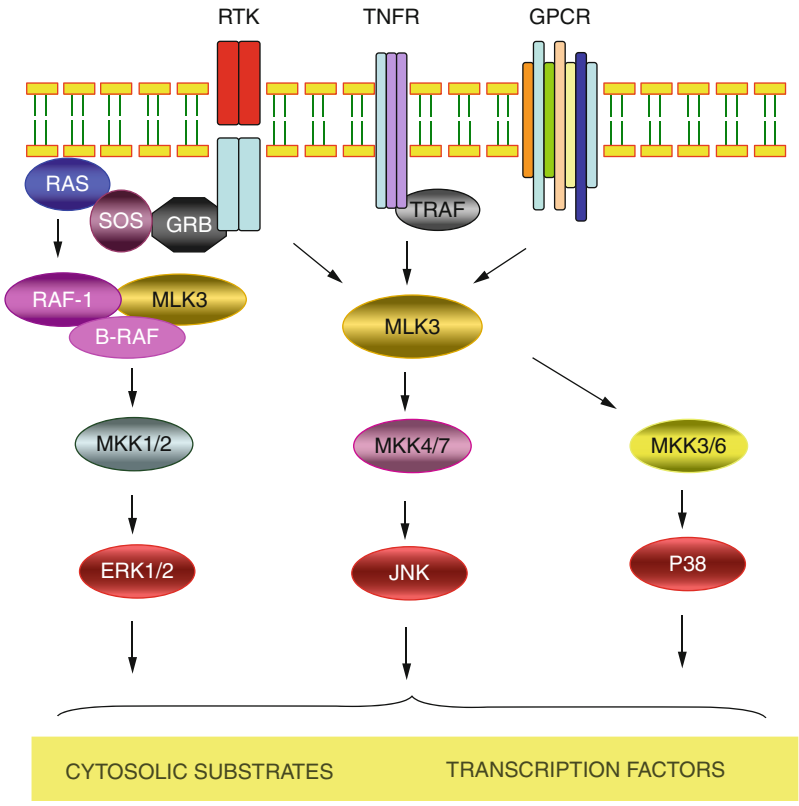
MLK3 signals through multiple mitogen-activated protein kinase (MAPK) pathways, including JNK, p38 MAPK, and ERK. MAPK pathways are three-tiered



MLK3, Fig. 2 Model for MLK3 activation by small GTPases, Cdc42 and Rac. Autoinhibition of MLK3 is maintained by an interaction between SH3 domain of MLK3 and a proline-containing sequence located between the LZ region and CRIB motif. The binding of GTP-bound GTPase(s) through the CRIB motif disrupts the autoinhibition, promotes dimerization and trans-autophosphorylation at Thr 277 and Ser 281 ensues

cascades wherein a MAP3K phosphorylates and activates a MAP2K which, in turn, phosphorylates and activates the terminal MAPK. Activated MAPKs have both nuclear and cytosolic substrates. MLK3, also known as MAP3K11, activates the JNK pathway by phosphorylation-mediated activation of MKK4/MKK7; it activates the p38 MAPK pathway through phosphorylation-mediated activation of MKK3/▶ MKK6. Whereas catalytic activity of MLK3 is required for activation of the JNK and p38 MAPK pathways, MLK3 acts as an indispensable scaffold required for B-Raf-mediated ERK activation (reviewed in Kyriakis 2007). Scaffold proteins, like the JIPs and POSH, are able to bind MLK3 as well as downstream MKKs and MAPKs, and may serve to localize, organize, or assemble MLK3 pathway complexes at specific subcellular locations (reviewed in Dhanasekaran et al. 2007).

Substantial evidence indicates that MLK3 can signal through multiple receptor types, including receptor tyrosine kinases, cytokine receptors, and G-protein-coupled receptors (GPCRs) (Fig. 3), but the details of these signaling pathways are largely unknown. The cytokine, tumor necrosis factor-alpha (TNF), is the best-described stimulus for activating MLK3 signaling to JNK. The TNF receptor-associated factor 2 (TRAF2) complexes with both the TNF receptor and MLK3 and is critical for TNF-induced JNK activation (Sondarva et al. 2010). It should be noted that other MAP3Ks, such as ASK, have also been implicated in TNF-induced JNK activation. Receptor tyrosine kinase signaling also involves MLK3, since silencing of MLK3 prevents epidermal growth factor (EGF)-induced activation of JNK, p38 MAPK, and ERK (reviewed in Kyriakis 2007). MLK3 can be activated by stimulation of cells with carbachol, a chemical ligand for G-protein-coupled acetylcholine receptors (Swenson-Fields et al. 2008). In this context, MLK3 complexes with the Rho guanine nucleotide



MLK3, Fig. 3 Role of MLK3 in signaling through cell surface receptors to MAPKs. MLK3-mediated signaling pathways downstream of various receptors are shown. *EGFR* epidermal growth factor receptor, *TNFR* tumor necrosis factor receptor, *GPCR* G-protein-coupled receptor. Activated MAPKs can phosphorylate cytosolic substrates or enter the nucleus to phosphorylate nuclear substrates, including transcription factors that regulate gene expression. See text for details

exchange factor (GEF), p63RhoGEF, to inhibit RhoA activation. Finally free fatty acids can activate MLK3 and signaling to JNK, implicating MLK3 in insulin resistance (Jaeschke and Davis 2007).

MLK3 in Cancer

Deregulation of signal transduction pathways drives development of human malignancies. MLK3 has been shown to be overexpressed in breast cancer cell lines compared with non-tumorigenic mammary epithelial cells, suggesting that MLK3 might contribute to acquisition of malignant phenotypes in breast cancer (Chen et al. 2010). Ectopic expression of wild-type MLK3 causes cellular transformation of immortalized fibroblasts (reviewed in Kyriakis 2007) and promotes a malignant phenotype of mammary epithelial spheroids in 3D culture (Chen et al. 2010). MLK3 silencing or inhibition can inhibit proliferation in some, but not all, tumor cell lines, perhaps depending upon the oncogenic signaling signature in those cells (reviewed in Schachter et al. 2006; Kyriakis 2007).

Several lines of evidence demonstrate a critical role of MLK3 in migration and invasion of cancer cells of epithelial origin. Induced expression of MLK3 promotes migration of poorly invasive breast cancer cells and invasion of mammary epithelial cells (Chen et al. 2010). Small interfering RNA-mediated silencing of MLK3 blocks migration of highly invasive breast, lung, and gastric carcinoma cells, indicating an essential function for MLK3 in migration and/or invasion of a broad array of epithelial-derived tumor cells (Chen et al. 2010; Mishra et al. 2010; Swenson-Fields et al. 2008). A major mechanism by which MLK3 controls migration and invasion is through activation of JNK and its downstream transcription factor AP-1, leading to expression of genes that promote invasion or epithelial-to-mesenchymal transition, such as MMP-7, fra-1, vimentin, and N-cadherin (Chen et al. 2010; Mishra et al. 2010; Shintani et al. 2008). An AP-1-independent role in which MLK3 blocks activation of RhoA has been identified in lung carcinoma cells (Swenson-Fields et al. 2008). The inactivation of the neurofibromatosis-2 tumor suppressor gene is associated with the formation of benign brain tumors.

NF-2/merlin can interact with MLK3 to inhibit its activity, preventing proliferation and invasion of Schwann cells (Zhan et al. 2011).

Missense mutations in *mlk3* have been identified in human gastrointestinal cancers and are significantly associated with microsatellite instability (MSI) phenotype in mismatch repair-deficient gastrointestinal carcinomas. The identified mutations are found in different functional domains of MLK3, including the SH3 domain (Y99C), the kinase domain (A165S; P252H), and the COOH-terminal proline-rich region (R799C; P840L). When ectopically expressed in fibroblasts, these mutant forms of MLK3 are more transforming and tumorigenic than wild-type MLK3 (Velho et al. 2010). How these specific mutations affect MLK3 signaling activities is yet to been determined.

MLK3 Is Implicated in Neurodegenerative Diseases

Numerous neurotoxic insults can induce JNK activation and mitochondria-mediated apoptosis. MLK3 has been implicated as an upstream mediator of JNK in neuronal cell death in several experimental systems, primarily based on the use of dominant negative forms of MLK3 and the pan-MLK inhibitors, CEP-1347 and CEP-11004. For example, deprivation of nerve growth factor (NGF) induces JNK activation and apoptosis in PC12 cells and in cultured superior cervical rat ganglia, which can be attenuated by blocking MLK activity (reviewed in Wang et al. 2004). Studies in rodents support a role for MLK3 in apoptosis in response to cerebral ischemia-reperfusion, a model for stroke (Zhang et al. 2009). MLK3 has also been linked to JNK activation in kainate-induced neurotoxicity, with the scaffolding protein PSD-95 interacting with both the GluR6 receptor and MLK3 (reviewed in Gallo and Johnson 2002). Finally, substantial evidence has accumulated for MLK3 in JNK activation and neuronal death in cell-based and in vivo models of Parkinson's disease that use the dopaminergic selective neurotoxin MPTP or its derivative MPP+, respectively (reviewed in Wang et al. 2004). In response to treatment with MPTP/MPP+, the MLK inhibitor CEP-1347 was able to suppress JNK activation and increase survival of dopaminergic neurons. These studies suggested that CEP-1347 might be a promising therapeutic for treating patients with Parkinson's disease. CEP-1347 ultimately progressed to Stage II/III clinical trials, but failed to delay progression of patients with early stage Parkinson's disease (Parkinson Study Group PRECEPT Investigators 2007).

MLKs in Inflammation

MLK3 signaling is important in the production of pro-inflammatory proteins. In the context of ▶ interferon-gamma-activated macrophages, MLK signals to p38 MAPK increasing the mRNA levels of TNF-α and interferon inducible protein 10, presumably through increased mRNA stability. In microglia activated by the bacterial endotoxin, lipopolysaccharide (LPS), an MLK or JNK inhibitor reduces AP-1-mediated transcription of TNF-α (reviewed in Handley et al. 2007). In primary cortical astrocytes, activated by a mixture of pro-inflammatory cytokines, MLK signaling to both p38 MAPK and JNK has been implicated in the induction of inflammation-responsive genes (Falsig et al. 2004).

Thus, there is considerable interest in MLK inhibitors as anti-inflammatory drugs, particularly in attenuating neuroinflammation associated with HIV infection. Recently, the MLK inhibitor, CEP-1347, was shown to prevent the production of cytokines and chemokines in HIV-infected human macrophages and to elicit anti-inflammatory and neuroprotective effects in mouse models of HIV-1 encephalitis (Eggert et al. 2010).

Summary

MLK3 is an intracellular serine/threonine kinase, which belongs to a larger family of related kinases that are evolutionarily conserved in metazoans. The kinase activity of MLK3 is autoinhibited through its SH3 domain. The small GTPases, Cdc42 and Rac, can bind to MLK3, disrupting autoinhibition and promoting zipper-mediated dimerization and subsequent transphosphorylation within the kinase domain to yield active MLK3. MLK3 signals through multiple cell surface receptors, including receptor tyrosine kinases, cytokine receptors, and heterotrimeric G-protein-coupled

receptors. MLK3 contributes to the activation of multiple MAPK pathways, functioning as a MAP3K to activate the JNK and p38 MAPK pathways, and as a scaffold in B-Raf-mediated ERK activation. Although soon after its identification, MLK3 was implicated in neuronal apoptosis, emerging data indicates that MLK3 is a critical player in many pathophysiological processes, including cancer cell migration and invasion as well as inflammation. Thus MLK inhibitors may prove valuable in multiple therapeutic arenas.

References

Chen J, Miller EM, Gallo KA. MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells. Oncogene. 2010;29:4399–411.

Dhanasekaran DN, Kashef K, Lee CM, Xu H, Reddy EP. Scaffold proteins of MAP-kinase modules. Oncogene. 2007;26:3185–202. Review.

Du Y, Böck BC, Schachter KA, Chao M, Gallo KA. Cdc42 induces activation loop phosphorylation and membrane targeting of mixed lineage kinase 3. J Biol Chem. 2005;280:42984–93.

Eggert D, Dash PK, Gorantla S, Dou H, Schifitto G, Maggirwar SB, Dewhurst S, Poluektova L, Gelbard HA, Gendelman HE. Neuroprotective activities of CEP-1347 in models of neuroAIDS. J Immunol. 2010;184:746–56.

Falsig J, Pörzgen P, Lotharius J, Leist M. Specific modulation of astrocyte inflammation by inhibition of mixed lineage kinases with CEP-1347. J Immunol. 2004;173:2762–70.

Gallo KA, Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol. 2002;3:663–72. Review.

Handley ME, Rasaiyaah J, Chain BM, Katz DR. Mixed lineage kinases (MLKs): a role in dendritic cells, inflammation and immunity? Int J Exp Pathol. 2007;88:111–26. Review.

Jaeschke A, Davis RJ. Metabolic stress signaling mediated by mixed-lineage kinases. Mol Cell. 2007;27:498–508.

Kyriakis JM. The integration of signaling by multiprotein complexes containing Raf kinases. Biochim Biophys Acta. 2007;1773:1238–47. Review.

Mishra R, Barthwal MK, Sondarva G, Rana B, Wong L, Chatterjee M, Woodgett JR, Rana A. Glycogen synthase kinase-3beta induces neuronal cell death via direct phosphorylation of mixed lineage kinase 3. J Biol Chem. 2007;282:30393–405.

Mishra P, Senthivinayagam S, Rangasamy V, Sondarva G, Rana B. Mixed lineage kinase-3/JNK1 axis promotes migration of human gastric cancer cells following gastrin stimulation. Mol Endocrinol. 2010;24:598–607.

Parkinson Study Group PRECEPT Investigators. Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. Neurology. 2007;69:1480–90.

Schachter KA, Liou GY, Du Y, Gallo KA. Mlk3. UCSD nature molecule pages. 2006. doi:10.1038/mp.a001551.01.

Shintani Y, Fukumoto Y, Chaika N, Svoboda R, Wheelock MJ, Johnson KR. Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1. J Cell Biol. 2008;180:1277–89.

Sondarva G, Kundu CN, Mehrotra S, Mishra R, Rangasamy V, Sathyanarayana P, Ray RS, Rana B, Rana A. TRAF2-MLK3 interaction is essential for TNF-alpha-induced MLK3 activation. Cell Res. 2010;20:89–98.

Swenson-Fields KI, Sandquist JC, Rossol-Allison J, Blat IC, Wennerberg K, Burridge K, Means AR. MLK3 limits activated Galphaq signaling to Rho by binding to p63RhoGEF. Mol Cell. 2008;32:43–56.

Velho S, Oliveira C, Paredes J, Sousa S, Leite M, Matos P, Milanezi F, Ribeiro AS, Mendes N, Licastro D, Karhu A, Oliveira MJ, Ligtenberg M, Hamelin R, Carneiro F, Lindblom A, Peltomaki P, Castedo S, Schwartz Jr S, Jordan P, Aaltonen LA, Hofstra RM, Suriano G, Stupka E, Fialho AM, Seruca R. Mixed lineage kinase 3 gene mutations in mismatch repair deficient gastrointestinal tumors. Hum Mol Genet. 2010;19:697–706.

Wang LH, Besirli CG, Johnson Jr EM. Mixed-lineage kinases: a target for the prevention of neurodegeneration. Annu Rev Pharmacol Toxicol. 2004;44:451–74. Review.

Zhan Y, Modi N, Stewart AM, Hieronimus RI, Liu J, Gutmann DH, Chadee DN. Regulation of mixed lineage kinase 3 is required for Neurofibromatosis-2-mediated growth suppression in human cancer. Oncogene. 2011;30:781–9.

Zhang Q-G, Wang R, Hana D, Dong Y, Branna DW. Role of Rac1 GTPase in JNK signaling and delayed neuronal cell death following global cerebral ischemia. Brain Res. 2009;1265:138–47.

MLK-like Mitogen-Activated Protein Triple Kinase (MLTK)

► MLTK

MLK-Related Kinase (MRK)

► MLTK

MLT

► MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1)

MLTK

Rosamaria Ruggieri
Oncology & Cell Biology, The Feinstein Institute for Medical Research, Manhasset, NY, USA

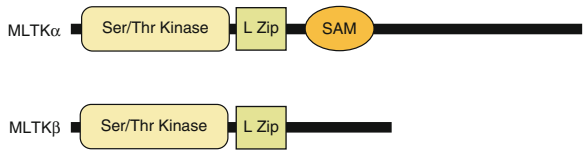
Synonyms

Mixed lineage kinase 7 (MLK7); MLK-like mitogen-activated protein triple kinase (MLTK); MLK-related kinase (MRK); Sterile-alpha motif and leucine zipper–containing kinase AZK (ZAK)

Historical Background

MLTK is a protein kinase that belongs to the family of mitogen-activated protein kinase kinase kinases (MAPKKK) with closer homology to the MLK proteins. It was isolated as ZAK by low-stringent hybridization to the Ste20 kinase (Liu et al. 2000): as MLK7, a gene highly expressed in the heart (Bloem et al. 2001); as MLTK, a gene induced by overexpression of active ERK (Gotoh et al. 2001); and as MRK, a human gene that acts as a MAPKKK in a yeast functional screen (Gross et al. 2002). The MLTK gene encodes two splice variants: MLTKα and MLTKβ of 91.7 kDa and 51.3 kDa calculated molecular mass, respectively. They share the N-terminus kinase and leucine zipper domains, while they diverge in the C-terminal region. MLTKα has a sterile α motif (SAM), whereas MLTKβ has a shorter C-terminus with a highly acidic region (Fig. 1).

The MLTK mRNAs are ubiquitously expressed, with higher levels in heart and skeletal muscle. With the exception of liver, MLTKβ is the more abundant of the two forms in most tissues (Gross et al. 2002). MLTK is found both in the cytoplasm and in the nucleus. Both splice forms have putative nuclear exclusion sequences, which suggest that the proteins shuttle between the cytoplasm and the nucleus. Blocking a nuclear export sequence receptor leads, in fact, to accumulation of MLTKβ in the nucleus (Gotoh et al. 2001). Activation of MLTK requires an autophosphorylation step (Gotoh et al. 2001), which occurs in the kinase loop region (Gross et al. 2002).

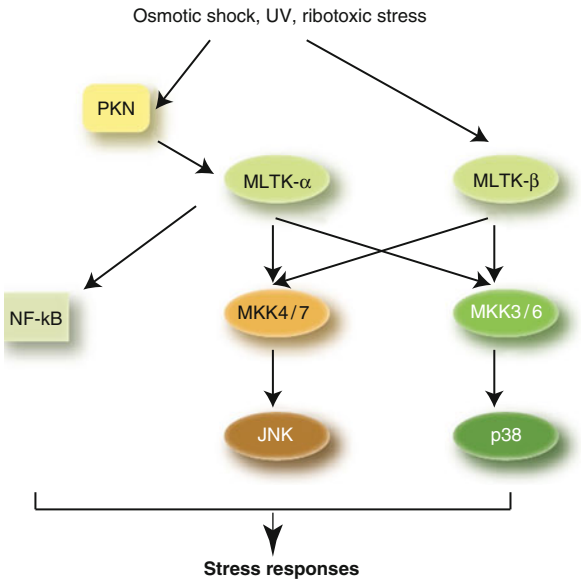


MLTK, Fig. 1 MLTK proteins structure. Schematic illustration of protein structure. *Ser/Thr kinase*, serine/threonine kinase; *L Zip*, Leucine Zipper; *SAM*, sterile α motif

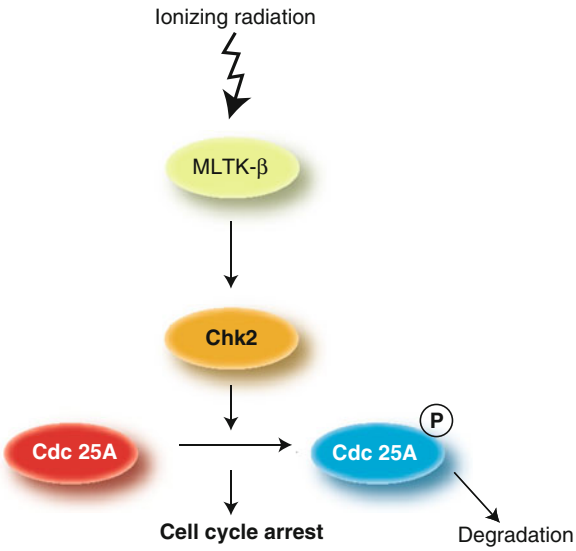
Despite sharing an identical kinase domain, there is some evidence suggesting that the two splice variants have distinct functions (see below).

Stress Response to IR, Anisomycin, UV, Osmotic Stress, Shiga Toxin, and Ricin

A variety of stressors activate both MLTKα and MLTKβ. They include osmotic stress (Gotoh et al. 2001; Takahashi et al. 2003; Mao et al. 2004), ultraviolet (UV) (Wang et al. 2005) and ionizing radiation (IR) (Gross et al. 2002; Tosti et al. 2004), and ribotoxic stress caused by anisomycin (Wang et al. 2005) and bacterial toxins like Shiga toxin and ricin (Jandhyala et al. 2008). When overexpressed in cells, both forms phosphorylate MKK4 and MKK7 to activate the Jun C-terminus kinase, JNK, and phosphorylate MKK3 and MKK6 to activate the p38 MAPK pathway (Gotoh et al. 2001, 2002; Yang 2002). The response to osmotic shock appears to be mediated by the protein kinase PKN, a member of the ▶ PKR family, upstream of MLTKα (Takahashi et al. 2003). A similar activation of MLTKβ has not been shown. Upon overexpression, MLTK also leads to activation of the MAP kinases ERK2 and ERK5 and the transcription factor ▶ NF-κB (Liu et al. 2000; Gotoh et al. 2001; Bloem et al. 2001; Gross et al. 2002) (Fig. 2). These responses may have protective and adaptive functions, unless the damage is too great, in which case, cell death is triggered. Apoptosis, a form of cell death, in fact, has been observed in some cases upon overexpression of MLTKα (Liu et al. 2000), and inhibition of MLTKβ improves cell viability after treatment with Shiga toxin and ricin (Jandhyala et al. 2008). MLTK proteins also mediate apoptotic responses following treatment with chemotherapeutic drugs like doxorubicin. This effect, however, appears to be limited only to normal cells and not to cancer cells (Sauter et al. 2010), which suggests that combining inhibition of MLTK with doxorubicin



MLTK, Fig. 2 MLTK signaling pathways stimulated by stress. Following treatment with the indicated stimuli, MLTK proteins activate the JNK and p38 MAP kinases and the transcription factor NF-κB, leading to stress responses



MLTK, Fig. 3 Role of MLTK-β in the response to DNA damage. Double-strand DNA damage induced by ionizing radiation activates MLTKβ, which in turn phosphorylates and activates Chk2. This protein phosphorylates and inactivates the phosphatase CDC25A, leading to cell cycle arrest

may increase the therapeutic window of this chemotherapeutic treatment.

In response to DNA damage caused by IR, MLTKβ phosphorylates the cell cycle check point protein Chk2 and contributes to cell cycle arrest in the S and G2 phases of the cell cycle (Tosti et al. 2004). Downregulation of MLTKβ by RNA interference, or by a dominant negative MLTKβ mutant, in irradiated cells prevents cell cycle arrest and leads to increased cell death (Gross et al. 2002; Tosti et al. 2004) (Fig. 3). MLTKα, but not MLTKβ, interacts with and phosphorylates histone H3, which is involved in chromatin remodeling (Choi et al. 2005).

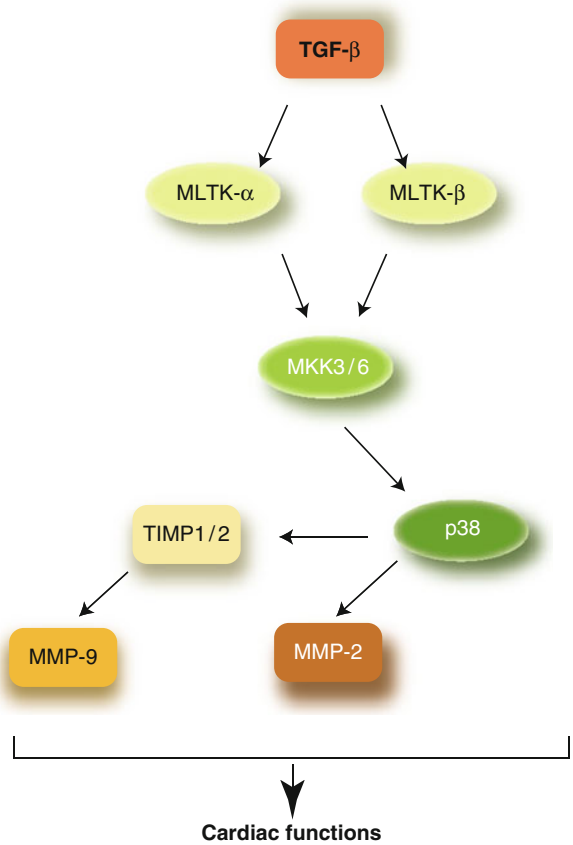
Cardiac Functions

The high levels of expression in heart and skeletal muscle suggest that MLTK proteins have an important role in these tissues. Overexpression of MLTK in cardiac myocytes or cardiomyoblasts causes hypertrophic growth, accompanied by increased protein synthesis and altered fetal gene expression (Bloem et al. 2001; Huang et al. 2004a). These effects are induced by TGF-β signals that are mediated by MLTK (Huang et al. 2004b). Overexpression of MLTKα in cardiomyoblasts increases metalloproteinase tissue inhibitor 1/2 (TIMP1/2) levels, which in turn reduce MMP-9 activity. MMP2 is also activated downstream of p38. These metalloproteinases have been suggested to contribute to cardiac fibrosis (Cheng et al. 2009) (Fig. 4).

The molecular events described above may explain the phenotypes observed in mice when MLTKβ is overexpressed in the heart. These animals, while they appear normal, show impaired cardiac functions under resting conditions and are less able to withstand cardiac stress. When challenged with β-adrenergic stress, provided by treatment with isoproterenol, the transgenic mice undergo a significant increase in mortality (Christe et al. 2004). Although these studies point to an important role for MLTK in the adaptive response to cardiac stress, mutations or alterations in expression levels of MLTK in the heart have not been investigated yet.

Regulation of the Actin Cytoskeleton

A clear distinction between MLTKα and MLTKβ functions has been observed upon overexpression of



MLTK, Fig. 4 MLTK pathway in the heart. Overexpression or stimulation of MLTK proteins by TGF-β leads to p38 activation, which contributes to cardiac changes via stimulation of the metalloproteases MMP2 and MMP9

these proteins in fibroblast cells. High levels of MLTKα, but not MLTKβ, cause disruption of the actin cytoskeleton that leads to cell rounding (Gotoh et al. 2001). As these proteins share an identical kinase domain, hence have the same enzymatic activity, it is likely that their different functions are mediated by their variant C-termini, which could be responsible for differential protein interactions and participation in different protein complexes.

Cancer

Conflicting observations have been reported regarding the role of MLTK proteins in cancer. In skin epidermal cells, overexpression of MLTKα induces proliferation and neoplastic transformation. These cells form fibrosarcoma tumors upon injection in athymic mice (Cho et al. 2004). In contrast, overexpression of MLTKα in lung cancer cells inhibits cell proliferation both in vitro and in vivo (Yang et al. 2010). Overexpression of MLTKα also causes cell cycle arrest in fibroblast cells (Yang 2002). The extent to which these opposite effects are caused by tissue-dependent responses or differences in expression levels used in the two studies is not clear. Elevated MLTK mRNA has been observed in multiple myeloma (Eisenberger et al. 2008); however, the relevance of this change to this disease is unclear.

Summary

The MLTK proteins are activated by a variety of stressors and may contribute to cellular adaptation to stress or cell death depending on the degree of damage. However, their role is not restricted to controlling apoptosis via the stress-activated MAP kinase proteins JNK and p38. Indeed, there is evidence that MLTK proteins are involved in additional functions like cell cycle arrest after ionizing radiation and cardiac adaptation to stress.

References

Bloem LJ, Pickard TR, Acton S, Donoghue M, Beavis RC, Knierman MD, et al. Tissue distribution and functional expression of a cDNA encoding a novel mixed lineage kinase. J Mol Cell Cardiol. 2001;33:1739–50.

Cheng YC, Kuo WW, Wu HC, Lai TY, Wu CH, Hwang JM, et al. ZAK induces MMP-2 activity via JNK/p38 signals and reduces MMP-9 activity by increasing TIMP-1/2 expression in H9c2 cardiomyoblast cells. Mol Cell Biochem. 2009;325:69–77.

Cho YY, Bode AM, Mizuno H, Choi BY, Choi HS, Dong Z. A novel role for mixed-lineage kinase-like mitogen-activated protein triple kinase alpha in neoplastic cell transformation and tumor development. Cancer Res. 2004;64:3855–64.

Choi HS, Choi BY, Cho YY, Zhu F, Bode AM, Dong Z. Phosphorylation of Ser28 in histone H3 mediated by mixed lineage kinase-like mitogen-activated protein triple kinase alpha. J Biol Chem. 2005;280:13545–53.

Christe M, Jin N, Wang X, Gould KE, Iversen PW, Yu X, et al. Transgenic mice with cardiac-specific over-expression of MLK7 have increased mortality when exposed to chronic beta-adrenergic stimulation. J Mol Cell Cardiol. 2004;37:705–15.

Eisenberger S, Ackermann K, Voggenreiter G, Sultmann H, Kasperk C, Pyerin W. Metastases and multiple myeloma generate distinct transcriptional footprints in osteocytes in vivo. J Pathol. 2008;214:617–26.

Gotoh I, Adachi M, Nishida E. Identification and characterization of a novel MAP kinase kinase kinase, MLTK. J Biol Chem. 2001;276:4276–86.
Gross EA, Callow MG, Waldbaum L, Thomas S, Ruggieri R. MRK, a mixed lineage kinase-related molecule that plays a role in gamma-radiation-induced cell cycle arrest. J Biol Chem. 2002;277:13873–82.
Huang CY, Chueh PJ, Tseng CT, Liu KY, Tsai HY, Kuo WW, et al. ZAK re-programs atrial natriuretic factor expression and induces hypertrophic growth in H9c2 cardiomyoblast cells. Biochem Biophys Res Commun. 2004a;324:973–80.
Huang CY, Kuo WW, Chueh PJ, Tseng CT, Chou MY, Yang JJ. Transforming growth factor-beta induces the expression of ANF and hypertrophic growth in cultured cardiomyoblast cells through ZAK. Biochem Biophys Res Commun. 2004b;324:424–31.
Jandhyala DM, Ahluwalia A, Obrig T, Thorpe CM. ZAK: a MAP3Kinase that transduces Shiga toxin- and ricin-induced proinflammatory cytokine expression. Cell Microbiol. 2008;10:1468–77.
Liu TC, Huang CJ, Chu YC, Wei CC, Chou CC, Chou MY, et al. Cloning and expression of ZAK, a mixed lineage kinase-like protein containing a leucine-zipper and a sterile-alpha motif. Biochem Biophys Res Commun. 2000;274:811–6.
Mao X, Bravo IG, Cheng H, Alonso A. Multiple independent kinase cascades are targeted by hyperosmotic stress but only one activates stress kinase p38. Exp Cell Res. 2004;292:304–11.
Sauter KA, Magun EA, Iordanov MS, Magun BE. ZAK is required for doxorubicin, a novel ribotoxic stressor, to induce SAPK activation and apoptosis in HaCaT cells. Cancer Biol Ther. 2010;10:1–9.
Takahashi M, Gotoh Y, Isagawa T, Nishimura T, Goyama E, Kim HS, et al. Regulation of a mitogen-activated protein kinase kinase kinase, MLTK by PKN. J Biochem (Tokyo). 2003;133:181–7.
Tosti E, Waldbaum L, Warshaw G, Gross EA, Ruggieri R. The stress kinase MRK contributes to regulation of DNA damage checkpoints through a p38gamma-independent pathway. J Biol Chem. 2004;279:47652–60.
Wang X, Mader MM, Toth JE, Yu X, Jin N, Campbell RM, et al. Complete inhibition of anisomycin and UV radiation but not cytokine induced JNK and p38 activation by an aryl-substituted dihydropyrrolopyrazole quinoline and mixed lineage kinase 7 small interfering RNA. J Biol Chem. 2005;280:19298–305.
Yang JJ. Mixed lineage kinase ZAK utilizing MKK7 and not MKK4 to activate the c-Jun N-terminal kinase and playing a role in the cell arrest. Biochem Biophys Res Commun. 2002;297:105–10.
Yang JJ, Lee YJ, Hung HH, Tseng WP, Tu CC, Lee H, et al. ZAK inhibits human lung cancer cell growth via ERK and JNK activation in an AP-1-dependent manner. Cancer Sci. 2010;101:1374–81.

MMAC

► PTEN

mMMK3b

► Mek3

Mnk (MAP Kinase-Interacting Kinase)

► p38 MAPK Family of Signal Transduction Proteins

mob-1

► CXCL10

Molecule Possessing Ankyrin Repeats Induced by Lipopolysaccharide (MAIL)

► IkBz

Molecule Possessing Ankyrin-Repeats Induced by Lipopolysaccharide

► IkBz

Monocytic Leukemia Zinc-Finger Protein

► MOZ and MORF Lysine Acetyltransferases

Monopolar Spindle 1 (Mps1)

Matthew L. H. Chu[1] and Patrick A. Eyers[2]
[1]Department of Structural Biology, Stanford University School of Medicine, Stanford, CA, USA
[2]YCR Institute for Cancer Studies, University of Sheffield, Sheffield, UK

Synonyms

Destruction-box, D-box; Monopolar spindle 1, Mps1; Spindle assembly checkpoint, SAC; Spindle pole body, SPB

Historical Background

Mps1 was first identified in the budding yeast *Saccharomyces cerevisiae* and named for the monopolar spindles that form in the *mps1* mutant strain (reviewed in Fisk et al. 2004). These spindles are generated as a result of the absence of spindle pole body (SPB) duplication; mutant yeast cells consequently undergo a "monopolar" mitosis. Four years later, the *mps1* gene was shown to encode an essential dual-specificity protein kinase, and a fission yeast homolog, termed Mph1, was also identified in *Schizosaccharomyces pombe* (Fisk et al. 2004). Library screening with antibodies to phosphotyrosine also identified a human protein kinase termed TTK/PYT, and a mouse kinase termed Esk; these were later recognized as vertebrate Mps1 orthologs. In addition, *Xenopus*, zebrafish and *Drosophila* Mps1 orthologs have also been characterized (Fisk et al. 2004). Indeed, the Mps1 family of protein kinases has now recognized in all eukaryotic phyla for which sequence data exist, except in the *Caenorhabditis elegans* genome, where its function may be redundant or replaced by a distinct protein kinase (Winey and Huneycutt 2002; Fisk et al. 2004).

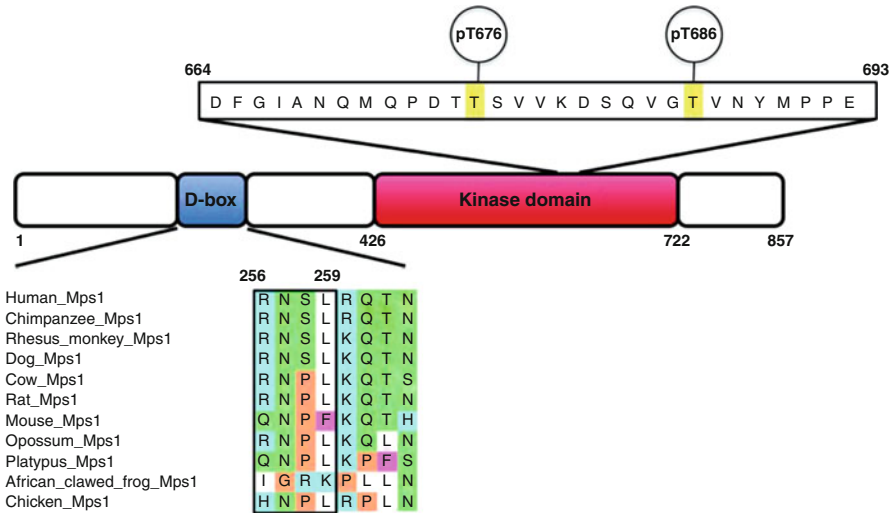
The domain structure of Mps1 is relatively simple (Fig. 1), with a large N-terminal region containing a Destruction- (D-) box, and a region that exhibits some homology to other mitotic kinases, likely to be important for subcellular targeting. The C-terminus harbors a kinase domain of the "non-RD" type, which exhibits dual-specificity kinase activity in vitro (Tyler et al. 2009).

Functions of Mps1

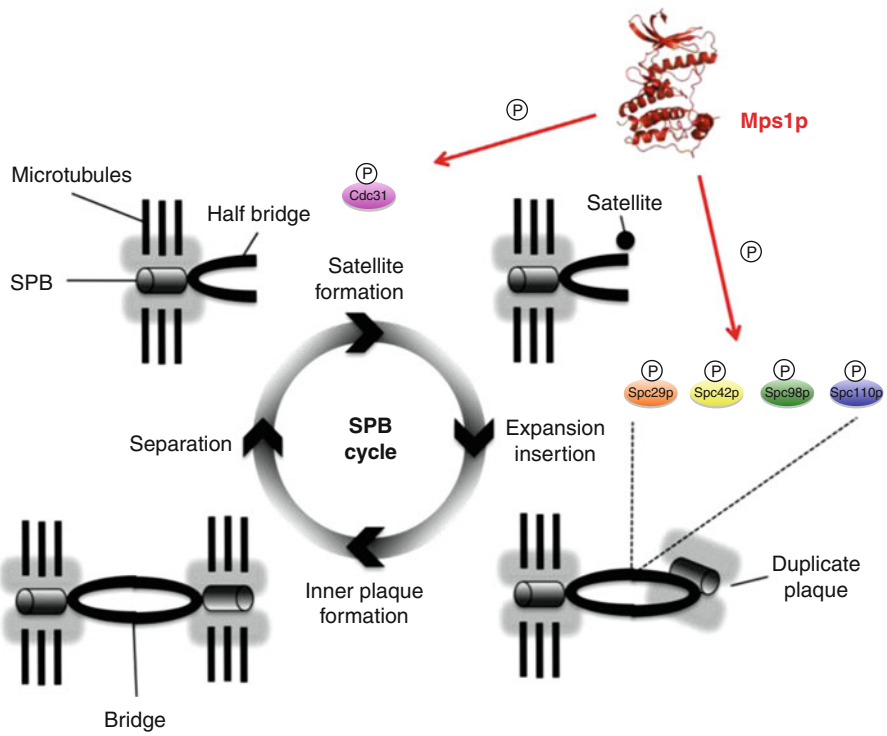
SPB duplication in budding yeast was the first identified function of Mps1p (Fisk et al. 2004). The SPB of yeast cells is duplicated during late G1 phase in the cell cycle to form the two poles of the mitotic spindle (Fig. 2). The *mps1p* gene is required for the transition from satellite-bearing to side-by-side SPBs. Loss of function caused by the original *mps1p* gene mutation (*mps1-1*) produces a single, large SPB with enlarged half-bridge (monopolar mitosis) (Fisk et al. 2004), and an additional series of alleles revealed that Mps1 has additional roles in SPB duplication (Winey and Huneycutt 2002). The unduplicated SPB observed by electron microscopy in *mps1-1* cells has a unique morphology, suggesting that Mps1p is required for SPB duplication (Fisk et al. 2004).

Furthermore, Mps1p localizes to SPBs (Fisk et al. 2004), and chemical genetic-induced inactivation of Mps1 revealed a defect in SPB duplication, suggesting that Mps1 has an important role in this process (Jones et al. 2005). Mps1p regulates SPB duplication by phosphorylating SPB components, including Spc98p, Spc110p, Spc42p, and Spc29p, which are all substrates of Mps1p in vitro and in vivo (Winey and Huneycutt 2002; Holinger et al. 2009; Fig. 2). Recently, the phosphorylation of Spc29p in G1/S phase was suggested to be required for recruiting the SPB membrane insertion machinery complex Mps2-Bbp1 to the newly formed SPB to facilitate its insertion into the nuclear envelope (Araki et al. 2010). An additional Mps1 substrate, the yeast centrin Cdc31, was also identified and its phosphorylation was shown to regulate its binding to the essential half bridge protein Kar1 (Araki et al. 2010). Although Mps1p is essential for SPB duplication in budding yeast, the fission yeast *S. pombe* Mps1 ortholog, Mph1, does not seem to have such a function (Winey and Huneycutt 2002).

The well-characterized requirement for Mps1p in SPB duplication in budding yeast implies that vertebrate Mps1 proteins might also function in centrosome duplication, analogous to those described for yeast Ipl (Aurora) and vertebrate Cdc5 (Polo) kinases. The mouse Mps1 enzyme was the first vertebrate Mps1 shown to regulate centrosome duplication. Mouse Mps1 localizes to the centrosome throughout the cell cycle in NIH 3T3 cells, and its overexpression induces centrosome reduplication, whereas overexpression of a catalytically inactive mutant (D637A) prevents centrosome duplication (reviewed in Fisk et al. 2004). Conflicting data have been reported as to the localization of human Mps1 and its possible roles in centrosome duplication. Stucke et al. demonstrated that human Mps1 is not required for centrosome duplication (Stucke et al. 2002), and did not find localization of human Mps1 at centrosomes or evidence that the kinase has a role in the centrosome cycle, despite the use of various functional analyses, including antibody microinjection, small interfering RNA (siRNA), and overexpression of wild-type and kinase-dead human Mps1. This result was unexpected owing to the very high sequence similarity (90%) between the mouse and human Mps1 proteins (Winey and Huneycutt 2002; Fisk et al. 2003). In contrast, separate studies showed



Monopolar Spindle 1 (Mps1), Fig. 1 Schematic representation of full-length human Mps1. Destruction-box (D-box) and kinase domain of Mps1 are denoted in *blue* and *red*, respectively. The D-box sequence (RNSL) is conserved in most mammalian species (bottom sequences, adapted from Cui et al. 2010). Conserved amino acids are indicated in *black* bars. The sequences were colored according to residue type: *Orange* small, *Red* negatively charged, *Blue* positively charged, *Green* polar, *White* hydrophobic, *Pink* aromatic. The activation segment sequence is indicated (*top*) and the important Mps1 autophosphorylation sites Thr 676 and Thr 686 (pT676 and pT686) are highlighted in *yellow*



Monopolar Spindle 1 (Mps1), Fig. 2 Mps1 regulates budding yeast spindle pole body (SPB) duplication. During the budding yeast cell cycle, the satellite appears on the cytoplasmic face of the half bridge during SPB duplication, and is thought to be the precursor of the new spindle pole body. The Mps1p gene is required for the transition from satellite-bearing to side-by-side SPBs and the phosphorylation of SPB components Cdc31, Spc29p, Spc42p, Spc98p, and Spc110p depend on Mps1p kinase activity

that human Mps1 does localize to centrosomes during interphase using distinct polyclonal Mps1 antibodies (reviewed in Fisk et al. 2004). Tyler et al. (2009) also reported centrosomal localization of phosphorylated human Mps1 in mitotic HeLa and DLD1 cells using RNA interference (RNAi)-validated phosphospecific

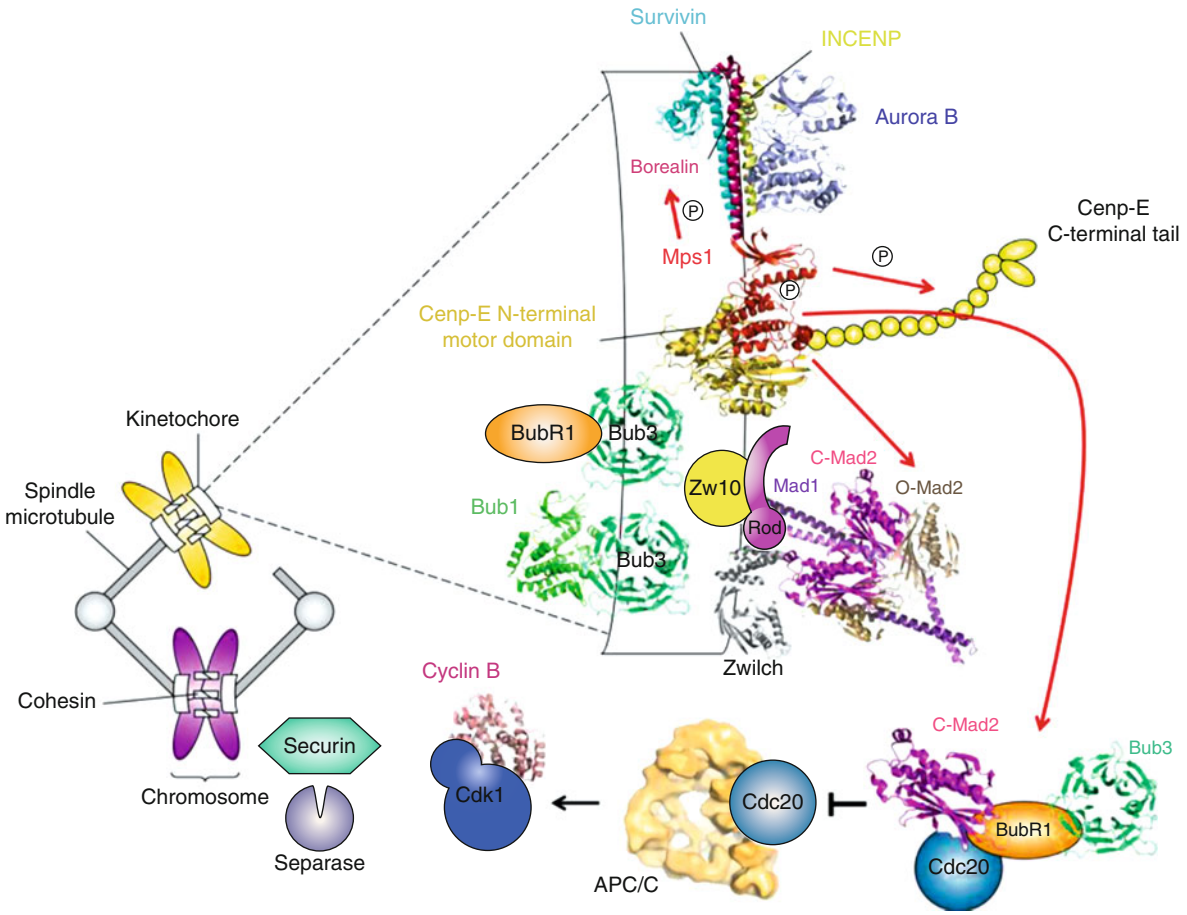
antibodies. Moreover, Fisk et al. showed that overexpression of human Mps1 causes centrosome reduplication, whereas overexpression of a kinase-dead mutant or siRNA depletion prevents normal centrosome duplication in various cell types, including NIH 3T3 cells (Fisk et al. 2003). Additional data further suggested that Mps1-dependent centrosome duplication lies downstream of Cyclin-Dependent Kinase-2 (CDK2) (Kasbek et al. 2007; Kasbek et al. 2009). Mps1-associated acceleration of centrosome duplication may also depend on the presence of phosphorylated mortalin (Kanai et al. 2007). Thus, despite controversy, vertebrate Mps1 protein kinases – including the human ortholog – do seem to regulate aspects of centrosome duplication.

The role of Mps1 in the SAC has been studied extensively. The checkpoint function of Mps1 was first reported when mutant *mps1-1* yeast cells were shown to inappropriately segregate their DNA (Fisk et al. 2004). The *mps1-1* mutant also overrides a nocodazole-induced mitotic checkpoint, further suggesting that Mps1p is essential in the SAC (Fisk et al. 2004). Mps1 localizes to the kinetochore through a complex series of protein-substrate interactions involving kinetochore components such as Mad1p, Ndc80-Hec1, and CENP-E (Fisk et al. 2004; Vigneron et al. 2004; Zhao and Chen 2006; Tighe et al. 2008). Notably, Mps1 also interacts with the APC and is destabilized by APC/C-Cdc20 for mitosis exit after anaphase in budding yeast (Palframan et al. 2006) and in human cells (Cui et al. 2010).

A chemical genetic study clearly revealed that inactivation of yeast Mps1 generates defects in the SAC (Jones et al. 2005). Moreover, the small-molecule inhibitor cincreasin blocks this checkpoint by inhibiting Mps1p (Dorer et al. 2005). The localization of Mps1p to kinetochores, observed by immunofluorescence and immunoelectron microscopy, is also consistent with its function in the SAC (Winey and Huneycutt 2002). Consistently, Mps1p phosphorylates a kinetochore protein termed Mad1p when the SAC is activated in yeast. The overexpression of Mps1p induces hyperphosphorylation of Mad1p and leads to cell cycle arrest in mitosis with morphologically normal spindles (Hardwick et al. 1996). In budding yeast, mutation in any of the checkpoint genes, *mad1-3* and *bub1-3*, blocks the ability of overexpressed Mps1p to arrest the cell cycle, indicating that Mps1p is involved in the Mad- and Bub-dependent SAC pathway and may lie "high up" in the cascade (Winey and Huneycutt 2002). Mps1p-dependent phosphorylation of Ndc80/HEC1 is also likely to be important for SAC activation at kinetochores (Kemmler et al. 2009).

Xenopus Mps1 is also required for the SAC and depletion of Mps1 leads to failure of the SAC in *Xenopus* egg extracts (reviewed in Fisk et al. 2004). Human Mps1 was subsequently shown by antibody microinjection and siRNA approaches to be required for mitotic checkpoint arrest in response to microtubule depolymerization (Fisk et al. 2003). Zebrafish Mps1 was also found to be required for SAC signaling during wound regeneration downstream of Fibroblast Growth Factor (FGF) (see Fisk et al. 2004). In fact, *Xenopus* Mps1, mouse Mps1, human Mps1, and *Drosophila* Mps1 all localize to kinetochores, probably through the noncatalytic N-terminus, implying a conserved N-terminal kinetochore function of vertebrate Mps1. Megator (Mtor), the *Drosophila* counterpart of the translocated promoter region of the human nuclear pore complex protein, promotes the recruitment of *Drosophila* Mps1 to unattached kinetochores and mediates normal mitotic duration and the SAC response (Lince-Faria et al. 2009). In *Xenopus* extracts, Mps1 is required for the recruitment of CENP-E, Mad1, and Mad2 to kinetochores. In human cells, the Hec1-Ndc80-Nuf2 complex is essential for the recruitment of human Mps1 to kinetochores (Fisk et al. 2004), as is the human PRP4 kinase, whose ablation by RNAi overrides the SAC (Montembault et al. 2007). In turn, human Mps1 is physically or catalytically required for the recruitment of the SAC components Mad1 and Mad2 (Fisk et al. 2003; Tighe et al. 2008; Xu et al. 2009).

Autophosphorylation of human Mps1 and modulation of its kinase activity have been extensively studied, and activity is required for SAC function (Mattison et al. 2007; Kang et al. 2007; Tighe et al. 2008; Jelluma et al. 2008a). Analysis using various chemical inhibitors demonstrated that Mps1 kinase activity is required to recruit other SAC components, including Bub1, BubR1, Bub3, Mad1, Mad2, and the Rod-Zw10-Zwilch complex (reviewed in Lan and Cleveland 2010) (Fig. 3). Mechanistically, Mps1 maintains the recruitment of the inactive open Mad2 conformer (O-Mad2) at unattached kinetochores to the stably bound Mad1-active closed Mad2 (C-Mad2) complex. Mps1 dimerizes and become activated by autophosphorylation at kinetochores followed by



Monopolar Spindle 1 (Mps1), Fig. 3 Mps1 functions in the SAC. Mps1 kinase activity is required to recruit the spindle assembly checkpoint proteins Aurora B, Bub1, Bub3, BubR1, CENP-E, Mad1, Mad2, and the Rod-Zw10-Zwilch complex to unattached kinetochores (white sections, expanded diagram indicated by *dashed lines*). The kinase activities of Aurora B, BubR1, and Mps1 are essential for checkpoint signaling. Aurora B binds and phosphorylates INCENP, which forms the chromosomal passenger complex with Survivin and Borealin. Mps1 phosphorylates Borealin and the CENP-E C-terminal tail; Mps1 also undergoes autophosphorylation. Mps1 maintains the recruitment of O-Mad2 at unattached kinetochores to the stably bound Mad1-C-Mad2 template. Activated C-Mad2, BubR1, and Bub3 form the mitotic checkpoint complex, which tightly associates with Cdc20, preventing it from activating the APC/C and thereby inhibiting ubiquitylation and degradation of securin and cyclin B. Separase, the protease that cleaves the securing that hold sister chromatids together, is inhibited by binding to securin. Thus, anaphase onset and mitotic exit are blocked. The crystal structures of some of these checkpoint proteins have been solved and are shown in the figure (produced using *Pymol*): Aurora B-INCENP – PDB ID 2BFX; Borealin-INCENP-Survivin – PDB ID 2QFA; Bub1 – PDB ID 3E7E; Bub3 – PDB ID 1YFQ; Cyclin B – PDB ID 2B9R; CENP-E N-terminal motor domain – PDB ID 1T5C; Mad1-Mad2 – PDB ID 1GO4; Mad2 – PDB ID 2V64; Mps1 – PDB ID 2ZMC; Zwilch – PDB ID 3IF8. The structure of the APC/C has recently been revealed using single-particle electron microcopy (EMD-1816) and is shown in the figure

rapid release into the cytosol (Kang et al. 2007). Cytosolic Mps1 kinase activity also promotes the assembly of the APC/C-Cdc20 inhibitory complex formed between BubR1, Bub3, and C-Mad2 (Lan and Cleveland 2010).

Mps1 was shown to phosphorylate Borealin (also called cell division cycle-associated protein-8, or Cdc8) to control the kinase activity of another mitotic kinase, Aurora B, thereby helping to coordinate kinetochore attachment and correct merotelic attachment error (only one kinetochore attached to both poles) alongside SAC signaling (Jelluma et al. 2008b). However, recent studies have also shown that the kinetochore localization of Mps1 may depend on Aurora

B activity, suggesting that Aurora B may indeed act upstream of Mps1 (Lan and Cleveland 2010). Mps1 also phosphorylates the kinesin-related motor protein CENP-E, which relieves its autoinhibition and is likely to contribute to M-phase chromosome congression (Espeut et al. 2008) (Fig. 3).

Like many other protein kinases that control mitotic progression, Mps1 has other functions beyond those described for centrosome duplication and the SAC. A chemical and genetic approach revealed that inactivation of Mps1p generates defects in mitotic spindle formation, sister kinetochore positioning at metaphase, and chromosome segregation during anaphase, implying a multifunctional requirement for Mps1p at the kinetochore in mitotic spindle assembly and function (Jones et al. 2005). Mutation analysis of a small subset of Mps1p phosphorylation sites on a kinetochore component, Dam1p, suggested that Mps1p and Ipl1p (Aurora B) are required for coupling kinetochores to plus ends of microtubules in budding yeast (Shimogawa et al. 2006). The analysis of Mps1p function in yeast meiosis further revealed that Mps1p is required for chromosome segregation and spore wall formation (Winey and Huneycutt 2002). Additionally, *Drosophila* Mps1 is required for the arrest of cell cycle progression in response to hypoxia (Pandey et al. 2007) and has an important role in meiosis in female flies by regulating processes that are crucial for ensuring the proper segregation of nonexchange chromosomes (Gilliland et al. 2005; Gilliland et al. 2007). A hypomorphic Mps1 mutation also causes aneuploidy in zebrafish embryos, indicating the disastrous consequences of defects in Mps1 function in vertebrate germ-cell meiosis (Poss et al. 2004). Notably, a more recent study has shown that Mps1 is a target of microRNA miR-133 in zebrafish, during fin regeneration downstream of the FGF receptor (Yin et al. 2008).

Depletion of human Mps1 by siRNA causes mitotic catastrophe in the absence of microtubule poisons, including a high incidence of unaligned chromosomes at metaphase, large numbers of lagging chromosomes in anaphase, and failure of cytokinesis (reviewed in Fisk et al. 2004). Recent studies also suggested that the kinase activity of human Mps1 is required for proper chromosome alignment and accurate chromosome segregation. Analysis using various chemical inhibitors showed that inhibition of Mps1 kinase activity prevents correction of syntelic attachments (both kinetochores of a mitotic chromatid pair are attached to the same pole) (reviewed in Lan and Cleveland 2010), which agree with the previous finding in Mps1-depleted cells using shRNA (Jelluma et al. 2008b; Tighe et al. 2008). Mps1 also phosphorylates the Bloom syndrome gene product BLM at Ser 144, which is important for ensuring accurate chromosome segregation, and its deregulation may contribute to cancer (Leng et al. 2006).

Interestingly, human Mps1 may also participate in regulation of the G2-M DNA structure checkpoint by directly phosphorylating CHK2 on Thr 68 (Wei et al. 2005). Studies have also shown that human Mps1 controls nuclear targeting of c-Abl tyrosine kinase by 14-3-3-coupled phosphorylation at Thr 735 of c-Abl in response to oxidative stress (Nihira et al. 2008), and that a constitutively active version of the oncogenic B-Raf protein could regulate the level of Mps1, thus influencing the spindle checkpoint, in human melanoma cells (Cui and Guadagno 2008). Mps1 might also influence p53-dependent postmitotic signaling mechanisms through phosphorylation of the tumor suppressor p53 (Huang et al. 2008).

Regulation of Mps1

In the context of yeast SPB duplication, genetic evidence indicates that Mps1p requires molecular chaperones for its function. The kinase activity of Mps1p is reduced in *cdc37*-mutant strains, implying that Cdc37 provides a client chaperone function that promotes Mps1p activity for SPB duplication (Winey and Huneycutt 2002). Cdc28 (CDK1) also phosphorylates Mps1p on Thr 29 to maintain Mps1p levels for regulating Spc42p phosphorylation and assembly during SPB duplication (Jaspersen et al. 2004). The regulation of vertebrate Mps1 function has been reported with respect to centrosome duplication during S phase. The kinase activity of the G1-S regulator CDK2 is believed to be required for regulating Mps1 activity. In particular, human Mps1 is phosphorylated by CDK2-cyclin E in vitro at Ser 436 and Thr 453; this observation has also been made with mouse and *Xenopus* Mps1 (Fisk et al. 2004; Grimison et al. 2006). Furthermore, one major function for CDK2 in centrosome duplication is to prevent the proteasome-mediated degradation of Mps1 during S phase (Fisk et al. 2004; Kasbek et al. 2007). Kasbek et al. (2007) further showed that phosphorylation at Thr 468 of

Mps1 by CDK2-cyclinA regulates the accumulation of Mps1 at centrosomes. Moreover, in *Xenopus*, mitogen-activated protein kinase (MAPK) phosphorylates *Xenopus* Mps1 on Ser 844, regulating its kinetochore localization (Zhao and Chen 2006). In human somatic cells or melanoma cells, B-Raf kinase signaling promotes phosphorylation and kinetochore localization of Mps1 through the MAPK pathway (Cui and Guadagno 2008; Borysova et al. 2008).

Studies in fission and budding yeast have shown that protein phosphatase-1 gamma regulates the SAC silencing mechanism by dephosphorylation of yeast checkpoint components, including Aurora B (Vanoosthuyse and Hardwick 2009; Pinsky et al. 2009). In human cells, Mps1 enzyme autoactivation (autophosphorylation) and substrate phosphorylation increases during G2, becoming maximal during mitosis (Stucke et al. 2002; Kang et al. 2007). Notably, Mps1 phosphorylation and activity are enhanced after activation of the SAC (Stucke et al. 2002), and Mps1 occupies an upstream position in the yeast SAC signaling cascade (Fisk et al. 2004). Identifying the protein phosphatase(s) that regulate Mps1 and/or inactivate the SAC in human cells is therefore an important challenge for the future. Human Mps1 phosphorylation and catalytic activity are enhanced by experimental activation of the SAC with nocodazole (Stucke et al. 2002). However, how these mitotic phosphorylation events contribute to the regulation of Mps1 activity during mitosis is not yet clear, although autophosphorylation may contribute to activation through changes in localization and/or substrate binding, or through the positioning of catalytic residues for productive catalysis. Recombinant Mps1 produced in bacteria is active when assessed by in vitro kinase assay and shows significant autophosphorylation and substrate phosphorylation when affinity-purified or immunopurified from prokaryotic or eukaryotic sources (Mattison et al. 2007; Kang et al. 2007; Jelluma et al. 2008b; Tyler et al. 2009).

The conserved activation-segment residues, Thr 676 and Thr 686, were identified as important autophosphorylation sites for the regulation of human Mps1 activity in vitro and in vivo (Mattison et al. 2007; Kang et al. 2007; Jelluma et al. 2008a; Tyler et al. 2009) (Fig. 1), and both of these sites are near-stoichiometrically modified in vitro (Johnson et al. 2009). Numerous other sites of autophosphorylation have been identified, including putative kinetochore targeting sites in the human N terminus at Thr 12 and Ser 15 (Xu et al. 2009). Tyler et al. (2009) used a combined mass-spectrometric, mutational, and phosphospecific antibody approach to identify a series of novel Mps1 autophosphorylation sites, several of which map to regions of the catalytic domain outside the activation segment. Although the in vivo function of many of these phosphorylation events is not yet known, they are important in regulating Mps1 autophosphorylation and activity in vitro (Kang et al. 2007; Tyler et al. 2009). In addition, a phosphorylated C-terminal extension is important for localization of *Xenopus* Mps1 to kinetochores in M-phase extracts (Zhao and Chen 2006); this conserved serine residue (Ser 844 in *Xenopus* Mps1 and Ser 794 in mouse Mps1) is also stoichiometrically autophosphorylated by Mps1 in vitro (Johnson et al. 2009) and highly phosphorylated on endogenous human Mps1 during mitosis (Tyler et al. 2009).

In addition to roles in centrosome duplication and the SAC, human Mps1 mRNA and protein expression are negatively regulated by the p53 protein after DNA damage (Bhonde et al. 2006). The lack of suppression of human Mps1 by p53 may contribute to DNA damage-induced apoptosis in cells (Bhonde et al. 2006). Human Mps1 activates CHK2 by phosphorylating CHK2 at Thr 68 after DNA damage. To maintain the DNA checkpoint control, activated CHK2 in turn phosphorylates Mps1 at Thr 288 and stabilizes the kinase, thus forming a positive regulatory loop (Yeh et al. 2009).

Mps1 protein levels are cell-cycle regulated and highest in rapidly proliferating tissues (Winey and Huneycutt 2002). CDK2 prevents the proteasome-mediated degradation of Mps1 during S phase (Fisk and Winey 2001), and phosphorylation of Mps1 at Thr 468 by CDK2-cyclinA regulates the accumulation of Mps1 at centrosomes in yeast (Kasbek et al. 2007). In addition, CHK2 phosphorylates Mps1 on Thr 288 and controls stability after DNA damage (Yeh et al. 2009). Several lines of indirect evidence suggest that the phosphorylation of Mps1 at Ser 844 is important for targeting and concentrating *Xenopus* Mps1 and Mad1 to the kinetochore and permitting M-phase checkpoint function (Zhao and Chen 2006), although this modification is clearly not important for *Xenopus* (Zhao and Chen 2006) or human (Tyler et al. 2009) Mps1 catalytic activity.

In anaphase, yeast Mps1p is regulated by the anaphase-promoting complex/cyclosome APC/C-Cdc20

complex. When the SAC is activated in metaphase, Mps1p acts through the checkpoint pathway to inhibit APC/C-Cdc20 activity and increase Mps1p stability, whereas in anaphase Mps1p is destabilized by APC/C-Cdc20 for mitosis exit (Palframan et al. 2006). Mps1p and APC/C-Cdc20 mutually inhibit each other to create this double-negative feedback loop (Palframan et al. 2006). However, it is still not known how yeast Mps1p localization is regulated. In human cells, Mps1 is also targeted for degradation by the APC/C-ubiquitin-proteasome pathway during late mitosis and G1 phase. A single D-box sequence, RNSL motif, was recently identified within the N-terminal region of human Mps1, which is recognized by APC/C-Cdc20 or APC/C-Cdh1 and is conserved in most mammalian species (Cui et al. 2010) (Fig. 1). The same study demonstrated that human Mps1 is a target of the APC/C-Cdc20 and APC/C-Cdh1 ubiquitin ligases, which undergoes proteolysis during anaphase through G1 phase to allow for proper centrosome duplication and cell cycle progression.

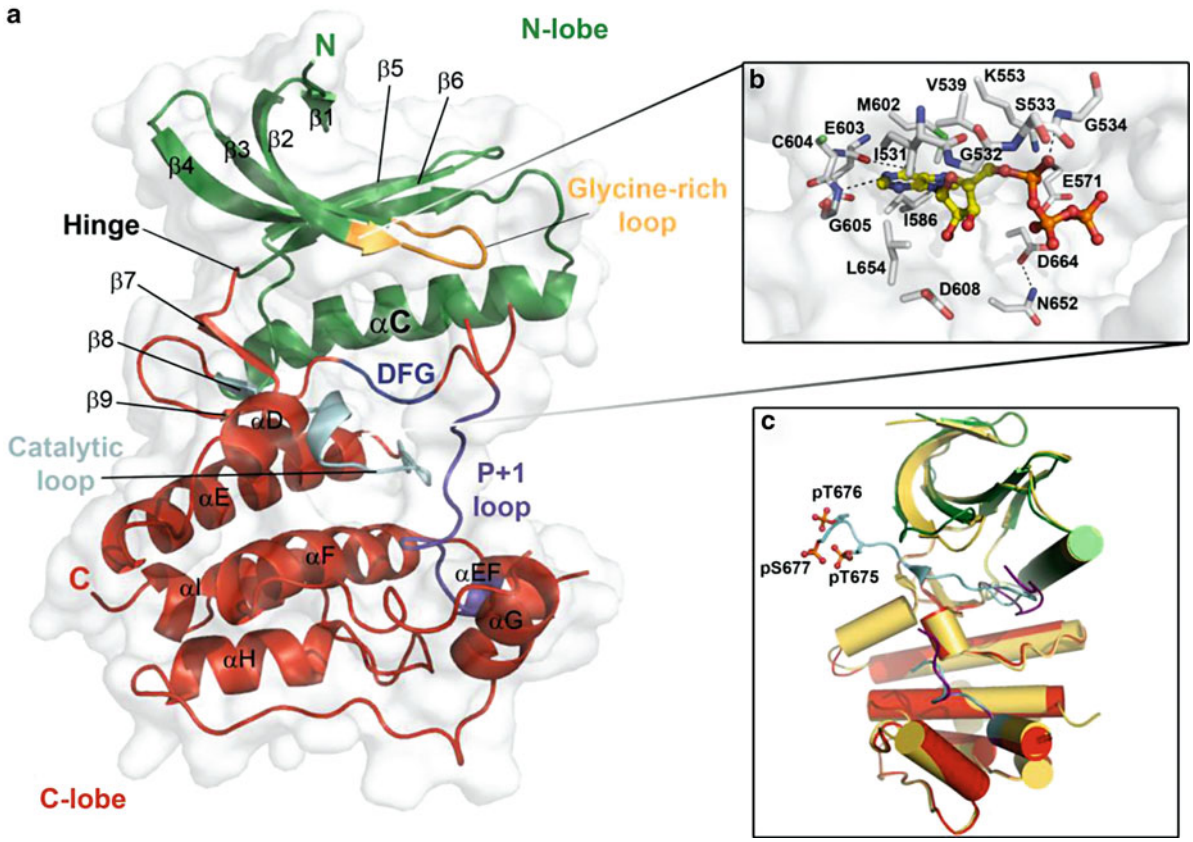
Mps1 and Disease

As a central mitotic kinase, it is not surprising that human Mps1 mRNA is found at high levels in freshly isolated malignant tissues and cancer cell lines, including breast cancer, cervical carcinoma, choriocarcinoma, hematopoietic cells, lung cancer, melanoma, neuroblastoma, ovarian cancer, and testicular tumors (Winey and Huneycutt 2002). The first data to implicate human Mps1 in tumorigenesis came with the observation that its mRNA is overexpressed in gastric cancer tissue, as well as in colon, kidney and lung cancers and bronchogenic carcinoma (Iwase et al. 1993). However, no studies have yet examined expression of Mps1 protein in parallel. Notably, Mps1 was identified in an siRNA screen of the human kinome as a modulator of cancer cell sensitivity to the microtubule-stabilizing agent, paclitaxel (Swanton et al. 2007). Dual roles of Mps1 in chromosome alignment and the SAC might be particularly important for its ability to synergize with therapeutic concentrations of paclitaxel in transformed cells (Janssen et al. 2009). These findings also indicate that Mps1 inhibition might be useful in treating patients with paclitaxel-resistant cancer, perhaps as part of a synthetic lethal strategy (Kaelin 2005). Notably, heterozygous Mps1 mutations have recently been identified in microsatellite-unstable colorectal cancers although these Mps1 mutants did not promote SAC weakening or override mitotic arrest (Niittymaki et al. 2011). By virtue of its importance in SAC signaling and its regulation of the Aurora B signaling pathway through Borealin (Jelluma et al. 2008b; Bourhis et al. 2009), Mps1 inhibition or modulation of its degradation kinetics might represent a way to sensitize or kill rapidly dividing cells (Kops et al. 2004; Kasbek et al. 2009).

Small Molecule Mps1 Inhibitors

Before 2010, only three small molecules – cincreasin (Dorer et al. 2005), SP600125 (Schmidt et al. 2005), and 1NM-PP1 (Jones et al. 2005; Tighe et al. 2008) – had been described to inhibit cellular yeast and human Mps1, respectively. Genetic and biochemical data suggest that Mps1p is a target of cincreasin, but this compound is a weak inhibitor of Mps1p, with a relatively high half-maximal inhibitory concentration of about 700 μm in an in vitro kinase assay (Dorer et al. 2005). The cell-permeant ATP analogue inhibitor 1NM-PP1 inhibits a modified M600G/A Mps1 allele and can be used to probe Mps1 function in yeast or human cells (Jones et al. 2005; Tighe et al. 2008). The ATP-competitive JNK1 inhibitor SP600125 is also a potent inhibitor of human Mps1 function. SP600125 completely inhibits Mps1 activity at 10 μm in vivo and in vitro (Schmidt et al. 2005); in contrast, it inhibits JNK1 at a half-maximal inhibitory concentration of 40 nM and is the first-choice JNK inhibitor for cellular studies (Bennett et al. 2001). Unfortunately, SP600125 also inhibits several other kinases in vitro, so its use as a specific inhibitor of Mps1 activity is prone to validation problems (Bain et al. 2007; Tighe et al. 2008). Nevertheless, SP600125 has also emerged as a potentially useful screening tool for the discovery of ATP-dependent Mps1 inhibitors through the development of fluorescent Mps1 ligand-displacement assays (Chu et al. 2010).

In 2010, six groups reported seven distinct small-molecule inhibitors of Mps1 (reviewed in Lan and Cleveland 2010; Colombo et al. 2010). Using these different chemical inhibitors, these studies demonstrated that Mps1 kinase activity is indispensable for accurate chromosome segregation through its recruitment of SAC proteins to kinetochores, formation of the



Monopolar Spindle 1 (Mps1), Fig. 4 Crystal structure of Mps1 catalytic domain. (**a**) Ribbon representation of the WT-apo Mps1 catalytic domain (PDB ID 2ZMC; Chu et al. 2008). Characteristic key features important for substrate binding and catalysis are labeled as follows: glycine loop (*orange*), aC helix, catalytic loop (*cyan*), activation segment with DFG motif (*blue*), and P + 1 loop (*purple*). (**b**) Detailed view of Mps1-ATP co-crystal structure, showing ATP bound in the hydrophobic cleft (PDB ID 3HMN; Chu et al. 2010). The residues that interact with ATP are depicted as *sticks* and ATP is depicted as *ball-and-sticks*. Catalytic residues are not in typical "active-form" orientations (conserved ion-pair Lys-553 and Glu-571 is disengaged, Asp-608 and Asn-652 in the catalytic loop, and Asp-664 at the DFG-motif do not interact with the ATP). (**c**) Superposition of unphosphorylated (*red* and *green*; PDB ID 3HMN; Chu et al. 2010) and phosphorylated Mps1 (*gold*, PDB ID 3H9F; Kwiatkowski et al. 2010) (90°-view of Fig. 4a). The activation segment of the unphosphorylated and phosphorylated Mps1 are colored in *purple* and *cyan*, respectively, and phospho-Thr-675, phospho-Thr-676, phospho-Ser-677 are shown as *ball-and-sticks*. The figure was produced using Pymol

APC/C-Cdc20 inhibitory mitotic checkpoint complex, and correction of erroneous microtubule attachments. However, contradictory results were described among the studies: two papers proposed that Mps1 acts upstream of Aurora B to correct syntelic attachments, while three other papers found that the kinetochore localization of Mps1 depends on Aurora B activity, suggesting that Aurora B acts upstream of Mps1 (reviewed by Lan and Cleveland 2010). An ultimate goal of Mps1 inhibitor discovery is the design of novel therapeutic agents for proliferative disorders such as cancer. Initial studies have shown that the purine analogues, 23-dMB-PP1, Mps1-IN-1, and Mps1-IN-2, all kill cultured tumor cells by direct targeting of Mps1. Colombo et al. (2010) have also described a selective, orally bioavailable, Mps1 inhibitor (NMS-P715) that reduces cancer cell proliferation and inhibits tumor growth in a preclinical model.

Crystal Structures of Human Mps1 Catalytic Domain: Insights into Mps1 Structural Biology

The first published crystal structures of the human Mps1 catalytic domain (amino acids 424–791) were

unphosphorylated catalytic domain deletions of a wild-type apo form (amino acids 510–857) and an inactive T686A mutant complexed with SP600125 at 3.14 Å and 2.88 Å, respectively (Chu et al. 2008). The catalytic domain of Mps1 adopts the classical fold of protein kinases, with the activation loop packing against the N-terminal lobe, where it induces conformational changes indicative of a catalytically inactive form of the enzyme (Fig. 4a). The structure of the complex with SP600125 shows the inhibitor bound in the ATP-binding site and highlights distinct structural features in the active-site cleft that might be exploited for rational drug design. Chu et al. (2010) also determined co-crystal structures with ATP (2.7 Å, Fig. 4b), the aspecific model kinase inhibitor staurosporine (2.4 Å) and a low-affinity quinazoline drug-like fragment termed Compound 4 (2.3 Å), which extended the knowledge of the Mps1 nucleotide binding site architecture. A 2.7-Å K553R Mps1 mutant apo-structure has also been reported (Wang et al. 2009). Despite the similar fold in other published Mps1 structures, Wang et al. identified two lysine residues, Lys 708 and Lys 710, in the loop between the αEF and αF helices that are essential for in vitro substrate recruitment and maintenance of high levels of kinase activity through Mps1 autophosphorylation. Although the C terminus of Mps1 is structurally disordered, Sun et al. (2010) recently showed that this tail is important for Mps1 substrate binding and transphosphorylation (but without affecting autophosphorylation). Most interestingly, it is also essential for SAC activation.

Most recently, Kwiatkowski et al. (2010) and Colombo et al. (2010) also reported Mps1 co-crystal structures with the kinase inhibitors, Mps1-IN-1 (2.74 Å), Mps1-IN-2 (2.6 Å), and NMS-P715 (3.1 Å), which give new insights for the design of Mps1-specific inhibitors. Significant structural difference is also found in the Mps1-IN-2 Mps1 complex. While the activation segments are absent in all other Mps1 crystal structures, it becomes ordered in the presence of this compound. Indeed, three phosphorylation sites can be observed, including Thr 675, Thr 676, and Ser 677, although the overall conformation of the catalytic domain is similar to those reported for unphosphorylated Mps1 structures (r.m.s.d. = 0.6 Å, Fig. 4c). This questions the function of the phosphorylation of these residues for Mps1 activation and the potential stabilization of a dimeric transphosphorylation complex.

Summary

Mps1 is a conserved eukaryotic dual-specificity protein kinase with an important role in centrosome duplication, SAC, chromosome alignment, and other critical cell cycle processes. Mps1 phosphorylates a growing family of cell cycle-modified proteins, and its own signaling function is controlled by phosphorylation, ubiquitination, and transient subcellular targetting. Inhibition of Mps1 activity by small molecules, either alone or as part of a multitherapy regime, might represent a novel therapeutic strategy for proliferative diseases such as cancer, where its central role as a cell cycle orchestrator makes it necessary for cell survival.

References

Araki Y, Gombos L, Migueleti SP, Sivashanmugam L, Antony C, Schiebel E. N-terminal regions of Mps1 kinase determine functional bifurcation. J Cell Biol. 2010;189:41–56.

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. Biochem J. 2007;408:297–315.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA. 2001;98:13681–6.

Bhonde MR, Hanski ML, Budczies J, Cao M, Gillissen B, Moorthy D, Simonetta F, Scherubl H, Truss M, Hagemeier C, Mewes HW, Daniel PT, Zeitz M, Hanski C. DNA damage-induced expression of p53 suppresses mitotic checkpoint kinase hMps1: the lack of this suppression in p53MUT cells contributes to apoptosis. J Biol Chem. 2006;281:8675–85.

Borysova MK, Cui Y, Snyder M, Guadagno TM. Knockdown of B-Raf impairs spindle formation and the mitotic checkpoint in human somatic cells. Cell Cycle. 2008;7:2894–901.

Bourhis E, Lingel A, Phung Q, Fairbrother WJ, Cochran AG. Phosphorylation of a borealin dimerization domain is required for proper chromosome segregation. Biochemistry. 2009;48:6783–93.

Chu ML, Chavas LM, Douglas KT, Eyers PA, Tabernero L. Crystal structure of the catalytic domain of the mitotic checkpoint kinase Mps1 in complex with SP600125. J Biol Chem. 2008;283:21495–500.

Chu ML, Lang Z, Chavas LM, Neres J, Fedorova OS, Tabernero L, Cherry M, Williams DH, Douglas KT, Eyers PA. Biophysical and X-ray crystallographic analysis of Mps1 kinase inhibitor complexes. Biochemistry. 2010;49:1689–701.

Colombo R, Caldarelli M, Mennecozzi M, Giorgini ML, Sola F, Cappella P, Perrera C, Depaolini SR, Rusconi L, Cucchi U, Avanzi N, Bertrand JA, Bossi RT, Pesenti E, Galvani A, Isacchi A, Colotta F, Donati D, Moll J. Targeting the mitotic checkpoint for cancer therapy with NMS-P715, an inhibitor of MPS1 kinase. Cancer Res. 2010;70:10255–64.

Cui Y, Guadagno TM. B-Raf(V600E) signaling deregulates the mitotic spindle checkpoint through stabilizing Mps1 levels in melanoma cells. Oncogene. 2008;27:3122–33.

Cui Y, Cheng X, Zhang C, Zhang Y, Li S, Wang C, Guadagno TM. Degradation of the human mitotic checkpoint kinase Mps1 is cell cycle-regulated by APC-cCdc20 and APC-cCdh1 ubiquitin ligases. J Biol Chem. 2010;285:32988–98.

Dorer RK, Zhong S, Tallarico JA, Wong WH, Mitchison TJ, Murray AW. A small-molecule inhibitor of Mps1 blocks the spindle-checkpoint response to a lack of tension on mitotic chromosomes. Curr Biol. 2005;15:1070–76.

Espeut J, Gaussen A, Bieling P, Morin V, Prieto S, Fesquet D, Surrey T, Abrieu A. Phosphorylation relieves autoinhibition of the kinetochore motor Cenp-E. Mol Cell. 2008;29:637–43.

Fisk HA, Winey M. The mouse Mps1p-like kinase regulates centrosome duplication. Cell. 2001;106:95–104.

Fisk HA, Mattison CP, Winey M. Human Mps1 protein kinase is required for centrosome duplication and normal mitotic progression. Proc Natl Acad Sci USA. 2003; 100:14875–80.

Fisk HA, Mattison CP, Winey M. A field guide to the Mps1 family of protein kinases. Cell Cycle. 2004; 3:439–42.

Gilliland WD, Wayson SM, Hawley RS. The meiotic defects of mutants in the Drosophila mps1 gene reveal a critical role of Mps1 in the segregation of achiasmate homologs. Curr Biol. 2005;15:672–7.

Gilliland WD, Hughes SE, Cotitta JL, Takeo S, Xiang Y, Hawley RS. The multiple roles of mps1 in Drosophila female meiosis. PLoS Genet. 2007;3:e113.

Grimison B, Liu J, Lewellyn AL, Maller JL. Metaphase arrest by cyclin E-Cdk2 requires the spindle-checkpoint kinase Mps1. Curr Biol. 2006;16:1968–73.

Hardwick KG, Weiss E, Luca FC, Winey M, Murray AW. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. Science. 1996;273: 953–56.

Holinger EP, Old WM, Giddings Jr TH, Wong C, Yates III JR, Winey M. Budding yeast centrosome duplication requires stabilization of SPC29 via MPS1-mediated phosphorylation. 2009;284(19):12949-55.

Huang H, Hittle J, Zappacosta F, Annan RS, Hershko A, Yen TJ. Phosphorylation sites in BubR1 that regulate kinetochore attachment, tension, and mitotic exit. J Cell Biol. 2008;183:667–80.

Iwase T, Tanaka M, Suzuki M, Naito Y, Sugimura H, Kino I. Identification of protein-tyrosine kinase genes preferentially expressed in embryo stomach and gastric cancer. Biochem Biophys Res Commun. 1993;194: 698–705.

Janssen A, Kops GJ, Medema RH. Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells. Proc Natl Acad Sci USA. 2009;106:19108–13.

Jaspersen SL, Huneycutt BJ, Giddings Jr TH, Resing KA, Ahn NG, Winey M. Cdc28/Cdk1 regulates spindle pole body duplication through phosphorylation of Spc42 and Mps1. Dev Cell. 2004;7:263–74.

Jelluma N, Brenkman AB, McLeod I, Yates III JR, Cleveland DW, Medema RH, Kops GJ. Chromosomal instability by inefficient Mps1 auto-activation due to a weakened mitotic checkpoint and lagging chromosomes. PLoS One. 2008a;3:e2415.

Jelluma N, Brenkman AB, van den Broek NJ, Cruijsen CW, van Osch MH, Lens SM, Medema RH, Kops GJ. Mps1 phosphorylates Borealin to control Aurora B activity and chromosome alignment. Cell. 2008b;132:233–46.

Johnson H, Eyers CE, Eyers PA, Beynon RJ, Gaskell SJ. Rigorous determination of the stoichiometry of protein phosphorylation using mass spectrometry. J Am Soc Mass Spectrom. 2009;20:2211–20.

Jones MH, Huneycutt BJ, Pearson CG, Zhang C, Morgan G, Shokat K, Bloom K, Winey M. Chemical genetics reveals a role for Mps1 kinase in kinetochore attachment during mitosis. Curr Biol. 2005;15:160–5.

Kaelin Jr WG. The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer. 2005;5:689–98.

Kanai M, Ma Z, Izumi H, Kim SH, Mattison CP, Winey M, Fukasawa K. Physical and functional interaction between mortalin and Mps1 kinase. Genes Cells. 2007; 12:797–810.

Kang J, Chen Y, Zhao Y, Yu H. Autophosphorylation-dependent activation of human Mps1 is required for the spindle checkpoint. Proc Natl Acad Sci USA. 2007;104:20232–7.

Kasbek C, Yang CH, Yusof AM, Chapman HM, Winey M, Fisk HA. Preventing the degradation of mps1 at centrosomes is sufficient to cause centrosome reduplication in human cells. Mol Biol Cell. 2007;18:4457–69.

Kasbek C, Yang CH, Fisk HA. Mps1 as a link between centrosomes and genomic instability. Environ Mol Mutagen. 2009;50(8):654–65.

Kemmler S, Stach M, Knapp M, Ortiz J, Pfannstiel J, Ruppert T, Lechner J. Mimicking Ndc80 phosphorylation triggers spindle assembly checkpoint signalling. EMBO J. 2009;28: 1099–110.

Kops GJ, Foltz DR, Cleveland DW. Lethality to human cancer cells through massive chromosome loss by inhibition of the mitotic checkpoint. Proc Natl Acad Sci USA. 2004;101: 8699–704.

Kwiatkowski N, Jelluma N, Filippakopoulos P, Soundararajan M, Manak MS, Kwon M, Choi HG, Sim T, Deveraux QL, Rottmann S, Pellman D, Shah JV, Kops GJ, Knapp S, Gray NS. Small-molecule kinase inhibitors provide insight into Mps1 cell cycle function. Nat Chem Biol. 2010;6:359–68.

Lan W, Cleveland DW. A chemical tool box defines mitotic and interphase roles for Mps1 kinase. J Cell Biol. 2010;190:21–4.

Leng M, Chan DW, Luo H, Zhu C, Qin J, Wang Y. MPS1-dependent mitotic BLM phosphorylation is important for chromosome stability. Proc Natl Acad Sci USA. 2006;103:11485–90.

Lince-Faria M, Maffini S, Orr B, Ding Y, Claudia F, Sunkel CE, Tavares A, Johansen J, Johansen KM, Maiato H. Spatiotemporal control of mitosis by the conserved spindle matrix protein Megator. J Cell Biol. 2009;184:647–57.

Mattison CP, Old WM, Steiner E, Huneycutt BJ, Resing KA, Ahn NG, Winey M. Mps1 activation loop autophosphorylation enhances kinase activity. J Biol Chem. 2007;282:30553–61.

Montembault E, Dutertre S, Prigent C, Giet R. PRP4 is a spindle assembly checkpoint protein required for MPS1, MAD1, and

MAD2 localization to the kinetochores. J Cell Biol. 2007;179:601–9.
Nihira K, Taira N, Miki Y, Yoshida K. TTK/Mps1 controls nuclear targeting of c-Abl by 14-3-3-coupled phosphorylation in response to oxidative stress. Oncogene. 2008;27:7285–95.
Niittymaki I, Gylfe A, Laine L, Laakso M, Lehtonen HJ, Kondelin J, Tolvanen J, Nousiainen K, Pouwels J, Jarvinen H, Nuorva K, Mecklin JP, Makinen M, Ristimaki A, Orntoft TF, Hautaniemi S, Karhu A, Kallio MJ, Aaltonen LA. High frequency of TTK mutations in microsatellite-unstable colorectal cancer and evaluation of their effect on spindle-assembly checkpoint. Carcinogenesis. 2011;32:305–11.
Palframan WJ, Meehl JB, Jaspersen SL, Winey M, Murray AW. Anaphase inactivation of the spindle checkpoint. Science. 2006;313:680–4.
Pandey R, Heeger S, Lehner CF. Rapid effects of acute anoxia on spindle kinetochore interactions activate the mitotic spindle checkpoint. J Cell Sci. 2007;120:2807–18.
Pinsky BA, Nelson CR, Biggins S. Protein phosphatase 1 regulates exit from the spindle checkpoint in budding yeast. Curr Biol. 2009;19:1182–7.
Poss KD, Nechiporuk A, Stringer KF, Lee C, Keating MT. Germ cell aneuploidy in zebrafish with mutations in the mitotic checkpoint gene mps1. Genes Dev. 2004;18:1527–32.
Schmidt M, Budirahardja Y, Klompmaker R, Medema RH. Ablation of the spindle assembly checkpoint by a compound targeting Mps1. EMBO Rep. 2005;6:866–72.
Shimogawa MM, Graczyk B, Gardner MK, Francis SE, White EA, Ess M, Molk JN, Ruse C, Niessen S, Yates III JR, Muller EG, Bloom K, Odde DJ, Davis TN. Mps1 phosphorylation of Dam1 couples kinetochores to microtubule plus ends at metaphase. Curr Biol. 2006;16:1489–501.
Stucke VM, Sillje HH, Arnaud L, Nigg EA. Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. EMBO J. 2002;21:1723–32.
Sun T, Yang X, Wang W, Zhang X, Xu Q, Zhu S, Kuchta R, Chen G, Liu X. Cellular abundance of Mps1 and the role of its carboxyl terminal tail in substrate recruitment. J Biol Chem. 2010;285:38730–9.
Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E, Elustondo F, Chang J, Temple J, Ahmed AA, Brenton JD, Downward J, Nicke B. Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. Cancer Cell. 2007;11:498–512.
Tighe A, Staples O, Taylor S. Mps1 kinase activity restrains anaphase during an unperturbed mitosis and targets Mad2 to kinetochores. J Cell Biol. 2008;181:893–901.
Tyler RK, Chu ML, Johnson H, McKenzie EA, Gaskell SJ, Eyers PA. Phosphoregulation of human Mps1 kinase. Biochem J. 2009;417:173–81.
Vanoosthuyse V, Hardwick KG. A novel protein phosphatase 1-dependent spindle checkpoint silencing mechanism. Curr Biol. 2009;19:1176–81.
Vigneron S, Prieto S, Bernis C, Labbe JC, Castro A, Lorca T. Kinetochore localization of spindle checkpoint proteins: who controls whom? Mol Biol Cell. 2004;15:4584–96.
Wang W, Yang Y, Gao Y, Xu Q, Wang F, Zhu S, Old W, Resing K, Ahn N, Lei M, Lui X. Structural and mechanistic insights into dMps1 Kinase Activation. J Cell Mol Med. 2009;13:1679–94.
Wei JH, Chou YF, Ou YH, Yeh YH, Tyan SW, Sun TP, Shen CY, Shieh SY. TTK/hMps1 participates in the regulation of DNA damage checkpoint response by phosphorylating CHK2 on threonine 68. J Biol Chem. 2005;280:7748–57.
Winey M, Huneycutt BJ. Centrosomes and checkpoints: the MPS1 family of kinases. Oncogene. 2002;21:6161–9.
Xu Q, Zhu S, Wang W, Zhang X, Old W, Ahn N, Liu X. Regulation of kinetochore recruitment of two essential mitotic spindle checkpoint proteins by Mps1 phosphorylation. Mol Biol Cell. 2009;20:10–20.
Yeh YH, Huang YF, Lin TY, Shieh SY. The cell cycle checkpoint kinase CHK2 mediates DNA damage-induced stabilization of TTK/hMps1. Oncogene. 2009;28:1366–78.
Yin VP, Thomson JM, Thummel R, Hyde DR, Hammond SM, Poss KD. Fgf-dependent depletion of microRNA-133 promotes appendage regeneration in zebrafish. Genes Dev. 2008;22:728–33.
Zhao Y, Chen RH. Mps1 phosphorylation by MAP kinase is required for kinetochore localization of spindle-checkpoint proteins. Curr Biol. 2006;16:1764–9.

Monopolar Spindle 1, Mps1

► Monopolar Spindle 1 (Mps1)

MORF

► MOZ and MORF Lysine Acetyltransferases

MORF Lysine Acetyltransferase

► MOZ and MORF Lysine Acetyltransferases

MOX44

► CD53

MOZ

► MOZ and MORF Lysine Acetyltransferases

MOZ and MORF Lysine Acetyltransferases

Jiang-Ping Zhang[1,2], Xiaoyu Du[1,2] and Xiang-Jiao Yang[1,2,3]
[1]The Rosalind & Morris Goodman Cancer Research Center, McGill University, Québec, Canada
[2]Department of Medicine, McGill University Health Center, Montréal, Québec, Canada
[3]Departments of Biochemistry and Anatomy & Medicine, McGill University Health Center, Montréal, Québec, Canada

Synonyms

MOZ lysine acetyltransferase: MOZ; Monocytic leukemia zinc-finger protein; MYST3; Lysine (K) acetyltransferase 6A; KAT6A
MORF lysine acetyltransferase: MORF; MOZ-related factor; MYST4; Querkopf; Lysine (K) acetyltransferase 6B; KAT6B

Historical Background

MOZ is a founding member of the MYST (*MOZ*, *YBF2*, *SAS2*, and *TIP60*) family of lysine acetyltransferases (Borrow et al. 1996). It was identified in 1996 through positional cloning of the reciprocal chromosomal translocation t(8;16)(p11;p13) associated with a subset of acute myeloid leukemia (AML) (Borrow et al. 1996). A few years later, the acetyltransferase activity was formally demonstrated and mapped to the MYST domain (Champagne et al. 2001; Kitabayashi et al. 2001a). Human MORF was identified in BLAST search against expressed sequence tag databases for additional MYST proteins (Champagne et al. 1999). Mouse *Morf* was identified as *Querkopf*, a mutant allele causing craniofacial abnormalities (Thomas et al. 2000). MORF is highly homologous to MOZ (Fig. 1).

Domain Organization of MOZ and MORF

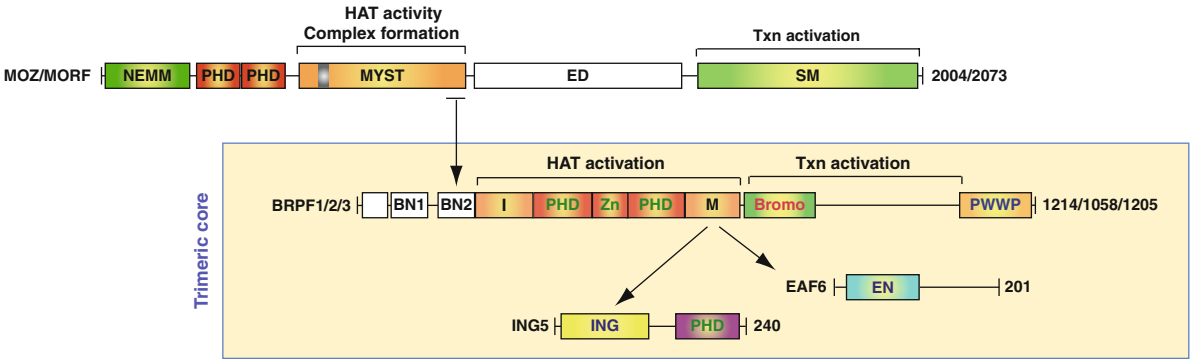
MOZ and MORF are large proteins (~250 kDa) with multiple functional domains (Fig. 1). The MYST domain contains a C2HC nucleosome-binding zinc finger and the histone acetyl transferase (HAT) core. The MYST domain of MOZ is able to acetylate histone H2A, H3 and H4, Runx1, and MOZ itself (Champagne et al. 2001; Kitabayashi et al. 2001a), but cannot acetylate nucleosomal histones (Champagne et al. 2001). MORF preferentially acetylates histones H3 and H4, and is capable to autoacetylate itself. When oligonucleosomes is used as substrates, MORF preferentially acetylated H4 (Champagne et al. 1999).

MOZ and MORF are members of the MYST family, which has five members in *Drosophila* or humans (Fig. 1). The MYST domain is the only structural feature common to all members of this family. MOZ and MORF show sequence similarity to the very N-terminal region and the MYST domain of Enok (Enoki mushroom), the most related *Drosophila* protein (Scott et al. 2001). Different from *Drosophila*, zebrafish possesses orthologs of MOZ and MORF. Thus, different from TIP60, MOF, and HBO1, both MOZ and MORF are vertebrate-specific.

Other protein domains of MOZ and MORF include an NEMM (N-terminal part of Enok, MOZ or MORF) domain, tandem PHD (plant homeodomain-linked) zinc fingers, a long acidic stretch, and a SM (serine/methionine-rich) region. The C-terminal part of the NEMM domain shows some sequence similarity to histones H1 and H5. The H15-like domain was shown to promote nuclear targeting (Kitabayashi et al. 2001a). The tandem PHD fingers are similar to those of Requiem and homologs (Borrow et al. 1996; Nabirochkina et al. 2002). The N terminal regions of MOZ and MORF possess strong transcriptional repression domains (Champagne et al. 1999). The SM domain constitutes an activation domain. The SM domain of MOZ is weaker than that of MORF, raising the interesting possibility that insertion of a PQ-stretch in the SM domain of MOZ reduces its activation potential (Champagne et al. 2001). The possession of multiple functional domains by MOZ and MORF suggests that they may regulate transcription in both acetylation-dependent and -independent manners.

MOZ and MORF Acetyltransferase Complexes

MOZ and MORF function as the catalytic subunits of an ING5 complexes (Doyon et al. 2006). ING5 is the



MOZ and MORF Lysine Acetyltransferases, Fig. 1 Schematic illustration of MOZ, MORF, and their association partners. A small region within the MYST domain of MOZ and MORF interacts with BRPF1 and paralogs, which utilize domain M for binding to ING5 and EAF6. ING5 and EAF6 form a trimeric core with BRPF1, -2, or -3. Association of ING5 with BRPF proteins promotes EAF6 interaction. The N-terminal domain of ING5 is sufficient for BRPF binding. The PHD fingers of ING5, BRPFs, MOZ, and MORF do not appear to be important for complex formation, but may mediate complex recruitment to specific chromatin domains. The bromodomain and PWWP domain of BRPF proteins may also recognize modified chromatin. *Txn*, transcription; *EN*, EAF6 N-terminal (Adapted from (Ullah et al. 2008)).

fifth member of the ING (inhibitor of growth) family of tumor suppressors (Soliman and Riabowol 2007). The other subunits of the complexes are EAF6 (homolog of yeast *E*sa1-*a*ssociated *f*actor 6) and BRPF1, -2, or -3 (*br*omodomain-*P*HD *f*inger protein 1, 2, or 3; Fig. 1) (Doyon et al. 2006). Different from MOZ and MORF alone, the complexes acetylate only histone H3 at lysine 14 (Doyon et al. 2006). BRPF1 binds to the MYST domains of MOZ and MORF, stimulates their acetyltransferase and coactivator activities, and bridges interaction with ING5 and EAF6 (Fig. 1) (Ullah et al. 2008). Coexpression of MOZ, BRPF1, and ING5 has a synergistic effect on the Runx2 promoter. The complexes may also be important for DNA replication (Doyon et al. 2006).

MOZ and MORF Act as Transcriptional Coactivators of Transcription Factors

Both MOZ and MORF are widely expressed in various mouse and human tissues (Borrow et al. 1996; Champagne et al. 1999; Katsumoto et al. 2006; Thomas et al. 2006), so similar to GCN5/PCAF and p300/CBP, they may function as coactivators for many transcription factors. Indeed, many lines of evidence have demonstrated that MOZ and MORF act as coactivators for different transcription factors such as Runxs, Hox, ETV6, PU.1, and p53; all of them play pivotal roles in different development and cellular processes. MOZ and MORF can bind to Runx2 (runt-related transcription factor 2) through the SM domain and potentiate Runx2-dependent transcriptional activation (Pelletier et al. 2002). Runx2 plays an important role in T cell lymphomagenesis (Pelletier et al. 2002), and is essential in controlling osteoblast differentiation and bone formation (Ducy et al. 2000).

MOZ and MORF in Animal Development

Consistent with the regulatory role of MOZ/MORF in regulating the activities of transcription factors that are essential in different developmental processes, studies on mutant fish and mouse models indicate that MOZ and MORF play key roles in hematopoiesis, skeletogenesis, neurogenesis, and other differentiation programs (Thomas et al. 2000, 2006; Miller et al. 2004; Crump et al. 2006; Katsumoto et al. 2006; Merson et al. 2006).

Homozygous *Moz* mutant mice lacking the Moz protein expression die at birth. Fetal liver hematopoietic cells from the *Moz* mutant fail to contribute to the hematopoietic system of recipients after transplantation, and display defects in the stem cell compartment (Thomas et al. 2006). $Moz^{-/-}$ mice without the expression of Moz generated by a separate group die around embryonic day 15 (E15). In $Moz^{-/-}$ E14.5 embryos, HSCs (hematopoietic stem cells) and progenitors are decreased, and maturation of erythroid cells is inhibited in Moz-deficient fetal liver that is due to the

synergistic effects of decreased expression of c-Mpl, HoxA9, and c-Kit (Katsumoto et al. 2006). Therefore, *Moz* is essential for the maintenance of hematopoietic stem cells.

A mouse strain carrying a point mutation that inactivates the HAT activity of the Moz protein remains alive during the gestation period, but about 40% of the homozygotes die within the first 6 months after birth. These mice exhibit significant defects in the number of HSCs and committed precursors and B-cell development defect. The reduced number of HSCs is caused by the failure of $HAT^{-/-}$ cells to expand. These results indicate that the HAT activity of Moz play a critical role in the proliferation and maintenance of hematopoietic precursors (Perez-Campo et al. 2009).

Studies on $Moz^{-/-}$ mutant mice (Thomas et al. 2006) also show that Moz is required for normal levels of H3K9 acetylation and gene expression at the Hox loci, and for correct specification of 19 body segments. In addition, Moz is required for recruitment of Ing5 and the H3 K4 methyltransferase Mll1 to the Hox loci (Voss et al. 2009). In zebrafish, it was found that Brpf1 and Moz cooperate to promote histone acetylation and maintenance of anterior Hox gene expression, and to determine pharyngeal segmental identities (Laue et al. 2008).

Homozygous mouse mutants for *Querkopf* fail to thrive in the postnatal period, display craniofacial abnormalities due to defects in the calvarial bones, and have defects in cerebral cortex development. (Thomas et al. 2000). Homozygous mice show smaller cerebral cortex, lack of large pyramidal cells in layer V of the cortex, and have reduction in the number of GAD67-positive interneurons throughout the cortex, suggesting that *Querkopf* is an essential in regulating cell differentiation in the cortex (Thomas et al. 2000). *Querkopf*-deficient mice also display defects in adult neurogenesis in vivo. Isolated neural stem/progenitor cells exhibit decreased self-renewal capacity and reduced ability to produce differentiated neurons. Thus, *Querkopf* is also essential for adult neurogenesis.

MOZ and MORF in Cancer and Other Diseases

MOZ fusion proteins caused by chromosomal rearrangement enable the transformation of non-self-renewing myeloid progenitors into leukemia stem cells (Huntly et al. 2004). Fusion partners of MOZ include (CREB-binding protein) CBP (Borrow et al. 1996), the CBP paralog p300 (Chaffanet et al. 2000; Kitabayashi et al. 2001b), the p300/CBP-interacting nuclear receptor coactivators TIF2 (transcription intermediary factor 2) (Carapeti et al. 1998; Liang et al. 1998), and nuclear receptor coactivators 2 (NCOA3) (Esteyries et al. 2008). *MORF* translocation is also associated with childhood AML or therapeutic myelodysplastic syndromes, in (Panagopoulos et al. 2001; Kojima et al. 2003). The *MORF* gene is also disrupted in multiple cases of uterine leiomyomata. *GCN5* is the fusion partner (Moore et al. 2004). Deregulation of gene expression resulting from these translocations may be involved in the pathogenesis. MOZ is involved in regulating cell cycle arrest in the G1 phase (Rokudai et al. 2009). DNA damage increases the level of a p53-MOZ complex. In $MOZ^{-/-}$ mouse fibroblasts, DNA damage fails to induce the expression of p21 and G1 cell cycle arrest. The leukemic fusion protein MOZ-CBP inhibits p53-mediated transcription. Thus, inhibition of p53/MOZ-mediated transcription contributes to leukemogenesis (Rokudai et al. 2009). In addition, the *MORF* gene has recently been shown to be mutated in three developmental disorders, including variants of Noonan syndrome and Ohdo syndrome, and Genitopatellar syndrome (Kraft et al. 2011; Clayton-Smith et al. 2011; Campeau et al. 2012). Strikingly, such patients all display characteristic bone defects, a phenotype that is somewhat consistent with what was observed in the *Querkopf* mutant mice (Thomas et al. 2000). It will be interesting to determine whether similar mutations are present in the *MOZ, BRPF* and *ING5* genes, and whether MOZ and MORF play a role in diseases other than cancer and bone defect disorders.

Summary

MOZ and MORF belong to the MYST family of histone acetyltransferases. Their multifunctional domains confer the function as co-regulators of transcription. They can regulate gene expression by acetylation-dependent and -independent manners. MOZ and MORF act as potential coactivators for different transcription factors such as Runxs, Hox, ETV6, PU.1, and p53, which play pivotal roles in different development and cellular processes. Accordingly, studies on mutant animal models indicate that MOZ and MORF are important for hematopoiesis, skeletogenesis, neurogenesis, body segment specification, and cell cycle control. MOZ and MORF are targets

of chromosome rearrangements that are associated with leukemia and other types of cancer. Deregulation of gene expression resulting from these translocations may be involved in tumor pathogenesis. Furthermore, the *MORF* gene is mutated in multiple syndromes with bone and various other abnormalities. Overall, both MOZ and MORF are enzymatic transcriptional coregulators that have important functions in various physiological and pathological processes.

Acknowledgments This research was supported by operating grants from CIHR, Canadian Cancer Society and MDEIE (to X.J.Y.).

References

Borrow J, Stanton Jr VP, Andresen JM, Becher R, Behm FG, Chaganti RS, Civin CI, Disteche C, Dube I, Frischauf AM, et al. The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nat Genet. 1996;14:33–41.

Campeau PM, Kim JC, Lu JT, Schwartzentruber JA, Abdul-Rahman OA, Schlaubitz S, Murdock DM, Jiang MM, Lammer EJ, Enns GM, Rhead WJ, Rowland J, Robertson SP, Cormier-Daire V, Bainbridge MN, Yang XJ, Gingras MG, Gibbs RA, Rosenblatt DS, Majewski J, Lee JB. Mutations in KAT6B, encoding a histone acetyltransferase, cause genitopatellar syndrome. Am J Hum Genet. 2012;90:282–9.

Carapeti M, Aguiar RC, Goldman JM, Cross NC. A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. Blood. 1998;91:3127–33.

Chaffanet M, Gressin L, Preudhomme C, Soenen-Cornu V, Birnbaum D, Pebusque MJ. MOZ is fused to p300 in an acute monocytic leukemia with t(8;22). Genes Chromosomes Cancer. 2000;28:138–44.

Champagne N, Bertos NR, Pelletier N, Wang AH, Vezmar M, Yang Y, Heng HH, Yang XJ. Identification of a human histone acetyltransferase related to monocytic leukemia zinc finger protein. J Biol Chem. 1999;274:28528–36.

Champagne N, Pelletier N, Yang XJ. The monocytic leukemia zinc finger protein MOZ is a histone acetyltransferase. Oncogene. 2001;20:404–9.

Clayton-Smith J, O'Sullivan J, Daly S, Bhaskar S, Day R, Anderson B, Voss AK, Thomas T, Biesecker LG, Smith P, Fryer A, Chandler KE, Kerr B, Tassabehji M, Lynch SA, Krajewska-Walasek M, McKee S, Smith J, Sweeney E, Mansour S, Mohammed S, et al. Whole-exome-sequencing identifies mutations in histone acetyltransferase gene KAT6B in individuals with the Say-Barber-Biesecker variant of Ohdo syndrome. Am J Hum Genet. 2011;89:675–81.

Crump JG, Swartz ME, Eberhart JK, Kimmel CB. Moz-dependent Hox expression controls segment-specific fate maps of skeletal precursors in the face. Development. 2006;133:2661–9.

Doyon Y, Cayrou C, Ullah M, Landry AJ, Cote V, Selleck W, Lane WS, Tan S, Yang XJ, Cote J. ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Mol Cell. 2006;21:51–64.

Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science. 2000;289:1501–4.

Esteyries S, Perot C, Adelaide J, Imbert M, Lagarde A, Pautas C, Olschwang S, Birnbaum D, Chaffanet M, Mozziconacci MJ. NCOA3, a new fusion partner for MOZ/MYST3 in M5 acute myeloid leukemia. Leukemia. 2008;22:663–5.

Huntly BJ, Shigematsu H, Deguchi K, Lee BH, Mizuno S, Duclos N, Rowan R, Amaral S, Curley D, Williams IR, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell. 2004;6:587–96.

Katsumoto T, Aikawa Y, Iwama A, Ueda S, Ichikawa H, Ochiya T, Kitabayashi I. MOZ is essential for maintenance of hematopoietic stem cells. Genes Dev. 2006;20:1321–30.

Kitabayashi I, Aikawa Y, Nguyen LA, Yokoyama A, Ohki M. Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. EMBO J. 2001a;20:7184–96.

Kitabayashi I, Aikawa Y, Yokoyama A, Hosoda F, Nagai M, Kakazu N, Abe T, Ohki M. Fusion of MOZ and p300 histone acetyltransferases in acute monocytic leukemia with a t(8;22)(p11;q13) chromosome translocation. Leukemia. 2001b;15:89–94.

Kojima K, Kaneda K, Yoshida C, Dansako H, Fujii N, Yano T, Shinagawa K, Yasukawa M, Fujita S, Tanimoto M. A novel fusion variant of the MORF and CBP genes detected in therapy-related myelodysplastic syndrome with t(10;16)(q22;p13). Br J Haematol. 2003;120:271–3.

Kraft M, Cirstea IC, Voss AK, Thomas T, Goehring I, Sheikh BN, Gordon L, Scott H, Smyth GK, Ahmadian MR, Trautmann U, Zenker M, Tartaglia M, Ekici A, Reis A, Dorr HG, Rauch A, Thiel CT. Disruption of the histone acetyltransferase MYST4 leads to a Noonan syndrome-like phenotype and hyperactivated MAPK signaling in humans and mice. J Clin Investig. 2011;121:3479–91.

Laue K, Daujat S, Crump JG, Plaster N, Roehl HH, Kimmel CB, Schneider R, Hammerschmidt M. The multidomain protein Brpf1 binds histones and is required for Hox gene expression and segmental identity. Development. 2008;135:1935–46.

Liang J, Prouty L, Williams BJ, Dayton MA, Blanchard KL. Acute mixed lineage leukemia with an inv(8)(p11q13) resulting in fusion of the genes for MOZ and TIF2. Blood. 1998;92:2118–22.

Merson TD, Dixon MP, Collin C, Rietze RL, Bartlett PF, Thomas T, Voss AK. The transcriptional coactivator Querkopf controls adult neurogenesis. J Neurosci. 2006;26:11359–70.

Miller CT, Maves L, Kimmel CB. MOZ regulates Hox expression and pharyngeal segmental identity in zebrafish. Development. 2004;131:2443–61.

Moore SD, Herrick SR, Ince TA, Kleinman MS, Dal Cin P, Morton CC, Quade BJ. Uterine leiomyomata with t(10;17) disrupt the histone acetyltransferase MORF. Cancer Res. 2004;64:5570–7.

Nabirochkina E, Simonova OB, Mertsalov IB, Kulikova DA, Ladigina NG, Korochkin LI, Buchman VL. Expression pattern of dd4, a sole member of the d4 family of transcription

factors in *Drosophila melanogaster*. Mech Dev. 2002; 114:119–23.
Panagopoulos I, Fioretos T, Isaksson M, Samuelsson U, Billstrom R, Strombeck B, Mitelman F, Johansson B. Fusion of the MORF and CBP genes in acute myeloid leukemia with the t(10;16)(q22;p13). Hum Mol Genet. 2001;10:395–404.
Pelletier N, Champagne N, Stifani S, Yang XJ. MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. Oncogene. 2002;21:2729–40.
Perez-Campo FM, Borrow J, Kouskoff V, Lacaud G. The histone acetyl transferase activity of monocytic leukemia zinc finger is critical for the proliferation of hematopoietic precursors. Blood. 2009;113:4866–74.
Rokudai S, Aikawa Y, Tagata Y, Tsuchida N, Taya Y, Kitabayashi I. Monocytic leukemia zinc finger (MOZ) interacts with p53 to induce p21 expression and cell-cycle arrest. J Biol Chem. 2009;284:237–44.
Scott EK, Lee T, Luo L. enok encodes a Drosophila putative histone acetyltransferase required for mushroom body neuroblast proliferation. Curr Biol. 2001;11:99–104.
Soliman MA, Riabowol K. After a decade of study-ING, a PHD for a versatile family of proteins. Trends Biochem Sci. 2007;32:509–19.
Thomas T, Voss AK, Chowdhury K, Gruss P. Querkopf, a MYST family histone acetyltransferase, is required for normal cerebral cortex development. Development. 2000;127:2537–48.
Thomas T, Corcoran LM, Gugasyan R, Dixon MP, Brodnicki T, Nutt SL, Metcalf D, Voss AK. Monocytic leukemia zinc finger protein is essential for the development of long-term reconstituting hematopoietic stem cells. Genes Dev. 2006;20:1175–86.
Ullah M, Pelletier N, Xiao L, Zhao SP, Wang K, Degerny C, Tahmasebi S, Cayrou C, Doyon Y, Goh SL, et al. Molecular architecture of quartet MOZ/MORF histone acetyltransferase complexes. Mol Cell Biol. 2008;28:6828–43.
Voss AK, Collin C, Dixon MP, Thomas T. Moz and retinoic acid coordinately regulate H3K9 acetylation, Hox gene expression, and segment identity. Dev Cell. 2009;17:674–86.

MOZ Lysine Acetyltransferase

► MOZ and MORF Lysine Acetyltransferases

MOZ-Related Factor

► MOZ and MORF Lysine Acetyltransferases

MPK-1 (*Caenorhabditis elegans*)

► ERK1/ERK2

Mpk3

► Mek3

MRC2

Julian Hendrik Gronau[1], Clare M. Isacke[2] and Justin Sturge[1]
[1]Division of Cancer, Department of Surgery & Cancer, Imperial College London, Hammersmith Hospital, London, UK
[2]Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, London, UK

Synonyms

CD280; Collagen-binding factor Endo180; C-type mannose receptor 2; Endo180; Endocytic receptor 180; Macrophage mannose receptor 2; Mannose receptor, C-type 2; Urokinase-type plasminogen activator receptor-associated protein (uPARAP)

Historical Background

The *MRC2* gene encodes the constitutively recycling type I transmembrane receptor Endo180 (Wu et al. 1996; Sheikh et al. 2000; Isacke et al. 1990), which is also cited in the literature as urokinase plasminogen activator receptor associated protein (uPARAP) following its identification as part of a trimolecular complex with pro-urokinase plasminogen activator (pro-uPA) and its receptor urokinase plasminogen activator receptor (► uPAR) (Behrendt et al. 2000). Endo180 is a member of the mannose receptor family, which also includes the mannose receptor (MR; CD206), M-type phospholipase A2 receptor, and DEC-205 (CD205) (East and Isacke 2002). The constitutive recycling of Endo180 between clathrin-coated pits at the plasma membrane and intracellular endosomal compartments is dependent on a dihydrophobic endocytosis motif and modulated by a conserved upstream acidic residue (Howard and Isacke 2002). Endo180 internalization is independent of a conserved tyrosine-based motif that is critical role for internalization to the other

three family members (Howard and Isacke 2002). Endo180 expression is regulated by transforming growth factor-beta receptor (TGF-beta Receptor) signaling via the Smad transcription factor pathway (Huijbers et al. 2010).

Endo180 functions as a signaling receptor that regulates the overall and tempero-spatial activation of the small Rho GTPases, Cdc42, Rac and Rho, which drive directed cell migration (chemotaxis) or random cell migration via pro-uPA-uPAR-dependent or independent mechanisms (Sturge et al. 2003, 2006). The factors responsible for Rho GTPase activation by Endo180 during its functional role as a chemotaxis receptor involves unidentified factors that promote guaninine nucleotide exchange (GDP → GTP) (see Fig. 1 and section "Endo180-Dependent Signaling in Cell Migration" for further details).

Endo180 Structure and Implications for Signaling

Endo180 has a similar domain structure to other members of the mannose receptor family and can be subdivided into 12 structural elements (see Fig. 1). The 150 kDa ectodomain of Endo180 is comprised of a cysteine-rich domain (CRD), a fibronectin type II–like domain (FNII) and eight repeated C-type lectin domains (CTLDs 1–8) and is modified by approximately 30 kDa of N-linked sugars (Wu et al. 1996; Sheikh et al. 2000; Behrendt et al. 2000). Following its transmembrane domain Endo180 has a short cytoplasmic tail, which in humans is comprised of 42 amino acid residues, including several putative phosphorylation sites (Isacke et al. 1990) (see Fig. 2 and section "Phosphorylation of Endo180" for more details). The three-dimensional structural arrangement of the amino terminal domains of Endo180 (CRD–FNII–CTLD1–2) has been modeled from single-particle electron microscopy analysis to reveal a hairpin loop structure (Rivera-Calzada et al. 2003) that is predicted to undergo conformational changes in response to ligand binding or microenvironmental factors (Boskovic et al. 2006) (see Fig. 1). The functional and biological context of these intramolecular alterations in Endo180 have not been determined but could modulate the intracellular signaling pathways activated downstream of Endo180. The identification of molecular partners that interact with the cytoplasmic tail of Endo180 and the receptor ectodomain will help to provide further insight about the biological context of the signaling events regulated by Endo180.

Endo180 Ligands and Implications for Signaling

Collagens

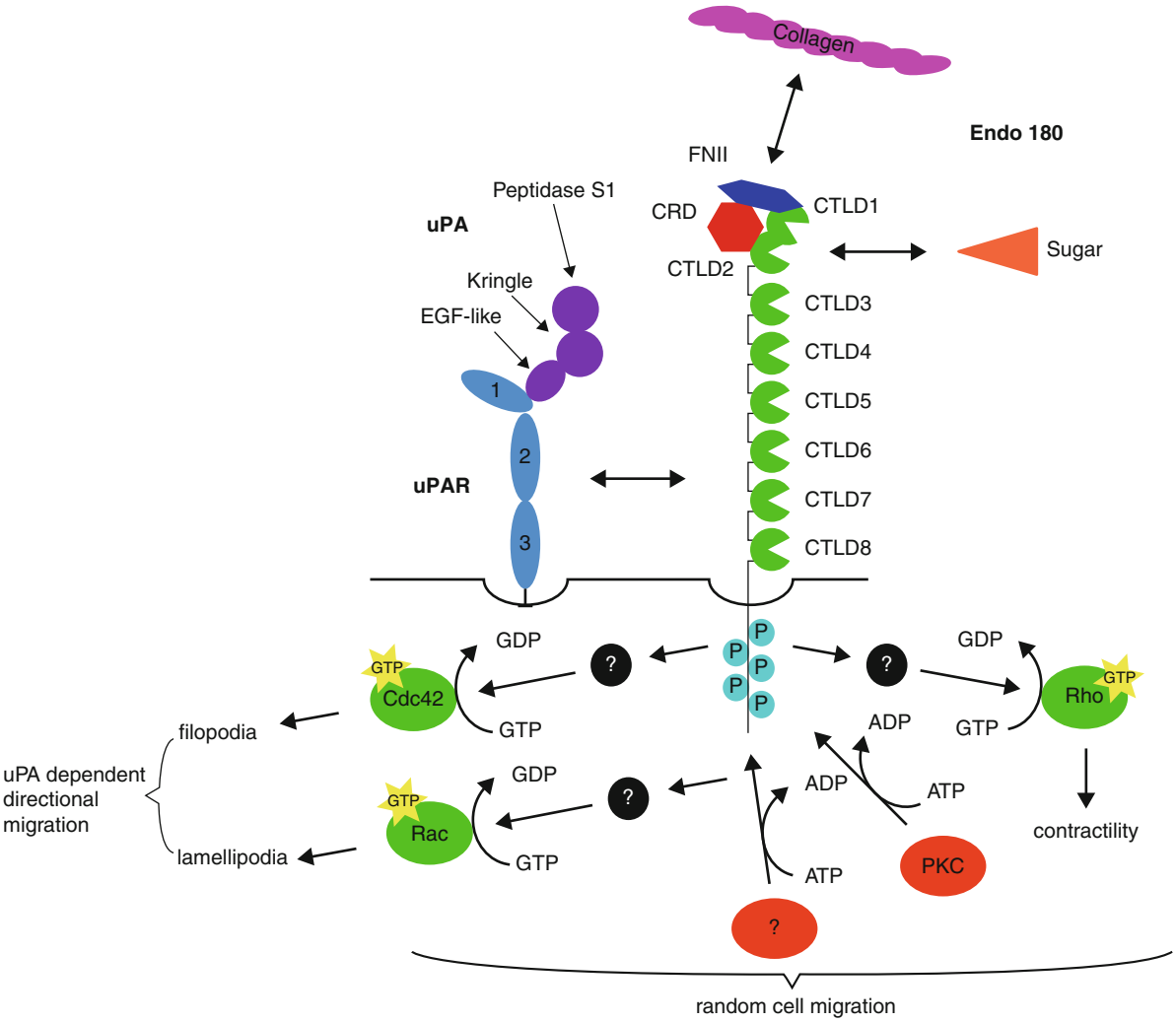
Endo180 interacts with different subtypes of collagen via its FNII domain (Wienke et al. 2003). For type I collagen this interaction occurs through the interaction of Endo180 with its C-terminal domain (Thomas et al. 2005). The binding of soluble collagen ligands at the cell surface results in their rapid internalization by Endo180 and subsequent transport to lysosomal compartments for degradation (Wienke et al. 2003; Engelholm et al. 2003; East et al. 2003), a function that has been implicated in the tissue remodeling process associated with cancer progression (Huijbers et al. 2010; Kogianni et al. 2009; Wienke et al. 2007) and tumor cell invasion (Huijbers et al. 2010). Another important function for this interaction is the promotion of cell-matrix adhesion (Sturge et al. 2006; Thomas et al. 2005). The intracellular signaling events activated by the engagement of Endo180 with either soluble or fibrillar forms of collagen have not been fully explored. To date, evidence suggests that the enhanced phosphorylation of ▶ myosin (regulatory) light chain-2 (MLC2) by the Endo180-Rho-Rho kinase (ROCK) signaling axis is independent of cell adhesion to collagen (Sturge et al. 2006).

Sugars

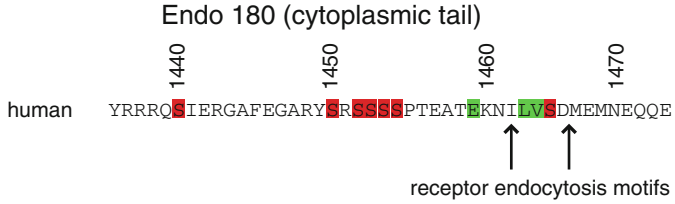
Endo180 has been shown to interact with various sugar moeities via calcium-dependent binding to its only functional C-type lectin-like domain, CTLD2 (East et al. 2002, 2003;). However, the impact of the lectin activity of Endo180 on its signaling function, or any of its other functions, has not yet been reported.

pro-uPA-uPAR

The trimolecular complex formed between Endo180, uPAR and pro-uPA on the cell surface (Behrendt et al. 2000) is implicated in the enhanced migratory behavior of tumor cells, during which, Endo180 can coordinate the activation of Rho GTPases (Cdc42, Rac, and Rho), cytoskeletal remodeling events



MRC2, Fig. 1 Endo180 domain structure, interaction partners, and signaling. Endo180 domains: N-terminal cysteine-rich domain (CRD); fibronectin type II domain (FNII); eight C-type lectin domains (CTLD1-8); a single transmembrane domain; and a short (42 amino acid residue) intracellular C-terminal domain. CRD, FNII, and CTLD1-2 form a hairpin loop structure at the receptor N-terminus. FNII binds collagen and CTLD2 binds sugars. Endo180 is a coreceptor of pro-uPA, which is comprised of a peptidase domain, kringle domain and EGF-like domain and uPAR, which is comprised of domains 1–3. Endo180 has at least five phosphorylation sites in its cytoplasmic tail with at least one of these sites characterized as a substrate for protein kinase C (PKC). Functional studies have shown that Endo180 regulates the overall and tempero-spatial activation of the small GTPases Cdc42, Rac, and Rho during pro-uPA dependent chemotaxis and random cell migration (Sturge et al. 2003, 2006)



MRC2, Fig. 2 Potential PKC-phosphorylation sites in Endo180. Potential serine targets for PKC-mediated phosphorylation are marked in red (Sheikh et al. 2000; Isacke et al. 1990). An E1464A mutation partially alters the internalization of Endo180, whereas L1468A/V1469A co-mutation blocks Endo180 internalization (Howard and Isacke 2002)

(filopodial, lamellipodial, and stress fiber dynamics), cell-matrix adhesion turnover and cell–cell adhesion disassembly (Sturge et al. 2003, 2006; Takahashi et al. 2010) (see section "Endo180-Dependent Signaling in Cell Migration" for further details). MR-dependent chemotactic responses in myoblasts (Jansen and Pavlath 2006) suggests that a common biological role exists for this related receptor; however, the molecular basis underlying the function of MR as a chemotaxis receptor and the chemotactic signaling events that are activated downstream of MR have not been determined.

Endo180–Dependent Signaling in Cell Migration

Ectopic overexpression of Endo180 in receptor null tumor cells results in their enhanced cell migration and chemotaxis (Sturge et al. 2003). In accordance, the genetic silencing of Endo180 by small interference RNA (siRNA) in invasive human tumor cell lines that express high endogenous levels of Endo180 (Sturge et al. 2003, 2006), or the targeted deletion of Endo180 in murine embryonic fibroblasts (Engelholm et al. 2003; East et al. 2003), results in reduced cell migration and adhesion. As stated previously there are three major signaling pathways that have been identified as downstream targets that can be modulated by Endo180 and can promote cell migration:

Endo180–Dependent Activation of Cdc42

The requirement for Endo180 in MDA-MB-231 metastatic breast cancer cells to sense a chemotactic gradient of pro-uPA was demonstrated by genetic silencing of Endo180 by siRNA in Dunn chemotaxis chamber assays (Sturge et al. 2003). The role of Endo180 in this chemotactic response was supported by a gain in directional migration toward a gradient of pro-uPA following its overexpression in non-invasive MCF7 breast cancer cells. These changes in chemotactic behavior are directly linked to altered dynamics of pro-uPA-dependent Cdc42 activation, which is suppressed by Endo180 silencing in MDA-MB-231 cells, enhanced in MCF7 cells that overexpress Endo180 and display increased filopodial protrusions in response to pro-uPA (Sturge et al. 2003). The activation of Cdc42 and detection of a pro-uPA gradient in cells that express an Endo180 mutant receptor that cannot be internalized indicates that the chemosensory function of Endo180 is independent of receptor endocytosis (Sturge et al. 2003).

Endo180–Dependent Activation of Rac

The rapid activation of Rac by uPA in MCF7 cells that overexpress Endo180 and the requirement for the internalization of Endo180 to activate Rac and promote an increase in migratory speed suggest the mechanism of Rac activation by Endo180 is distinct from that required for its activation of Cdc42 (Sturge et al. 2003). The localization of Endo180 to lamellipodia (Sturge et al. 2003) and the requirement of Endo180 for lamellipodial protrusion formation (Takahashi et al. 2010) indicates that it plays a critical role in Rac-dependent signaling, which drives cytoskeletal remodeling and cell migration.

Endo180–Dependent Activation of Rho-ROCK-MLC2

Endo180 is required for the diphosphorylation of serine 19/threonine 18 of MLC2 in MG63, MDA-MB-231, BE and HT-1080 cells and these signals are enhanced by the overexpression of Endo180 in MCF7 cells. Endo180 is also required for the phosphorylation of myosin light chain phosphatase-1 (MYPT1) and ▶ LIMK, which are common downstream targets of ROCK. Functional studies demonstrated that the Rho-ROCK-MLC2 diphosphorylation signaling axis is spatially localized by Endo180–containing endosomes for the promotion of focal adhesion disassembly at the rear of migrating cells (Sturge et al. 2006).

Phosphorylation of Endo180

Endo180 is phosphorylated by purified protein kinase C (PKC) in vitro and can be phosphorylated in vivo by treating cells with phorbol esters (Sheikh et al. 2000; Isacke et al. 1990). ^{32}Pi-labeling revealed that Endo180 contains at least three residues, which are constitutively phosphorylated in Flow 2000 embryonic lung diploid fibroblasts under normal growth conditions (Sheikh et al. 2000; Isacke et al. 1990). Treatment of Flow 2000 cells with phorbol 12-myristate 13-acetate (PMA) increased the phosphorylation of one of these sites and revealed two additional phosphorylation sites. All three PMA responsive phosphorylation sites have been identified as serine residues.

PKC purified from rat brain showed a strong preference to only one of these three phosphorylation sites in vitro. Endo180 has also been tested in vitro for a kinase activity against itself and a number of common substrates with negative results (Sheikh et al. 2000; Isacke et al. 1990). Endo180 has several putative phosphorylation sites in its cytoplasmic tail (see Fig. 2) but their functional role has not been confirmed.

Summary

Endo180 is an endocytic receptor that can signal to regulate the overall and tempero-spatial activation of the small Rho GTPases, Cdc42, Rac, and Rho (Sturge et al. 2003, 2006). Future work is required to: (a) elucidate the factors responsible for Rho GTPase activation by Endo180; (b) identify the intracellular partners of Endo180; (c) determine how conformational changes in the receptor ectodomain impact on receptor function and signaling, with a particular focus on the impact of collagen and uPA/uPAR-binding; and (d) investigate the influence of Endo180 phosphorylation on downstream signal transduction pathways.

References

Behrendt N, Jensen ON, Engelholm LH, Mortz E, Mann M, Dano K. A urokinase receptor-associated protein with specific collagen binding properties. J Biol Chem. 2000; 275:1993–2002.

Boskovic J, Arnold JN, Stilion R, Gordon S, Sim RB, Rivera-Calzada A, et al. Structural model for the mannose receptor family uncovered by electron microscopy of Endo180 and the mannose receptor. J Biol Chem. 2006;281:8780–7.

East L, Isacke CM. The mannose receptor family. Biochim Biophys Acta. 2002;1572:364–86.

East L, Rushton S, Taylor ME, Isacke CM. Characterization of sugar binding by the mannose receptor family member, Endo180. J Biol Chem. 2002;277:50469–75.

East L, McCarthy A, Wienke D, Sturge J, Ashworth A, Isacke CM. A targeted deletion in the endocytic receptor gene Endo180 results in a defect in collagen uptake. EMBO Rep. 2003;4:710–6.

Engelholm LH, List K, Netzel-Arnett S, Cukierman E, Mitola DJ, Aaronson H, et al. uPARAP/Endo180 is essential for cellular uptake of collagen and promotes fibroblast collagen adhesion. J Cell Biol. 2003;160:1009–15.

Howard MJ, Isacke CM. The C-type lectin receptor Endo180 displays internalization and recycling properties distinct from other members of the mannose receptor family. J Biol Chem. 2002;277:32320–31.

Huijbers IJ, Iravani M, Popov S, Robertson D, Al-Sarraj S, Jones C, et al. A role for fibrillar collagen deposition and the collagen internalization receptor Endo180 in glioma invasion. PLoS One. 2010;5:e9808.

Isacke CM, van der Geer P, Hunter T, Trowbridge IS. p180, a novel recycling transmembrane glycoprotein with restricted cell type expression. Mol Cell Biol. 1990; 10:2606–18.

Jansen KM, Pavlath GK. Mannose receptor regulates myoblast motility and muscle growth. J Cell Biol. 2006;174: 403–13.

Kogianni G, Walker MM, Waxman J, Sturge J. Endo180 expression with cofunctional partners MT1-MMP and uPAR-uPA is correlated with prostate cancer progression. Eur J Cancer. 2009;45:685–93.

Rivera-Calzada A, Robertson D, MacFadyen JR, Boskovic J, Isacke CM, Llorca O. Three-dimensional interplay among the ligand-binding domains of the urokinase-plasminogen-activator-receptor-associated protein, Endo180. EMBO Rep. 2003;4:807–12.

Sheikh H, Yarwood H, Ashworth A, Isacke CM. Endo180, an endocytic recycling glycoprotein related to the macrophage mannose receptor is expressed on fibroblasts, endothelial cells and macrophages and functions as a lectin receptor. J Cell Sci. 2000;113:1021–32.

Sturge J, Wienke D, East L, Jones GE, Isacke CM. GPI-anchored uPAR requires Endo180 for rapid directional sensing during chemotaxis. J Cell Biol. 2003;162:789–94.

Sturge J, Wienke D, Isacke CM. Endosomes generate localized Rho-ROCK-MLC2-based contractile signals via Endo180 to promote adhesion disassembly. J Cell Biol. 2006;175: 337–47.

Takahashi S, Yamada-Okabe H, Hamada K, Ohta S, Kawase T, Yoshida K, et al. Downregulation of uPARAP mediates cytoskeletal rearrangements and decreases invasion and migration properties in glioma cells. J Neurooncol. 2010. doi:10.1007/s11060-010-0398-z.

Thomas EK, Nakamura M, Wienke D, Isacke CM, Pozzi A, Liang P. Endo180 binds to the C-terminal region of type I collagen. J Biol Chem. 2005;280:22596–605.

Wienke D, MacFadyen JR, Isacke CM. Identification and characterization of the endocytic transmembrane glycoprotein Endo180 as a novel collagen receptor. Mol Biol Cell. 2003;14:3592–604.

Wienke D, Davies GC, Johnson DA, Sturge J, Lambros MB, Savage K, et al. The collagen receptor Endo180 (CD280) is expressed on basal-like breast tumor cells and promotes tumor growth in vivo. Cancer Res. 2007;67: 10230–40.

Wu K, Yuan J, Lasky LA. Characterization of a novel member of the macrophage mannose receptor type C lectin family. J Biol Chem. 1996;271:21323–30.

MRV Integration Site 1

► Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

Mrv1

► Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

Mrv1a

► Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

Mrvi1

► Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

MSK1

Antigone Lazou and Thomais Markou
School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece

Synonyms

90 kDa ribosomal protein S6 kinase 5; Mitogen- and stress-activated protein kinase 1; MSPK1; Nuclear mitogen- and stress-activated protein kinase-1; Ribosomal protein S6 kinase, 90kD, polypeptide 5; Ribosomal protein S6 kinase, polypeptide 5; RLPK; RLSK; Rps6ka5; Rsk-like (RSKL); Rsk-like protein kinase (RLPK)

Historical Background

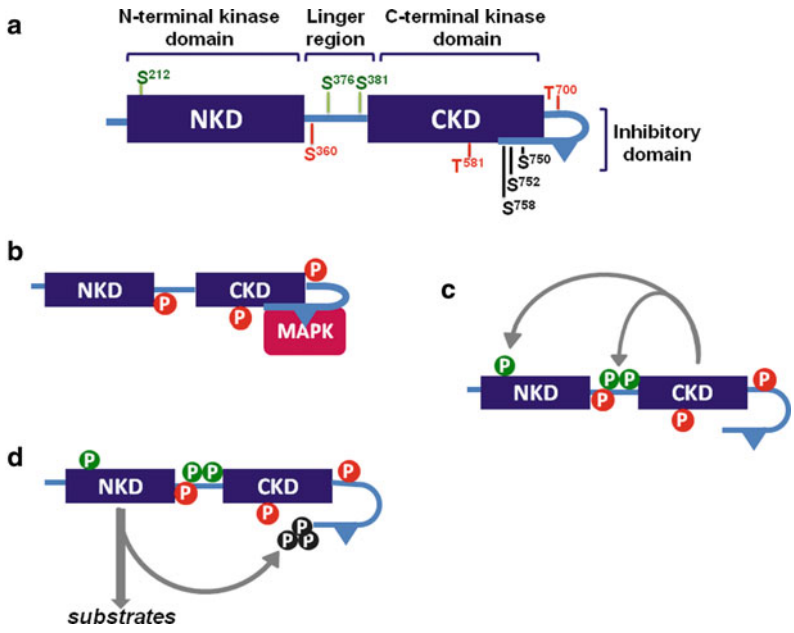
Mitogen- and stress-activated protein kinase (Msk) 1 and Msk2 are nuclear serine/threonine kinases that are widely expressed in tissues including heart, brain, placenta, lung, liver, kidney, and pancreas, with the highest levels observed in brain, muscle, and placenta (Deak et al. 1998). They belong to a group of structurally related kinases called the AGC kinase (cAMP-dependent, cGMP-dependent, and protein kinase C) family. This group includes p70 ribosomal S6 kinase (S6K), protein kinase B (PKB; also called Akt), protein kinase C-related kinase (Prk), ► p90 ribosomal S6 kinase (Rsk) and serum- and glucocorticoid-inducible kinase (Sgk) (Pearce et al. 2010).

Msk1 was identified through its homology to the N-terminal Rsk domain (Deak et al. 1998). Similarly to Rsk, Msk1 contains two kinase domains, connected with a linker region, in a single polypeptide (Deak et al. 1998). The N-terminal kinase domain is related to the AGC family of protein kinases (containing PKA, PKG, and PKC families), whereas the C-terminal kinase domain is related to the calmodulin-activated protein kinase family (Pearce et al. 2010). The C-terminal tail fulfills at least three functions: it contains a nuclear localization sequence and a mitogen-activated protein (MAP) kinase docking sequence, and can also act as an auto-inhibitory sequence (McCoy et al. 2007). Mutation of either the C- or N-terminal kinase domains in Msk1 is sufficient to block the phosphorylation of its substrates (Deak et al. 1998).

Because it can be activated through two different pathways (extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 MAP kinase), Msk1 is able to integrate signals from growth factors, pro-inflammatory cytokines (tumor necrosis factor-α (► TNF-α)) and cellular stress (ultraviolet (UV)-irradiation, hydrogen peroxide). Msk1 is predominantly found in the nucleus and it contains a nuclear localization sequence in the C-terminal region, but a portion is localized to the cytosol of mouse fibroblasts and HEK 293 cells (Deak et al. 1998).

Regulation of Msk1 Activity

In cells, Msk1 is activated via a complex series of phosphorylation and autophosphorylation reactions downstream of ERK1/2 or p38α-MAP kinase. Many stimuli have been shown to activate Msk1 in cell cultures, including UV irradiation, anisomycin, nerve growth factor (NGF), ► TNF-α, interleukin (IL)-1, lysophosphatidic acid, endothelin-1, α_1-adrenergic stimulation, arsenic trioxide, and oxidative stress. In addition, exercise has been shown to induce Msk1 activation in animal skeletal muscle and light activates Msk1 in the suprachiasmatic nucleus in the brain



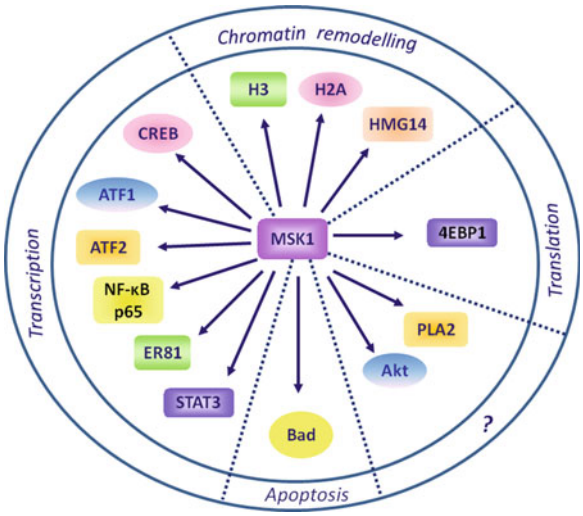
MSK1, Fig. 1 *Msk1 activation.* (**a**) Msk1 contains two kinase domains (C-terminal kinase domain, CKD and N-terminal kinase domaid, NKD) connected with a short linker region, and a C-terminal tail which contains a MAPK kinase docking sequence. Phosphorylation sites are shown. In the inactive Msk1, the C-terminal tail acts as an inhibitory domain of the CKD. (**b**) Active MAPKs (ERK1/2 or p38a-MAPK) bind to the docking domain in the C-terminal tail and phosphorylate S360, T581, and T700. (**c**) Phosphorylation of T700 promotes the dissociation of the inhibitory domain. The activated CKD phosphorylates S376 and S381 in the linker region and S212 in the NKD. (**d**) The NKD is activated and it is able to phosphorylate Msk1 substrates, or autophosphorylate Msk1 at S750, S752, and S758

(extensively reviewed in Arthur 2008; Vermeulen et al. 2009; Lazou and Markou 2010).

Msk1 is regulated by multiple phosphorylation sites (Fig. 1). In total, 13 phosphorylation sites have now been identified in Msk1 and six of these sites have significant roles in its activation. The role of the remaining sites is unclear, but phosphorylation of these residues may influence other processes such as localization or protein–protein interactions in vivo. Upstream ▶ MAP kinases, ERK1/2, and ▶ p38α, phosphorylate Msk1 on S360, T581, and T700 (Deak et al. 1998; McCoy et al. 2007). Phosphorylation of T581 is essential for Msk1 activation. On the other hand, mutation of S360 only partly reduces Msk1 activity and mutation of T700 does not affect it (McCoy et al. 2007). When phosphorylated at T581 and S360, the C-terminal kinase domain is activated and then autophosphorylates S376 and S381 in the linker region and S212 in N-terminal kinase domain. Of these three sites, S212 and S376 appear to be essential for Msk1 activation, as their mutation to alanine prevents kinase activation (McCoy et al. 2007). On the other hand, phosphorylation of S381 promotes N-terminal activation, probably through stabilization of phosphorylation of S376, but it is not essential for Msk1 activity. These autophosphorylation events activate the N-terminal kinase domain, allowing the phosphorylation of Msk1 substrates as well as the phosphorylation of S750, S752, and S758 in the C terminus of Msk1. Phosphorylation of the C-terminal sites is not required for Msk1 activation, and the function of this autophosphorylation is unknown (McCoy et al. 2007).

Msk1 Substrates

Msk1 is involved in the regulation of gene expression by phosphorylating transcription factors and chromatin-associated proteins. Furthermore, it has been shown that Msk1 phosphorylates proteins such as eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and Bad, thereby regulating translation initiation or apoptosis (Fig. 2; extensively reviewed in Arthur 2008; Vermeulen et al. 2009; Lazou and Markou 2010).



MSK1, Fig. 2 *Msk1 substrates*. ER81, H2A, and cPLA2 have not been validated in vivo

Transcription Factors

cAMP-responsive element (CRE) binding protein (► CREB) was the first Msk1 substrate to be identified (Deak et al. 1998). Since then, several studies using a variety of experimental settings, in vitro and in vivo, have shown phosphorylation of CREB (S133) and activation of its transcriptional activity by Msk1 in response to growth factors, phorbol esters, pro-inflammatory cytokines, G protein-coupled receptor agonists or cellular stresses (for review see Arthur 2008; Vermeulen et al. 2009; Lazou and Markou 2010). Phosphorylation of ► CREB by Msk1 leads to transcription of several immediate early genes that have CRE in their promoter including *c-fos*, *junB*, *Nur77*, *Mkp1*, *MUC5AC*, *Cox-2*, *IL-1β*, *ANF*, and *c-jun*. Msk1 also phosphorylates activating transcription factor 1 (ATF1) and activating transcription factor 2 (ATF2).

Another transcription factor that is a substrate for Msk1 is the nuclear factor (NF)-κB (Vermeulen et al. 2003; Vermeulen et al. 2009). In unstimulated cells NF-κB is found in the cytoplasm where it is kept inactive by binding to the inhibitory protein IκB. Stimulation with various agonists leads to the release of ► NF-κB and its translocation to the nucleus where further posttranslational modifications regulate its activity. Msk1 phosphorylates the p65/RelA subunit of NF-κB at S276 in response to ► TNF-α in fibroblasts, and in response to oxidative stress in skeletal myoblasts.

STAT3 (signal transducer and activator of transcription 3) and ER81 (E26 transformation-specific (ETS)-related protein 81; also called Ets transcript variant 1, Etv1) are also targeted by Msk1. Phosphorylation of STAT3 at S727 is mediated through Msk1 in JB6 cells in response to UVA irradiation and in erythroid cells in response to erythropoietin (Wierenga et al. 2003). The activity of the transcription factor ER81, which is involved in oncogenesis and breast tumor formation, is regulated by Msk1 via direct phosphorylation at S191 and S216. However, it should be noted that ER81 phosphorylation has not been validated in vivo.

Chromatin-Associated Proteins

Increasing evidence indicates an important role of Msk1 in remodeling chromatin structure and the epigenetic regulation of gene expression. Using H89 as an inhibitor of Msk1 activity, it has been shown that Msk1 phosphorylates histone H3 at S 10 and S 28 in response to phorbol esters (12-*O*-tetradecanoylphorbol-13-acetate (TPA)), epidermal growth factor (EGF), or anisomycin (Thomson et al. 1999). Studies using various types of cells from genetically modified animals provided further evidence for the role of Msk1 in H3 phosphorylation (for a review see Brami-Cherrier et al. 2009; Lazou and Markou 2010). However, the mechanism by which Msk1 is recruited to chromatin and directed to phosphorylated S10 or S28 in H3 is not known. It is not likely that Msk1 is pre-loaded onto immediate early gene promoters before induction, as it has been demonstrated that Msk1 is associated with the *IL-6* promoter after cells are stimulated (Vermeulen et al. 2003). Furthermore, E-26-like protein 1 (Elk-1) is required for the recruitment of ERK and Msk1 to the promoter of immediate early genes *c-fos* and *egr-1* (Zhang et al. 2008). On the other hand, using an in vitro system, it was recently shown that the recruitment of Msk1 and the subsequent phosphorylation of H3 on the *c-fos* chromatin requires CREB and, to a lesser extent, ATF1. Phosphorylation of CREB at S133 is essential for this process (Shimada et al. 2010).

In addition to H3, Msk1 also phosphorylates other chromatin-associated proteins such as nonhistone chromosomal protein HMGN1 (also called HMG-14) as well as histone H2A (Thomson et al. 1999; Brami-Cherrier et al. 2009).

Translation Factors

Msk1 has been implicated in the regulation of translational control by phosphorylating eukaryotic translation initiation factor 4E-BP1. 4E-BP1 binds to eIF4E in resting cells preventing formation of eIF-4 F complex which is essential for cap-dependent initiation of translation. UVB irradiation promotes phosphorylation of 4E-BP1 by Msk1 leading to the dissociation from eIF4E and the release of translational block after UVB irradiation (Liu et al. 2002).

Cell Death-Related Proteins

The ▶ Bcl-2 family member Bad has been also reported as Msk1 substrate. Bad is a pro-apoptotic protein and its phosphorylation promotes cell survival in many cell types. UVB irradiation promotes phosphorylation of Bad at S112 through Jnk1, Rsk2, and Msk1 (She et al. 2002). Furthermore, Msk1 mediates phosphorylation of neuronal Bad following Ca^{2+} influx (Clark et al. 2007).

Other Proteins

Msk1 phosphorylates ▶ cPLA2 at S727 in vitro and is also implicated in the translocation of cPLA2 to the membrane (Lazou and Markou 2010). However, it remains to be investigated whether cPLA2 is a real target of MSK1 in vivo. In mouse epidermal JB6 cells, the expression of Msk1 C-terminal kinase-dead mutant inhibited UVB-induced phosphorylation and activation of Akt, implicating Msk1 as the kinase responsible (Nomura et al. 2001).

Physiological Roles of Msk1

Inflammation

As mentioned above, Msk1 contributes to the transcriptional activation of ▶ NF-κB and ▶ CREB, two transcription factors with important roles in the regulation of inflammatory genes (Arthur 2008; Vermeulen et al. 2009). Furthermore, Msk1 controls the transcription of *COX-2* in response to TLR (toll-like receptor) signaling. Recently, it was reported that Msk1 induces the transcription of the MAP kinase phosphatase Dusp1 and the anti-inflammatory cytokines IL-10 and IL-1ra, which implies that Msk1 has a critical role in the negative feedback mechanisms required to reduce inflammation through TLR signaling (Ananieva et al. 2008).

Cell Death

There are controversial reports regarding the role of Msk1 in cell death. Activation of Msk1 by glutamatergic neurotransmission results in enhanced neuronal injury (Hughes et al. 2003). Furthermore, in human lymphoma B cells, the sequential activation of p38-MAP kinase and Msk1 leads to the Mn^{2+}-dependent activation of caspase-8 and cell death (El Mchichi et al. 2007). Although, expression of a dominant-negative mutant of Msk1 in these cells inhibits caspase-8 activation, the underlying mechanisms are not yet known. On the other hand, other studies suggest that Msk1 represents a pro-survival pathway. Decreased expression of Msk1 enhances arsenic trioxide-induced apoptosis of leukemic cells in vitro and in vivo (Kannan-Thulasiraman et al. 2006). Furthermore, Msk1 knockdown reduces Bad phosphorylation and enhances Noxa and Bim expression, leading to enhanced TGFβ-induced caspase-3 activity and cell death (van der Heide et al. 2011). In agreement with this latter study, Msk1 promotes cell survival by phosphorylating Bad in mouse epidermal cells (She et al. 2002). Thus, it is likely that the role of Msk1 is dependent on cell-type specific factors that are as yet undefined.

Cell Growth

Msk1 activity has also been implicated in cellular transformation. Using Msk1 dominant-negative mutants or small interfering RNA (siRNA) against Msk1, it was shown that Msk1 is required for TPA-induced or EGF-induced cellular transformation of JB6 Cl41 cells. This effect is mediated by phosphorylation of histone H3 at S 10 as well as AP-1 activation (Kim et al. 2008). Msk1 is also a positive regulator of epithelial cell proliferation (Schiller et al. 2006). Furthermore, pharmacological inhibition of Msk1 inhibits hypertrophic cell growth of cardiac myocytes (Lazou and Markou 2010).

Neuronal Plasticity

Several lines of evidence support the notion that Msk1 is involved in the mechanisms underlying neuronal plasticity in memory formation or as a response to drugs of abuse. It has been shown that Msk1 contributes to chromatin remodeling in striatal neurons and hippocampus resulting in long-term synaptic plasticity and memory formation (Brami-Cherrier et al. 2009). Furthermore, using Msk1-knockout mice, it was

demonstrated that Msk1 plays an important role in cocaine-induced transcriptional events and behavioral alterations. In these mice, phosphorylation of CREB and histone H3 as well as induction of c-*fos* and dynorphin in response to cocaine was prevented. In addition, a selective impairment of locomotor sensitization was observed. Msk1 deficiency was also shown to be responsible for the transcriptional dysregulation and striatal neurodegeneration in a mouse model of Huntington's disease (Brami-Cherrier et al. 2009).

Summary

Msk1 is a serine/threonine kinase that is activated in response to both growth factor and cellular stress stimuli. Msk1 belongs to a family of protein kinases that contain two protein kinase domains: an N-terminal kinase domain related to the AGC kinase family, and a C-terminal kinase domain related to the CaMK family, and it is activated via a complex series of phosphorylation and autophosphorylation reactions. Msk1 is predominantly located in the nucleus and has been shown to modulate gene expression by phosphorylating various substrates, including transcription factors and chromatin-associated proteins. Among the substrates that have been identified are CREB, ATF1, STAT3, the p65/RelA subunit of NF-κB and the immediate early genes *c-fos*, *junB*, *c-jun*, and *Nurr77*. In addition to transcription factors, Msk1 has been shown to phosphorylate the nucleosomal proteins histone H3 and nonhistone chromosomal protein HMG-14 as well as the eukaryotic translation initiation factor 4E-BP1 and the pro-apoptotic protein Bad. It seems that Msk1 plays a role in integrating the effects of diverse extracellular signals and it remains to be determined how it affects its downstream targets leading to the observed signal- and cell-type-dependent specificity. The full panoply of Msk1 physiological roles has not been elucidated yet, but substantial evidence implicates Msk1 in the regulation of cytokine production and the inflammatory response, as well as neuronal synaptic plasticity. In addition, recent work suggests that Msk1 can also be functionally involved in tumor initiation, cell growth, and cell death regulation. This evidence along with the growing number of newly identified Msk1 substrates makes this kinase a suitable target for therapeutic manipulation of several diseases.

References

Ananieva O, Darragh J, Johansen C, Carr JM, McIlrath J, Park JM, et al. The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. Nat Immunol. 2008;9:1028–36.

Arthur JS. MSK activation and physiological roles. Front Biosci. 2008;13:5866–79.

Brami-Cherrier K, Roze E, Girault JA, Betuing S, Caboche J. Role of the ERK/MSK1 signalling pathway in chromatin remodelling and brain responses to drugs of abuse. J Neurochem. 2009;108:1323–35.

Clark CJ, McDade DM, O'Shaughnessy CT, Morris BJ. Contrasting roles of neuronal MSK1 and Rsk2 in Bad phosphorylation and feedback regulation of Erk signalling. J Neurochem. 2007;102:1024–34.

Deak M, Clifton AD, Lucocq LM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J. 1998;17:4426–41.

El Mchichi B, Hadji A, Vazquez A, Leca G. p38 MAPK and MSK1 mediate caspase-8 activation in manganese-induced mitochondria-dependent cell death. Cell Death Differ. 2007;14:1826–36.

Hughes JP, Staton PC, Wilkinson MG, Strijbos PJ, Skaper SD, Arthur JS, Reith AD. Mitogen and stress response kinase-1 (MSK1) mediates excitotoxic induced death of hippocampal neurones. J Neurochem. 2003;86:25–32.

Kannan-Thulasiraman P, Katsoulidis E, Tallman MS, Arthur JS, Platanias LC. Activation of the mitogen- and stress-activated kinase 1 by arsenic trioxide. J Biol Chem. 2006;281:22446–52.

Kim HG, Lee KW, Cho YY, Kang NJ, Oh SM, Bode AM, Dong Z. Mitogen- and stress-activated kinase 1-mediated histone H3 phosphorylation is crucial for cell transformation. Cancer Res. 2008;68:2538–47.

Lazou A, Markou T. MSK1. UCSD-nature molecule pages. 2010. doi:10.1038/mp.a001562.01.

Liu G, Zhang Y, Bode AM, Ma WY, Dong Z. Phosphorylation of 4E-BP1 is mediated by the p38/MSK1 pathway in response to UVB irradiation. J Biol Chem. 2002;277:8810–6.

McCoy CE, Macdonald A, Morrice NA, Campbell DG, Deak M, Toth R, McIlrath J, Arthur JS. Identification of novel phosphorylation sites in MSK1 by precursor ion scanning MS. Biochem J. 2007;402:491–501.

Nomura M, Kaji A, Ma WY, Zhong S, Liu G, Bowden GT, Miyamoto KI, Dong Z. Mitogen- and stress-activated protein kinase 1 mediates activation of Akt by ultraviolet B irradiation. J Biol Chem. 2001;276:25558–67.

Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol. 2010;11:9–22.

Schiller M, Böhm M, Dennler S, Ehrchen JM, Mauviel A. Mitogen- and stress-activated protein kinase 1 is critical for interleukin-1-induced, CREB-mediated, c-fos gene expression in keratinocytes. Oncogene. 2006;25:4449–57.

She QB, Ma WY, Zhong S, Dong Z. Activation of JNK1, RSK2, and MSK1 is involved in serine 112 phosphorylation of Bad by ultraviolet B radiation. J Biol Chem. 2002;277:24039–48.

Shimada M, Nakadai T, Fukuda A, Hisatake K. cAMP-response element-binding protein (CREB) controls MSK1-mediated phosphorylation of histone H3 at the c-fos promoter in vitro. J Biol Chem. 2010;285:9390–401.

Thomson S, Clayton AL, Hazzalin CA, Rose S, Barratt MJ, Mahadevan LC. The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. EMBO J. 1999;18:4779–93.

van der Heide LP, van Dinther M, Moustakas A, ten Dijke P. TGFbeta activates mitogen- and stress-activated protein kinase-1 (MSK1) to attenuate cell death. J Biol Chem. 2011;286:5003–11.

Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G. Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). EMBO J. 2003;22:1313–24.

Vermeulen L, Vanden Berghe W, Beck IM, De Bosscher K, Haegeman G. The versatile role of MSKs in transcriptional regulation. Trends Biochem Sci. 2009;34:311–8.

Wierenga AT, Vogelzang I, Eggen BJ, Vellenga E. Erythropoietin-induced serine 727 phosphorylation of STAT3 in erythroid cells is mediated by a MEK-, ERK-, and MSK1-dependent pathway. Exp Hematol. 2003;31:398–405.

Zhang HM, Li L, Papadopoulou N, Hodgson G, Evans E, Galbraith M, et al. Mitogen-induced recruitment of ERK and MSK to SRE promoter complexes by ternary complex factor Elk-1. Nucleic Acids Res. 2008;36:2594–607.

MSPK1

▶ MSK1

MST098

▶ Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

MSTP098

▶ Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

mtDNA (Mitochondrial DNA)

▶ p38 MAPK Family of Signal Transduction Proteins

mTEF-1

▶ Tead

mTOR

Aaron M. Robitaille
Growth and Development, Biozentrum, University of Basel, Basel, Switzerland

Synonyms

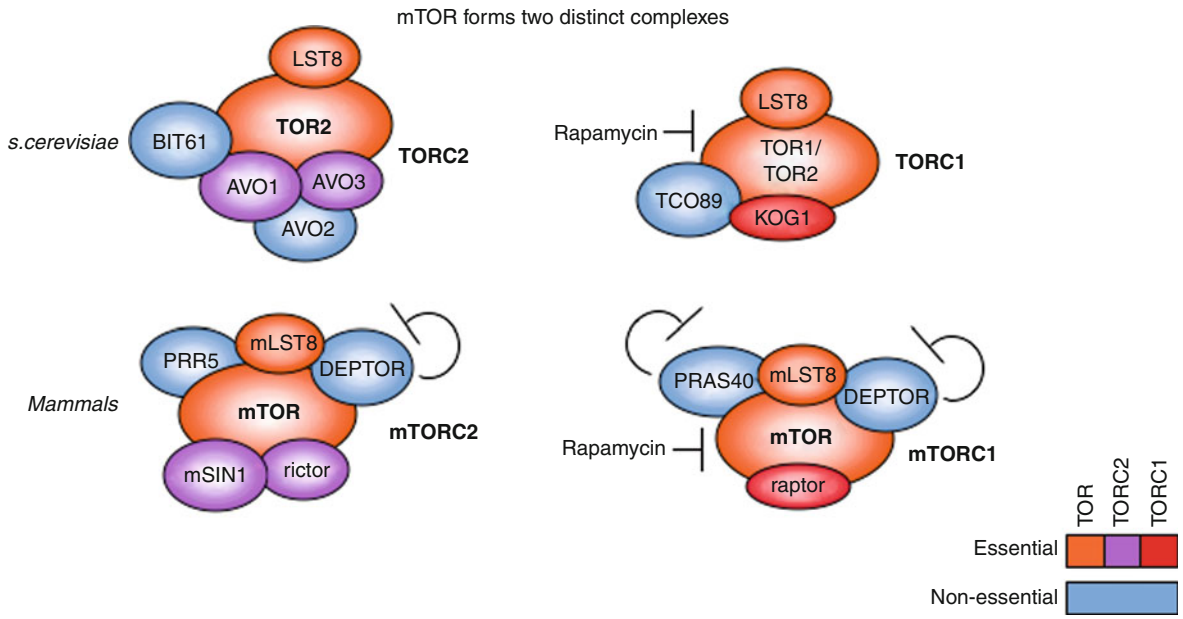
FKBP-rapamycin-associated protein (FRAP); mammalian Target of Rapamycin (mTOR); Rapamycin and FKBP12 Target-1 protein (RAFT1)

Historical Background

The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase structurally and functionally conserved from yeast to humans that positively regulates cell growth, proliferation, and survival, while inhibition of mTOR signaling extends lifespan (Harrison et al. 2009). In eukaryotes, mTOR is ubiquitously expressed and whole-organism knockout has demonstrated that it is essential for cell growth and viability. mTOR forms two multiprotein complexes, namely, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The macrolide rapamycin acutely inhibits mTORC1 but not mTORC2. mTORC1 and mTORC2 regulate functionally distinct, yet partially overlapping, signaling networks that collectively control the spatial and temporal regulation of cell growth.

Nutrients and growth factors activate mTORC1, whereas low cellular energy levels or stress inhibits mTORC1. Under favorable conditions for growth, mTORC1 is activated to promote translation, ribosome biogenesis, and inhibit autophagy. Thus, mTORC1 regulates when a cell grows. mTORC2 is activated by growth factors to promote cell survival and actin cytoskeleton remodeling in yeast, dictyostelium, and mammals. Thus, in broad terms, mTORC2 regulates where a cell grows.

As primary regulators of cell growth, the mTORC1 and mTORC2 signaling networks function as key regulatory nodes whose functions are important for development, aging, and have been linked to cancer and metabolic disorders. These signaling networks will be discussed in detail below:



mTOR, Fig. 1 mTORC1 and mTORC2: mTOR forms two distinct, multiprotein complexes, mTORC1 and mTORC2, which are structurally and functionally conserved from yeast to human

mTOR Complexes

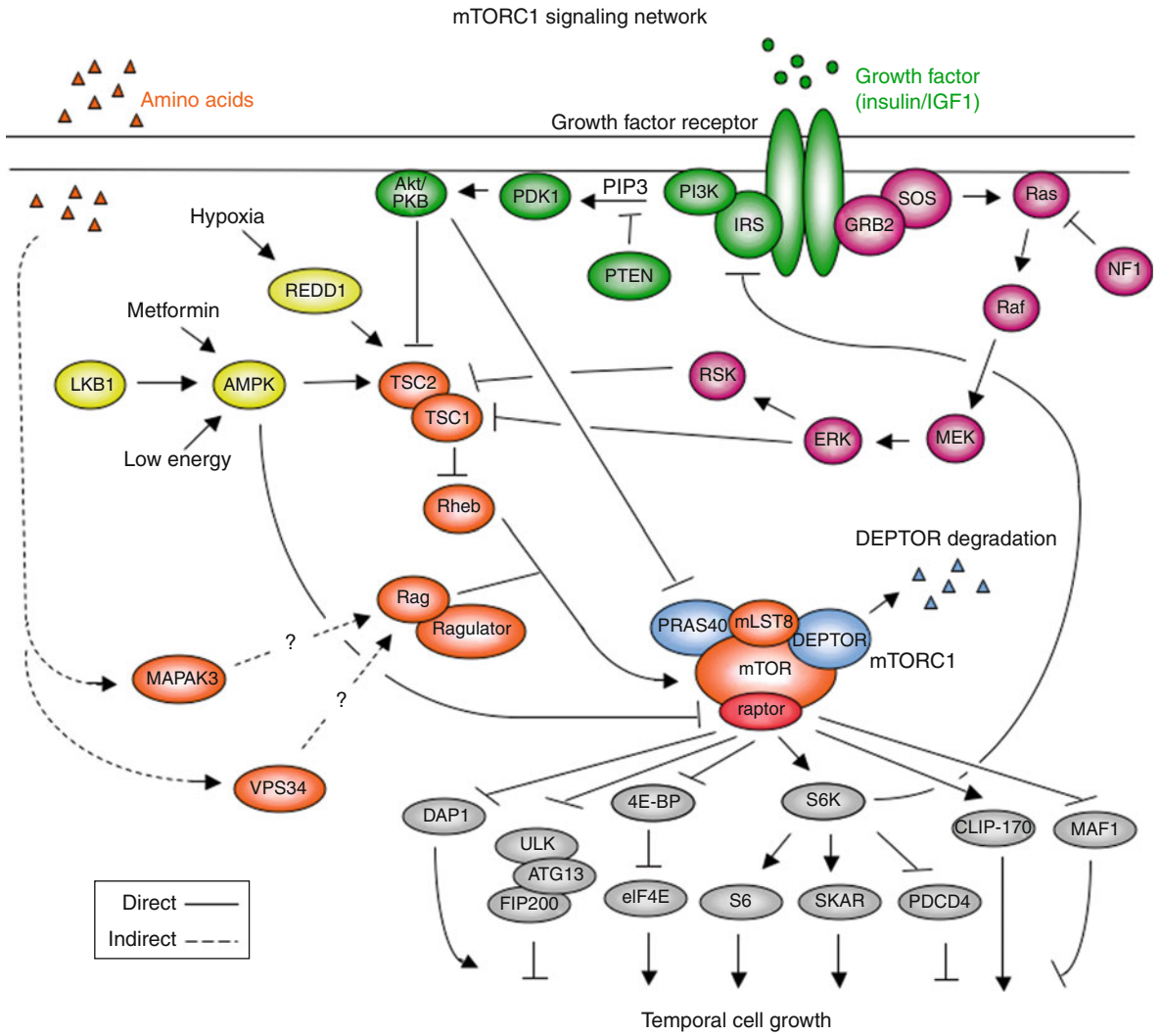
mTORC1 is multiprotein complex consisting of mTOR, Raptor, and mLST8 (also called GβL) (Fig. 1). Whole-body knockout of mTOR is embryonic lethal in mice. Homozygous mTOR−/− mice die at embryonic (E) day E5.5–E6. Similar to mTOR, Raptor−/− mice die at day E5.5–E6.5, suggesting that both mTORC1 components are required for progressing past the same stage of embryonic development. Additionally, mTORC1 associates with PRAS40 (proline-rich Akt/protein kinase B (PKB) substrate 40 kDa), and DEP-domain-containing mTOR-interacting protein (DEPTOR); ultimately this results in the negative regulation of mTORC1 activity (reviewed in Laplante and Sabatini 2009).

mTORC2 is composed of mTOR, Rictor, Sin1, mLST8, and PRR5/PRR5L (Protor 1/Protor 2) (reviewed in Cybulski and Hall 2009). PRR5 is not required for the interaction between mTOR, Rictor, Sin1, and mLST8. Deletion of Rictor or Sin1 is embryonic lethal, as mice die after day E10–E10.5. Loss of Sin1 disrupts Rictor's association with mTORC2. Interestingly, ablation of Rictor or Sin1 results in loss of Akt/PKB hydrophobic motif (HM) phosphorylation (at Ser473), yet the phosphorylation of several Akt/PKB targets is unaffected. This suggests either that phosphorylation of Ser473 may alter substrate specificity, or that in the absence of active Akt/PKB, other AGC kinases such as SGK, S6K, or RSK can phosphorylate a subset of Akt/PKB substrates. Surprisingly, although mLST8 is a component of both mTORC1 and mTORC2, mLST8 knockout mice die at day E10.5, phenotypically resembling Rictor−/− mice. Furthermore, mLST8 is required for Akt/PKB HM phosphorylation, but not S6K signaling. These data suggest that mLST8 has an essential role only in mTORC2.

In mice, tissue-specific knockouts of mTOR or mTORC1/mTORC2 components have demonstrated that mTOR plays a key role in regulating muscle atrophy, glucose metabolism, and lipid metabolism (reviewed in Polak and Hall 2009). mTORC1 and mTORC2 signal via distinct pathways to control a wide variety of cellular processes. These mTOR-regulated processes mediate the accumulation of cellular mass and, thereby, ultimately determine cell size.

The mTORC1 Signaling Network

Upstream regulation of mTORC1: Four major inputs control mTORC1: nutrients, such as amino acids; growth factors, such as insulin; cellular energy levels, such as the AMP: ATP ratio; and stress, such as



mTOR, Fig. 2 mTORC1 signaling network: amino acid and growth factor activation of mTORC1 promotes temporal cell growth and proliferation

hypoxia (reviewed in Taniguchi et al. 2006). mTORC1 integrates growth factors, cellular energy levels, and hypoxia through an upstream negative regulator called the tuberous sclerosis complex (TSC1/2), a heterodimeric GTPase-activating protein (Fig. 2). Growth factors inhibit TSC1/2 through the phosphoinositide 3-kinase (PI3K) pathway. Upon insulin stimuli, the insulin receptor substrate (IRS), and subsequently PI3K are recruited to the insulin receptor. The cellular level of phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3) is maintained by the antagonistic actions of PI3K and the lipid phosphatase PTEN. Akt/PKB and PDK1 are recruited to the plasma membrane via PI(3,4,5)P3, resulting in the phosphorylation and activation of Akt/PKB at the activation loop site by PDK1. TSC2 is then phosphorylated and functionally inactivated by Akt/PKB in response to insulin. In addition, Akt/PKB phosphorylates PRAS40 at Ser 247 and inactivates it, which leads to mTORC1 activation. mTORC1 can further phosphorylate PRAS40 at Ser 183 and Ser 221. Growth factor activation of mTORC1 also leads to the phosphorylation and subsequent degradation of DEPTOR, a negative regulation of mTORC1 activity (Peterson et al. 2009). The TSC1/2 complex is also inhibited by canonical MAPK signaling. Similar to Akt/PKB, active ERK or RSK can phosphorylate and inhibit TSC2 GAP activity.

In contrast to growth factors that inhibit TSC1/2, low cellular energy and stress activate TSC1/2 GTPase-activator protein (GAP) activity. Low energy (high AMP:ATP ratio) activates AMP-activated protein kinase (AMPK) to phosphorylate TSC2. This action inhibits mTORC1 by increasing TSC2 GAP activity toward Rheb, a Ras-like GTPase. In addition to phosphorylating TSC2, AMPK also inhibits mTORC1 by directly phosphorylating Raptor. Mutations of the AMPK upstream activating kinase, LKB1, result in hyperactive mTORC1 signaling, thereby linking LKB1 to the TSC1/2 mTORC1 pathway. Hypoxia inhibits mTORC1 signaling through the HIF1-mediated upregulation of two homologous proteins REDD1 and REDD2 (Regulated in Development and DNA damage response genes 1 and 2) REDD acts to activate TSC1/2, independently of LKB1-AMPK, in order to inhibit mTORC1. The stress and energy signaling pathways are likely to be further associated, as prolonged hypoxia leads to ATP depletion and activation of AMPK.

Inactivation of TSC1/2 then allows GTP-bound Rheb to activate mTORC1. However, amino acids are required for proper mTORC1 localization to a late endosome/lysosome compartment containing active Rheb. In contrast to growth factor stimulation, amino-acid activation of mTORC1 occurs independently of TSC1/2, as amino acid withdrawal also downregulates mTORC1 signaling in TSC2-deficient cells. Activation of mTORC1 by amino acids requires the Ras-like GTPases Rheb and Rag, both of which bind directly to mTORC1. The Rag GTPase heterodimer (RagA or RagB binding to RagC or RagD) mediates the localization of mTORC1 to a late endosomal/lysosomal compartment containing Rheb. Rag heterodimers are recruited to lysosomes via an aptly named protein complex, Ragulator, which is composed of the genes encoded by the MAPKSP1, ROBLD3, and c11orf59 (Sancak et al. 2010). Ragulator-to-Rag-to-mTORC1 binding then facilitates the activation of mTORC1 by GTP-bound Rheb. hVps34 has also been implicated in the amino-acid stimulation of mTORC1 in a TSC1/2-independent fashion. The hVps34 product phosphatidylinositol-3-phosphate (PI(3)P) may help recruit mTORC1 through an unidentified scaffold protein. However, in flies, Vps34 functions downstream of TOR. How amino acids GTP-load, and thereby activate the Rag GTPases to ultimately activate mTORC1 remains to be determined. Intriguingly, MAP4K3 acts upstream of Rag-mediated mTORC1 activation (Yan et al. 2010). Amino acids stimulate MAP4K3 activity to phosphorylate an unknown substrate upstream of the Rag/Ragulator complex, whereas under amino acid–starved conditions, PP2A T61 epsilon inhibits MAP4K3 and subsequent mTORC1 activation. Upon activation, mTORC1 controls protein synthesis, promotes ribosome biogenesis, and inhibits autophagy.

Downstream effectors of mTORC1: Translation: mTORC1 activates cap-dependent translation initiation and elongation by phosphorylating the eukaryotic initiation factor 4E (eIF4E)–binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase (S6K). The phosphorylation of 4E-BP1 prevents its binding to eIF4E, enabling eIF4E to then associate with eIF4G to stimulate translation initiation, whereas phosphorylation of S6K by mTORC1 and phosphoinositide-dependent kinase 1 (PDK1) is required for complete activation. Activated S6K promotes translation initiation by phosphorylating eIF4B, programmed cell death protein 4 (PDCD4), and eEF2 kinase (eEF2K). Phosphorylation of eIF4B and PDCD4 activates translation initiation, whereas phosphorylation of eEF2K upregulates translation elongation. S6K also promotes the translation efficiency of spliced mRNAs via S6K-Aly/REF-like substrate (SKAR) (Ma et al. 2008). SKAR associates with mRNAs in a splicing-dependent manner, where it then recruits activated S6K and thereby preferentially enhances translation of spliced mRNAs. Additionally, S6K phosphorylates 40S ribosomal protein S6, but the significance of this phosphorylation is unknown. mTORC1 associates with its substrates 4E-BP1 and S6K through Raptor and a TOR signaling (TOS) motif in 4E-BP1 and S6K. The TOS motif is a conserved five-amino-acid sequence (FEMDI and FDIDL in the C terminus of 4E-BP1 and in the N terminus of S6K, respectively) that is necessary for the phosphorylation of these proteins by mTORC1. However, TOS motifs have not been identified in recently discovered substrates, suggesting mTORC1 may bind to different substrates through distinct regions or alternative scaffold proteins.

Ribosome biogenesis: mTORC1 promotes the synthesis of ribosomes and transfer RNAs (tRNAs). Rapamycin blocks the biosynthesis of ribosomes by inhibiting transcription of RNA polymerase I (Pol I)–dependent rRNA genes, Pol II–dependent ribosomal protein genes (RP genes), and Pol III–dependent

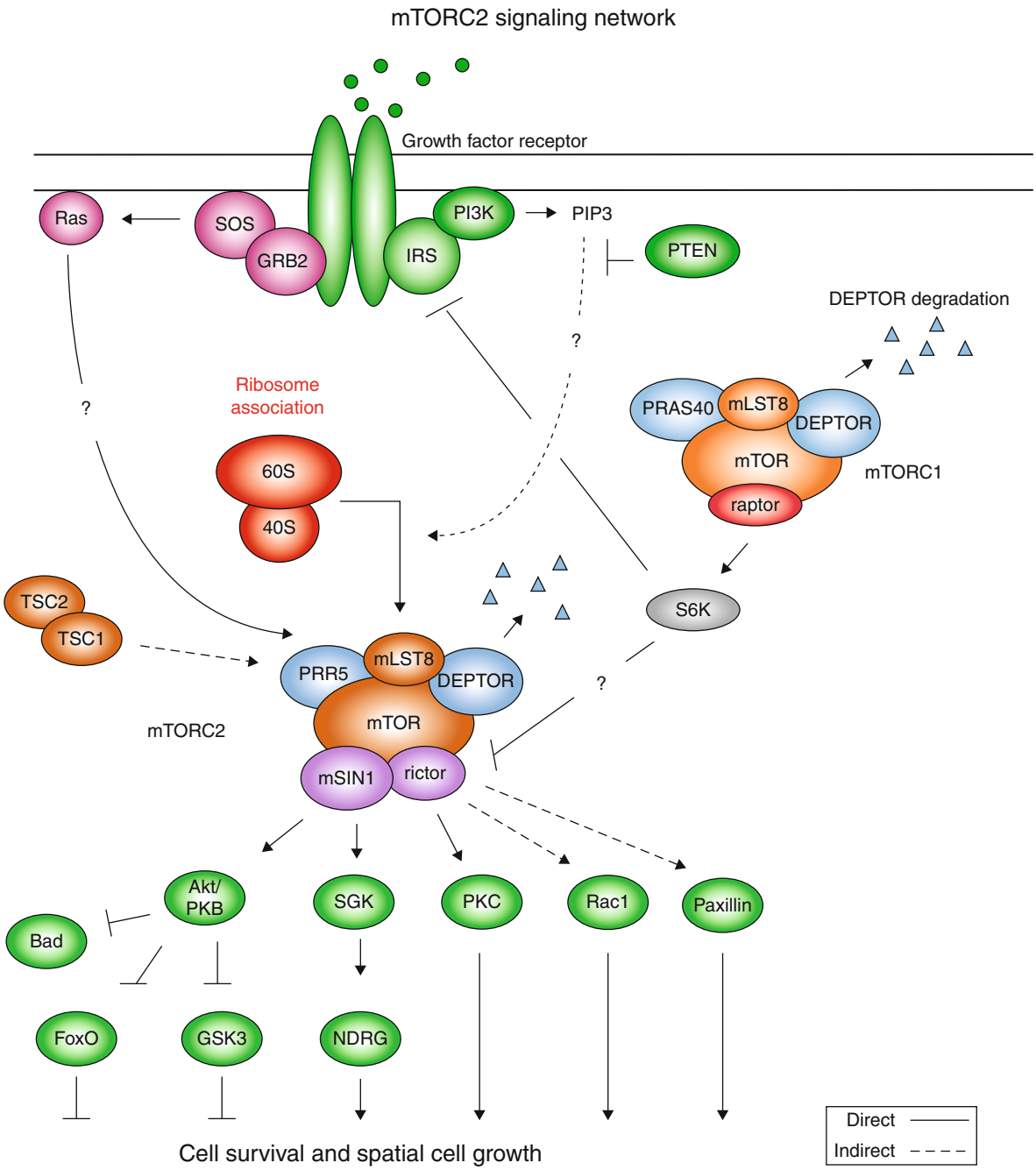
tRNA genes. mTOR controls Pol I via the essential transcription initiation factor TIF-1A (Transcriptional Intermediary Factor 1A). Rapamycin treatment leads to TIF-1A inactivation and thus impairs formation of the transcription initiation complex. Furthermore, TIF1A translocates from the nucleus to the cytoplasm upon rapamycin-mediated mTORC1 inactivation. In yeast, the forkhead-like transcription factor FHL1 functions as a TOR-dependent regulator of Pol II-dependent RP gene expression. mTORC1 regulates Pol III-mediated gene expression by directly phosphorylating and inhibiting MAF1, a Pol III transcriptional repressor. mTORC1 associates with TFIIIC, is recruited to Pol III-transcribed genes, and relieves MAF1-mediated repression, thus allowing Pol III transcription to occur (Michels et al. 2010).

Autophagy: mTORC1 negatively regulates macroautophagy, a starvation-induced catabolic process where bulk cytoplasm is enclosed in a double membrane structure and delivered to the vacuole for degradation. Rapamycin induces autophagy in yeast and human tissue culture, demonstrating the conserved role of mTORC1 as an inhibitor of autophagy. mTORC1 directly inhibits autophagy by phosphorylating and repressing a conserved protein complex composed of unc-51-like kinase 1 (ULK1), autophagy-related gene 13 (ATG13), and focal adhesion kinase family–interacting protein with a molecular weight of 200 kDa (FIP200) (Jung et al. 2009). mTORC1 also directly phosphorylates and represses DAP1 (Koren et al. 2010). Unlike the ULK1/ATG13/FIP200 complex, DAP1 is a negative regulator of autophagy. Thus via positive and negative regulation, mTORC1 activity tightly controls the absolute level of cellular autophagy. Additionally, dysregulation of mTORC1 may contribute to cancer cell survival, as tumor cells may temporarily activate autophagy to overcome nutrient deprivation under poor growth conditions. Through the mechanisms described above, mTORC1 regulates temporal cell growth.

The mTORC2 Signaling Network

Upstream regulation of mTORC2: In contrast to the detailed understanding of mTORC1 activation, a clear molecular mechanism underlying the activation of mTORC2 has been elusive until recently (reviewed in Robitaille and Hall 2008). In response to growth factors, mTORC2 promotes cell growth and proliferation by directly phosphorylating the hydrophobic motif of Akt/PKB and SGK. Growth factors signal to mTORC2 through the ▶ PI3K pathway (Fig. 3). Similar to the activation of mTORC1, upon insulin stimuli, the insulin receptor substrate (IRS) and subsequently PI3K are recruited to the insulin receptor. The cellular level of phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3) is maintained by the antagonistic actions of PI3K and the lipid phosphatase PTEN. Activation of PI3K promotes the association of mTORC2 with intact 80S ribosomes. The interaction between mTORC2 and the ribosome is required for mTORC2 activation, independent of protein translation (Zinzalla et al. 2011). How (PI(3,4,5)P3) promotes mTORC2 association with the ribosome and subsequent activation remains a mystery, but PI(3,4,5)P3-containing liposomes do not stimulate mTORC2's in vitro kinase activity. Growth factors activation of mTORC2 also leads to the phosphorylation and subsequent degradation of DEPTOR, a negative regulator of mTORC2 activity (Peterson et al. 2009). The TSC1/2 complex may also function upstream of mTORC2, but this activity is independent of TSC2 GAP activity. Loss of TSC1/2 inhibits the phosphorylation of mTORC2 substrates, while simultaneously hyperactivating mTORC1 and S6K. Active S6K can then directly phosphorylate Rictor and IRS, which is part of a negative feedback loop that attenuates insulin signaling. It is unclear to what degree the loss of TSC1/2, S6K-mediated Rictor phosphorylation, and IRS inhibition individually contribute to inhibition of mTORC2 activity. Recently, Ras has been shown to be upstream of mTORC2 independently of PI3K and conical MAPK signaling. In dictyostelium, RasC physically binds to TORC2 and activates it through an unknown mechanism (Charest et al. 2010). Mammalian Ras can also bind to Sin1 in vitro, but the physiological significance of this interaction has not been demonstrated. Additionally, mTORC2 and mTORC1 can bind phosphatidic acid (PA), suggesting that PA may mediate membrane localization of mTOR. Taken together, this data suggests that growth factors activate mTORC2 through multiple mechanisms, perhaps explaining why identifying a definitive upstream regulator has proven so elusive.

Downstream effectors of mTORC2: Unlike mTORC1, which can be specifically inhibited by rapamycin, mTORC2 is insensitive to acute rapamycin treatment. Thus, many of the downstream effectors and physiological functions of mTORC2 remain unknown.



mTOR, Fig. 3 mTORC2 signaling network: growth factor activation of mTORC2 promotes cell survival and spatial cell growth

Cell survival: mTORC2-deficient cells are sensitive to stress-induced apoptosis. mTORC2 promotes cell survival through the activation of Akt/PKB and serum and glucocorticoid-inducible kinase (SGK), two AGC kinases that have both distinct and overlapping substrates. Akt/PKB negatively regulates the pro-apoptotic protein BAD, while SGK regulates the phosphorylation of NDRG1, and both kinases negatively regulate FOXO. Interestingly, mTORC2 activity is required for prostrate tumorigenesis in PTEN null tumors, and not for normal prostate function (Guertin et al. 2009). This suggests that inhibiting

mTORC2-mediated cell survival would be attractive cancer therapeutic target with few side effects to healthy tissue.

Cytoskeleton remodeling: mTORC2 regulates actin cytoskeleton remodeling in yeast and is important for chemotaxis and cell migration in dictyostelium and mammals respectively (Charest et al. 2010). Furthermore, loss of mTORC2 alters actin polymerization and perturbs cell morphology. Thus, mTORC2 regulates where cell growth occurs. mTORC2-dependent cytoskeleton remodeling is likely mediated by the mTORC2 effectors PKCα, Paxillin, and Rac1, although the molecular mechanism by which mTORC2 regulates all these processes has not been determined.

AGC kinase activation and stability: mTORC1 and mTORC2 activate many members of the AGC kinase family (reviewed in Jacinto and Lorberg 2008). The AGC kinase activated by mTORC1 includes S6K, while mTORC2 regulates Akt/PKB, SGK, and PKCα. mTOR phosphorylates the hydrophobic motif (HM) in the AGC kinases to stimulate kinase activity. In S6K and SGK, phosphorylation of the HM creates a docking site for PDK1 and subsequent phosphorylation of the AGC kinase activation loop (reviewed in Pearce et al. 2010). mTOR also phosphorylates the turn motif (TM), at least in Akt/PKB and PKCα, to control stability of the kinase. In yeast, TOR phosphorylates and activates the AGC kinases Gad8, Ypk2, and Sch9. Future studies may reveal additional AGC kinases that are regulated by mTOR.

Open Questions and Summary

Expressional regulation of mTORC1: mTOR is classically thought to be ubiquitously expressed in eukaryotes. However, emerging research suggests mTORC1 may also be regulated at the level of expression. mTOR belongs to a subgroup of the atypical protein kinases, called [phosphoinositide 3-kinase]-related kinases (PIKK), which include Ataxia telangiectasia mutated (ATM); ATM and Rad3 related (ATR); DNA-dependent protein kinase (DNA-PK); suppressor with morphological effect on genitalia 1 (SMG1); and transformation/transcription domain–associated protein (TRRAP). All members of the PIKK family, including mTOR, interact with Tel2. These interactions positively regulate PIKK protein stability, but the physiological conditions under which Tel2 might regulate mTOR remain to be defined. Another recently identified regulator of mTOR protein stability is the tumor suppressor FBXW7. FBXW7 physically associates with mTOR and targets it for ubiquitination and degradation. Loss of FBXW7 increases the total amount of mTOR and subsequently the phosphorylation of S6K, indicating that FBXW7 is upstream of mTORC1. Depletion of FBXW7 did not affect the phosphorylation of Akt/PKB, suggesting that FBXW7 is not upstream of mTORC2. Further research is required to identify the physiological conditions when mTORC1 may be expressionally regulated.

Pharmacological inhibition of mTORC1 and mTORC2: Acute treatment with rapamycin, which forms a complex with FKBP12, binds to and allosterically inhibits mTORC1 but not mTORC2. In some cell lines, prolonged treatment with rapamycin (>24 h) indirectly inhibits mTORC2. Recently, several groups have independently developed active-site inhibitors of mTOR, which include PP242, Torin1, Ku-0063794, WAY-354, and AZD8055 (reviewed in Sparks and Guertin 2010). These compounds exhibit IC50 values in the low nanomolar range against mTORC1 and mTORC2. Furthermore, PP242 inhibited cancer cell proliferation to a greater extent than rapamycin (Janes et al. 2010). The increased antiproliferative affects of PP242 over rapamycin could be caused by inhibition of mTORC1 and mTORC2, rapamycin-resistant functions of mTORC1, or inhibition of an off-target kinase. mTOR active-site inhibitors will likely play an important role in the identification of novel mTOR functions and possibly as future cancer therapeutics.

mTORC1 and mTORC2 consensus motif: mTOR, as part of mTORC1 or mTORC2, can canonically phosphorylate two distinct target sites, a serine or threonine flanked by bulky hydrophobic (Φ) residues (Φ-pSer/Thr-Φ) and serine or threonine followed by a proline (pSer/Thr-Pro). Recent research in yeast and human cells to define the mTOR-regulated phosphoproteome revealed that mTORC1 can also phosphorylate serine followed by glutamine target sites (pSer-Gln) such as those in MAF1 (Huber et al. 2009 and Shor et al. 2010). Identification of additional mTORC1 and mTORC2 substrates may help to clarify the true mTOR consensus motif. Alternatively, mTOR may function as a promiscuous protein kinase to phosphorylate a wide variety of consensuses motifs. Under the latter model, a specific subunit of mTORC1 or mTORC2 would function as a scaffold protein to regulate substrate specificity.

Closing remarks: The mTORC1 and mTORC2 signaling networks have emerged as central controllers of cell growth that are important for development, aging, and diseases such as cancer and diabetes. The revelation that mTORC1 and mTORC2 regulate functionally distinct, yet partially overlapping signaling networks to collectively control the spatial and temporal cell growth has been a major conceptual advancement. Finally, the synthesis of mTORC1 and mTORC2 active-site inhibitors have the potential to be promising new therapeutic options to target mTOR dysregulation in cancer and metabolic disorders.

Acknowledgments I offer my regrets to our colleagues whose excellent work I could not described due to space limitations. I acknowledge the Werner-Siemens Foundation for funding support and M.N. Hall for advice and the general figure template.

Cross-References

▶ Phosphoinositide 3-kinase
▶ PTEN
▶ Ras (H-, K-, N-Ras)

References

Charest PG, Shen Z, Lakoduk A, Sasaki AT, Briggs SP, Firtel RA. A Ras signaling complex controls the RasC-TORC2 pathway and directed cell migration. Dev Cell. 2010;18(5):737–49.

Cybulski N, Hall MN. TOR complex 2: a signaling pathway of its own. Trends Biochem Sci. 2009;34(12):620–7.

Guertin DA, Stevens DM, Saitoh M, Kinkel S, Crosby K, Sheen JH, et al. mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. Cancer Cell. 2009;15(2):148–59.

Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460(7253):392–5.

Huber A, Bodenmiller B, Uotila A, Stahl M, Wanka S, Gerrits B, et al. Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. Genes Dev. 2009;23(16):1929–43.

Jacinto E, Lorberg A. TOR regulation of AGC kinases in yeast and mammals. Biochem J. 2008;410(1):19–37.

Janes MR, Limon JJ, So L, Chen J, Lim RJ, Chavez MA, et al. Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. Nat Med. 2010;16(2):205–13.

Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell. 2009;20(7):1992–2003.

Koren I, Reem E, Kimchi A. DAP1, a novel substrate of mTOR, negatively regulates autophagy. Curr Biol. 2010;20(12):1093–8.

Laplante M, Sabatini DM. mTOR signaling at a glance. J Cell Sci. 2009;122(Pt 20):3589–94.

Ma XM, Yoon SO, Richardson CJ, Julich K, Blenis J. SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs. Cell. 2008;133(2):303–13.

Michels AA, Robitaille AM, Buczynski-Ruchonnet D, Hodroj W, Reina JH, Hall MN, et al. mTORC1 directly phosphorylates and regulates human MAF1. Mol Cell Biol. 2010;30(15):3749–57.

Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol. 2010;11(1):9–22.

Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell. 2009;137(5):873–86.

Polak P, Hall MN. mTOR and the control of whole body metabolism. Curr Opin Cell Biol. 2009;21(2):209–18.

Robitaille AM, Hall MN. mTOR. UCSD-Nature Molecule Pages. 2008. Available from: http://www.signaling-gateway.org/molecule/query?afcsid=A000094. Accessed 15 Oct 2008.

Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010;141(2):290–303.

Shor B, Wu J, Shakey Q, Toral-Barza L, Shi C, Follettie M, and Yu K. Requirement of the mTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. JBC. 2010;285:15380–15392.

Sparks CA, Guertin DA. Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. Oncogene. 2010;29(26):3733–44.

Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006;7(2):85–96.

Yan L, Mieulet V, Burgess D, Findlay GM, Sully K, Procter J, et al. PP2A T61 epsilon is an inhibitor of MAP4K3 in nutrient signaling to mTOR. Mol Cell. 2010;37(5):633–42.

Zinzalla V, Stracka D, Oppliger W, Hall MN. Activation of mTORC2 by association with the ribosome. Cell. 2011;144(5):757–768.

MTSG1, GK1, and AT2R Binding Protein of 50 kDa (ATBP50)

▶ MTUS1/ATIP

MTUS1 Isoform 1 (ATIP3)

▶ MTUS1/ATIP

MTUS1 Isoform 2 (ATIP3b)

▶ MTUS1/ATIP

MTUS1 Isoform 3 (ATIP4)

▶ MTUS1/ATIP

MTUS1 Isoform 4 (ATIP2)

▶ MTUS1/ATIP

MTUS1 Isoform 5 (ATIP1)

▶ MTUS1/ATIP

MTUS1/ATIP

Simon N.S. Louis, Naghmeh Varghayee, Laurie T.C. Chow and William J. Louis
Clinical Pharmacology Unit, Department of Medicine Austin Health/Northern Health, University of Melbourne, Heidelberg, VIC, Australia

Synonyms

Angiotensin II type 2 receptor interacting protein (ATIP); MTSG1, GK1, and AT2R binding protein of 50 kDa (ATBP50); MTUS1 isoform 1 (ATIP3); MTUS1 isoform 2 (ATIP3b); MTUS1 isoform 3 (ATIP4); MTUS1 isoform 4 (ATIP2); MTUS1 isoform 5 (ATIP1)

Historical Background

Following its initial identification in Japan in 1999 (Nagase et al. 1999), mitochondrial tumor suppressor 1 (MTUS1/ATIP) (2011), a putative tumor suppressor gene, has been independently identified by research groups from Japan (Kinjo et al. 2000), Germany (Seibold et al. 2003; Wruck et al. 2005), and France (Nouet et al. 2004). As a result, MTUS1/ATIP has been designated a variety of different names; therefore, the nomenclature used to describe MTUS1/ATIP in publications can be confusing.

MTUS1/ATIP was first identified in 1999 following the sequencing of 100 cDNA clones of unknown human genes from two sets of size-fractionated human adult and fetal brain cDNA libraries (Nagase et al. 1999). At this time, it was designated KIAA1288 (Genbank Accession Number: ABO33114).

In 2000, a second Japanese group independently identified MTUS1/ATIP following large-scale genomic DNA sequencing of chromosome 8p21.3 (Kinjo et al. 2000), where a loss of heterozygosity is associated with progression in a range of human cancers including breast, colorectal, hepatocellular, pancreatic, lung, and prostate cancers (Seibold et al. 2003). They designated the gene GK1 and following Northern blot analysis identified ubiquitous expression of 7.0- and 4.4-kb gene transcripts.

Then in 2003, a German group, who were investigating gene expression during induction of cellular differentiation and quiescence, using a combination of 3D collagen I cell culture and differential display reverse transcriptase-polymerase chain reaction (RT-PCR), identified that MTUS1, which they designated MTGS1, was transiently upregulated during initiation of cellular differentiation (Seibold et al. 2003).

In 2004, a group at the Cochin Institute in Paris (Nouet et al. 2004) identified a 354 bp insert that encoded an open reading frame of 118 amino acids using the yeast two-hybrid cDNA cloning system and the last 52 amino acids of the human AT2-receptor as a bait to screen a mouse fetal cDNA library. This was designated ATIP-ID, which stands for interacting domain of the AT2-receptor-interacting protein. ATIP-ID interacted with the C-terminal intracellular tail of the AT2-receptor, but not with the AT1-receptor or with the C-terminal domains of other G-coupled protein receptors such as β2-adrenergic or ▶ bradykinin receptor. In total, five MTUS1/ATIP variants, which are derived from a single gene by alternate promoter utilization and exon/intron splicing, were identified. Co-immunoprecipitation studies identified a constitutive interaction between the C-terminal end of the AT2-receptor and ATIP.

Finally in 2005, a second German group independently identified an interaction between the AT2-receptor and MTUS1, which they designated ATBP50 and described it as an AT2-receptor binding protein of 50 kDa.

Localization

The most commonly expressed MTUS1 transcripts, MTUS1 isoform 1 (ATIP3a), MTUS1 isoform 2 (ATIP3b), MTUS1 isoform 5 (ATIP1), and MTUS1 isoform 3 (ATIP4) are differentially distributed throughout the body (Table 1) and their translated sequences contain consensus motifs for localization to different subcellular compartments (cytosol, nucleus, and plasma membrane for isoform 5/ATIP1, isoforms 1 and 2 (ATIP3), and isoform 4/ATIP4, respectively), suggesting that the various transcripts may possess a wide range of physiological functions. By contrast, MTUS1 isoform 6/ATIP2 is expressed at very low levels or cannot be detected in all of the tissues examined to date (<1%) (Di Benedetto et al. 2006a).

MTUS1 isoform 5/ATIP1 is the major transcript found in the brain, excepting the cerebellum (Di Benedetto et al. 2006a). Moreover, it has also been detected in organs, which contain high levels of AT2-receptor expression, such as in the female reproductive (placenta breast ovary, uterus) and the heart.

MTUS1 isoforms 1 and 2 (ATIP3) are the major transcripts found in all other organs of the body. Both transcripts show a similar pattern of distribution in human tissues; however isoform 1 is the predominantly expressed isoform in most tissues (Di Benedetto et al. 2006a).

Expression of mRNA for MTUS1 isoform 4/ATIP4 is localized to the brain, predominantly in the fetal brain, and it is particularly abundant in the cerebellum (Di Benedetto et al. 2006a). Interestingly, the cerebellum is the only region in the brain where the AT2-receptor has been consistently detected.

Although MTUS1 isoform 5/ATIP1 has been previously localized to the mitochondrial region of the cytoplasm (Kinjo et al. 2000; Seibold et al. 2003), only recently has the cellular localization of MTUS1 isoform 1/ATIP3a been established. Using a combination of microtubule co-sedimentation assays and immunohistochemical techniques, isoform 1/ATIP3, in contrast to isoform 5/ATIP1, has been identified as a microtubule-associated protein (Rodrigues-Ferreira et al. 2009).

MTUS1/ATIP, Table 1 MTUS1 variants and the corresponding ATIP variant

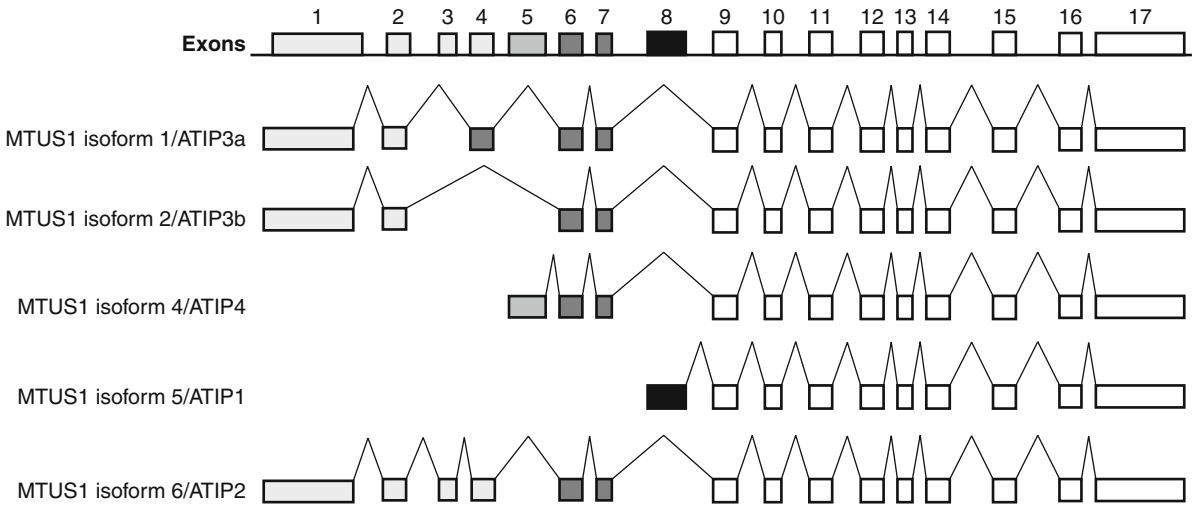
MTUS1 variant	Corresponding ATIP variant
MTUS1	ATIP
MTUS1 isoform 1	ATIP3a
MTUS1 isoform 2	ATIP3b
MTUS1 isoforms 1 and 2	ATIP3
MTUS1 isoform 4	ATIP4
MTUS1 isoform 5	ATIP1
MTUS1 isoform 6	ATIP2

Structure

Full-length human MTUS1 isoform 5/ATIP1, the first isoform fully characterized by the French group, comprises 1977 nucleotides (GenBank accession number AF293357) and encodes a 436-amino acid polypeptide and shares approximately 86% homology with mouse MTUS1 isoform 5/ATIP1 (Nouet et al. 2004).

The proteins encoded by mouse and human MTUS1 isoform 5/ATIP1 are mainly hydrophilic and contain no transmembrane domains. A major component of isoform 5/ATIP1 comprises a large coiled-coil domain, which contains two leucine zippers, and a high proportion of basic residues with a stretch of 30 C-terminal residues rich in proline, serine/threonine, and arginine. Protein analysis using an antibody-specific ATIP-ID revealed four apparent molecular weights of 30, 60, 120, and 180 kDa in a range of human tissues, which are thought to correspond to the various MTUS1/ATIP homologues (Nouet et al. 2004).

Comparison of the ATIP-ID nucleotide sequence in the Genbank database identified sequences that contained the homologous 354 bp region in uterus, brain, and fetal brain and these were designated ATIP2 (MTUS1 isoform 6), ATIP3 (isoforms 1 and 2), and ATIP4 (isoform 4), respectively, by the French group. Further structural analysis of the human MTUS1/ATIP gene (Genbank NT_030747) identified 17 coding exons encoding five transcripts through alternative splicing and these were designated ATIP1 (isoform 5), ATIP2 (isoform 6), ATIP3a (isoform 1), ATIP3b (isoform 2),



MTUS1/ATIP, Fig. 1 Schematic representation of the 17 coding exons of human MTUS1/ATIP and the corresponding exons contained in MTUS1 isoform 1 (ATIP3a), isoform 2 (ATIP3b), isoform 4 (ATIP4), isoform 5 (ATIP1), and isoform 6 (ATIP2) (Adapted from Di Beneditto et al. 2006a)

and ATIP4 (isoform 4) (Di Benedetto et al. 2006a) (Fig. 1).

All of the MTUS1/ATIP isoforms exhibit the same C-terminal domain capability to interact with the AT2-receptor (Nouet et al. 2004). MTUS1 isoform 5/ATIP1, isoforms 1/ATIP3a and 2/ATIP3b, and isoform 4/ATIP4, which correspond to murine ATBP50, ATBP135, and ATBP60, respectively (Wruck et al. 2005), share the same 3′ exons (exons 9–17) but contain differing 5′ exons (exons 8, 1, and 5, respectively). Moreover, isoforms 1/ATIP3a, 2/ATIP3b, and 4/ATIP4 are splice variants and all use exons 1, 2, 6, and 7, while in addition isoforms 1/ATIP3a and 4/ATIP4 contain exons 4 and 3, respectively.

The sequence of exon 4, which is present in human MTUS1 isoform 1/ATIP3a but not isoform 2/ATIP3b, does not exist in the coding region of either rat or mouse ATIP3, indicating that this gene, and the protein it encodes, are not present in these species (Krezel et al. 2011).

MTUS1 Function and Interaction with the AT2-Receptor

Initial studies examining the function of the most studied MTUS1 isoform, isoform 5/ATIP1, identified that recombinant expression of isoform 5/ATIP1 into MIA PaCa-2 pancreatic tumor cells, which did not endogenously express MTUS1/ATIP, inhibited cell proliferation, as measured by a 30% reduction in uptake of a thymidine analogue, bromodeoxyuridine (Seibold et al. 2003).

In 2004 it was reported, following parallel studies in wild-type monkey kidney fibroblasts (COS) cells and COS cells stably transfected with the AT2-receptor (COS-AT2), that transient transfection of isoform 5/ATIP1, into both cell lines resulted in a 50% reduction in EGF-induced ERK2 phosphorylation in AT2-receptor expressing cells but not in wild-type cells, suggesting that expression of the AT2-receptor is a necessary component of MTUS1 isoform 5 signaling cascade. Further studies, in stably transfected Chinese hamster ovary (CHO) cells, identified that overexpression of isoform 5/ATIP1 in the presence of AT2-receptors inhibited not only EGF-mediated ERK2 phosphorylation but also inhibited the effects of other growth factors, including insulin and basic fibroblast growth factor (Nouet et al. 2004). Additionally, following stimulation of isoform 5/AT2-receptor transfected CHO cells with insulin, autophosphorylation of the insulin receptor beta-chain migrating at 97 kDa was reduced compared with CHO cells transfected with an empty pcDNA3 vector (Nouet et al. 2004), i.e., it interferes at the initial step of insulin receptor signaling. It must also be noted that these studies were conducted in the absence of AT2-receptor activation, suggesting that overexpression of isoform 5/ATIP1 mimics the

activation of this receptor (Elbaz et al. 2000), while only slightly modifying the expression and affinity of AT2-receptors in these cells (Nouet et al. 2004).

Further evidence supporting the possible interdependence between MTUS1 isoform 5/ATIP1 and AT2-receptor function was provided in the form of siRNA studies in N1E-115 neuroblastoma cells (Wruck et al. 2005). These studies indicated that knockdown of MTUS1 isoform 5 not only reduced the ability of AT2-receptor activation to inhibit ERK2 phosphorylation and cell proliferation, which has recently been confirmed in studies of HVEC normal human umbilical vein epithelial cells (Zuern et al. 2010), it also increased AT2-receptor expression at the endoplasmic reticulum and significantly reduced the expression of these receptors at the cell surface, indicating that the receptor was not being transported to the cell surface(Wruck et al. 2005).

MTUS1 Other Potential Signaling Partners

As identified in the previous section, the activity of MTUS1 isoform 5/ATIP1, at least, is dependent on co-expression of the AT2-receptor. Beyond this little is known concerning the intracellular interactions of MTUS1/ATIP or the mechanism by which it inhibits ERK phosphorylation. Initially it was thought that MTUS1/ATIP did not act as a phosphatase (Nouet et al. 2004) or interact directly with protein tyrosine phosphatase 1 (SHP-l), a soluble protein tyrosine phosphatase that inhibits growth factor receptor signaling. However, more recently, it has been demonstrated that following activation of the AT2-receptor, ATIP interacts with SHP-1 to induce cell differentiation in primary cultures of rat neural cells (Li et al. 2007). Moreover, the ATIP/SHP-1 complex translocates into the nucleus and increases expression of one of the ubiquitin-conjugating enzyme variants, MMS2, which prevents neural damage and enhances neural cell differentiation (Li et al. 2007).

Pharmacological Regulation of MTUS1 Activity

In PC12W rat pheochromocytoma cells, which endogenously express both the AT2-receptor and MTUS1 isoform 5/ATIP1, AT2-receptor stimulation results in a "continuous increase" in isoform 5/ATIP1 mRNA levels, which commenced 30 min following receptor activation (Wruck et al. 2005).

Similarly, poly(ADP-ribose) polymerase-1 (PARP-1), which is implicated in the development of both cardiovascular and neuronal disease, is also thought to play a role in MTUS1 gene transcription, as PARP inhibition and PARP-1 ablation suppresses MTUS1 isoform 5/ATIP1 expression and promoter activity, whereas, they increased AT2-receptor expression at the plasma membrane (Reinemund et al. 2009). These findings appear to contradict the earlier work of Wruck et al. whereby isoform 5/ATIP1 knockdown decreased AT2-expression at the cell surface and this issue remains to be resolved.

EGF stimulation has also been shown to significantly decrease MTUS1/ATIP and MTUS1 isoform 5/ATIP1 mRNA expression by 45–65% in two prostate cancer cell lines (Louis et al. 2010).

MTUS1 in Cancer

Although MTUS1/ATIP was initially thought to be ubiquitously expressed (Kinjo et al. 2000), there is now a growing body of evidence indicating that a loss or downregulation of MTUS1/ATIP mRNA expression occurs with the development of a wide range of cancers, including cancers of the pancreas (Seibold et al. 2003), ovary (Pils et al. 2005), breast (Rodrigues-Ferreira et al. 2009; Frank et al. 2007; Chanrion et al. 2008), and colon (Zuern et al. 2010). For instance, Seibold *et al*. demonstrated an inverse correlation between MTUS1 mRNA expression and cellular differentiation and proliferation in pancreatic cancer cell lines and, as previously mentioned, demonstrated that recombinant expression of isoform 5 in MIA PaCa-2 cells inhibited cell proliferation (Seibold et al. 2003).

Similarly, QPCR studies examining control tissues containing ovarian tissues and cysts, 58 ovarian tumor biopsies, and 38 ovarian cancer cell lines identified that MTUS1 expression was significantly lower ($P = 0.004$) in primary ovarian carcinoma compared with control tissues (Pils et al. 2005).

Moreover, MTUS1 expression was also found to be significantly downregulated in cancers of the colon, compared to the corresponding normal tissues, at both the protein and mRNA level. However, no mutations in the MTUS1 coding or promoter sequences

could be identified. Similarly, only five nucleotide substitutions in the MTUS1/ATIP gene were detected in 109 primary hepatocellular carcinoma tumors and cell lines (Di Benedetto et al. 2006b), suggesting that the MTUS1/ATIP gene may not be a major target for mutation in either colon cancer or hepatocellular carcinoma. By contrast, a copy number variant, which lacks exon 4 of MTUS1/ATIP (i.e., MTUS1 isoform 2/ATIP3b), has been identified in breast cancers and this variation is significantly associated with a decreased risk for both familial and high-risk familial breast cancers (Frank et al. 2007; Hinds et al. 2006).

In addition, a recent study has identified that isoform 1/ATIP3a is the predominantly altered MTUS1/ATIP isoform in invasive breast cancer and that it is significantly reduced in highly proliferative breast carcinomas of poor clinical outcome. In addition, they have demonstrated that isoform 1/ATIP3a re-expression inhibits tumor cell proliferation both *in vitro* and *in vivo* and its overexpression delays the progression of mitosis by extension of the metaphase (Rodrigues-Ferreira et al. 2009).

Studies in human prostate cancer cell lines that express functional AT2-receptors (Chow et al. 2008) indicate that in a model of early stage, androgen-dependent prostate cancer, LNCaP cells, in which MTUS1/ATIP expression was knocked down using siRNA techniques the ability of EGF to stimulate DNA synthesis was potentiated, whereas, it had no effect on the basal rate of DNA synthesis. By contrast, MTUS1/ATIP knockdown in a model of late-stage, androgen-independent, prostate cancer, PC3 cells, resulted in a significantly increased basal rate of thymidine incorporation and stimulation of the cells with EGF did not increase DNA synthesis beyond this already heightened level of activation (Louis et al. 2010). Further studies examined the transient overexpression of MTUS1 isoform 5/ATIP1 in PC3 cells, as it expresses relatively low endogenous levels of ATIP. In these studies not only was the basal rate of ERK2 phosphorylation diminished but the ability of EGF to stimulate this pathway was also attenuated (Louis et al. 2010).

Of particular interest in prostate cancer however, is the re-expression of MTUS1/ATIP in the neoplastic epithelial cells in high-grade prostatic intraepithelial neoplasia, the premalignant phase of prostate cancer (Louis et al. 2007), suggesting that the downregulation of MTUS1/ATIP expression identified in other cancers may be preceded by an initial re-expression early in the malignant process, which may be of value in diagnosing the premalignant stage of a number of cancers in particular prostate cancer (Louis et al. 2011).

Summary

MTUS1/ATIP is a putative tumor suppressor gene, which localizes at 8p22 and encodes a family of five proteins as a result of alternate promoter utilization and exon/intron splicing. All five proteins contain a C-terminal domain which can interact with the AT2-receptor. MTUS 1 isoform 1/ATIP3a is the predominantly expressed variant throughout the body, excepting the brain where isoform 5/ATIP1 is the major transcript. The subcellular localization of MTUS1 isoform 1/ATIP3a has only recently been identified in the microtubules of SK-MES, HeLa, RPE1,MDA-MB-231, and MCF7 cells, whereas, isoform 5/ATIP1 has been identified in both the mitochondria and Golgi matrix. Relatively little is currently known concerning the action of MTUS1/ATIP; however, isoform 5/ATIP1, the most studied variant to date, has been shown to inhibit growth factor-induced ERK2 phosphorylation and cell proliferation in both normal and cancer cell lines; possibly by acting directly on growth factor receptors or via interaction with SHP-1. By contrast, isoform 1/ATIP3a inhibits tumor cell proliferation by extension of the metaphase, thereby, delaying the progression of mitosis. Studies indicate that MTUS1 isoform 5/ATIP1 and isoform 1/ATIP3a are downregulated in a range of human cancers and a mutation in isoform 1/ATIP3a has been associated with a decreased risk for both familial and high-risk familial breast cancers. Although there is much to be learned regarding the function and signaling pathways of MTUS1/ATIP, the information available indicates that it may play an important inhibitory role in cell proliferation and therefore may prove a potential therapeutic target in not only cancer but also cardiovascular disease.

References

Chanrion M, Negre V, Fontaine H, Salvetat N, Bibeau F, Mac Grogan G, Mauriac L, Katsaros D, Molina F, Theillet C, Darbon JM. A gene expression signature that can predict the recurrence of tamoxifen-treated primary breast cancer. Clin Cancer Res. 2008;14(6):1744–52.

Chow L, Rezmann L, Immamura K, Wang L, Catt K, Tikellis C, Louis WJ, Frauman AG, Louis SNS. Functional angiotensin II

type 2 receptors inhibit growth factor signalling in LNCaP and PC3 prostate cancer cell lines. Prostate. 2008;68(6):651–60.
Di Benedetto M, Bieche I, Deshayes F, Vacher S, Nouet S, Collura V, Seitz I, Louis S, Pineau P, Amsellem-Ouazana D, Couraud PO, Strosberg AD, Stoppa-Lyonnet D, Lidereau R, Nahmias C. Structural organization and expression of human MTUS1, a candidate 8p22 tumor suppressor gene encoding a family of angiotensin II AT2 receptor-interacting proteins, ATIP. Gene. 2006a;380(2):127–36.
Di Benedetto M, Pineau P, Nouet S, Berhouet S, Seitz I, Louis S, Dejean A, Couraud PO, Strosberg AD, Stoppa-Lyonnet D, Nahmias C. Mutation analysis of the 8p22 candidate tumor suppressor gene ATIP/MTUS1 in hepatocellular carcinoma. Mol Cell Endocrinol. 2006b;252(1–2):207–15.
Elbaz N, Bedecs K, Masson M, Sutren M, Strosberg AD, Nahmias C. Functional trans-inactivation of insulin receptor kinase by growth-inhibitory angiotensin II AT2 receptor. Mol Endocrinol. 2000;14(6):795–804.
Frank B, Bermejo JL, Hemminki K, Sutter C, Wappenschmidt B, Meindl A, Kiechle M, Bugert P, Schmutzler RK, Bartram CR, Burwinkel B. Copy number variant in the candidate tumor suppressor gene MTUS1 and familial breast cancer risk. Carcinogenesis. 2007;28(7):1442–5.
Hinds DA, Kloek AP, Jen M, Chen X, Frazer KA. Common deletions and SNPs are in linkage disequilibrium in the human genome. Nat Genet. 2006;38(1):82–5.
Kinjo T, Isomura M, Iwamasa T, Nakamura Y. Molecular cloning and characterization of two novel genes on chromosome 8p21.3. J Hum Genet. 2000;45(1):12–7.
Krezel MA, Rezmann LA, Varghayee N, Pete J, Frauman AG, Louis SN. Gene sequencing and tissue expression of unknown isoforms of an angiotensin II type 2 receptor interacting protein, ATIP, in the rat. Biosci Biotechnol Biochem. 2011;75(3):414–8.
Li JM, Mogi M, Tsukuda K, Tomochika H, Iwanami J, Min LJ, Nahmias C, Iwai M, Horiuchi M. Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1. Mol Endocrinol. 2007;21(2):499–511.
Louis SN, Wang L, Chow L, Rezmann LA, Imamura K, MacGregor DP, Casely D, Catt KJ, Frauman AG, Louis WJ. Appearance of angiotensin II expression in non-basal epithelial cells is an early feature of malignant change in human prostate. Cancer Detect Prev. 2007;31(5):391–5.
Louis SN, Chow L, Rezmann L, Krezel MA, Catt KJ, Tikellis C, Frauman AG, Louis WJ. Expression and function of ATIP/MTUS1 in human prostate cancer cell lines. Prostate. 2010;70(14):1563–74.
Louis SNS, Chow LTC, Varghayee N, Rezmann LA, Frauman AG, Louis WJ. The expression of MTUS1/ATIP and its major isoforms, ATIP1 and ATIP3, in human prostate cancer. Cancer. 2011;3(4):3824–57.
MTUS1 microtubule associated tumor suppressor 1 (Homo sapiens) (database on the Internet). NCBI National Center for Biotechnology Information (cited updated on 7 Aug 2011). Available from: http://www.ncbi.nlm.nih.gov/gene/57509.
Nagase T, Ishikawa K, Kikuno R, Hirosawa M, Nomura N, Ohara O. Prediction of the coding sequences of unidentified human genes. XV. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. DNA Res. 1999;6(5):337–45.
Nouet S, Amzallag N, Li JM, Louis S, Seitz I, Cui TX, Alleaume AM, Di Benedetto M, Boden C, Masson M, Strosberg AD, Horiuchi M, Couraud PO, Nahmias C. Trans-inactivation of receptor tyrosine kinases by novel angiotensin II AT2 receptor-interacting protein, ATIP. J Biol Chem. 2004;279(28):28989–97.
Pils D, Horak P, Gleiss A, Sax C, Fabjani G, Moebus VJ, Zielinski C, Reinthaller A, Zeillinger R, Krainer M. Five genes from chromosomal band 8p22 are significantly down-regulated in ovarian carcinoma: N33 and EFA6R have a potential impact on overall survival. Cancer. 2005;104(11):2417–29.
Reinemund J, Seidel K, Steckelings UM, Zaade D, Klare S, Rompe F, Katerbaum M, Schacherl J, Li Y, Menk M, Schefe JH, Goldin-Lang P, Szabo C, Olah G, Unger T, Funke-Kaiser H. Poly(ADP-ribose) polymerase-1 (PARP-1) transcriptionally regulates angiotensin AT2 receptor (AT2R) and AT2R binding protein (ATBP) genes. Biochem Pharmacol. 2009;77(12):1795–805.
Rodrigues-Ferreira S, Di Tommaso A, Dimitrov A, Cazaubon S, Gruel N, Colasson H, Nicolas A, Chaverot N, Molinie V, Reyal F, Sigal-Zafrani B, Terris B, Delattre O, Radvanyi F, Perez F, Vincent-Salomon A, Nahmias C. 8p22 MTUS1 gene product ATIP3 is a novel anti-mitotic protein underexpressed in invasive breast carcinoma of poor prognosis. PLoS One. 2009;4(10):e7239.
Seibold S, Rudroff C, Weber M, Galle J, Wanner C, Marx M. Identification of a new tumor suppressor gene located at chromosome 8p21.3–22. FASEB J. 2003;17(9):1180–2.
Wruck CJ, Funke-Kaiser H, Pufe T, Kusserow H, Menk M, Schefe JH, Kruse ML, Stoll M, Unger T. Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated Golgi protein. Arterioscler Thromb Vasc Biol. 2005;25(1):57–64.
Zuern C, Heimrich J, Kaufmann R, Richter KK, Settmacher U, Wanner C, Galle J, Seibold S. Down-regulation of MTUS1 in human colon tumors. Oncol Rep. 2010;23(1):183–9.

Mucolipins, TRPML1-3

► TRP (Transient Receptor Potential Cation Channel)

Mucosa-Associated Lymphoid Tissue Translocation Gene 1

► MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1)

MUK

► DLK (Dual Leucine Zipper-Bearing Kinase)

Murine Leukemia Viral (v-Raf-1) Oncogene Homolog 1 (3611-MSV)

▶ RAF-1 (C-RAF)

Murine Retrovirus Integration Site 1 Homolog

▶ Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate

Murine Sarcoma 3611 Oncogene 1

▶ RAF-1 (C-RAF)

MYB

▶ c-Myb

Myc

Anna Frenzel and Marie Arsenian Henriksson
Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden

Synonyms

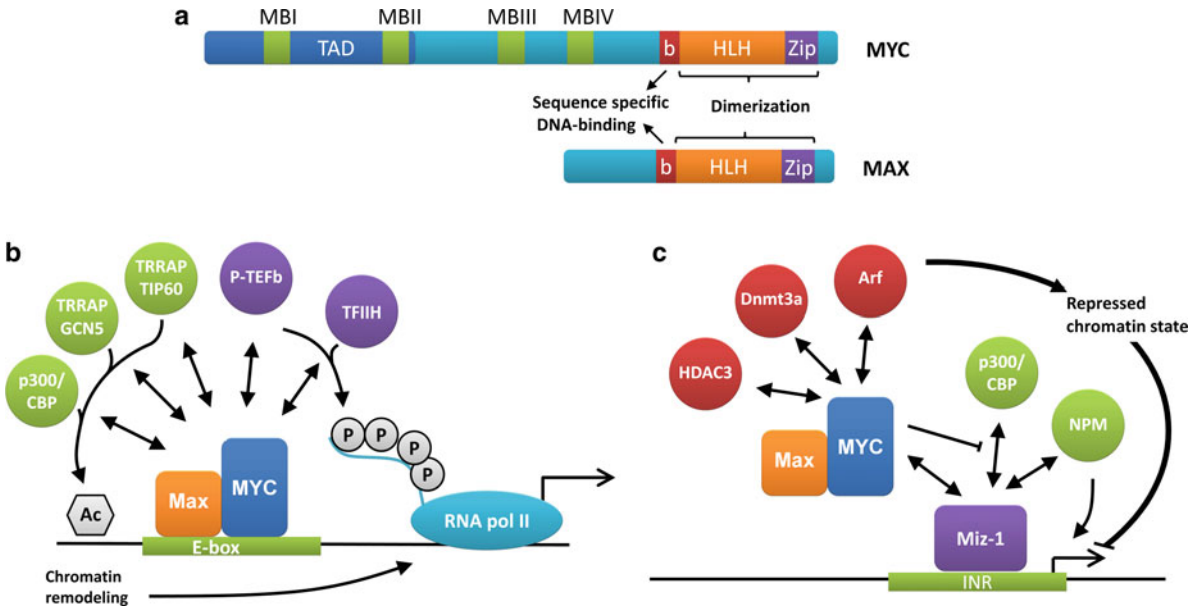
c-MYC; v-myc myelocytomatosis viral oncogene homolog

Historical Background

The first *MYC* gene was identified in the late 1970s in the avian acute leukemia virus MC29. This virus was known to cause a range of malignancies and the sequence in the viral genome responsible for the transforming capacity was named v-*myc* (short for viral myelocytomatosis, a leukemia caused by the virus). In 1979, a cellular homologue was identified in many species and was subsequently called c-*MYC*, where "c" denotes cellular. In contrast to other oncogenes known at the time, *MYC* did not seem to be activated by point mutations in the coding sequence. Instead studies in the early 1980s led to the identification of three novel mechanisms of oncogene activation: insertional mutagenesis (virus integration into the host genome at or near proto-oncogenes resulting in high levels of expression driven by the viral promoter), chromosomal translocation, and gene amplification (see also "MYC in Cancer" below). Following the discovery of c-*MYC*, it was realized that it was member of a larger family of oncogenes. In 1983, a new *MYC* gene was found to be amplified in neuroblastoma and was named *MYCN* where the "N" denotes neuroblastoma. Amplification of *MYCN* still remains one of the most important prognostic factors in neuroblastoma and is associated with aggressive disease and very poor prognosis. Two years later, yet another *MYC* family gene, *MYCL*, was identified in gene amplifications in small cell lung cancer. Since their discovery, studies of the MYC proteins have unveiled their fundamental roles in the development and function of normal tissues and have led to new knowledge about cancer biology (Wasylishen and Penn 2010).

The Transcription Factor MYC

Mammalian genomes contain three related genes, c-*MYC*, *MYCN*, and *MYCL* (here collectively referred to as *MYC*), which are structurally and functionally conserved. The founding member of the family, c-*MYC* is the best studied of the genes but many aspects of c-*MYC* biology and function hold true also for *MYCN* and *MYCL* (Eilers and Eisenman 2008). The MYC protein (MYC) is a transcription factor that can both activate and repress specific genes (Adhikary and Eilers 2005). It contains an N-terminal transactivation domain (TAD) and a C-terminal basic helix-loop-helix leucine zipper (bHLH-Zip) motif responsible for dimerization and DNA binding (Fig. 1a; Cowling and Cole 2006). To bind DNA MYC needs to heterodimerize with the small bHLH-Zip MYC-associated protein X (Max) (Lüscher and Larsson 1999). Max in turn can also interact with another group of bHLH-Zip-containing proteins, namely, Mxd1-4, Mnt, and Mga, but these heterodimers instead repress transcription and thus act



Myc, Fig. 1 *MYC structure and function in gene transactivation and repression.* (**a**) Schematic depiction of the structure of MYC and Max proteins indicating important functional domains. *MB* MYC box, *TAD* transactivation domain, *b* basic region, *HLH* helix-loop-helix, *Zip* Leucine-zipper. (**b**) Transactivation mediated by the MYC-Max dimer binding to the E-box sequence. Recruitment of HAT complexes leading to an open chromatin state, as well as MYC-promoted phoshphorylation of the C-terminal domain of RNA pol II, resulting in stimulated transcriptional elongation is shown. (**c**) MYC-mediated repression of Miz-1-induced transcription. The MYC-Max complex interacts with Miz-1 bound to the core promoter, resulting in displacement of co-activators of Miz-1 and instead recruiting factors mediating a repressed chromatin state

as antagonists of MYC. The MYC TAD contains two conserved regions, which are important for the transactivation and transforming activity of the protein referred to as MYC box (MB) I and MBII, respectively. MBII is mediating the interaction of MYC with several of its cofactors for transcriptional activation and repression, such as transformation/transcription domain-associated protein (TRAPP) and histone acetyl transferases (HATs). Located within MBI are residues that are important for regulation of MYC activity and turnover (see "Regulation of MYC Activity" below). Two additional conserved regions, MBIII and MBIV, are located in the central part of the MYC protein and are important for its full transforming activity (Cowling and Cole 2006).

To activate transcription, the MYC-Max heterodimer binds to the conserved E-box sequence core motif 5′-CANNTG, where 5′-CACGTG is the preferred sequence (Fig. 1b). MYC then recruits different cofactors in order to drive transcription and can stimulate histone acetylation at promoters by recruiting TRAPP-containing complexes with either GCN5 or TIP60 HATs or the CBP/p300 acetyl transferase (Fig. 1b). Histone acetylation opens the chromatin and provides docking sites for additional proteins that promote transcription (Cowling and Cole 2006). In addition, MYC also stimulates transcriptional elongation through recruitment of P-TEFb and TFII-H, which in turn phosphorylate the C-terminal domain of RNA pol II, allowing its release from the promoter and subsequent elongation (Fig. 1b; Cole and Cowling 2008). Furthermore, MYC can repress the transcription of specific genes by binding to other transcription factors in core promoters and inhibiting their transactivation activity (Herkert and Eilers 2010). The most studied example is the POZ-domain containing protein Miz-1, but other factors, including Sp1, can also be bound and inhibited by MYC. Several negative regulators of the cell cycle are among the transcriptional targets of Miz-1 including the cyclin-dependent kinase inhibitors (CKIs) p21 and p15. By binding to Miz-1, MYC can displace co-activators, such as CBP/p300 and instead recruit the DNA methylase Dnmt3, histone deacetylase 3 (HDAC3) and p14/ARF. This in turn leads to deacetylation of histones

as well as methylation of DNA and histones promoting a repressed chromatin state (Fig. 1c; Herkert and Eilers 2010). Recently it has also been recognized that regulation of specific microRNAs is another means whereby MYC indirectly regulates the expression of many genes (Bui and Mendell 2010). In addition to RNA pol II–mediated transcription of mRNA, MYC can also stimulate the transcription by RNA pol I and RNA pol III (Adhikary and Eilers 2005; van Riggelen et al. 2010). RNA pol I mediates the transcription of ribosomal RNA (rRNA) from rDNA. MYC promotes RNA pol I activity by binding E-boxes in rDNA promoter regions where it associates with the RNA pol I–specific promoter selectivity factor SL1. By binding to TFIIIB, an RNA pol III–specific transcription factor, MYC can also directly activate RNA pol III–mediated the transcription of 5S rRNA and tRNA.

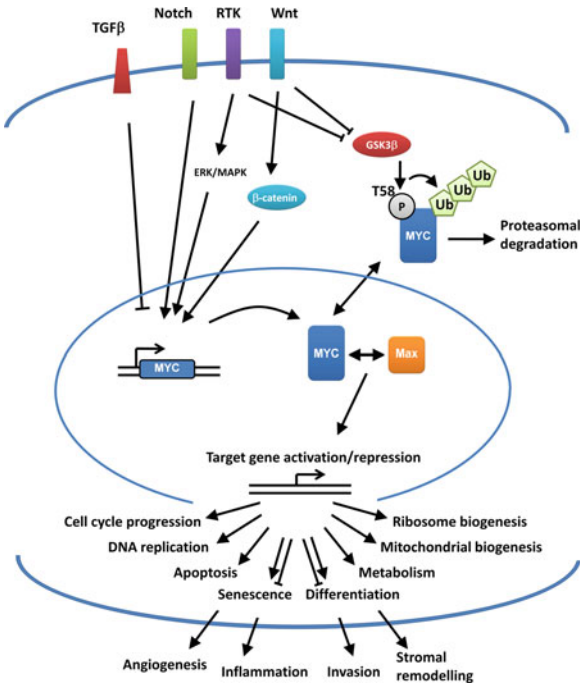
Several transcription-independent functions have been reported for MYC, including mRNA cap methylation and DNA replication (Cole and Cowling 2008). Cap methylation occurs during early stages of mRNA transcription, where the mRNA is first capped with an inverted guanosine and this cap is subsequently methylated. Uncapped mRNA is rapidly degraded and methylation of the cap is necessary for binding of translation factors. MYC stimulates the recruitment of enzymes responsible for both capping and methylation to specific mRNAs (Cole and Cowling 2008). In this way, the level of a protein can be increased by MYC, independent of any effects on transcription. A direct role for MYC at origins of replication has also been suggested. Defining the direct actions of MYC in regulating replication is not straightforward since MYC also stimulates DNA replication through transcription-dependent mechanisms. However, several components of the pre-replicative complex have been found to be direct interaction partners of MYC. Furthermore, MYC overexpression increased the number of origins of replication, even in the presence of transcription inhibitors, and could influence the rate of DNA synthesis in a *Xenopus* system, which supports cell cycle–regulated DNA replication in the absence of transcription and new protein synthesis (Herold et al. 2009). Recently, a cytosolic function of a cleavage product of the MYC protein was described (Conacci-Sorrell and Eisenman 2011). ▶ Calpain-mediated cleavage generates an N-terminal version of MYC, MYC-Nick, that lacks nuclear localization signal and was shown to promote differentiation of muscle cells.

MYC in Cell Biology

Although some transcription-independent activities have been reported for MYC, it acts primarily as a transcriptional regulator that exerts its biological properties through induction or repression of specific genes. The fact that MYC is a relatively poor transactivator and induces many of its targets only about twofold has complicated global analyses to identify target genes. This relatively weak MYC-induced gene regulation frequently drowns in the noise inherent to the methods applied, or fall below the cutoff values used. Still, MYC has profound effects on cell behavior and cell fate. This is likely achieved by modulation of the activity of an entire pathway or even of multiple cooperating pathways. MYC regulates specific groups of genes that are involved in metabolism, protein synthesis, cell cycle regulation, cell adhesion, and cytoskeleton composition (Fig. 2; Dang et al. 2006).

Early on, MYC's crucial role in driving cell proliferation was noted (Henriksson and Lüscher 1996; Wasylishen and Penn 2010). It was found to be an intermediate early response gene after serum stimulation and ectopic expression of MYC promoted cell cycle entry of quiescent cells. The effect of MYC on the cell cycle is achieved through repression of cell cycle checkpoint proteins and CKIs, including p21, p15, p27, and ▶ GADD45. MYC also activates several proteins that are important for the progression of the cell cycle including cyclins D1, D2, E1, A2 as well as CDK4, CDC25A E2F1 and E2F2 (Dang et al. 2006; Eilers and Eisenman 2008).

MYC drives cell division not only through regulation of cell cycle checkpoints but also by stimulating biosynthesis of macromolecules, allowing the doubling of cell mass required for every cell division. It is a direct regulator of ribosome biogenesis and thereby stimulates protein synthesis (van Riggelen et al. 2010). Through activation of the transcriptional activity of all three RNA polymerases, MYC stimulates synthesis of tRNAs, ribosomal RNAs (rRNA) and proteins, enzymes responsible for catalyzing the processing of rRNA, ribosome assembly, and export from the nucleus as well translation initiation and elongation factors. MYC also stimulates mitochondrial biogenesis and controls the activity of many metabolic pathways and the uptake of nutrients required to fuel these. Both glucose and glutamine transporters as well as several key enzymes in the glycolysis and glutaminolysis



Myc, Fig. 2 *MYC regulation and biological functions. MYC* gene expression is controlled downstream of a multitude of signaling pathways, including, Wnt/β-catenin, ▶ Notch, receptor tyrosine kinases (RTK), and tumor growth factor β (TGFβ). MYC activity is also regulated at the level of protein turnover. Through regulation of gene expression, MYC influences many intracellular and extracellular processes as indicated

pathways are direct transcriptional targets (Dang et al. 2006; Dang et al. 2009). Furthermore, MYC has a broad role in driving biosynthetic reactions that are required to supply macromolecules to support cell growth and stimulates the production of amino acids, ribose sugars, and purine nucleotides.

Somewhat surprising when it first was discovered, MYC overexpression can also cause apoptosis (Wasylishen and Penn 2010). This is the result if MYC overexpression is present together with anti-proliferative signals and can be rescued by specific survival factors (Hoffman and Liebermann 2008). Abrogation of the apoptotic signaling by MYC strongly contributes to cancer development. MYC can promote apoptosis through ▶ p53-dependent and p53-independent functions (Hoffman and Liebermann 2008). Overexpression of *MYC* can be sensed by ARF, which in turn activates ▶ p53 through inhibition of HDM2. High expression of *MYC* also promotes DNA damage and genomic instability. This may be the reason for activation of the ATM and ATR kinases, which also contributes to ▶ p53 activation in response to MYC. There is evidence showing that MYC can alter the balance of pro- and anti-apoptotic members of the Bcl2 family, in parallel with or independent of ▶ p53 activation. For example, the expression of the anti-apoptotic proteins Bcl2 and Bclx can be repressed, while the pro-apoptotic protein Bim can be induced by overexpression of *MYC* (Hoffman and Liebermann 2008). In addition, multiple proteins involved in the death-receptor pathway can be regulated directly or indirectly by MYC, resulting in increased sensitivity to apoptosis induced by ligation of death receptors such as the Fas and TRAIL receptors.

Regulation of MYC Activity

MYC activity is tightly regulated by developmental and mitogenic signals. The transcript and protein have short half-lives, allowing rapid adjustments of levels and thereby MYC activity. Many signaling pathways are involved in the transcriptional regulation of the gene. Transcription of *MYC* is, for example, induced downstream of signaling by ▶ Notch, Wnt/β-catenin, and receptors tyrosine kinases (Fig. 2). Several of these pathways interact with each other and there are multiple levels of regulation in each pathway. Thus *MYC* expression is controlled by a very complex network of intracellular signaling pathways allowing for tight regulation of gene activity. In addition, the cellular, developmental, and microenvironmental context will affect expression (see Wierstra and Alves 2008 for an extensive review).

Protein turnover is another means of regulating the expression level of MYC. Phosphorylation of two N-terminal sites, Serine 62 (S62) and Threonine 58 (T58) within the MBI has been shown to have an important regulatory function for protein stability (Hann 2006; Vervoorts et al. 2006). Importantly, mutations of these residues have been found in Burkitt lymphomas and other lymphomas and are associated with stabilized MYC protein, increased transforming activity and impaired ability to induce apoptosis (Hann 2006). Phosphorylation of S62 and T58 are interdependent since T58 require prior S62 phosphorylation. S62 phosphorylation stabilizes the MYC protein while phosphorylation of T58 promotes its degradation. Several kinases have been implicated in the phosphorylation of S62 including MEK/ERK, JNK, and CDK1 (Hann 2006). Once S62 is phosphorylated, MYC can

be recognized by GSK3β, which phosphorylates T58 resulting in the targeting of MYC for proteasomal degradation. Upon T58 phosphorylation, S62 is dephosphorylated. T58 phosphorylated MYC can then be recognized by the F-box protein FBW7, which is a subunit of the SKP1-CUL1-F-box protein (SCF) complex that stimulates polyubiquitylation and subsequent proteasomal degradation of MYC (Hann 2006; Vervoorts et al. 2006). Ras signaling is believed to stabilize MYC through activation of Raf/► MEK/ERK leading to S62 phosphorylation, and at the same time inhibiting T58 phosphorylation through activation of ► PI3K signaling, which in turn inhibits the activity of GSK3β (Hann 2006). Another ubiquitin ligase, SKP2, can when recruited to MYC-regulated promoters mediate the ubiquitylation of MYC. This results first in transcriptional activation followed by proteasomal degradation of MYC. Furthermore, MYC can be phosphorylated at multiple additional sites, be ubiquitylated by HectH9, and is also subject to acetylation (Vervoorts et al. 2006).

Development, Differentiation, and Stem Cells

The importance of MYC activity during development has been demonstrated by gene targeting in mice, where loss of either c-*myc* or *mycn* is embryonic lethal. c-$myc^{-/-}$ embryos die at embryonic day 10.5 due to severe hematopoietic as well as placental defects, while $mycn^{-/-}$ embryos succumb to neuroectodermal and heart abnormalities between embryonic day 10.5 and 11.5. Mice lacking *mycl* on the other hand appear normal (Laurenti et al. 2009). It is likely that c-*myc* and *mycn* can at least partly compensate for the absence of each other during development since $max^{-/-}$ mice die already at embryonic day 6.5 (Laurenti et al. 2009).

It was early recognized that MYC expression negatively correlated with terminal differentiation of tissues and that MYC expression could prevent terminal differentiation. The role of MYC in differentiation now appears more complex and the presence of MYC is important for many steps in differentiation in various tissues, including the hematopoietic system and the skin (Eilers and Eisenman 2008; Laurenti et al. 2009).

Furthermore, MYC function seems to be required for embryonic stem (ES) cell self-renewal. MYC was also one of the factors in the first described four-factor reprogramming of somatic cells to induced pluripotent stem (iPS) cells. While not strictly necessary for iPS generation, MYC has been found to enhance the reprogramming of cells. This has been linked to its capabilities to stimulate proliferation and to inhibit differentiation as well as to its ability to modify epigenetic patterns. Taken together, MYC can promote an open chromatin state and an ES cell-like chromatin landscape (Laurenti et al. 2009).

MYC in Cancer

Deregulated expression of one of the *MYC* family genes is detected in a wide range of human cancers and has in many instances been associated with aggressive, poorly differentiated tumors. Activation of *MYC* in cancers can be achieved through different means and is in most cases not the consequence of activating point mutations, but rather through increased expression. At the genomic level translocations or amplifications of the *MYC* genes occur. Translocation of *MYC* to one of the immunoglobulin loci is the hallmark of Burkitt lymphoma, but also occurs at variable frequency in other lymphomas and leukemias (Vita and Henriksson 2006). Amplification of one of the *MYC* genes is found in a range of solid tumors, including breast cancer, prostate cancer, neuroblastoma, and lung cancer (Vita and Henriksson 2006; Albihn et al. 2010). Overexpression of MYC can also be achieved through deregulation of upstream signaling pathways, which regulate *MYC* transcription and/or MYC protein stability. Furthermore, mutations leading to enhanced translation, or mutations affecting specific residues in MYC, such as T58, which are involved in the regulation of MYC protein turnover can be the cause of enhanced MYC protein levels in tumors. In fact overexpression of MYC is one of the most common events coupled with tumorigenesis (Pelengaris and Khan 2003; Albihn et al. 2010).

Early in vitro experiments defining the oncogenic properties of MYC revealed that its constitutive overexpression in rat embryo fibroblasts (REFs) lead to immortalization and prevented exit from cell cycle. MYC's contribution to tumorigenesis has been attributed mainly to its ability to promote tumor cell proliferation without the requirements of exogenous mitogenic signals resulting in uncontrolled proliferation. Overexpression of MYC can also promote

tumorigenesis through its effects on metabolism, cell growth, and metastasis as well as on the tumor microenvironment, for example, through the regulation of angiogenic factors (Pelengaris and Khan 2003; Sodir and Evan 2009). The tumor promoting effect of MYC was also verified in vivo in transgenic mice, first in the *Eμ-myc* model where MYC is overexpressed in B cells resulting in lymphoma, and then in many other tissues (Pelengaris and Khan 2003).

High levels of MYC expression promote apoptosis. While contradictory to its tumor promoting function, this has come to be viewed as an intrinsic tumor suppressor function to guard against uncontrolled proliferation. The same holds true for other oncogenes, such as E2F. Cooperation in transformation is seen between MYC and other oncoproteins, for example, Ras and Bcl2 or loss of tumor suppressor proteins such as ▶ p53 and ARF that counteracts the apoptosis signaling induced by MYC. Another tumor suppressor function that can be affected by MYC is senescence. MYC can suppress senescence induced by oncoproteins such as Ras and BRAF. However, under certain circumstances, MYC can also induce senescence (Larsson and Henriksson 2010).

Conditional transgenic models where *MYC* overexpression can be turned on and off have shown that inactivation of MYC can lead to tumor regression (Felsher 2010). Some tumors are dependent on the sustained expression of MYC, so-called oncogene addiction, which implicates MYC as a therapeutic target at least in some tumor types. MYC inactivation in tumors can lead to proliferative arrest, differentiation, senescence, and/or apoptosis. In addition, inhibition of MYC can also affect the tissue microenvironment and tumor-induced angiogenesis. Studies in a collection of conditional transgenic models of MYC-driven tumors of various tissue origins have shown that the efficiency and reversibility of the effects of MYC inhibition depends on the cellular, genetic, and epigenetic contexts (Felsher 2010). Approaches to target MYC include strategies that interfere with MYC expression such as antisense and RNA interference, inhibition of downstream MYC target genes and small molecules that disrupt MYC-Max interaction (Vita and Henriksson 2006; Larsson and Henriksson 2010; Prochownik and Vogt 2010). MYC has for several reasons been regarded as an impossible target for therapy and development of small targeting molecules has lagged behind. Interfering with protein-protein interactions instead of the classical targeting strategy of gain-of-function mutants in cancer-associated kinases has been deemed difficult. Recent development of other protein interaction–targeting small molecule compounds, including nutlins, which disrupts the ▶ p53-HDM2 interaction, and ABT-737 that targets the interaction between specific Bcl2 family members, shows however that this strategy indeed is possible and can be successful (Prochownik and Vogt 2010). Concerns have also been raised regarding the effect of inhibition of MYC in normal tissues. One recent study of systemic MYC inhibition in mice with the conditional expression of the MYC inhibitor Omomyc indicates, however, that the effects on normal tissues are mild and can be tolerated (Sodir and Evan 2009). Different screening strategies have lead to the identification of MYC-Max disrupting small molecules (Prochownik and Vogt 2010). Although these compounds need to be further developed, they give hope for the continued efforts to develop compounds that could be used clinically to target MYC in human cancer.

Summary

The MYC protein is a transcription factor, which appears to act as a global regulator of gene transcription that controls many important normal and neoplastic cellular processes. The studies of MYC have in fact greatly contributed to our understanding of fundamental mechanisms of cell growth, cell death, and development and will likely continue to do so. Furthermore, the oncogenic role of MYC in tumor development is well established and deregulated *MYC* is involved in many human cancers. This has stimulated the idea that inhibition of MYC could be an attractive strategy for development of novel cancer therapies. This notion is further encouraged by recent progress in the development of MYC-targeting small molecules.

References

Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol. 2005; 6:635–45.

Albihn A, Johnsen JI, Henriksson MA. MYC in oncogenesis and as a target for cancer therapies. Adv Cancer Res. 2010;107:163–224.

Bui TV, Mendell JT. Myc: Meastro of MicroRNAs. Genes Cancer. 2010;1:568–75.

Cole MD, Cowling VH. Transcription-independent functions of MYC: regulation of translation and DNA replication. Nat Rev Mol Cell Biol. 2008;9:810–15.
Conacci-Sorrell M, Eisenman RN. Post-translational control of Myc function during differentiation. Cell Cycle. 2011;10:604–10.
Cowling VH, Cole MD. Mechanism of transcriptional activation by the Myc oncoproteins. Semin Cancer Biol. 2006;16:242–52.
Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. Semin Cancer Biol. 2006;16:253–64.
Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. Clin Cancer Res. 2009;15:6479–83.
Eilers M, Eisenman RN. Myc's broad reach. Genes Dev. 2008;22:2755–66.
Felsher DW. MYC inactivation elicits oncogene addiction through both tumor cell-intrinsic and host-dependent mechanisms. Genes Cancer. 2010;1:597–604.
Hann SR. Role of post-translational modifications in regulating c-Myc proteolysis, transcriptional activity and biological function. Semin Cancer Biol. 2006;16:288–302.
Henriksson M, Lüscher B. Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv Cancer Res. 1996;68:109–82.
Herkert B, Eilers M. Transcriptional repression: the dark side of Myc. Genes Cancer. 2010;1:580–6.
Herold S, Herkert B, Eilers M. Facilitating replication under stress: an oncogenic function of MYC? Nat Rev Cancer. 2009;9:441–4.
Hoffman B, Liebermann DA. Apoptotic signaling by c-MYC. Oncogene. 2008;27:6462–72.
Larsson LG, Henriksson MA. The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy. Exp Cell Res. 2010;316:1429–37.
Laurenti E, Wilson A, Trumpp A. Myc's other life: stem cells and beyond. Curr Opin Cell Biol. 2009;21:844–54.
Lüscher B, Larsson LG. The basic region/helix-loop-helix/leucine zipper domain of Myc proto-oncoproteins: function and regulation. Oncogene. 1999;18:2955–66.
Pelengaris S, Khan M. The c-MYC oncoprotein as a treatment target in cancer and other disorders of cell growth. Expert Opin Ther Targets. 2003;7:623–42.
Prochownik EV, Vogt PK. Therapeutic targeting of Myc. Genes Cancer. 2010;1:650–9.
Sodir NM, Evan GI. Nursing some sense out of Myc. J Biol. 2009;8:77.
van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and protein synthesis. Nat Rev Cancer. 2010;10:301–9.
Vervoorts J, Luscher-Firzlaff J, Luscher B. The ins and outs of MYC regulation by posttranslational mechanisms. J Biol Chem. 2006;281:34725–9.
Vita M, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol. 2006;16:318–30.
Wasylishen AR, Penn LZ. Myc: the beauty and the beast. Genes Cancer. 2010;1:532–41.
Wierstra I, Alves J. The c-myc promoter: still MysterY and challenge. Adv Cancer Res. 2008;99:113–333.

MyD88

► MyD88, Myeloid Differentiation Primary Response Gene 88
► Toll-like Receptor Adaptor Protein Family Members

MyD88, Myeloid Differentiation Primary Response Gene 88

Shaherin Basith, Balachandran Manavalan and Sangdun Choi
Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Synonyms

MyD88; Myeloid differentiation primary response gene 88

Historical Background

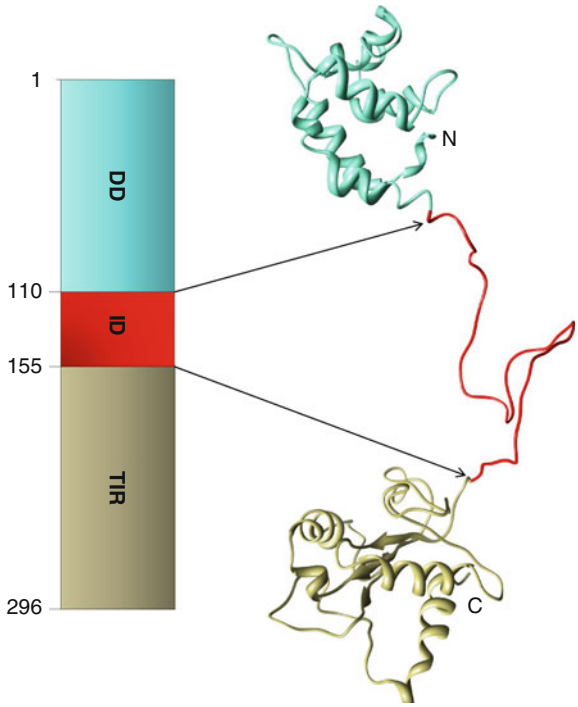
Myeloid differentiation primary response gene 88 (MyD88) was originally discovered and cloned by Liebermann and Hoffman in 1990 as one of 12 different mRNA transcripts that were induced in M1 myeloblastic leukemia cells upon activation with lung-conditioned medium or recombinant interleukin (IL)-6 (Lord et al. 1990). The "MyD" portion of the name stands for myeloid differentiation, while "88" refers to the gene number in the list of induced genes (Lord et al. 1990). At the time of its discovery, the MyD88 sequence showed no homology with other sequences available in the databases and contained no recognizable protein motifs. In 1994, the C-terminal portion of MyD88 was found to be similar to a conserved stretch of ~200 amino acids in the intracellular regions of the *Drosophila* Toll receptor and the mammalian interleukin-1 receptor (IL-1R) (Hultmark 1994), and was thus referred to as the TLR (Toll-like receptor)- and IL-1R-related (TIR) domain. The N-terminal portion of MyD88 encodes the death domain (DD) (Feinstein et al. 1995) that was originally identified in apoptosis-promoting proteins. By 1997,

the function of MyD88 had been determined. Specifically, MyD88 was first shown to be involved in type 1 interleukin-1 receptor (IL-1R1) signaling and subsequently in all TLRs (Hultmark 1994) signaling except for TLR3 response and ▶ TLR4-mediated late response. The MyD88 adapter protein links members of the TLR and IL-1R superfamily to the downstream activation of nuclear factor-κB (▶ NF-κB) and mitogen-activated protein kinases (MAPKs). Although originally identified as a myeloid-differentiation marker, MyD88 is known to play an essential role in the innate immune response of insects and mammals. The generation of MyD88-deficient mice as well as the identification of MyD88-related proteins and regulators of MyD88 signaling have revealed new and important insights into the functions of MyD88 (Janssens and Beyaert 2002).

MyD88 Localization and Structure

MyD88 transcript is detected in most tissues including the heart, brain, spleen, lung, liver, muscle, kidney, and testis, as well as in the T, B, and myeloid cell lines. MyD88 is localized in distinct condensed particles scattered throughout the cytoplasm, reportedly in organelles yet to be identified (Nishiya et al. 2007).

MyD88 is a 296 amino acid cytoplasmic adaptor protein that relays signals from IL-1, IL-18, IFNγ, IL-33, and most TLRs. MyD88 has a clear-cut modular structure composed of three main domains that are encoded by five exons (Hardiman et al. 1997). The first exon encodes the N-terminal DD, which mediates downstream interactions with the IRAK family of kinases. Exon 2 encodes the short linker ID, while the last three exons encode the C-terminal TIR domain, which mediates the interaction of MyD88 with other TIR domain receptors. The N-terminal DD is related to a motif that was originally defined as the region of similarity between the cytoplasmic tails of the FAS/Apol/CD95 and TNF (tumor necrosis factor) receptors that is required for cytotoxic signaling by the so-called death receptors (Tartaglia et al. 1993). Conversely, the MyD88 C-terminal TIR domain is homologous with the cytoplasmic signaling regions of the *Drosophila* Toll receptor and the IL-1 receptor complex. To date, the entire structure of MyD88 has not been solved. However, the crystal structure of the MyD88 DD in complex with DDs of IRAK4 (interleukin-1 receptor associated kinase 4)-IRAK2 (PDB ID: 3MOP) was recently solved by Chang et al. (Lin et al. 2010) and the NMR solution structures of the TIR domain of MyD88 (PDB ID: 2Z5V and 2J67) were solved by two independent research groups (Ohnishi et al. 2009). The entire structure of MyD88 is depicted in Fig. 1.



MyD88, Myeloid Differentiation Primary Response Gene 88, Fig. 1 *Representation of protein domains found in the MyD88 adaptor protein*. (**a**). Schematic representation of MyD88 protein domains. MyD88 contains a Toll/interleukin-1 receptor (TIR) domain, an intermediary domain (ID), and a death domain (DD). The N-terminal (N) DD is shown in *aquamarine color*, ID is shown in *red color*, and the C-terminal (C) TIR domain is represented by *khaki color*. (**b**). *Ribbon* representation of the entire structure of MyD88, where the N-terminal DD represents the crystal structure solved by Chang et al. (Lin et al. 2010) and the C-terminal TIR domain represents the NMR solution structure solved by Ohnishi et al. (2009). The ID domain shown is the modeled structure generated using the MOE program (Molecular Operating Environment: http://www.chemcomp.com/software.htm)

The importance of the MyD88 DD is that it interacts with IRAKs, including IRAK1, IRAK2, IRAK4, and IRAK-M (IL-1R-associated kinase M), which are characterized by an N-terminal DD and a carboxy-terminal Ser/Thr kinase or kinase-like domain. The ensuing pathway eventually activates transcription factors NF-κB, activator protein (AP)-1 and interferon regulatory factors (IRFs) to elicit anti-pathogen

responses and inflammation (Akira et al. 2006). The crystal structure of the DD of MyD88 consists of six helices (H1–H6) with a short H3 and an extraordinarily long H6 from residue 99 to the end of the construct at residue 117, which includes part of the ID (residues 110–154). For the loop regions, MyD88 has the longest H1–H2 loop, shortest H3–H4 loop, and longest H4–H5 loop (Lin et al. 2010). The MyD88 DD structure explains the disruptive phenotypes of mutations ΔE52 and L93P in children suffering from life-threatening pyogenic bacterial infections (von Bernuth et al. 2008). Structure-based mutagenesis studies identified critical residues of MyD88 DD (V43, A44, E52, Y58, I61, and R62). In particular, E52 and Y58 were found to be involved in IRAK4 recruitment and NF-κB signaling (Loiarro et al. 2009).

Following the MyD88 DD is an ID. Alternative splicing of MyD88 results in a variant that lacks the ID – MyD88 short (MyD88s), which is only expressed in the spleen and brain. When overexpressed in HEK293 cells, MyD88s is able to bind IRAK, but it does not activate NF-κB because it cannot induce IRAK phosphorylation. Hence, it has been suggested that MyD88 ID plays a potent role in the differential activation of distinct (NF-κB versus JNK-dependent) transcriptional programs (Janssens et al. 2003).

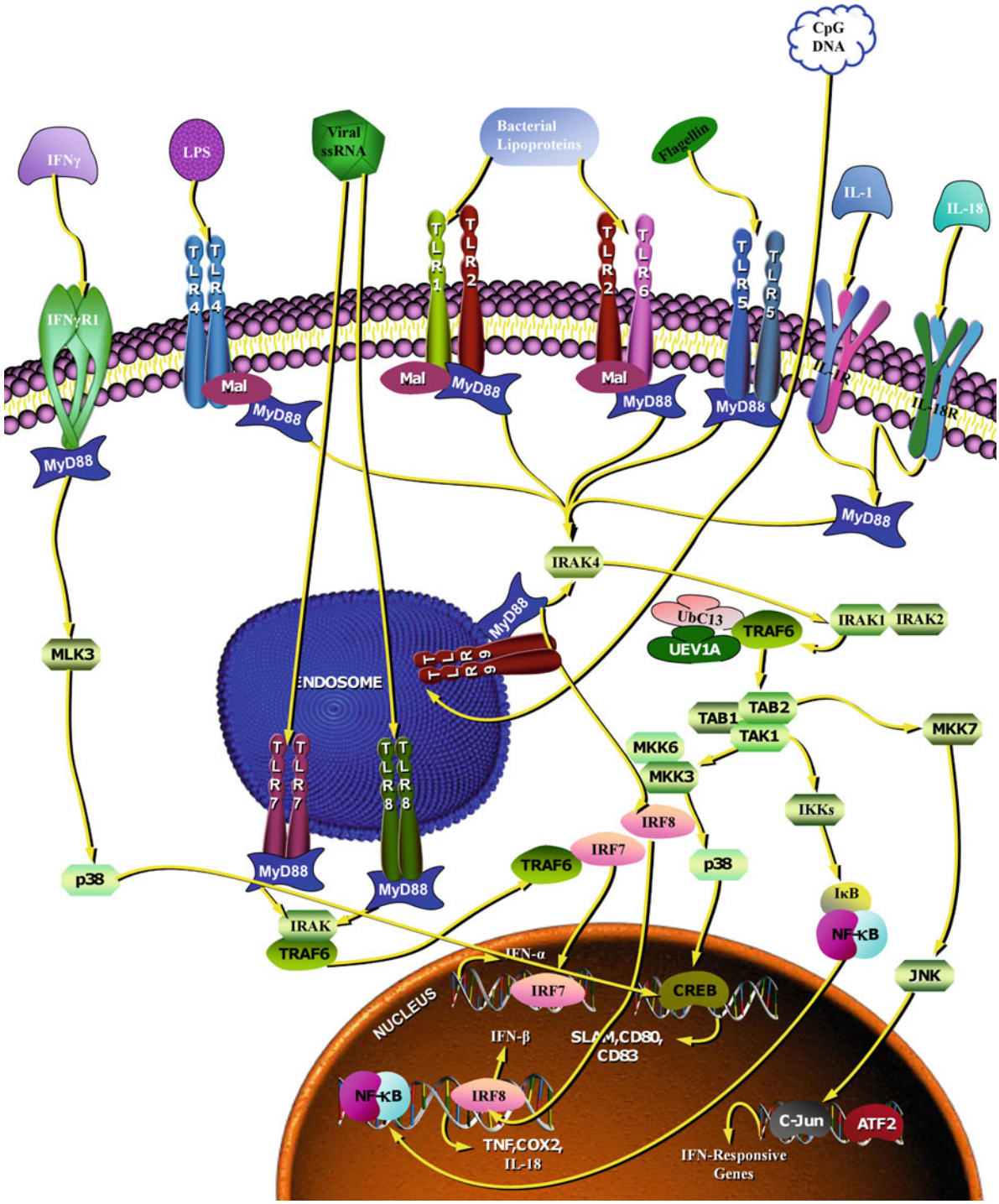
The C-terminal portion of MyD88 contains a TIR domain that mediates homo- and heterotypic protein interactions during signal transduction. The NMR solution structure of the MyD88 TIR domain (residues 157–296) identified by Ohnishi et al. comprised a central five-stranded parallel β-sheet (βA-βE) surrounded by four α-helices (αA-αC and αE) (Ohnishi et al. 2009). Rossi et al. also released the solution structure of the TIR domain of human MyD88 in the Protein Data Bank (PDB). The overall folding was identical to that reported by Ohnishi, despite minor differences. MyD88-TIR was found to reside in a monomeric state, as determined by the size-exclusion chromatography. However, the DD including the ID of MyD88 existed in a dimeric state. Therefore, the reported MyD88 dimerization was likely mediated by DD + ID and not by the TIR domain. TIR domains in TLRs, IL receptors, and the adaptors MyD88 and ▶ Mal (MyD88-adaptor like protein)/TIRAP (TIR domain containing adaptor protein) contain three conserved boxes (boxes 1, 2 and 3), which are required for signaling (Li et al. 2005). A mutagenesis study conducted by Jiang et al. in conjunction with previous docking studies revealed that the BB loop and Poc site (I179N) in the TIR domain are critical for responses to most TLR ligands and for all other MyD88-dependent TIR signaling events (Jiang et al. 2006).

Universal Role of MyD88 in Signaling

A great deal has been learnt about the role of MyD88 in signaling pathways. In terms of signaling, the situation regarding MyD88 has become more complex. MyD88 is considered to be a critical component in the signaling cascades mediated by all TLRs, IL-1R, IL-18R, IFNγ, and IL-33 (Kakkar and Lee 2008; Wesche et al. 1997; Muzio et al. 1997; Burns et al. 1998; Medzhitov et al. 1998) (Fig. 2).

MyD88 adapter protein links members of the TLR and IL-1R superfamily to the downstream activation of NF-κB and MAPKs. The extracellular domains of the TLRs and IL-1Rs are divergent; however, their intracellular domains share a high degree of similarity and activate similar signaling cascades upon stimulation. It is generally accepted that MyD88 is recruited to all members of the TLR/IL-1R family. The signaling in IL-1R and TLRs is initiated by the direct recruitment of MyD88 to the activated receptor complex. However, in the case of TLR2 and 4 signaling, a bridging adaptor, Mal, is required for MyD88 recruitment. Members of the IRAK family are recruited immediately downstream of MyD88. IRAK4 appears to be the MyD88-proximal kinase, which in turn recruits IRAK1. IRAK2 can also be found in the MyD88 complex (although this has only been shown when IRAK2 is overexpressed). A key downstream target for IRAK1 is believed to be TNF-receptor-associated factor 6 (▶ TRAF6) which, through the recruitment of transforming-growth-factor-β-activated kinase 1 (TAK1) and TAK1-binding protein 2 (TAB2), and the ubiquitylating factors, ubiquitin-conjugating enzyme E2 variant 1 isoform A (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13), ultimately engages with the upstream kinases for p38 and JNK and with the inhibitor of NF-κB kinase (IKK) complex, leading to NF-κB activation.

In addition to the MyD88 pathway, which results in NF-κB translocation, there is a cell-specific pathway that is required for the induction of type I IFNs by TLR7, 8 and 9. MyD88 has also been shown to be essential for activation of IRF7 by these TLRs, which leads to IFNα production (Honda et al. 2005). Furthermore, a complex comprising MyD88, IRAK1, IRAK4,



MyD88, Myeloid Differentiation Primary Response Gene 88, Fig. 2 *Overview of MyD88-dependent signaling pathways*. The MyD88-dependent signaling pathway is utilized by all TLRs (with the exception of TLR3 and certain signals of TLR4), IL-1R, IL-18R, and IL-33. As the name suggests, the adapter molecule, MyD88 is the key mediator of this pathway, and its main role is activation of NF-κB (a hallmark of MyD88 signaling). MyD88 possesses its own C-terminal TIR domain, which drives the heterodimerization of the adaptor with the activated receptor. The N-terminal DD of MyD88 then recruits the IRAK1 and IRAK4 kinases. IRAK4 phosphorylates and activates IRAK1, which in turn initiates autophosphorylation

TRAF6, and IRF7 has been detected, with IRF7 being phosphorylated by IRAK1. This phosphorylation appears to be a key role of IRAK1 in TLR7, 8 and 9 signaling, as the IRF7 response is totally abolished in IRAK1-deficient cells, whereas NF-κB activation is only partially impaired (Uematsu et al. 2005). In the case of ▶ TLR9, IRF7 activation seems to require a stable interaction between MyD88 and the TIR domain of TLR9, which occurs on the cytosolic side of endosomes (Honda et al. 2005). These studies have expanded our understanding of the role of MyD88 in signaling during the host defense response.

MyD88 also interacts with ▶ IRF5 and IRF1 and is required for the activation of these IRFs. IRF5 was found to be crucial for the induction of pro-inflammatory cytokines and type I IFNs by all TLRs tested (Takaoka et al. 2005). The association of MyD88 with IRF1 appears to be required for the translocation of IRF1 to the nucleus in myeloid DCs, and IRF1 is required for the induction of several TLR-dependent genes in these cells. IFNγ is required to induce IRF1, which might be the basis for the priming effect of IFNγ on TLR action. Another intriguing link to the IFNγ system is the reported association between MyD88 and IFNγ receptor 1 (IFNγR1) (Sun and Ding 2006). MyD88 recruits mixed-lineage kinase 3 (MLK3) downstream of IFNγR1, which in turn activates p38. Therefore, an alternative signaling pathway for MyD88 that does not involve TIR-domain-containing receptors has been revealed.

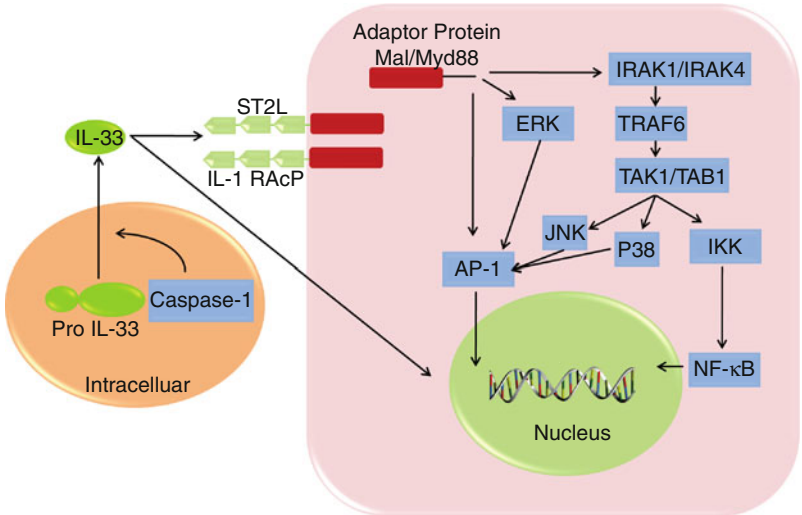
Involvement of MyD88 in IL-33 signaling has recently been clarified (Fig. 3). IL-33 appears to bind a receptor complex composed of ST2L (transmembrane bound form of ST2) and IL-1RAcP (IL-1 receptor accessory protein). In general, upon activation of a Toll-like receptor/IL-1-receptor superfamily member, the transmembrane receptor's TIR domain dimerizes with the TIR domain of cytosolic adaptor molecules. The adaptor proteins, MyD88 and the associated protein IRAKs activate downstream MAPKs through TRAF6 signaling, which in turn activates AP-1 through c-Jun N-terminal kinases (JNKs). TRAF6 also activates the inhibitor of NF-κB kinase (IKK) complex, leading to the downstream liberation of active NF-κB from the complex. IL-33 signaling appears to share many of these properties, and events downstream of IL-33 stimulation may include phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, JNKs, and activation of NF-κB (Kakkar and Lee 2008).

An additional TLR-independent function of MyD88 was reported in CD95 (also known as FAS) signaling. CD95 signaling enhances IL-1R1 signaling by redirecting FADD (FAS-associated death domain) from a complex with MyD88 to the death domain of CD95, thereby allowing IL-1R1 to signal through MyD88 (Ma et al. 2004).

Antagonists of MyD88 Signaling

Although positive regulation of NF-κB, AP1, and IRFs via MyD88-dependent signaling has been extensively studied, recent studies have begun to unravel how these pathways are negatively regulated. The molecules that have been shown to negatively regulate MyD88-dependent signaling include MyD88s, transforming growth factor-β (TGF-β), IRAK-M, SIGIRR (single immunoglobulin IL-1-receptor-related molecule), and ST2L (Fig. 4).

MyD88, Myeloid Differentiation Primary Response Gene 88, Fig. 2 (continued) and recruits TRAF6. TRAF6 together with IRAK dissociates from the activated receptor and binds to the preformed complex of TAK1/TAB1/TAB2. TAK1 is a MAP3K involved in the activation of IκB kinase (IKK). Activation of IKK appears to require atypical polyubiquitination. The TRAF6/TAK1/TAB1/TAB2 complex associates with the heterodimeric ubiquitin-conjugating-enzyme UBC13/UEV1A. This results in modification of TRAF6 with the lysin63-linked polyubiquitin chain, which leads to IKK activation, IκB phosphorylation, ubiquitination, and degradation. NF-κB released from IκB translocates to the nucleus and switches on the transcription of a large number of proinflammatory genes. TAK1 is also responsible for activation of p38 and JNKs. MyD88 also couples to IRF5 and IRF1. In the case of TLR2 and TLR4 signaling, a bridging adaptor, Mal, is required for MyD88 recruitment. In the case of TLR7, 8 and 9, the MyD88-IRAK4 pathway also leads through TRAF6 to the activation of IRF7. Finally, IFNγR1 can also engage with MyD88, leading to activation of p38 through MLK3. MyD88, Myeloid differentiation primary response gene 88; IL-1R, Interleukin-1 receptor; NF-κB, nuclear factor-κB; IRAK4, IL-1R-associated kinase 4; TRAF6, tumor-necrosis-factor-receptor-associated factor 6; TAK1, transforming-growth-factor-β-activated kinase; TAB1, TAK1 binding protein; MAP3K, mitogen-activated-protein 3 kinase; UEV1A, ubiquitin-conjugating enzyme E2 variant 1 isoform A; UBC13, ubiquitin-conjugating enzyme 13; IFNγR1, interferon-γ-receptor 1; MLK3, mixed-lineage kinase 3

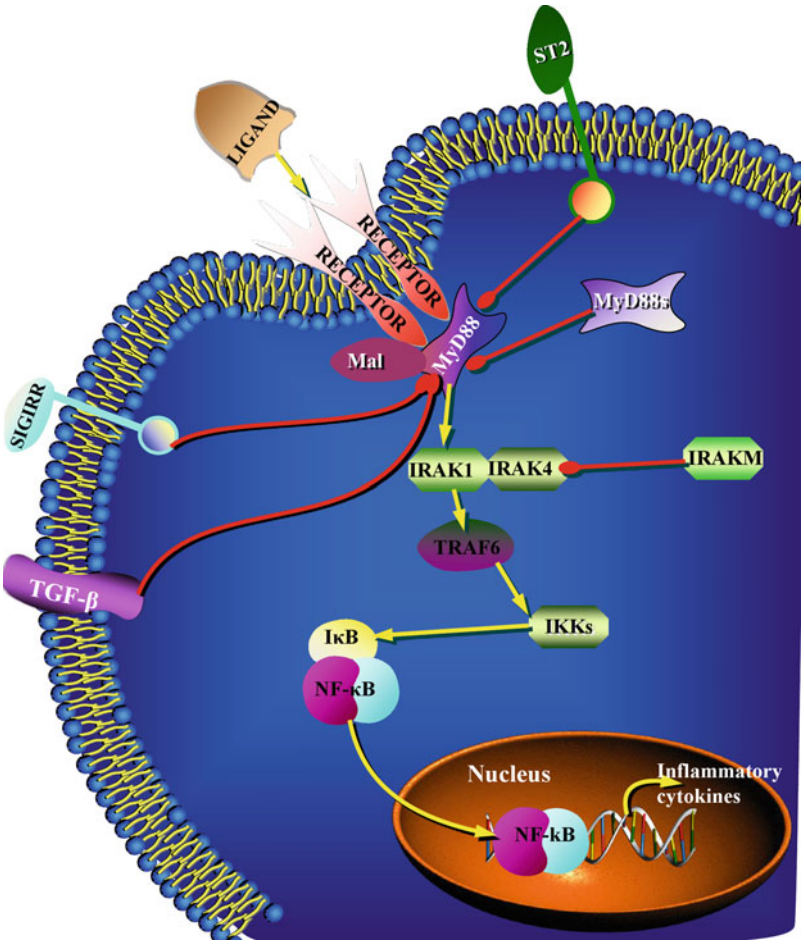


MyD88, Myeloid Differentiation Primary Response Gene 88, Fig. 3 *A model for IL-33/ST2 signaling*. MyD88-dependent pathway of Toll-like receptor signaling involves TIR dimerization between the receptor and the Mal. Recruitment of MyD88 and downstream activation of TRAF6 via IRAK proteins results in TRAF6-mediated activation of the IKK complex and liberation of NF-κB from the complex. Free NF-κB is then able to bind DNA and act as a gene transcription regulator. IL-33 signaling appears to share many of these properties, and events downstream of IL-33 stimulation may include phosphorylation of ERK 1/2, p38 MAPK, and JNKs and activation of NF-κB. IL-33 binds to its receptor complex, which is composed of ST2L and IL-1RAcP. Subsequent sequestering of the adaptor proteins, MyD88 and Mal, results in modulation of IRAK-mediated TRAF6 activation and subsequent MAPK and IKK/NF-κB activation. The nature of this modulation of NF-κB activity by IL-33 is complex. MyD88, Myeloid differentiation primary response gene 88; IL-33, Interleukin-33 receptor; NF-κB, nuclear factor-κB; IKK, inhibitor of NF-κB kinase; IRAK, IL-1R-associated kinase; TRAF6, tumor-necrosis-factor-receptor-associated factor 6; MAPK, mitogen-activated-protein kinase; IL-1RAcP, Interleukin-1 receptor accessory protein

MyD88s, an alternatively spliced variant form of MyD88 that lacks the ID, is induced in monocytes following stimulation with LPS (lipopolysaccharide). Overexpression of MyD88s inhibits IL-1- and LPS-induced NF-κB activation, but not TNF-induced NF-κB activation (Janssens et al. 2002). The mechanism of inhibition has been elucidated and shown to involve altered interactions in the downstream signaling pathway. Overexpression of MyD88s favors formation of MyD88s-MyD88 heterodimers, which are recruited to the receptor in favor of full-length MyD88 homodimers; however, the mechanism responsible for this process is not clear. In the presence of MyD88s-MyD88 heterodimers, IRAK1 was still recruited, presumably through a DD interaction with MyD88s, but was no longer phosphorylated. IRAK4 causes IRAK1 phosphorylation, and the presence of MyD88 is essential for this effect. MyD88s inhibits the ability of IRAK4 to phosphorylate IRAK1 because, unlike full-length MyD88, MyD88s does not interact with IRAK4. Although IRAK4 recruitment primarily requires the DD of MyD88, it also requires the ID for its binding with MyD88; therefore, the kinase is not recruited to the receptor complex (Janssens et al. 2003). This indicates that MyD88s might be involved in a negative-feedback regulatory mechanism to control excessive TLR-mediated signaling. Although MyD88s is inhibitory with respect to NF-κB signaling, overexpression of this adaptor still activates transcription factor AP1, implying an important role for alternative splicing in the fine-tuning of TLR responses (Janssens et al. 2003).

Another molecule that has been shown to negatively regulate MyD88-dependent signaling is TGF-β. Naiki et al. showed that TGF-β blocked NF-κB activation and cytokine production in response to TLR2, 4 and 5 ligands by decreasing MyD88 protein, but not mRNA levels (Naiki et al. 2005). TGF-β was found to cause the ubiquitination of MyD88 and an observed decrease in MyD88 protein levels. Furthermore, a protease inhibitor was found to abolish this effect, suggesting that TGF-β causes the polyubiquitination of MyD88, resulting in its proteasomal degradation.

Another negative regulator of MyD88 signaling is IRAK-M, which is primarily found in cells of



MyD88, Myeloid Differentiation Primary Response Gene 88,

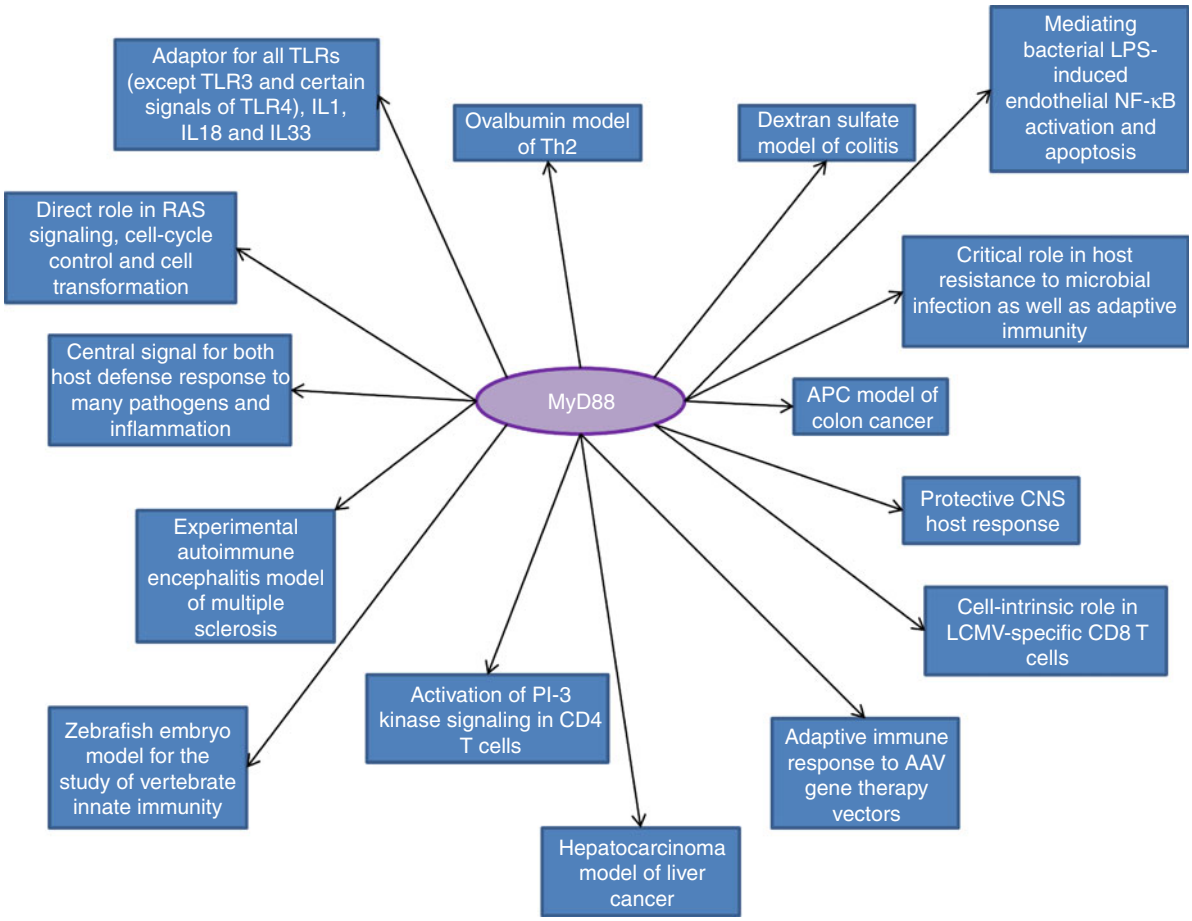
Fig. 4 *Negative regulation of MyD88-dependent signaling.* MyD88-dependent signaling pathways are negatively regulated by several molecules that are induced by the stimulation of receptors. IRAK-M inhibits the dissociation of the IRAK1-IRAK4 complex from the receptor. MyD88s blocks the association of IRAK4 with MyD88. TGF-β blocks NF-κB activation and cytokine production in response to TLR2, 4 and 5 ligands by decreasing MyD88 protein. The TIR-domain-containing receptors, SIGIRR and ST2L, have also been shown to negatively regulate MyD88-dependent signaling. IRAK-M, IL-1R-associated kinase M; MyD88s, MyD88 short; TGF-β, transforming growth factor-β; SIGIRR, single immunoglobulin IL-1-receptor-related molecule; ST2L, transmembrane bound form of ST2

monomyeloic origin such as monocytes and is induced upon TLR stimulation (Wesche et al. 1999). IRAK-M acts to prevent the dissociation of IRAK1 and IRAK4, which results in IRAK1 being unable to interact with TRAF6 and therefore unable to induce a signaling cascade. IRAK-M deficient cells exhibited increased cytokine production upon TLR/IL-1 stimulation and bacterial challenge.

Another important negative regulatory mechanism for MyD88-dependent signaling involves the transmembrane proteins, ST2L and SIGIRR (both members of the TIR superfamily). ST2L interacts with MyD88 and Mal and sequesters MyD88-dependent NF-κB activation by TLR2, 4 and 9 signaling pathways (Brint et al. 2004). SIGIRR binds to TLR4 and IRAK and terminates the downstream TLR signaling pathways (Wald et al. 2003). These findings indicate that MyD88 serves as an important point of control for signaling through various receptors.

Multiple Roles of MyD88

Studies conducted in the past few years have firmly established the central role of MyD88 adaptor in inflammation and immunity (Fig. 5). As mentioned above, much of the recent scientific literature has focused on the role of MyD88 with respect to TLR signaling leading to the activation of several signaling cascades including the NF-κB pathway, resulting in the production of proinflammatory cytokines/chemokines and upregulation of co-stimulatory and adhesion molecules involved in both innate and adaptive immune responses. MyD88 is also essential for transducing signals from IL-1R family members, including IL-1R, IL-18R, and several other receptors. Furthermore, Andrew et al. identified the role of MyD88 in the activation of PI-3 kinase signaling in $CD4^+$ T cells, thereby enabling CpG oligodeoxynucleotide-mediated co-stimulation (Gelman et al. 2006).



MyD88, Myeloid Differentiation Primary Response Gene 88, Fig. 5 *Diverse roles of MyD88.* MyD88 is essential for signaling by IL-1, IL-18, IL-33, and all TLRs (except TLR3 and certain TLR4 signals) and is a central signal for many processes involving host defense to infection, inflammation, and cancer. MyD88 is required for inflammation in the ovalbumin model of Th2 responses, the collagen-induced arthritis model of rheumatoid arthritis (RA), the dextran sulfate model of colitis, the APC model of colon cancer, a hepatocarcinoma model, and the experimental autoimmune encephalitis model of multiple sclerosis (MS). MyD88 plays a potent cell-intrinsic role in lymphocytic *choriomeningitis* virus (LCMV)-specific CD8 T cells, activation of PI-3 kinase signaling in $CD4^+$ T cells, and a direct role in RAS signaling, cell cycle control, and cell transformation, implicating its role in tumorigenesis via proinflammatory mechanisms. MyD88 also plays an important role in innate immune signaling mechanisms during vertebrate embryogenesis in a zebrafish embryo model. TLR9-MyD88 pathway is known to be critical for adaptive immune responses to adeno-associated virus (AAV) gene therapy vectors in mice. Bovine Mal and MyD88 are essential to mediation of bacterial LPS-induced endothelial NF-κB activation and apoptosis. MyD88 also establishes a protective CNS host response during the early stages of brain abscess development, thus exhibiting its central role in the responses of microglia to PAMPs

The overall importance of MyD88 is demonstrated by the results of many studies involving MyD88-deficient mice. Targeted disruption of the MyD88 gene results in loss of TLR, IL-1, IL-33, and IL-18-mediated functions (Adachi et al. 1998). MyD88-deficient mice have been subjected to many models of infection, inflammation, and recently cancer (Takeuchi et al. 2000a; Naugler et al. 2007; Rakoff-Nahoum and Medzhitov 2007), and the results have shown that MyD88 is essential for the inflammation seen in models of airway hyperreactivity, colitis, and APC model of colon cancer and hepatocarcinoma (Naugler et al. 2007; Rakoff-Nahoum and Medzhitov 2007; Araki et al. 2005; Piggott et al. 2005). MyD88-deficient mice fail to generate pro-inflammatory and Th1 responses when stimulated with TLR ligands (Schnare et al. 2001). Additionally, these mice are highly susceptible to infection by a wide variety of different pathogens, including *Staphylococcus aureus*

(Takeuchi et al. 2000b), *Listeria monocytogenes* (Seki et al. 2002), *Toxoplasma gondii* (Scanga et al. 2002), and *Mycobacterium tuberculosis* (Shi et al. 2003). MyD88-deficient mice are also highly susceptible to *Leishmania major* infection associated with a polarized Th2 response (Muraille et al. 2003). An impaired production of proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* is conspicuous in mice lacking functional MyD88 (Campos et al. 2004). MyD88 has generally been considered to indirectly regulate adaptive immune responses by controlling inflammatory cytokine production and antigen (Ag) presentation in innate immune cells; however, Rahman et al. identified an unappreciated cell-intrinsic role of MyD88 in lymphocytic *choriomeningitis* virus (LCMV)-specific CD8 T cells (Rahman et al. 2008). Their results demonstrated the importance of MyD88-dependent signals for supporting the survival of the cells and sustained accumulation. MyD88-dependent signaling is also required for the control of *ehrlichial* infection via a potent role in immediate activation of the innate immune system and inflammatory cytokine production, as well as in activation of the adaptive immune system at a later stage by providing for optimal Th1 immune responses (Koh et al. 2010).

MyD88 contributes to regulation of cell proliferation and differentiation in human adipose tissue-derived mesenchymal stem cells (hASCs) (Yu et al. 2008). Astrid et al. demonstrated that the innate immune response of the developing embryo involves MyD88-dependent signaling by utilizing the zebrafish embryo as a model for a study of vertebrate innate immunity (van der Sar et al. 2006). The TLR9-MyD88 pathway has also been shown to be critical for adaptive immune responses to adeno-associated virus (AAV) gene therapy vectors in mice (Zhu et al. 2009). Both MAVS and MyD88 are known to be essential for innate immunity, but not cytotoxic T lymphocyte response against respiratory *syncytial* virus (Bhoj et al. 2008), thereby providing an example of a normal and effective adaptive immune response in the absence of innate immunity. Cates et al. demonstrated that bovine Mal and MyD88 are essential for mediation of bacterial LPS-induced endothelial NF-κB activation and apoptosis (Cates et al. 2009). Furthermore, Tammy et al. showed an essential role enacted by MyD88 in establishment of a protective CNS host response during the early stages of brain abscess development (Esen and Kielian 2006), thus exhibiting a central role for MyD88 in the responses of microglia to pathogen-associated molecular patterns (PAMPs).

Recently, Isabelle et al. demonstrated that, in addition to its role in inflammation, MyD88 played a crucial direct role in RAS signaling, cell cycle control, and cell transformation, thus demonstrating its potent role in tumorigenesis via proinflammatory mechanisms (Coste et al. 2010). Bettina et al. showed that both MyD88 and TRIF are nonredundant signaling pathways involved in early endotoxin-induced rodent ileus, and that MyD88 is the essential adaptor molecule involved in the transduction of early TLR4-induced ileus and inflammatory signaling (Buchholz et al. 2010). These findings have led to the conclusion that MyD88 plays a critical role in host resistance to microbial infection, inflammation, and innate and adaptive immunity.

Summary

Numerous reports conducted in the past few years have established MyD88 as a universal and essential signaling adaptor molecule that is critical for an effective immune response against a wide range of microbial pathogens. MyD88 fulfills important functions in both innate and adaptive immunity, inflammation, and programmed cell death. However, much remains to be learned about the in vivo functions of MyD88 in higher organisms. Future studies should address the prospect of a detailed molecular account of the MyD88 interaction at the DD, ID, and TIR levels with receptors and adaptor proteins. Moreover, the underlying factors involved in MyD88 direct/indirect (via the use of other adaptors) interaction with receptors following ligand stimulation have yet to be elucidated. Joined efforts among researchers will help us to understand the molecular mechanisms by which MyD88 functions in cellular processes. MyD88-related findings have proven to be fruitful in terms of improving our knowledge of the molecular basis for innate immunity and inflammation, and thus we can anticipate further discoveries in the coming years.

Acknowledgments This work was supported by the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (2012016803).

References

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998;9(1):143–50.

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783–801.

Araki A, Kanai T, Ishikura T, Makita S, Uraushihara K, Iiyama R, et al. MyD88-deficient mice develop severe intestinal inflammation in dextran sodium sulfate colitis. J Gastroenterol. 2005;40(1):16–23.

Bhoj VG, Sun Q, Bhoj EJ, Somers C, Chen X, Torres JP, et al. MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus. Proc Natl Acad Sci USA. 2008;105(37):14046–51.

Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, et al. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. Nat Immunol. 2004;5(4):373–9.

Buchholz BM, Billiar TR, Bauer AJ. Dominant role of the MyD88-dependent signaling pathway in mediating early endotoxin-induced murine ileus. Am J Physiol Gastrointest Liver Physiol. 2010;299(2):G531–8.

Burns K, Martinon F, Esslinger C, Pahl H, Schneider P, Bodmer JL, et al. MyD88, an adapter protein involved in interleukin-1 signaling. J Biol Chem. 1998;273(20):12203–9.

Campos MA, Closel M, Valente EP, Cardoso JE, Akira S, Alvarez-Leite JI, et al. Impaired production of proinflammatory cytokines and host resistance to acute infection with Trypanosoma cruzi in mice lacking functional myeloid differentiation factor 88. J Immunol. 2004;172(3):1711–8.

Cates EA, Connor EE, Mosser DM, Bannerman DD. Functional characterization of bovine TIRAP and MyD88 in mediating bacterial lipopolysaccharide-induced endothelial NF-kappaB activation and apoptosis. Comp Immunol Microbiol Infect Dis. 2009;32(6):477–90.

Coste I, Le Corf K, Kfoury A, Hmitou I, Druillennec S, Hainaut P, et al. Dual function of MyD88 in RAS signaling and inflammation, leading to mouse and human cell transformation. J Clin Invest. 2010;120(10):3663–7.

Esen N, Kielian T. Central role for MyD88 in the responses of microglia to pathogen-associated molecular patterns. J Immunol. 2006;176(11):6802–11.

Feinstein E, Kimchi A, Wallach D, Boldin M, Varfolomeev E. The death domain: a module shared by proteins with diverse cellular functions. Trends Biochem Sci. 1995;20(9):342–4.

Gelman AE, LaRosa DF, Zhang J, Walsh PT, Choi Y, Sunyer JO, et al. The adaptor molecule MyD88 activates PI-3 kinase signaling in CD4+ T cells and enables CpG oligodeoxynucleotide-mediated costimulation. Immunity. 2006;25(5):783–93.

Hardiman G, Jenkins NA, Copeland NG, Gilbert DJ, Garcia DK, Naylor SL, et al. Genetic structure and chromosomal mapping of MyD88. Genomics. 1997;45(2):332–9.

Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, et al. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature. 2005;434(7036):1035–40.

Hultmark D. Macrophage differentiation marker MyD88 is a member of the Toll/IL-1 receptor family. Biochem Biophys Res Commun. 1994;199(1):144–6.

Janssens S, Beyaert R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. Trends Biochem Sci. 2002;27(9):474–82.

Janssens S, Burns K, Tschopp J, Beyaert R. Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. Curr Biol. 2002;12(6):467–71.

Janssens S, Burns K, Vercammen E, Tschopp J, Beyaert R. MyD88S, a splice variant of MyD88, differentially modulates NF-kappaB- and AP-1-dependent gene expression. FEBS Lett. 2003;548(1–3):103–7.

Jiang Z, Georgel P, Li C, Choe J, Crozat K, Rutschmann S, et al. Details of toll-like receptor:adapter interaction revealed by germ-line mutagenesis. Proc Natl Acad Sci USA. 2006;103(29):10961–6.

Kakkar R, Lee RT. The IL-33/ST2 pathway: therapeutic target and novel biomarker. Nat Rev Drug Discov. 2008;7(10):827–40.

Koh YS, Koo JE, Biswas A, Kobayashi KS. MyD88-dependent signaling contributes to host defense against ehrlichial infection. PLoS One. 2010;5(7):e11758.

Li C, Zienkiewicz J, Hawiger J. Interactive sites in the MyD88 Toll/interleukin (IL) 1 receptor domain responsible for coupling to the IL1beta signaling pathway. J Biol Chem. 2005;280(28):26152–9.

Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. Nature. 2010;465(7300):885–90.

Loiarro M, Gallo G, Fanto N, De Santis R, Carminati P, Ruggiero V, et al. Identification of critical residues of the MyD88 death domain involved in the recruitment of downstream kinases. J Biol Chem. 2009;284(41):28093–103.

Lord KA, Hoffman-Liebermann B, Liebermann DA. Complexity of the immediate early response of myeloid cells to terminal differentiation and growth arrest includes ICAM-1, Jun-B and histone variants. Oncogene. 1990;5(3):387–96.

Ma Y, Liu H, Tu-Rapp H, Thiesen HJ, Ibrahim SM, Cole SM, et al. Fas ligation on macrophages enhances IL-1R1-Toll-like receptor 4 signaling and promotes chronic inflammation. Nat Immunol. 2004;5(4):380–7.

Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, et al. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell. 1998;2(2):253–8.

Muraille E, De Trez C, Brait M, De Baetselier P, Leo O, Carlier Y. Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response. J Immunol. 2003;170(8):4237–41.

Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997;278(5343):1612–5.

Naiki Y, Michelsen KS, Zhang W, Chen S, Doherty TM, Arditi M. Transforming growth factor-beta differentially inhibits MyD88-dependent, but not TRAM- and TRIF-dependent, lipopolysaccharide-induced TLR4 signaling. J Biol Chem. 2005;280(7):5491–5.

Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science. 2007;317(5834):121–4.

Nishiya T, Kajita E, Horinouchi T, Nishimoto A, Miwa S. Distinct roles of TIR and non-TIR regions in the subcellular localization and signaling properties of MyD88. FEBS Lett. 2007;581(17):3223–9.

Ohnishi H, Tochio H, Kato Z, Orii KE, Li A, Kimura T, et al. Structural basis for the multiple interactions of the MyD88

TIR domain in TLR4 signaling. Proc Natl Acad Sci USA. 2009;106(25):10260–5.
Piggott DA, Eisenbarth SC, Xu L, Constant SL, Huleatt JW, Herrick CA, et al. MyD88-dependent induction of allergic Th2 responses to intranasal antigen. J Clin Invest. 2005;115(2):459–67.
Rahman AH, Cui W, Larosa DF, Taylor DK, Zhang J, Goldstein DR, et al. MyD88 plays a critical T cell-intrinsic role in supporting CD8 T cell expansion during acute lymphocytic choriomeningitis virus infection. J Immunol. 2008;181(6):3804–10.
Rakoff-Nahoum S, Medzhitov R. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science. 2007;317(5834):124–7.
Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY, et al. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. J Immunol. 2002;168(12):5997–6001.
Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol. 2001;2(10):947–50.
Seki E, Tsutsui H, Tsuji NM, Hayashi N, Adachi K, Nakano H, et al. Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice. J Immunol. 2002;169(7):3863–8.
Shi S, Nathan C, Schnappinger D, Drenkow J, Fuortes M, Block E, et al. MyD88 primes macrophages for full-scale activation by interferon-gamma yet mediates few responses to *Mycobacterium tuberculosis*. J Exp Med. 2003; 198(7):987–97.
Sun D, Ding A. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. Nat Immunol. 2006;7(4):375–81.
Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by toll-like receptors. Nature. 2005;434(7030): 243–9.
Takeuchi O, Takeda K, Hoshino K, Adachi O, Ogawa T, Akira S. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. Int Immunol. 2000a;12(1):113–7.
Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol. 2000b;165(10):5392–6.
Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell. 1993;74(5):845–53.
Uematsu S, Sato S, Yamamoto M, Hirotani T, Kato H, Takeshita F, et al. Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. J Exp Med. 2005;201(6):915–23.
van der Sar AM, Stockhammer OW, van der Laan C, Spaink HP, Bitter W, Meijer AH. MyD88 innate immune function in a zebrafish embryo infection model. Infect Immun. 2006;74(4):2436–41.
von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, et al. Pyogenic bacterial infections in humans with MyD88 deficiency. Science. 2008;321(5889):691–6.
Wald D, Qin J, Zhao Z, Qian Y, Naramura M, Tian L, et al. SIGIRR, a negative regulator of toll-like receptor-interleukin 1 receptor signaling. Nat Immunol. 2003;4(9):920–7.
Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity. 1997;7(6):837–47.
Wesche H, Gao X, Li X, Kirschning CJ, Stark GR, Cao Z. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. J Biol Chem. 1999; 274(27):19403–10.
Yu S, Cho HH, Joo HJ, Bae YC, Jung JS. Role of MyD88 in TLR agonist-induced functional alterations of human adipose tissue-derived mesenchymal stem cells. Mol Cell Biochem. 2008;317(1–2):143–50.
Zhu J, Huang X, Yang Y. The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. J Clin Invest. 2009;119(8):2388–98.

Myd88: Myeloid Differentiation Primary Response Gene (88)

▶ TLR5

MyD88-5

▶ Toll-like Receptor Adaptor Protein Family Members

Myd88-Adapter-Like

▶ Toll-like Receptor Adaptor Protein Family Members

Myeloid DAP-12-Associating Lectin-1

▶ CLEC5A

Myeloid Differentiation Primary Response Gene 88

▶ MyD88, Myeloid Differentiation Primary Response Gene 88
▶ Toll-like Receptor Adaptor Protein Family Members

MYLK (Myosin Light Chain Kinase)

Thomas J. Lukas[1] and Vladimir P. Shirinsky[2]
[1]Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, IL, USA
[2]Institute of Experimental Cardiology, Russian Cardiology Research Center of the Ministry of Health, Moscow, Russia

Synonyms

MLCK; MLCK108; MLCK210; MYLK1; Nonmuscle myosin light chain kinase; smMLCK; Smooth muscle myosin light chain kinase

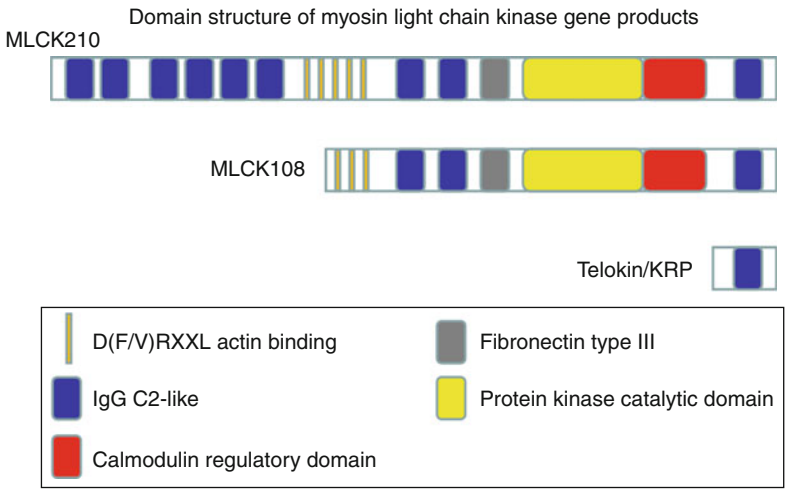
Historical Background

The gene for myosin light chain kinase encodes three proteins: MLCK210, MLCK108, and -telokin/kinase-related protein (KRP). The first protein discovered was MLCK108 (Mr = 110–140 kDa) as a major cytoplasmic component of smooth muscle and responsible for smooth muscle contractility through the phoshorylation of the regulatory light chain of myosin (Kamm and Stull 2001; Lukas et al. 1998). MLCK210 (Mr = 210–220 kDa) has an amino-terminal extension containing additional protein-binding elements (Fig. 1). It was discovered ~15 years later before the gene for MYLK was characterized from chicken (Birukov et al. 1998) and humans (Lazar and Garcia 1999). Both MLCKs are Ca^{2+} -calmodulin-dependent enzymes. Telokin/KRP is an independently expressed non-kinase gene product containing the C-terminus of MLCK, and functions as a ▶ myosin-binding and filament-stabilizing protein (Shirinsky et al. 1993). Telokin/KRP is primarily expressed in smooth muscle and modulates contractility by regulating the access of protein kinases to myosin light chains (Shcherbakova et al. 2010). It has also been implicated in activation of the myosin light chain phosphatase by an unknown mechanism (Choudhury et al. 2004).

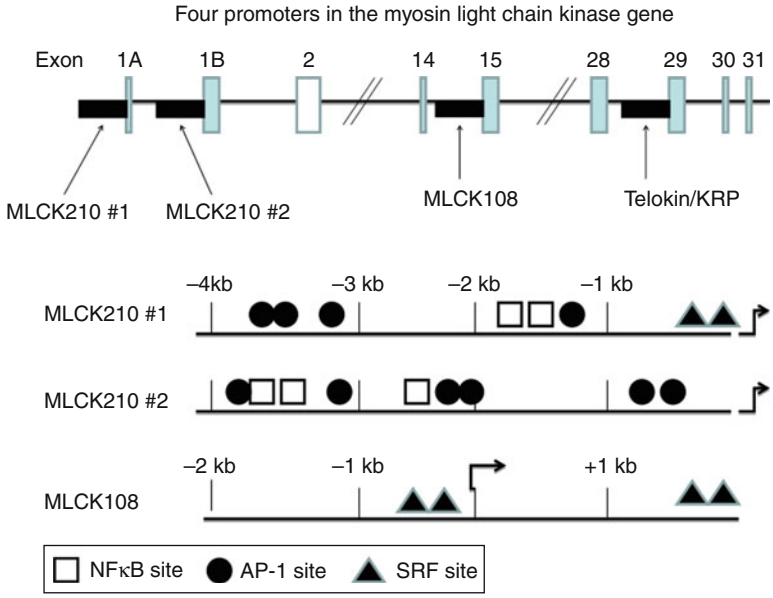
MLCK-Domain Structure and Human Genetics

As shown in Fig. 1, MLCK is a multidomain protein. MLCK108 has amino-terminal actin-binding motifs, a protein kinase catalytic domain, calmodulin regulatory domain, and the myosin-binding (telokin/KRP) domain. Within the amino-terminal domains are IgG C2-like domain motifs and fibronectin-like motifs that may be mediators of other protein–protein interactions within the cell. Similarly, the amino-terminal extension found in the MLCK210 isoform has six additional IgG domains and actin-binding motifs (Kamm and Stull 2001). The additional actin-binding motifs in MLCK210 allow stronger interactions with the actin cytoskeleton (Kamm and Stull 2001), while the expanded IgG domain motifs may be associated with binding to other proteins such as tubulin (Kudryashov et al. 2004), Macrophage migration inhibition factor (MIF) (Wadgaonkar et al. 2005), Arrested in development 1(ARD1) (Shin et al. 2009), and Supervillin (Takizawa et al. 2007).

Single nucleotide polymorphisms (SNPs) in the MYLK gene (Human chromosome 3q21) have been associated with two conditions: severe inflammatory lung disease, including asthma, and sepsis (Gao et al. 2007). It is interesting that African-derived and Caucasian populations differ in the frequency of various polymorphisms, which may make them risk factors in one population, but not the other. For example, a haplotype containing SNP rs9361070, which is within the promoter region of MLCK108, was negatively associated with asthma (Decreased risk) in both American and Caribbean families. However, the same haplotype conferred risk for severe sepsis (Gao et al. 2007). Microarray studies of mRNA from blood cells in the various patient population indicated that there was a decrease in MLCK expression associated with the minor allele of SNP rs9361070. This may explain the reduced risk of asthma, because MLCK expression is upregulated in asthmatic airway tissue (Stephens et al. 2007). African Americans are also at higher risk for glaucoma and there is an increased expression of MLCK108 in the optic nerve head astrocytes of African Americans compared to Caucasian populations, although specific polymorphisms have not been identified with disease. On the other hand, MLCK210 is upregulated in glaucomatous astrocytes of both populations (Lukas et al. 2008). Finally, upregulation of MLCK210 is associated with inflammatory bowel disease, but as with glaucoma, no polymorphisms have been associated with disease (Blair et al. 2006).



MYLK (Myosin Light Chain Kinase), Fig. 1 Domain structure of myosin light chain kinase gene products. This image has a schematic depicting each protein and its domain structure

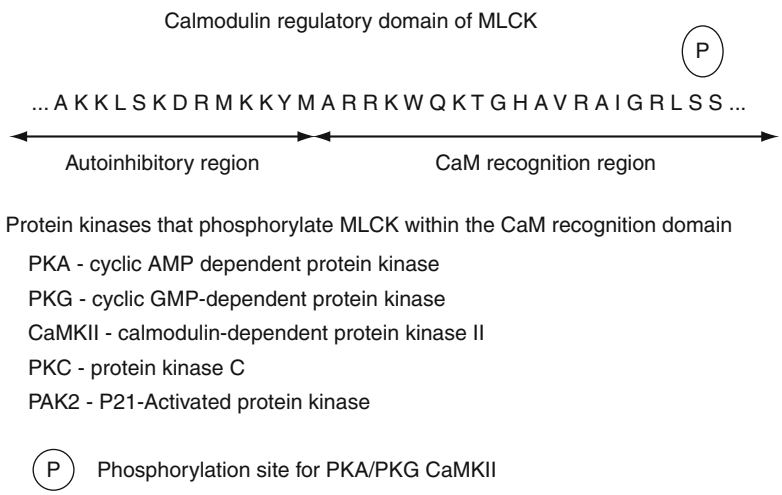


MYLK (Myosin Light Chain Kinase), Fig. 2 Promoters within the myosin light chain kinase gene. *Upper* part of the figure describes where the four promoters are located. The *bottom* part of the figure has the approximate location of transcription factor binding motifs within MLCK210 and MLCK108 promoters

Expression of MLCK Isoforms

MLCK108 is expressed in most tissues, with lower levels appearing in embryonic cells and precursors. MLCK210 specific expression occurs predominantly in endothelial cells of blood vessels and epithelial cells of the intestine and lung (Blue et al. 2002). Primary cells such as those from smooth muscle often exhibit decreased expression of MLCK108 when placed in tissue culture (Blue et al. 2002).

MLCK108, MLCK210, and telokin/KRP expression are independently regulated. Figure 2 illustrates the relationships among the transcripts produced from the MYLK. The promoter for MLCK108 contains several serum response factor (SRF) elements that are often associated with smooth muscle gene expression. Promoter analysis indicates that myocardin, an SRF activator, is necessary for high-level expression of MLCK108 and telokin/KRP in smooth muscle. GATA-6, an SRF repressor may be responsible for the downregulation of MLCK108 and telokin/KRP in nonmuscle tissues (Herring et al. 2006). In addition to SRF other trans-acting factors that affect MLCK108 and/or telokin/KRP expression include thyrotroph

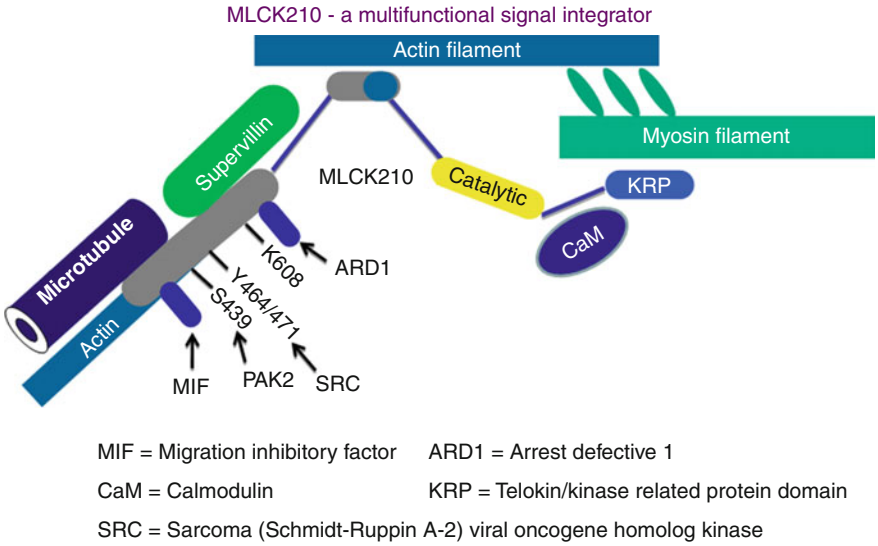


MYLK (Myosin Light Chain Kinase), Fig. 3 Calmodulin regulatory domain of MLCK. This domain contains the autoinhibitory and calmodulin recognition region and a listing of protein kinases that modulate Ca^{2+} -calmodulin binding through phosphorylation at the C-terminal end of this region. The legend indicates the protein kinases that phosphorylate one of the two serines within the RLSS sequence

embryonic factor (TEFB), FoxQ1 and Hox proteins. These factors bind to AT-rich regions adjacent to the SRF sites (Herring et al. 2006). In the case of MLCK210, the promoter is more complex because there are two alternate start sites for exon 1 each containing its own upstream promoter (Fig. 2). MLCK210 expression in epithelial cell types is inducible by tissue necrosis factor alpha (▶ TNFα) (Graham et al. 2006). However, two types of transcriptional motifs are involved. In the upstream promoter sequence of MLCK210 (Transcript #1 –Exon 1A) there are at least two NFκB motifs and at least four AP-1 sites in either promoter region (Fig. 2). These are important for the induction of MLCK210 by ▶ TNFα in epithelial cells. However, the relative importance of NFκB compared to AP-1 transcriptional activity is dependent on the extent of cell differentiation with NFκB declining and AP-1 increasing with cell confluence (Graham et al. 2006). The promoter for MLCK210 transcript #1 also contains two SP1 motifs at −275 and −218 bp upstream of the transcriptional start site that are responsible for basal MLCK210 promoter activity (Graham et al. 2006). Detailed promoter activity analysis for transcript #2 (Exon 1B) has not yet been reported but the presence of a common set of transcription factor binding motifs suggests that this activity will be similar to the Exon 1 promoter.

MLCK and Signal Transduction

As mentioned earlier, MLCK108 is one of the kinases responsible for Ca^{2+}-stimulated contraction of smooth muscle (Kamm and Stull 2001). Ca^{2+} calmodulin-dependent activation is further modulated by phosphorylation at serine residues within the calmodulin regulatory region (Fig. 3). This region has two subdomains that function to maintain the kinase in an autoinhibited state that is released when calmodulin binds and activates the enzyme (Lukas et al. 1998). Site-directed mutagenesis studies established the importance of selected charged residues within the autoinhibitory- and calmodulin-binding domains with respect to autoinhibition and calmodulin binding. Calmodulin-dependent protein kinase II, ▶ PAK2, and cyclic nucleotide (cyclic AMP, cyclic GMP)-dependent protein kinases are capable of phosphorylating sites within the calmodulin-binding domain (Fig. 3) and decrease the Ca^{2+} sensitivity of MLCK activation (Kamm and Stull 2001). MLCK210 is also phosphorylated by PAK2 at Ser-439, but this has not been associated with decreased MLCK activity (Kamm and Stull 2001). On the other hand, phosphorylation of MLCK by MAP Kinase stimulates activity in cultured tumor cells. A unique tyrosine phosphorylation site (Y464) is found in one splice form of MLCK210 and tyrosine phosphorylation of MLCK210 in cultured cells has been observed after stimulation with a tyrosine phosphatase inhibitor or constitutively activated epidermal growth factor (EGF) receptor (Kamm and Stull 2001). Although this site is distal from the calmodulin and kinase catalytic domains, tyrosine phosphorylation slightly increases MLCK210 activity (Kamm and Stull 2001).



MYLK (Myosin Light Chain Kinase), Fig. 4 MLCK210 contains multiple interaction sites for cytoskeletal and regulatory proteins. Shown in this figure are selected binding partners for MLCK210 that interact with MLCK210 as well as those shared by MLCK108 (Actin, CaM, and myosin)

MLCK210 as a Signal Integrator

The interactions of MLCK210 with various kinases as well as with specific proteins within the amino-terminal tail suggest that MLCK210 may coordinate (integrate) signaling from other cellular processes (Kudryashov et al. 2004). This may be particularly important in the cell types that express MLCK210 and contain mostly actin and little myosin, so that MLCK catalytic activity contributes toward changes in cell morphology and/or migration rather than a contractile event as found in smooth muscle. A summary of such signal integrations is illustrated in Fig. 4. Clearly, not all of these interactions happen in every tissue/cell type. However, selected sets of interactions and signaling events that regulate MLCK localization and activity are likely to play a role in specific cellular processes.

Interactions between MLCK210, MIF, and actomyosin fibers of the cytoskeleton may be important in endothelial cells involved in an inflammatory response (Wadgaonkar et al. 2005). Likewise, recruitment and activation of Pyk2 tyrosine kinase by MLCK210 amino-terminal tail is required for full activation of β_2 integrins and neutrophil transmigration through microvascular wall in sepsis-induced lung inflammation model (Xu et al. 2008). Membrane-associated scaffolding protein supervillin also directly interacts with MLCK210 N-terminus as well as with myosin II. Through these interactions, supervillin apparently modulates myosin II activation by MLCK210 and contributes to myosin II assembly during cell spreading (Takizawa et al. 2007).

MYLK (Myosin Light Chain Kinase), Table 1 Predicted Serine (S) and Threonine (T) phosphrylation sites in MLCK210

Site	Kinase	Score
S-10	PKC	0.81
T-185	PKC	0.71
S-239	PKC	0.86
S-298	PKC	0.71
S-324	PKC	0.84
T-430	PKC	0.82
S-482	PKA	0.72
T-599	PKC	0.89
S-605	PKC	0.86
S-609	PKA	0.80
T-643	PKC	0.73
S-733	PKC	0.76

Sites were found by analyzing the MLCK210 amino acid sequence using the NetPhos 1.0 Web site (http://www.cbs.dtu.dk/services/NetPhosK/) using a cutoff of 0.7 and no additional filtering. Only predicted sites within the N-terminal extension domain are shown

In addition to the identified sites at tyrosine 464/471, and serine 439 (Fig. 4), MLCK210 has multiple predicted phosphorylation sites in the N-terminal tail domain that may dynamically regulate localization and function of this protein (Table 1). For example, phosphorylation of an MLCK210 N-terminal tail construct in vitro alters the actin filament binding and phosphomimiking mutant of MLCK210 N-terminal tail has reduced binding to the cytoskeleton in cultured

cells (V.P. Shirinsky, unpublished data). In another study, the N-terminal domain of MLCK210 is differentially phosphorylated and localized in mitotic and interphase cultured cells suggesting that phosphorylation (by the kinase Aurora B) may impact MLCK210 cellular localization (Dulyaninova and Bresnick 2004). Similarly, ARD1 is a lysine acetylase that targets a site (Lys-608) in a Ca^{2+}-dependent fashion (Shin et al. 2009). Acetylation of MLCK210 reduces myosin light chain phosphorylation (Shin et al. 2009). Therefore, the signal integrating functions of MLCK210 are likely to by dynamically regulated through post-translational modifications such as acetylation and phosphorylation.

Summary: Future Work on MLCK Function

Details of the involvement of MLCK, particularly MLCK210 in cellular processes that mediate cell migration, epithelial, and endothelial barrier functions are still emerging. Population genetics studies are needed to determine whether specific variants in MLCK210 are associated with increased risk for specific conditions such as glaucoma and inflammatory bowel disease. Two complex promoters for MLCK210 are suggestive that tight regulation of its expression is key to cellular homeostasis in particular cell phenotypes. Alterations of MLCK210 expression in multiple human diseases such as cancer, lung injury, glaucoma, and inflammatory bowel disease indicate that MLCK210 is a potential target for pharmacological and/or genetic intervention. Additionally, cell interactions of the unique N-terminal tail region in MLCK210 with multiple cytoskeletal and signal transduction proteins offers opportunities for specific intervention at the level of protein–protein interactions. Finally, the posttranscriptional regulation of MLCK gene product expression through reagents such as siRNA and endogenous miRNAs will contribute to the array of tools used to modulate MLCK210 activities.

References

Birukov KG, Schavocky JP, Shirinsky VP, Chibalina MV, Van Eldik LJ, Watterson DM. Organization of the genetic locus for chicken myosin light chain kinase is complex: multiple proteins are encoded and exhibit differential expression and localization. J Cell Biochem. 1998;70(3):402–13.

Blair SA, Kane SV, Clayburgh DR, Turner JR. Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease. Lab Invest. 2006;86(2):191–201.

Blue EK, Goeckeler ZM, Jin Y, Hou L, Dixon SA, Herring BP, et al. 220- and 130-kDa MLCKs have distinct tissue distributions and intracellular localization patterns. Am J Physiol Cell Physiol. 2002;282(3):C451–60.

Choudhury N, Khromov AS, Somlyo AP, Somlyo AV. Telokin mediates Ca2+−desensitization through activation of myosin phosphatase in phasic and tonic smooth muscle. J Muscle Res Cell Motil. 2004;25(8):657–65.

Dulyaninova NG, Bresnick AR. The long myosin light chain kinase is differentially phosphorylated during interphase and mitosis. Exp Cell Res. 2004;299(2):303–14.

Gao L, Grant AV, Rafaels N, Stockton-Porter M, Watkins T, Gao P, et al. Polymorphisms in the myosin light chain kinase gene that confer risk of severe sepsis are associated with a lower risk of asthma. J Allergy Clin Immunol. 2007;119(5):1111–8.

Graham WV, Wang F, Clayburgh DR, Cheng JX, Yoon B, Wang Y, et al. Tumor necrosis factor-induced long myosin light chain kinase transcription is regulated by differentiation-dependent signaling events. Characterization of the human long myosin light chain kinase promoter. J Biol Chem. 2006;281(36):26205–15.

Herring BP, El Mounayri O, Gallagher PJ, Yin F, Zhou J. Regulation of myosin light chain kinase and telokin expression in smooth muscle tissues. Am J Physiol Cell Physiol. 2006;291(5):C817–27.

Kamm KE, Stull JT. Dedicated myosin light chain kinases with diverse cellular functions. J Biol Chem. 2001;276(7):4527–30.

Kudryashov DS, Stepanova OV, Vilitkevich EL, Nikonenko TA, Nadezhdina ES, Shanina NA, et al. Myosin light chain kinase (210 kDa) is a potential cytoskeleton integrator through its unique N-terminal domain. Exp Cell Res. 2004;298(2):407–17.

Lazar V, Garcia JG. A single human myosin light chain kinase gene (MLCK; MYLK). Genomics. 1999;57(2):256–67.

Lukas TJ, Mirzoeva S, Watterson DM. Calmodulin-regulated protein kinases. In: Van Eldik LJ, Watterson DM, editors. Calmodulin and signal transduction. New York: Academic; 1998. p. 66–168.

Lukas TJ, Miao H, Chen L, Riordan S, Li W, Crabb AM, et al. Susceptibility to glaucoma: differential comparison of the astrocyte transcriptome from glaucomatous African American and Caucasian American donors. Genome Biol. 2008;9(7):R111.

Shcherbakova OV, Serebryanaya DV, Postnikov AB, Schroeter MM, Zittrich S, Noegel AA, et al. Kinase-related protein/telokin inhibits Ca2+−independent contraction in Triton-skinned guinea pig taenia coli. Biochem J. 2010;429(2):291–302.

Shin DH, Chun YS, Lee KH, Shin HW, Park JW. Arrest defective-1 controls tumor cell behavior by acetylating myosin light chain kinase. PLoS ONE. 2009;4(10):e7451.

Shirinsky VP, Vorotnikov AV, Birukov KG, Nanaev AK, Collinge M, Lukas TJ, et al. A kinase related protein stabilizes unphosphorylated smooth muscle myosin minifilaments in the presence of ATP. J Biol Chem. 1993;268:16578–83.

Stephens NL, Cheng ZQ, Fust A. Sensitized airway smooth muscle plasticity and hyperreactivity: a review. Can J Physiol Pharmacol. 2007;85(7):679–85.

Takizawa N, Ikebe R, Ikebe M, Luna EJ. Supervillin slows cell spreading by facilitating myosin II activation at the cell periphery. J Cell Sci. 2007;120(Pt 21):3792–803.

Wadgaonkar R, Dudek SM, Zaiman AL, Linz-McGillem L, Verin AD, Nurmukhambetova S, et al. Intracellular interaction of myosin light chain kinase with macrophage migration inhibition factor (MIF) in endothelium. J Cell Biochem. 2005;95(4):849–58.

Xu J, Gao XP, Ramchandran R, Zhao YY, Vogel SM, Malik AB. Nonmuscle myosin light-chain kinase mediates neutrophil transmigration in sepsis-induced lung inflammation by activating beta2 integrins. Nat Immunol. 2008;9(8):880–6.

MYLK1

▶ MYLK (Myosin Light Chain Kinase)

Myo10

▶ Myosin X

MYO3A

▶ Myosin III

MYO3B

▶ Myosin III

myoIII$_{Lim}$

▶ Myosin III

Myosin I (Myo1)

Lynne M. Coluccio
Boston Biomedical Research Institute, Watertown, MA, USA

Nomenclature

The nomenclature of myosins I is confusing, although there is general agreement in the naming of myosins I in vertebrates (Gillespie et al. 2001). The names used in vertebrates, however, do not correspond to those used in lower organisms. The following names have been used to refer to class I myosins:

In yeast: Myo3, Myo5

In *Acanthamoeba*: Myosin IA-C

In *Dictyostelium*: MyoA-F, K

In *Caenorhabditis elegans*: Myo1A or HUM-1; HUM-5

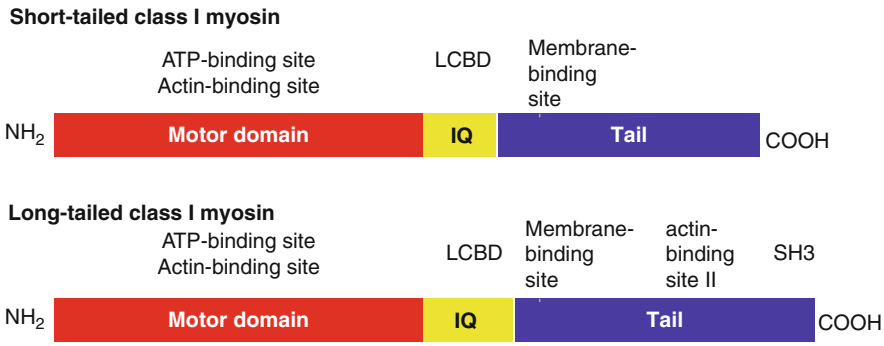
In *Drosophila:* Myo1A (Myo31DF, CG7438); Myo1B (Myo61F, CG9155)

In vertebrates: Myo1a-h; (MyoA-H in humans)

Myo1a (Brush border myosin I, 110kDa-calmodulin complex)
Myo1b (myr 1, myosin-Iα, 130-kDa myosin I)
Myo1c (myr 2, myosin-Iβ,110-kDa myosin I)
Myo1d (myr 4, myosin-Iγ, 105-kDa myosin I)
Myo1e (myr 3, human myosin-1C)
Myo1f
Myo1g
Myo1h

Historical Background

Myosin 1 is the largest of ~35 different classes of proteins that comprise the myosin superfamily, a collection of actin-associated molecular motor proteins that use the energy from ATP hydrolysis to translocate actin filaments. In the early 1970s Pollard and Korn identified in the soil amoeba, *Acanthamoeba,* the first myosin I, a relatively short, single polypeptide chain with an actin-activated ATPase activity resembling that of thick filament-forming myosin (II) from skeletal muscle, which was already known. A major difference was that unlike muscle myosin, the *Acanthamoeba* protein was single-headed and nonfilamentous. Subsequently, the first myosin I in vertebrates, the 110K-calmodulin complex or brush border myosin I (renamed Myo1a), was identified by several different research groups. In vertebrates, Myo1a forms lateral links that connect the core bundle of actin filaments in intestinal microvilli to the microvillar membrane. It is now known that multiple class I myosins are expressed in cells including two class I myosins in yeast (Myo3 and Myo5); three class I myosins in *Acanthamoeba* (myosin IA-C); 7 class I myosins in the slime mold, *Dictyostelium* (MyoA-F, K); 2 in the nematode, *Caenorhabditis elegans* (Myo1A or HUM-1; HUM-5); 2 class I myosins in the fly, *Drosophila melanogaster*



Myosin I (Myo1), Fig. 1 Schematic of short-tailed and long-tailed class I myosins showing the differences in tail domains. In addition to the membrane-binding domain found in short-tailed myosins I, long-tailed class I myosins contain in some cases an ATP-insensitive actin-binding site and an SH3 domain found in signaling proteins

(Myo1A and Myo1B); and 8 class I myosins in humans (Myo1A-H), and that these myosins are involved in a variety of different cellular events. More comprehensive recent reviews with extensive references are available (Coluccio 2008; Kim and Flavell 2008).

Myosin Structure

Myosins I are single polypeptide chains of 110–140 kDa containing a motor domain, a light-chain-binding domain (LCBD), and a tail region.

As with all myosins, the motor domains of class I myosins contain the nucleotide- and actin-binding sites. They share many of the highly conserved amino acid residues found in myosin II and other myosins. So far, the only available atomic structure of a class I myosin is that of the motor domain of *Dictyostelium* MyoE (Kollmar et al. 2002), which resembles the available solved structures of other myosin motor domains, although the surface loops differ in length and the position of the lever arm is 30° further up.

The LCBD of class I myosins is an alpha-helical region containing one or more repeats of ~29 amino acids called IQ regions for the isoleucine and glutamine residues that are normally present. The LCBDs of class I myosins bind calmodulin or light chains that resemble calmodulin. Alternate splicing can occur in the LCBD, e.g., mammalian Myo1b has 6 IQ regions, but is alternatively spliced in the cell to 4-IQ and 5-IQ forms. The sequences of the IQ domains vary among isoforms, as does the affinity of the light chains for the specific IQ domain, suggesting that the myosins might not have a full complement of light chains under all intracellular conditions.

Although the C-terminal tails of class I myosins are diverse, they all contain a membrane-binding domain with different affinities for specific phosphoinositides, which might account for differential localization of myosins I in cells (Fig. 1). Unlike short-tailed myosins I which contain only the membrane-binding domain, other class I myosins are long-tailed, which contain in some cases an ATP-insensitive actin-binding site and a Src-homology 3 (SH3) domain common to many proteins involved in signal transduction. In yeast, the SH3 domain binds proline-rich regions in the WASP-like adapter protein, Bee1p/Las17, and the actin-binding protein, verpolin (Vrp1p), which is analogous to human WASP-interacting protein (WIP). Verpolin is important in the localization of the yeast class I myosins, Myo3 and Myo5, to cortical structures found at sites of polarized growth and actin organization. Myo3/Myo5 and Bee1p bind and stimulate the actin-nucleating properties of the Arp2/3 complex (Anderson et al. 1998). The amoeboid myosins I also regulate actin assembly, but through a mechanism different from that of yeast. The SH3 domains of MyoB and MyoC from *Dictyostelium* bind a linker protein known as CARMIL (an analogous protein, Acan125, is found in *Acanthamoeba*), which associates with the Arp2/3 complex and capping protein to mediate actin assembly (Jung et al. 2001).

Phylogenetic trees show that class I myosins, which probably resemble the first myosin, are related evolutionarily and can be divided into four subclasses based on the amino acid sequences of their motor

domains. The amoeboid myosins I and long-tailed vertebrate Myo1e and Myo1f constitute subclass 1. Vertebrate Myo1a and Myo1b constitute subclass 2. Vertebrate Myo1c and Myo1h constitute subclass 3. Vertebrate Myo1d and Myo1g, along with *Drosophila* 1A and *C. elegans* HUM-1 and HUM-5, constitute subclass 4.

Biochemical and Mechanical Properties

Actin activates the Mg^{2+}-ATPase activity of the lower eukaryotic class I myosins 60-fold to a level similar to that of rabbit skeletal muscle myosin II; however, the ATPase activity of vertebrate class I myosins is only modestly activated by actin. Kinetic analyses indicate that class I myosins spend most of their time weakly bound to actin (i.e., they have a low duty ratio). Activation of the steady-state ATPase activity of myosins I can show a hyperbolic relationship with increasing actin concentration (e.g., Myo1a, Myo1b, Myo1c) or be triphasic (e.g., *Acanthamoeba* IA and IB, vertebrate Myo1e) with activation at low actin concentrations, inhibition at moderate actin concentrations, and further activation at high actin concentrations. This triphasic behavior was originally attributed to the presence of an ATP-insensitive actin-binding site in the tail; however, Myo1e does not have an ATP-insensitive actin-binding site, so the reason for this triphasic behavior remains unclear (see El-Mezgueldi and Bagshaw 2008).

Single-molecule mechanical studies showed that the interaction of myosin I with actin occurs in two parts representing release of inorganic phosphate followed by ADP release (Veigel et al. 1999; Batters et al. 2004). These results together with results from structural studies showing that class I myosins undergo an ADP-induced conformational change and kinetic studies showing that the change in fluorescence when ATP is added to pyrene actin-myosin I is biphasic, consisting of a fast phase followed by a slow phase, led to the hypothesis that some class I myosins are sensitive to strain (Coluccio and Geeves 1999). This hypothesis is supported by other single-molecule studies showing that the rate of detachment of Myo1b from actin decreases 75-fold under tension (Laakso et al. 2008). Furthermore, membrane tension increases in cells overexpressing class I myosins (Nambiar et al. 2009).

Regulation

Phosphorylation of a single serine or threonine between the ATP- and actin-binding sites in the heavy chain of the *Acanthamoeba* and *Dictyostelium* myosins I regulates their actin-activated Mg^{2+}-ATPase and motor activities. Phosphorylation by the myosin I heavy chain kinase, MIHCK, which is a p21-activated kinase (PAK) enhances the ATPase activity 40–80 fold (Brzeska et al. 1997). In vertebrate myosins I, negatively charged glutamate or aspartate is present at the corresponding site, known as the TEDS site after the one-letter codes for the amino acids found at the site, indicating that these myosins I are constitutively active (Bement and Mooseker 1995).

The vertebrate class I myosins translocate actin filaments in vitro albeit slowly. Although the ATPase activity is higher in calcium vs. EGTA, the rate of actin translocation is less in buffers containing calcium. This inhibition is reversed in the presence of exogenous calmodulin suggesting that calcium causes calmodulin dissociation from the LCBD thereby compromising its function as a lever arm and ability to support motility. In the case of Myo1c, calmodulin dissociation might reveal sites that are then available to interact with receptors on hair cells in the inner ear (Cyr et al. 2002).

The steady-state ATPase activity of a fragment of mammalian Myo1c consisting of the motor domain and first IQ domain, $Myo1c^{1IQ}$, is relatively insensitive to calcium; however, individual steps in the cyclic interaction of $Myo1c^{1IQ}$ with actin including the ATP hydrolysis step (7-fold inhibition) and ADP release (10-fold acceleration) are sensitive to calcium, which would result in acceleration in the detachment of the Myo1c crossbridge and lengthening of the lifetime of the detached M-ATP state (Adamek et al. 2008). This modulation by calcium could have important implications, especially for Myo1c function in the inner ear, where mechanotransduction involves changes in free calcium concentration as channels open and close in response to sound and vibration.

Function

Yeast in which one of the two class I myosins, Myo3 and Myo5, is deleted have no phenotype, although cells in which both myosins are deleted round up and exhibit an accumulation of intracellular vesicles and

sensitivity to osmotic shock. The three myosin I isoforms from *Acanthamoeba* – myosin 1A, 1B, and 1C – have both distinct and overlapping localization patterns and functions. Each is associated with the cell membrane, but only myosin 1C is found at contractile vacuoles. Amoebae loaded with anti-myosin 1C, but not anti-myosin 1B, become large with vacuoles and lyse in response to osmotic shock suggesting that myosin 1C is involved in the expulsion of water from contractile vacuoles. *Dictyostelium* amoebae are viable when any one of the seven myosin I genes expressed in that organism is deleted demonstrating functional redundancy among the isoforms. Amoebae lacking MyoA or MyoB show defects in speed of locomotion, formation of pseudopods, and directionality of movement. Cells, in which two or three isoforms are deleted, are deficient in pseudopod formation, motility, pinocytosis, membrane ruffling, endocytosis, secretion, and cortical tension (Ostap and Pollard 1996).

The two myosin I isoforms in *Drosophila*, Myo1A and Myo1B, are most closely related to mammalian Myo1d and Myo1c, respectively and are implicated in left-right symmetry (Hozumi et al. 2006; Spéder et al. 2006). Although both isoforms are expressed in brush border of the midgut, Myo1b is found along the length of the apical microvilli and is required for integrity of the brush border and resistance to bacterial infection (Hegan et al. 2007).

Significant progress in understanding the roles of the class I myosins in mammalian cells is being made. Although there are defects in the morphology of the microvillar membrane, mice in which expression of Myo1a is knocked out show little change in phenotype indicating that other myosins substitute for the loss of Myo1a (Benesh et al. 2010). Unlike Myo1a, which is found predominantly in intestine, Myo1b, which is most closely related to Myo1a, is ubiquitously expressed. Overexpression of Myo1b affects the distribution of endocytotic compartments suggesting that Myo1b plays a role in endocytosis (Raposo et al. 1999). Myo1c, also found in a variety of different cell types, supports the insulin-induced exocytic fusion of vesicles containing GLUT4 in adipocytes (Bose et al. 2002). In the specialized hair cells of the inner ear, Myo1c mediates adaptation, a process by which stimulated cells remain sensitive to new stimuli (Holt et al. 2002). In *Xenopus* eggs Myo1c plays a role in the compensatory endocytosis of cortical granule membranes after secretion (Sokac et al. 2006). Nuclear Myo1c, which differs from Myo1c by the presence of an N-terminal extension, supports RNA synthesis (Hofmann et al. 2006), although the exact mechanism is unknown. Myo1d plays a role in endocytic membrane trafficking (Huber et al. 2000). Mice in which Myo1e, which is associated with podocytes in kidney, is knocked out show signs of kidney disease (Krendel et al. 2009). Myo1f is expressed predominantly in the spleen, lymph, thymus, and lung; and neutrophils from Myo1f knockout mice exhibit decreased motility and increased adhesion making the mice more susceptible to infection (Kim et al. 2006). Knock-down experiments show that Myo1g, which is expressed exclusively in hematopoietic cells, regulates cell elasticity (Olety et al. 2009).

Summary

Myosins I are a diverse group of single-headed, actin- and membrane-associated molecular motors with roles in a variety of cytoskeletal-membrane events.

References

Adamek N, Coluccio LM, Geeves MA. Calcium sensitivity of the cross-bridge cycle of Myo1c, the adaptation motor of the inner ear. Proc Natl Acad Sci USA. 2008;105:5710–5.

Anderson BL, Boldogh I, Evangelista M, Boone C, Greene LA, Pon LA. The Src homology domain 3 (SH3) of a yeast type I myosin, Myo5p, binds to verprolin and is required for targeting to sites of actin polarization. J Cell Biol. 1998;141(6):1357–70.

Batters C, Arthur CP, Lin A, Porter J, Geeves MA, Milligan RA, et al. Myo1c is designed for the adaptation response in the inner ear. EMBO J. 2004;23:1433–40.

Bement WM, Mooseker MS. TEDS rule: a molecular rationale for differential regulation of myosins by phosphorylation of the heavy chain head. Cell Motil Cytoskeleton. 1995;31(2):87–92.

Benesh AE, Nambiar R, McConnell RE, Mao S, Tabb DL, Tyska MJ. Differential localization and dynamics of class I myosins in the enterocyte microvillus. Mol Biol Cell. 2010;21(6):970–8.

Bose A, Guilherme A, Robida SI, Nicoloro SM, Zhou QL, Jiang ZY, et al. Glucose transporter recycling in response to insulin is facilitated by myosin Myo1c. Nature. 2002;420(6917):821–4.

Brzeska H, Knaus UG, Wang ZY, Bokoch GM, Korn ED. p21-activated kinase has substrate specificity similar to Acanthamoeba myosin I heavy chain kinase and activates Acanthamoeba myosin I. Proc Natl Acad Sci USA. 1997;94(4):1092–5.

Coluccio LM. Myosin I. ProteinsCell Reg. 2008;7:95–124.

Coluccio LM, Geeves MA. Transient kinetic analysis of the 130-kDa myosin I (myr 1 gene product) from rat liver: A myosin I designed for maintenance of tension? J Biol Chem. 1999;274:21575–80.

Cyr JL, Dumont RA, Gillespie PG. Myosin-1c interacts with hair-cell receptors through its calmodulin-binding IQ domains. J Neurosci. 2002;22(7):2487–95.
El-Mezgueldi M, Bagshaw CR. The myosin family: biochemical and kientic properties. Proteins Cell Reg. 2008;7:55–93.
Gillespie PG, Albanesi JP, Bähler M, Bement WM, Berg JS, Burgess DR, et al. Myosin-I nomenclature. J Cell Biol. 2001;155(5):703–4.
Hegan PS, Mermall V, Tilney LG, Mooseker MS. Roles for *Drosophila melanogaster* myosin 1B in maintenance of enterocyte brush-border structure and resistance to the bacterial pathogen *Pseudomonas entomophila*. Mol Biol Cell. 2007;18:4625–36.
Hofmann WA, Vargas GM, Ramchandran R, Stojiljkovic L, Goodrich JA, de Lanerolle PA. Nuclear myosin I is necessary for the formation of the first phosphodiester bond during transcription initiation by RNA polymerase II. J Cell Biochem. 2006;99:1001–9.
Holt JR, Gillespie SKH, Provance DW, Shah K, Shokat KM, Corey DP, et al. A chemical-genetic strategy implicates myosin-1c in adaptation by hair cells. Cell. 2002;108(3):371–81.
Hozumi S, Maeda R, Taniguchi K, Kanai M, Shirakabe S, Sasamura T, et al. An unconventional myosin in *Drosophila* reverses the default handedness in visceral organs. Nature. 2006;440:798–802.
Huber LA, Fialka I, Paiha K, Hunziker W, Sacks DB, Bähler M, et al. Both calmodulin and the unconventional myosin Myr4 regulate membrane trafficking along the recycling pathway of MDCK cells. Traffic. 2000;1:494–503.
Jung G, Remmert K, Wu X, Volosky JM, Hammer 3rd JA. The *Dictyostelium* CARMIL protein links capping protein and the Arp2/3 complex to type I myosins through their SH3 domains. J Cell Biol. 2001;153(7):1479–97.
Kim SV, Flavell RA. Myosin I: from yeast to human. Cell Mol Life Sci. 2008;65:2128–37.
Kim SV, Mehal WZ, Dong X, Heinrich V, Pypaert M, Mellman I, et al. Modulation of cell adhesion and motility in the immune system by Myo1f. Science. 2006;314:136–9.
Kollmar M, Dürrwang U, Kliche W, Manstein DJ, Kull FJ. Crystal structure of the motor domain of a class-I myosin. EMBO J. 2002;21(11):2517–25.
Krendel M, Kim SV, Willinger T, Wang T, Kashgarian M, Flavell RA, et al. Disruption of Myosin 1e promotes podocyte injury. J Am Soc Nephrol. 2009;20(1):86–94.
Laakso JM, Lewis JH, Shuman H, Ostap EM. Myosin I can act as a molecular force sensor. Science. 2008;321(5885):133–6.
Nambiar R, McConnell RE, Tyska MJ. Control of cell membrane tension by myosin-I. Proc Natl Acad Sci USA. 2009;106(29):11972–7.
Olety B, Wälte M, Honnert U, Schillers H, Bähler M. Myo1g is a haematopoietic specific myosin that localises to the plasma membrane and regulates cell elasticity. FEBS Lett. 2010;584:493–499.
Ostap EM, Pollard TD. Overlapping functions of myosin-I isoforms? [comment]. J Cell Biol. 1996;133(2):221–4.
Raposo G, Cordonnier MN, Tenza D, Menichi B, Dürrbach A, Louvard D, et al. Association of myosin I alpha with endosomes and lysosomes in mammalian cells. Mol Biol Cell. 1999;10(5):1477–94.
Sokac AM, Schietroma C, Gundersen CB, Bement WM. Myosin-1c couples assembling actin to membranes to drive compensatory endocytosis. Dev Cell. 2006;11:629–40.
Spéder P, Ádám G, Noselli S. Type 1D unconventional myosin controls left-right asymmetry in *Drosophila*. Nature. 2006;440:803–7.
Veigel C, Coluccio LM, Jontes JD, Sparrow JC, Milligan RA, Molloy JE. The motor protein myosin-I produces its working stroke in two steps [see comments]. Nature. 1999;398(6727):530–3.

Myosin II, "Conventional" Myosin

► Myosins

Myosin III

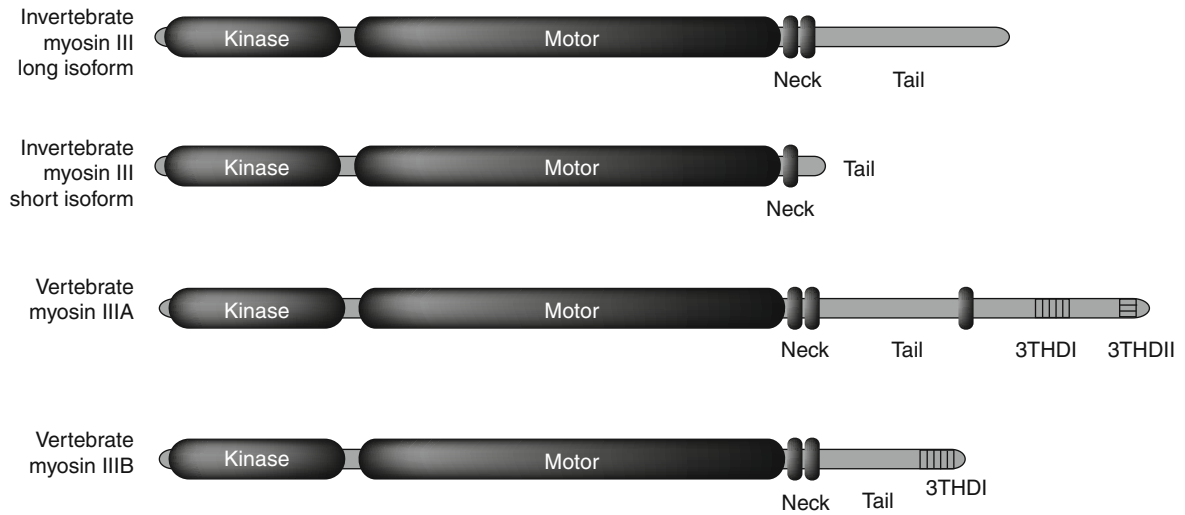
Shigeru Komaba
Boston Biomedical Research Institute, Watertown, MA, USA

Synonyms

LpMYO3; MYO3A; MYO3B; myoIII$_{Lim}$; NINAC (p174, p132)

Historical Background

Class III myosins are one of many classes of ► myosin motor proteins. The first class III myosin, *ninaC* (neither inactivation nor afterpotential C), was identified in *Drosophila* as a gene responsible for abnormal phototransduction (Montell and Rubin 1988). This novel class of myosin is unique in having an N-terminal putative kinase domain joined to a myosin motor domain. The second class III myosin was isolated from the horseshoe crab *Limulus polyphemus* as a phosphoprotein regulated by a circadian clock; it is exclusively expressed in photoreceptors (Battelle et al. 1998). Subsequently, human class III myosin, MYO3A, was isolated by degenerated polymerase chain reaction (PCR) and rapid amplification of cDNA ends PCR from retina and a retinal pigment epithelial cell line (Dose and Burnside 2000).



Myosin III, Fig. 1 Diagram of invertebrate myosin III, vertebrate myosin IIIA, and vertebrate myosin IIIB. Class III myosin consists of a kinase domain, a motor domain, a neck domain with IQ motifs, and a tail domain

A shorter class III isoform, MYO3B, encoded by a different gene from MYO3A, was also identified (Dose and Burnside 2002).

Structure

Drosophila has a single myosin III gene that expresses two alternative isoforms, p174 (long isoform) and p132 (short isoform) (Fig. 1). The long isoform has two IQ motifs, whereas the short isoform has a single IQ motif. The myosin III gene in horseshoe crab encodes a single isoform with a single IQ motif and a short tail. Vertebrates possess two genes that code for two distinct isoforms, myosin IIIA and myosin IIIB (Fig. 1). Similar to other myosin classes, myosin III consists of a motor domain, a neck domain, and a tail domain in addition to an N-terminal kinase domain. There is a large diversity in amino acid sequence of the motor domain among class III myosins, e.g., there is only 30% homology between myosin III from human and *Drosophila*. The neck region binds 1–4 light chains such as calmodulin. Vertebrate myosin IIIA has an additional 1–5 calmodulin-binding site in the tail. There are two conserved sequences in the tail domain of vertebrate myosin III, class III tail homology domain I (3THDI), and class III tail homology domain II (3THDII) (Dose et al. 2003). 3THDI is present in both myosin IIIA and myosin IIIB. 3THDII is located at the C-terminus of myosin IIIA and is an ATP-independent actin-binding domain, which is found in ▶ myosin light chain kinase (Erickson et al. 2003). Heavy meromyosin-like human myosin III consisting of a kinase domain, a motor domain, a neck region, and a partial tail domain is monomeric (Komaba et al. 2003), which is consistent with the observation that human myosin III does not have a long coiled-coil region with which to form dimers.

Biochemical and Biophysical Properties

Given its sequence similarity to other myosins, it was predicted that class III myosins function as molecular motors, enzymes characterized by the ability to interact with actin to produce mechanical force by hydrolyzing ATP. Hicks et al. demonstrated that *Drosophila* myosin III in the soluble fraction of a retinal homogenate binds to actin in an ATP-sensitive manner (Hicks et al. 1996), although to date there has been no report demonstrating that purified *Drosophila* myosin III has ATPase activity. The sequence of the phosphate-binding loop or P-loop, known as a nucleotide-binding motif, consists of GESGAGKT in many myosins; however, the sequence of *Drosophila* myosin III is

GESYSGKS. Indeed, myosin III from horseshoe crab binds actin, but lacks ATPase activity (Kempler et al. 2007). Amino acids which form a salt bridge, E^{459}-R^{238} in the case of *Dictiostelium* myosin II, are essential for ATP hydrolysis. Horseshoe crab myosin III has H^{487} at the equivalent position for R^{238}. The first experimental evidence to demonstrate that myosin III is an actual motor protein was reported in 2003 (Komaba et al. 2003). The authors showed that human myosin IIIA missing the tail domain has an ATPase activity with Vmax of 0.34 s^{-1} and is capable of gliding actin filament at 0.11 μm/s, which is relatively slower compared to many other myosins. Kinetic analysis demonstrated that the dephosphorylated motor domain of human myosin IIIA spends the majority of its time during the ATP hydrolysis cycle attached to actin suggesting that myosin III moves along actin cables without dissociating (Kambara et al. 2006).

Phosphorylation/Regulation

Kinase activity in a class III myosin was originally observed in expressed myosin III from *Drosophila*, where the kinase domain phosphorylates various substrates including itself (Ng et al. 1996); however, the biochemical or physiological role of autophosphorylation of *Drosophila* myosin III remains unclear. *Drosophila* myosin III is phosphorylated in the p174 tail domain by protein kinase C and this phosphorylation is required for normal phototransduction, although the effect of phosphorylation on the biochemical properties of myosin III has not been shown (Li et al. 1998).

Horseshoe crab myosin III is also autophosphorylated and phosphorylated by protein kinase A (PKA) in loop 2, which is at the interface of actin binding (Kempler et al. 2007), suggesting that the interaction between myosin III and actin is modulated by cAMP-mediated phosphorylation and/or autophosphorylation.

Human myosin IIIA also undergoes autophosphorylation in the motor domain, which significantly reduces its affinity for actin (Kambara et al. 2006) resulting in a decrease in the duty ratio (Komaba et al. 2009). Deletion of the kinase domain affects both the biochemical and cell biological properties of myosin IIIA. A kinase domain-deletion mutant has a twofold higher Vmax and fivefold higher affinity for actin, compared to that of myosin IIIA with the kinase domain (Dose et al. 2008). Erickson et al. reported that striped bass myosin IIIA localizes to the cytoplasm and to the tip of filopodia in HeLa cells and that deletion of the kinase domain reduced cytoplasmic localization while enhancing localization at the tips of filopodia, suggesting that the kinase domain inhibits localization to filopodial tips (Erickson et al. 2003).

Physiological Function

Deletion of *Drosophila ninaC* causes abnormal phototransduction and light- and age-dependent retinal degeneration (Porter et al. 1992). Deletion of the motor domain leads to a change in the subcellular distribution of myosin III and a phenotype indistinguishable from that of a null mutant, while mutations in the kinase domain result in defects in normal phototransduction, but no retinal degeneration (Porter and Montell 1993). The study using temperature-sensitive mutants demonstrated that the motor domain is required for maintenance of the retinal structure, but not for normal phototransduction (Porter and Montell 1993).

Myosin III is a major calmodulin-binding protein and the distribution of calmodulin in the retina of *Drosophila* is dependent on myosin III expression (Porter et al. 1993). In the mutant lacking p174, which is specifically localized to rhabdomeres, microvilli-packed structures in photoreceptor cells, calmodulin does not concentrate in rhabdomeres, whereas deletion of p132, the cell body–specific isoform, results in a decrease in cytoplasmic calmodulin.

Recent studies suggested that myosin III is involved in the transport of signaling molecules that undergo light-dependent translocation between the rhabdomere and the cell body in *Drosophila* photoreceptors. Upon light illumination, visual arrestin Arr2 translocates into rhabdomeres from the cell body and inactivates rhodopsin. Arr2 binds to myosin III mediated by phosphoinositides and this translocation is hindered in myosin III null and p132-lacking mutants (Lee and Montell 2004). However, another group demonstrated that light-dependent translocation of Arr2 does not require myosin III (Satoh and Ready 2005).

$G_q\alpha$, an activator of phospholipase Cβ, translocates from the rhabdomere to the cell body in response to light stimulation and returns to the rhabdomere in the

dark. The rate of translocation from the rhabdomere to the cell body in myosin III null mutants is similar to that of wild type; however, upon return to the dark, transport to the rhabdomere is significantly slower in myosin III mutants (Cronin et al. 2004).

Localization of transient receptor potential-like (TRPL) channels in the rhabdomere of myosin III mutants raised in the dark resembles that of wild type, whereas in orange light translocation to the cell body is partially inhibited in myosin III null mutants (Meyer et al. 2006).

One of the binding partners of myosin III in *Drosophila* is INAD, a scaffolding protein for signalplex consisting of phospholipase C, protein kinase C, and calmodulin. Disruption of binding between myosin III and INAD leads to defects in the termination of the photoresponse (Wes et al. 1999).

Vertebrate myosin IIIA is expressed at high levels in the retina and at lower level in the brain and testis. It localizes to the calycal processes of rod and cone photoreceptors (Dose et al. 2003).

Transgene expression of GFP-tagged myosin IIIA in *Xenopus* rod photoreceptors produces abnormal calycal processes and causes subsequent rod degeneration (Lin-Jones et al. 2004). Transgene-induced long and thick calycal processes contain a larger number of actin bundles, suggesting that myosin III regulates the organization of the actin-cytoskeleton in the rod photoreceptors.

Mutations in myosin III found in an Israeli family cause nonsyndromic progressive hearing loss, but have no effect on vision, although myosin III is strongly expressed in retina (Walsh et al. 2002). Myosin IIIA localizes at the tip of stereocilia, which are actin protrusions found on the sensory hair cells in the inner ear, in the thimble-like pattern (Schneider et al. 2006). Overexpression of GFP-tagged full-length myosin IIIA localizes similar to endogenous myosin IIIA and does not change stereocilia structure; however a mutant lacking the kinase domain accumulates at a higher level at the tip and leads to elongation of the stereocilia and bulging of the tip (Schneider et al. 2006). Myosin IIIA colocalizes with espin 1, an actin-bundling protein, at stereocilia tips (Salles et al. 2009). Espin 1 interacts with 3THD1 of myosin IIIA and when coexpressed with myosin IIIA produces longer stereocilia suggesting that myosin IIIA regulates the length of stereocilia together with espin 1. Normal vision and late-onset deafness in patients with myosin IIIA mutations suggests that myosin IIIB, which has 3THDI but not 3THDII compensates for myosin IIIA in photoreceptors and partially in the inner ear.

Summary

Myosin III is a unique myosin in that it has a kinase domain at the N-terminus of its motor domain. Physiological functions of class III myosins have not been fully uncovered, especially in vertebrates; however, the production of model animals such as myosin III knockout or transgenic mice will provide key information regarding the roles of myosin III.

References

Battelle BA, Andrews AW, Calman BG, Sellers JR, Greenberg RM, Smith WC. A myosin III from Limulus eyes is a clock-regulated phosphoprotein. J Neurosci. 1998;18(12):4548–59.

Cronin MA, Diao F, Tsunoda S. Light-dependent subcellular translocation of Gqalpha in *Drosophila* photoreceptors is facilitated by the photoreceptor-specific myosin III NINAC. J Cell Sci. 2004;117(Pt 20):4797–806.

Dose AC, Burnside B. Cloning and chromosomal localization of a human class III myosin. Genomics. 2000;67(3):333–42.

Dose AC, Burnside B. A class III myosin expressed in the retina is a potential candidate for Bardet-Biedl syndrome. Genomics. 2002;79(5):621–4.

Dose AC, Hillman DW, Wong C, Sohlberg L, Lin-Jones J, Burnside B. Myo3A, one of two class III myosin genes expressed in vertebrate retina, is localized to the calycal processes of rod and cone photoreceptors and is expressed in the sacculus. Mol Biol Cell. 2003;14(3):1058–73.

Dose AC, Ananthanarayanan S, Moore JE, Corsa AC, Burnside B, Yengo CM. The kinase domain alters the kinetic properties of the myosin IIIA motor. Biochemistry. 2008;47(8):2485–96.

Erickson FL, Corsa AC, Dose AC, Burnside B. Localization of a class III myosin to filopodia tips in transfected HeLa cells requires an actin-binding site in its tail domain. Mol Biol Cell. 2003;14(10):4173–80.

Hicks JL, Liu X, Williams DS. Role of the ninaC proteins in photoreceptor cell structure: ultrastructure of ninaC deletion mutants and binding to actin filaments. Cell Motil Cytoskeleton. 1996;35(4):367–79.

Kambara T, Komaba S, Ikebe M. Human myosin III is a motor having an extremely high affinity for actin. J Biol Chem. 2006;281(49):37291–301.
Kempler K, Toth J, Yamashita R, Mapel G, Robinson K, Cardasis H, et al. Loop 2 of limulus myosin III is phosphorylated by protein kinase A and autophosphorylation. Biochemistry. 2007;46(14):4280–93.
Komaba S, Inoue A, Maruta S, Hosoya H, Ikebe M. Determination of human myosin III as a motor protein having a protein kinase activity. J Biol Chem. 2003;278(24):21352–60.
Komaba S, Watanabe S, Umeki N, Sato O, Ikebe M. Effect of phosphorylation in the motor domain of human myosin IIIA on its ATP hydrolysis cycle. Biochemistry. 2009;49(17):3695–702.
Lee SJ, Montell C. Light-dependent translocation of visual arrestin regulated by the NINAC myosin III. Neuron. 2004;43(1):95–103.
Li HS, Porter JA, Montell C. Requirement for the NINAC kinase/myosin for stable termination of the visual cascade. J Neurosci. 1998;18(23):9601–6.
Lin-Jones J, Parker E, Wu M, Dose A, Burnside B. Myosin 3A transgene expression produces abnormal actin filament bundles in transgenic *Xenopus laevis* rod photoreceptors. J Cell Sci. 2004;117(Pt 24):5825–34.
Meyer NE, Joel-Almagor T, Frechter S, Minke B, Huber A. Subcellular translocation of the eGFP-tagged TRPL channel in *Drosophila* photoreceptors requires activation of the phototransduction cascade. J Cell Sci. 2006;119(Pt 12):2592–603.
Montell C, Rubin GM. The Drosophila ninaC locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. Cell. 1988;52(5):757–72.
Ng KP, Kambara T, Matsuura M, Burke M, Ikebe M. Identification of myosin III as a protein kinase. Biochemistry. 1996;35(29):9392–9.
Porter JA, Montell C. Distinct roles of the Drosophila ninaC kinase and myosin domains revealed by systematic mutagenesis. J Cell Biol. 1993;122(3):601–12.
Porter JA, Hicks JL, Williams DS, Montell C. Differential localizations of and requirements for the two Drosophila ninaC kinase/myosins in photoreceptor cells. J Cell Biol. 1992;116(3):683–93.
Porter JA, Yu M, Doberstein SK, Pollard TD, Montell C. Dependence of calmodulin localization in the retina on the NINAC unconventional myosin. Science. 1993;262(5136):1038–42.
Salles FT, Merritt Jr RC, Manor U, Dougherty GW, Sousa AD, Moore JE, et al. Myosin IIIa boosts elongation of stereocilia by transporting espin 1 to the plus ends of actin filaments. Nat Cell Biol. 2009;11(4):443–50.
Satoh AK, Ready DF. Arrestin1 mediates light-dependent rhodopsin endocytosis and cell survival. Curr Biol. 2005;15(19):1722–33.
Schneider ME, Dose AC, Salles FT, Chang W, Erickson FL, Burnside B, et al. A new compartment at stereocilia tips defined by spatial and temporal patterns of myosin IIIa expression. J Neurosci. 2006;26(40):10243–52.
Walsh T, Walsh V, Vreugde S, Hertzano R, Shahin H, Haika S, et al. From flies' eyes to our ears: mutations in a human class III myosin cause progressive nonsyndromic hearing loss DFNB30. Proc Natl Acad Sci USA. 2002;99(11):7518–23.
Wes PD, Xu XZ, Li HS, Chien F, Doberstein SK, Montell C. Termination of phototransduction requires binding of the NINAC myosin III and the PDZ protein INAD. Nat Neurosci. 1999;2(5):447–53.

Myosin X

Hiroshi Tokuo
Boston Biomedical Research Institute, Watertown, MA, USA

Synonyms

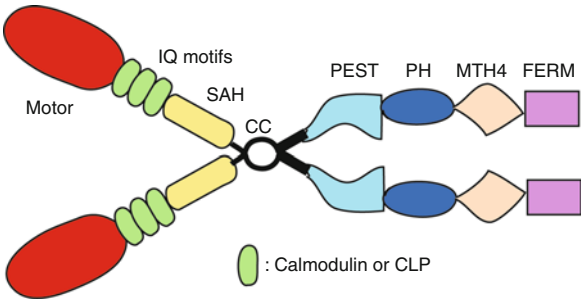
Myosin-10; Myo10

Historical Background

In 1994, myoX was first identified in a PCR screen designed to find novel ▶ myosins in the inner ear (Solc et al. 1994). The full-length cDNA sequences of myoX were determined in 2000 in mouse (Yonezawa et al. 2000), human, and cow (Berg et al. 2000), and the molecular structure and ubiquitous expression in tissues were reported (Berg et al. 2000, Yonezawa et al. 2000). The unique localization pattern of myoX in cultured cells at the tips of filopodia and in lamellipodia was identified in the same reports (Berg et al. 2000, Yonezawa et al. 2000). Subsequently, in 2001, it was shown that the head domain of myoX binds actin and hydrolyzes ATP to induce movement and force (Homma et al. 2001). In 2002, myoX was shown to participate in intrafilopodial movement and filopodia formation (Berg and Cheney 2002), and in phagocytosis (Cox et al. 2002). After these breakthroughs, studies of myoX were expanded to many different aspects by precise biochemical analyses or by identifying binding partners critical for exploring its functions in cells.

Molecular Structure

The N-terminal domain of myoX functions as a motor domain: It binds actin and hydrolyzes ATP to produce



Myosin X, Fig. 1 Schematic diagram of MyoX protein. The N-terminus of myoX functions as a motor domain. The motor domain is followed by a neck region. The predicted coiled-coil segment is present at the C-terminus of the neck region [22]; however, a recent study suggests that instead of forming a stable coiled-coil, this domain forms a stable α-helix (SAH) [23]. The C-terminus of the molecule, or tail, consists of four different domains: a PEST domain; three pleckstrin homology (PH) domains; a myosin tail homology 4 (MTH4) domain; and a band 4.1, Ezrin, Radixin, Moesin (FERM) domain

movement and force (Homma et al. 2001). The motor domain is followed by a neck region that contains three IQ motifs, which bind calmodulin or calmodulin-like light chains (Berg et al. 2000). In epithelial cells, myoX binds calmodulin-like protein (CLP), an epithelial-specific light chain that is expressed during differentiation. CLP expression increases myoX protein levels by stabilizing the molecule (Bennett et al. 2007). A predicted coiled-coil segment is present at the C-terminus of the neck region (Berg et al. 2000); however, it has been suggested that this domain does not form a stable coiled-coil, but instead forms a stable α-helix (SAH) (Knight et al. 2005). The C-terminal end of the molecule is the tail domain, which consists of four different domains: PEST, PH, MTH4, and FERM (Fig. 1). Classes VII, X, XII, and XV myosins share a conserved structural feature in their tail domains – a myosin tail homology 4 (MTH4) domain followed by a band 4.1, Ezrin, Radixin, Moesin (FERM) domain. Together, the myosins containing these domains comprise the MTH4-FERM superclass of myosins (Berg et al. 2001).

In addition to this full-length myoX, brain expresses a shorter form that lacks a motor domain (Sousa et al. 2006). This "headless" myoX might have a possible role as a natural dominant-negative regulator to suppress the function of full-length myoX in neurons.

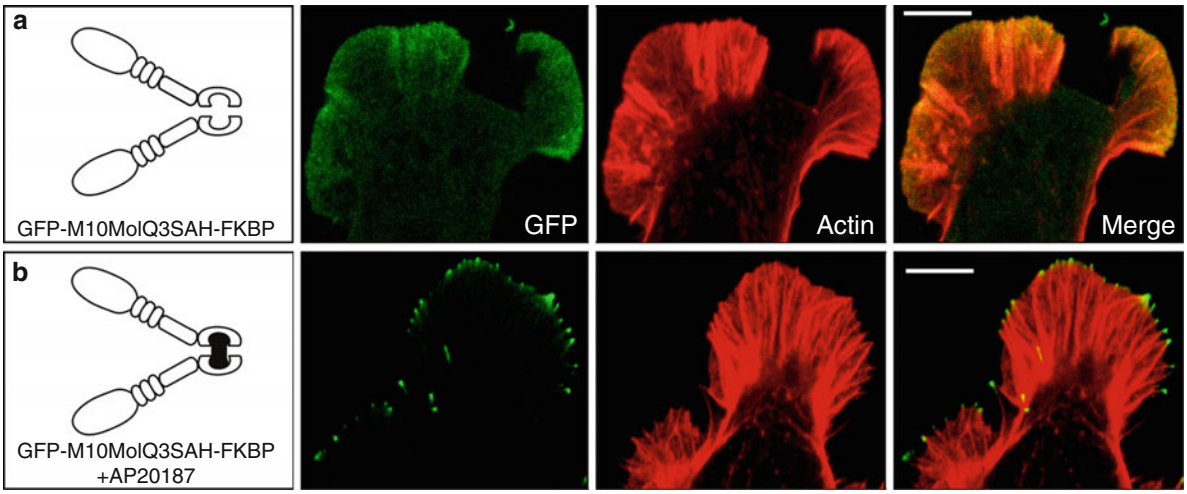
Intrafilopodial Motility

One of the most striking properties of myoX is its forward and rearward movements within filopodia (Berg and Cheney 2002). Recently, the velocity of the forward movement was calculated as ~600 nm/s in living cells using TIRF microscopy (Kerber et al. 2009), similar to the 340–780 nm/s reported for movement of individual myoX molecules on artificial actin bundles (Nagy et al. 2008). The rearward movement of myoX in filopodia is slow at 10–20 nm/s (Berg and Cheney 2002), presumably the rate of retrograde actin flow in filopodia. In these studies, only dimerized myoX showed intrafilopodial motility, suggesting that dimer formation of myoX is necessary for its proper movement in cells.

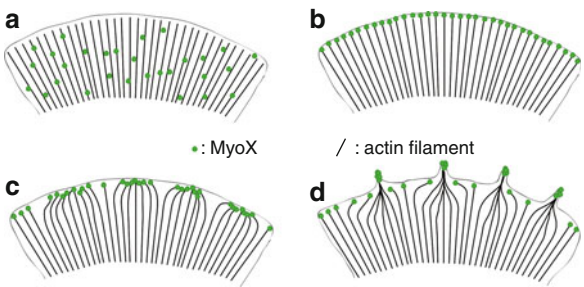
A forced dimer construct of tail-less myoX was first reported to preferentially select bundled actin for motility and showed poor processivity on single filaments in vitro (Nagy et al. 2008). The selectivity of actin tracks (single filaments or bundled) by myoX is controversial because a recent report shows the robust processivity of myoX on individual actin filaments (Sun et al. 2010). Sun et al. also report that myoX moves processively in a hand-over-hand manner with a left-hand helical walking path using single-molecule fluorescence techniques such as polTIRF, FIONA, and Parallax.

Filopodia Formation

MyoX has an important role in the formation of filopodia (Berg et al. 2000, Tokuo and Ikebe 2004, Bohil et al. 2006, Tokuo et al. 2007, Zhu et al. 2007), but the mechanism of filopodia induction by myoX is still largely unknown. Using a regulated dimerization technique (Tokuo et al. 2007), it was shown that the motor activity of myoX is itself critical for the initiation of filopodia formation. The regulated dimerization system (Ariad Pharmaceuticals) was based on the human FK506-binding protein variant (FKBP) and its small molecular ligands. An EGFP-tagged myoX construct was produced by fusing FKBP to the C-terminal end of the SAH domain (EGFP-M10MoIQ3SAH-FKBP, Fig. 2a). If two FKBPs are present, the dimer-inducing drug, AP20187, binds to both FKBPs, thus creating a dimer of the target molecule (Fig. 2b). After addition of dimerizer, EGFP-M10MoIQ3SAH-FKBP induced filopodia in transfected cells (Fig. 2b). These results support the following model (Fig. 3): (a) MyoX is present as a dimer and monomer in cells. Monomeric



Myosin X, Fig. 2 Dimerization of myoX is critical for filopodia formation. NIH3T3 cells were transfected with GFP-M10MoIQ3SAH-FKBP (*green*) and replated on fibronectin-coated cover slips with (**b**) or without (**a**) the dimerizer AP20187. *Red*: rhodamine-phalloidin staining



Myosin X, Fig. 3 The filopodia initiation model by dimerization of myoX. (**a**) MyoX is present as a dimer and a monomer in cells. Monomeric (single-headed) myoX does not localize at the edge of lamellipodia. (**b**) Once the dimer is produced, myoX moves to and concentrates at the tip of actin filaments presumably due to its ability to walk toward the barbed end of actin filaments. (**c**) The tips move laterally along the leading edge with actin filaments, and myoX motor activity plays a role in this process. (**d**) The lateral movement of myoX causes the barbed end of the actin filaments to converge, thus producing the base of filopodia, where actin polymerization might induce formation of parallel actin bundles

(single-headed) myoX does not localize at the edge of lamellipodia. (b) Once dimerization occurs, myoX moves to the tip of the actin filaments, presumably as a result of its ability to walk toward the barbed ends of actin filaments. (c) The tips move laterally along the leading edge with actin filaments, and the mechanical activity of myoX plays a role in this process. (d) The clustering of myoX causes convergence of the actin filaments into parallel bundles, thus producing the base of the filopodia. This hypothetical model is elicited from the observation that dimerized tail-less myoX induces microspikes or short unstable filopodia which is thought to be an initial step in filopodia formation. On the other hand, deletion of the MTH4-FERM region abolishes the formation of stable and elongated dorsal filopodia by myoX (Bohil et al. 2006). From these results, it is plausible that monomer-to-dimer transition of the motor and neck region controls initiation, and the tail region controls elongation and stabilization of filopodia formation.

TIRF microscopy was used to clarify the role of the tail domain in the mechanism of myoX-induced filopodia formation (Watanabe et al. 2010). MyoX was recruited to discrete sites at the leading edge where it assembles with exponential kinetics before filopodia extension. MyoX-induced filopodia showed repeated extension–retraction cycles with each extension of 2.4 μm, which was critical to produce long filopodia. FERM domain–deleted myoX moved to the tip as in wild type, but it was transported toward the cell body during filopodia retraction, did not undergo multiple extension–retraction cycles, and failed to produce long filopodia. Deletion of the FERM domain did not change movement at the single-molecule level with the same velocity of approximately 600 nm/s as wild type, suggesting that the myoX in filopodia moves without interacting with the attached membrane via the FERM domain. These

results suggest that the interaction of myoX and substrate-engaged integrin is necessary for phased elongation.

Regulating Molecules and Other Functions

Recent studies implicate myoX in a variety of cellular functions through its interactions with other molecules:

PIP_3: In leukocytes, myoX localizes to phagocytic cups with phosphatidylinositol-3,4,5-triphosphate (PIP_3) in a PI3K-dependent manner. Expression in macrophages of a tail domain of myoX inhibits phagocytosis (Cox et al. 2002). MyoX might provide a molecular link between PI3K and pseudopod extension during phagocytosis.

Ena/VASP: MyoX and VASP bind in vitro and in vivo, colocalize at the tips of filopodia, move together in filopodia, and there is a correlation between the length of filopodia and the concentration of VASP/myoX at the tips of filopodia (Tokuo and Ikebe 2004). These results suggest that myoX transports VASP to the tips of filopodia to support elongation by actin incorporation.

Microtubules: Through its MTH4-FERM domain, MyoX associates with microtubules. Expression of the tail domain or microinjection of anti-myoX antibodies disrupts nuclear anchoring, spindle assembly, and spindle-F-actin association in *Xenopus laevis* (Weber et al. 2004). These results indicate that during meiosis, myoX has a critical role in integrating the F-actin and microtubule cytoskeletons. MyoX is also essential for mitotic-spindle function (Woolner et al. 2008). Interaction with microtubules is also important in osteoclast function (McMichael et al. 2010). MyoX suppression by RNAi or overexpression of dominant-negative myoX (MTH4-FERM) leads to decreased sealing zone perimeter, motility, and resorptive capacity of osteoclasts. These results suggest that myoX plays a role in osteoclast attachment and podosome positioning by direct linkage of actin to the microtubule network.

Integrins: The FERM domain of myoX interacts with an NPXY motif within the cytoplasmic domain of β-integrin (Zhang et al. 2004). Knockdown of myoX results in decreased integrin-mediated cell adhesion to the extracellular matrix, and myoX is responsible for localization of integrins in filopodia. Localization of integrin at filopodial tips and filopodia elongation did not occur with myoX mutants deficient in integrin binding or with a β-integrin mutant deficient in myoX binding. These results suggest that binding of myoX to integrins allows tethering of the filopodial actin filaments to the extracellular matrix for stabilizing the structure of filopodia and elongation.

BMP6: In endothelial cells, myoX is a target gene of bone morphogenetic protein (BMP), and myoX colocalizes with the BMP6 receptor, ALK6, in a BMP6-dependent fashion. Other data indicate that myoX is required to guide endothelial migration toward BMP6 gradients via the regulation of filopodial function and amplification of BMP signals (Pi et al. 2007).

Netrin receptor: The FERM domain of myoX interacts with the cytoplasmic tail of the netrin receptors, DCC (deleted in colorectal cancer) and neogenin (Zhu et al. 2007). Netrins regulate axon path-finding, which is essential for proper wiring in the brain. Cortical explants derived from mouse embryos expressing dominant-negative myoX exhibit reduced neurite outgrowth in response to netrin-1. Inhibition of myoX in embryos causes impaired commissural neuronal axon projections in chicken brain. These results indicate that myoX regulates axon outgrowth and guidance in response to netrins (Zhu et al. 2007).

Using *Xenopus* as a model system to study the function of myoX in neurons, two reports show that knock-down of myoX expression results in retarded migration of cranial neural crest cells (Hwang et al. 2009, Nie et al. 2009). These results suggest that myoX has an essential function in neuronal development in vertebrates.

VE–cadherin: MyoX is directly associated with the VE–cadherin complex via a FERM domain. It colocalizes and moves synchronously with filopodial VE–cadherin. Expression of the FERM domain blocks the transportation of VE–cadherin along actin fibers, resulting in an almost total depletion of VE–cadherin at the cell edge. VE–cadherin trafficking along filopodia by myoX may be a pre-requisite for cell–cell junction formation in endothelial cells (Almagro et al. 2010).

Summary

MyoX functions both as a molecular transporter and a cytoskeletal regulator in cells.

References

Almagro S, Durmort C, Chervin-Petinot A, Heyraud S, Dubois M, Lambert O, et al. The motor protein myosin-X transports VE-cadherin along filopodia to allow the formation of early endothelial cell-cell contacts. Mol Cell Biol. 2010;30(7):1703–17.

Bennett RD, Mauer AS, Strehler EE. Calmodulin-like protein increases filopodia-dependent cell motility via up-regulation of myosin-10. J Biol Chem. 2007;282(5):3205–12.

Berg JS, Cheney RE. Myosin-X is an unconventional myosin that undergoes intrafilopodial motility. Nat Cell Biol. 2002;4(3):246–50.

Berg JS, Derfler BH, Pennisi CM, Corey DP, Cheney RE. Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin. J Cell Sci. 2000;113(Pt 19):3439–51.

Berg JS, Powell BC, Cheney RE. A millennial myosin census. Mol Biol Cell. 2001;12(4):780–94.

Bohil AB, Robertson BW, Cheney RE. Myosin-X is a molecular motor that functions in filopodia formation. Proc Natl Acad Sci USA. 2006;103(33):12411–6.

Cox D, Berg JS, Cammer M, Chinegwundoh JO, Dale BM, Cheney RE, et al. Myosin X is a downstream effector of PI (3)K during phagocytosis. Nat Cell Biol. 2002;4(7):469–77.

Homma K, Saito J, Ikebe R, Ikebe M. Motor function and regulation of myosin X. J Biol Chem. 2001;276(36):34348–54.

Hwang YS, Luo T, Xu Y, Sargent TD. Myosin-X is required for cranial neural crest cell migration in Xenopus laevis. Dev Dyn. 2009;238(10):2522–9.

Kerber ML, Jacobs DT, Campagnola L, Dunn BD, Yin T, Sousa AD, et al. A novel form of motility in filopodia revealed by imaging myosin-X at the single-molecule level. Curr Biol. 2009;19(11):967–73.

Knight PJ, Thirumurugan K, Xu Y, Wang F, Kalverda AP, Stafford 3rd WF, et al. The predicted coiled-coil domain of myosin 10 forms a novel elongated domain that lengthens the head. J Biol Chem. 2005;280(41):34702–8.

McMichael BK, Cheney RE, Lee BS. Myosin X regulates sealing zone patterning in osteoclasts through linkage of podosomes and microtubules. J Biol Chem. 2010;285(13):9506–15.

Nagy S, Ricca BL, Norstrom MF, Courson DS, Brawley CM, Smithback PA, et al. A myosin motor that selects bundled actin for motility. Proc Natl Acad Sci USA. 2008;105(28):9616–20.

Nie S, Kee Y, Bronner-Fraser M. Myosin-X is critical for migratory ability of Xenopus cranial neural crest cells. Dev Biol. 2009;335(1):132–42.

Pi X, Ren R, Kelley R, Zhang C, Moser M, Bohil AB, et al. Sequential roles for myosin-X in BMP6-dependent filopodial extension, migration, and activation of BMP receptors. J Cell Biol. 2007;179(7):1569–82.

Solc CK, Derfler BH, Duyk GM, Corey DP. Molecular cloning of myosins from the bullfrog saccular macula: a candidate for the hair cell adaptation motor. Aud Neurosci. 1994;1:63–75.

Sousa AD, Berg JS, Robertson BW, Meeker RB, Cheney RE. Myo10 in brain: developmental regulation, identification of a headless isoform and dynamics in neurons. J Cell Sci. 2006;119(Pt 1):184–94.

Sun Y, Sato O, Ruhnow F, Arsenault ME, Ikebe M, Goldman YE. Single-molecule stepping and structural dynamics of myosin X. Nat Struct Mol Biol. 2010;17(4):485–91.

Tokuo H, Ikebe M. Myosin X transports Mena/VASP to the tip of filopodia. Biochem Biophys Res Commun. 2004;319(1):214–20.

Tokuo H, Mabuchi K, Ikebe M. The motor activity of myosin-X promotes actin fiber convergence at the cell periphery to initiate filopodia formation. J Cell Biol. 2007;179(2):229–38.

Watanabe TM, Tokuo H, Gonda K, Higuchi H, Ikebe M. Myosin-X induces filopodia by multiple elongation mechanism. J Biol Chem. 2010;285:19605–14.

Weber KL, Sokac AM, Berg JS, Cheney RE, Bement WM. A microtubule-binding myosin required for nuclear anchoring and spindle assembly. Nature. 2004;431(7006):325–9.

Woolner S, O'Brien LL, Wiese C, Bement WM. Myosin-10 and actin filaments are essential for mitotic spindle function. J Cell Biol. 2008;182(1):77–88.

Yonezawa S, Kimura A, Koshiba S, Masaki S, Ono T, Hanai A, et al. Mouse myosin X: molecular architecture and tissue expression as revealed by northern blot and in situ hybridization analyses. Biochem Biophys Res Commun. 2000;271(2):526–33.

Zhang H, Berg JS, Li Z, Wang Y, Lang P, Sousa AD, et al. Myosin-X provides a motor-based link between integrins and the cytoskeleton. Nat Cell Biol. 2004;6(6):523–31.

Zhu XJ, Wang CZ, Dai PG, Xie Y, Song NN, Liu Y, et al. Myosin X regulates netrin receptors and functions in axonal path-finding. Nat Cell Biol. 2007;9(2):184–92.

M

Myosin-10

► Myosin X

Myosins

Lynne M. Coluccio
Boston Biomedical Research Institute,
Watertown, MA, USA

Synonyms

Myosin II, "conventional" myosin; other myosins, "unconventional" myosins

Historical Background

In 1864, Kühne named a protein extracted from nematode muscle in high salt *myosin* and in 1939, Engelhardt and Ljubimowa determined that this protein possessed an ATPase activity. Later studies

would determine that myosin is the major component of muscle thick filaments, and that its cyclic interactions with actin-containing thin filaments are the basis for muscle contraction. It would eventually be renamed myosin II to signify that the molecule is a dimer consisting of two polypeptide chains each with a globular head region and a long α-helical tail (Szent-Györgyi 2004). In 1969, a molecule resembling muscle myosin II was identified in slime mold, showing that myosin is a component of nonmuscle cells, too (Adelman and Taylor 1969). Subsequently, in 1973 a molecule with actin-activated ATPase activity resembling skeletal muscle myosin, although smaller in molecular weight and monomeric rather than filamentous, was isolated from *Acanthamoeba* and named ▶ myosin I (Pollard and Korn 1973). This would be the first of many studies leading to the recognition that myosins constitute a large structurally and functionally diverse family of actin-associated molecular motor proteins that use the energy from ATP hydrolysis to translocate actin filaments usually toward the preferred (or pointed) end of actin filaments (the exception is myosin VI). In fact phylogenetic analyses based on the N-terminal domains of 328 organisms show that there are as many as 35 different myosin classes (Odronitz and Kollmar 2007). After myosin I and myosin II, the different classes that constitute the myosin superfamily are named in order of discovery usually with Roman numerals. The largest class is that of the single-headed class I myosins, which probably most closely resemble the first myosin I, a molecule predicted to have consisted of only a motor domain with a nucleotide-binding site and an actin-binding site. Humans express myosins I, II, III, V, VI, VII, IX, X, XV, XVI, XVIII, and XIX, and mutations in myosins are associated with diseases including cardiac hypertrophic myopathies, deafness, and blindness. As of mid-2010, representatives from only a small subset of the 35 different myosin classes have been either purified from tissues or expressed in vitro and studied with biochemical and biophysical approaches. Some have been expressed in cells exogenously and studied with cell biological methods.

Structure

In general, myosins have an N-terminal head or motor domain consisting of a nucleotide-binding site and an actin-binding site, a neck or light-chain-binding domain (LCBD), which binds calmodulin or calmodulin-like molecules, and a C-terminal tail domain, which is highly diverse among myosins and is the site of regions involved in dimerization as well as membrane and cargo binding. The crystal structures of the motor domains of several different classes of myosins have been solved beginning with that of chicken skeletal muscle myosin II and are similar consisting of four main subdomains: the upper 50 kDa domain, the lower 50 kDa domain, a 7-stranded β-sheet known as the transducer, and the converter region (Holmes 2008; Sweeney and Houdusse 2010). These subdomains are surrounded by several structural elements that coordinate communication among the subunits. The upper and lower 50 kDa domains form a cleft that opens and closes as a function of nucleotide binding. The nucleotide-binding pocket, highly conserved among myosins, kinesins, and G-proteins, comprises the purine-binding, or P, loop on one side and the switch 1/P-loop on the other side. Myosin motor domains have surface loops, including loop 1, near the nucleotide-binding region, and loop 2, near the actin-binding face, that differ in length and overall charge among myosins and when mutated affect a variety of kinetic properties. The C-terminal end of the motor domain is the converter region, which rotates 60° in response to ATP binding and attaches to the LCBD. Several classes of myosins, such as myosins III, IX, XV, XVI, XVIII, and XVa, contain an N-terminal extension, which contains, e.g., a serine/threonine kinase domain in the case of myosin III and several ankyrin repeats in the case of myosin XVI.

The LCBD is an α-helical region containing one or more repeats of ~29 amino acids called IQ domains for the isoleucine and glutamine residues that are normally present. Myosins can have as few as one and as many as 17 IQ domains as in the case of the type 2 myosin from *Phytophthora ramorum*. The LCBD of myosin II binds a regulatory and essential light chain. The light chain of many myosins is the calcium-binding molecule calmodulin. The LCBD serves as a lever arm amplifying small structural changes in the motor domain. Myosin VI has a unique 53-amino-acid insert containing an unusual calmodulin-binding site between the motor domain and LCBD, which is responsible for redirecting the lever arm toward the minus end of actin resulting in the movement of myosin VI on actin in the direction opposite to that of other myosins (Bahloul et al. 2004).

The tail domains of myosins are highly variable in sequence and length and are involved in cargo and membrane binding as well as oligomerization of myosins (Mooseker and Foth 2008). As a consequence of long coiled-coil regions in their tails, myosins II associate to form bipolar filaments. Class V, VI, and X myosins dimerize to form two-headed molecules that move processively, i.e., take several steps along actin filaments without detaching, a property critical for carrying cargo long distances within the cell. In the case of myosin VI, dimerization is induced by binding to its adaptor proteins, optineurin and Dab2, following a conformational change that exposes dimerization sites within the myosin VI monomers (Phichith et al. 2009).

The tails of myosins contain different kinds of protein modules including those involved in protein–protein interactions such as SH3 (Src homology 3), MyTH4 and FERM, dilute, and PDZ-binding; and membrane-binding domains, such as PH domains. Myosins VII, X, XII, and XV are MYTH4-FERM myosins, defined by the presence in the tail of a Myosin Tail Homology 4 (MyTH4) domain followed by a band 4.1, Ezrin, Radixin, Moesin (FERM) domain. MyTH4-FERM myosins are implicated in mediating membrane-cytoskeleton interactions. The FERM domain in *Drosophila* myosin VIIa regulates its activity (Yang et al. 2009). In ATP, the tail of myosin VIIa is bent toward and interacts with the motor domain, but the molecule unfolds in the absence of ATP presumably as a consequence of actin binding by the FERM domain.

Biochemical and Mechanical Properties

Due to the similarity in structure of their motor domains, all myosins are Mg^{2+}-ATPases believed to operate by a common mechanism (El-Mezgueldi and Bagshaw 2008). The interaction of myosin with nucleotide, defined in the 1970s by Bagshaw and Trentham, is described by a seven-step scheme in which ATP binding to myosin occurs in two steps as monitored by tryptophan fluorescence (a binding step and a protein conformational change) followed by reversible ATP hydrolysis. Phosphate (Pi) release and ADP release then occur sequentially, each dissociation event preceded by a protein conformational change. The release of phosphate is activated by the presence of actin. Kinetic differences among myosins are due primarily to differences in rates of Pi and ADP release.

The kinetic interaction of ATP with the mammalian class I myosins when bound to actin is biphasic consisting of both a fast phase and a slow phase. The studies suggest that vertebrate myosins I must undergo a conformational change before ADP release can occur, which is supported by cryo-electron microscopy studies showing that myosins I undergo an ADP-induced conformational change. These data coupled with those from studies done with optical-tweezers transducers showing that the mechanical interaction of myosin I with actin occurs in two parts led to the idea that myosins I are strain-sensitive and complete their cycle only when strain is reduced (Coluccio and Geeves 1999). This notion is supported by single-molecule studies in which the rate of detachment from actin of mammalian Myo1b decreases 75-fold under tension (Laakso et al. 2008). Although first detected in vertebrate myosins I as a consequence of their slow kinetics (Veigel et al. 1999), the interaction with actin in two mechanical parts has now been seen with other myosins.

The ability of myosins to bind and move cargo long distances along actin filaments is crucial for intracellular transport and certain myosins are specially adapted structurally and functionally for this role. Processivity, the ability to take several steps along actin before dissociating, is usually associated with motors that have a high duty ratio, i.e., they spend most of their time attached to actin. Myosin V moves processively along actin filaments because of its high affinity for actin, which ensures that one of its two heads stays associated with the actin filament while the other is detached so that the motor does not diffuse away. Strain on the lead head causes it to stall until the rear head can detach from actin. Processivity of myosin V is also supported by its long LCBD, which allows the heads to bind actin at 36 nm intervals so that the molecule can walk along the longitudinal axis of the actin filament without the viscous drag that would occur if it had to spiral around the filament (Sellers and Weisman 2008). Myosin VI also moves processively (although in the opposite direction), but its step size varies from 25 to 36 nm, and although it carries cargo along the longitudinal axis of the actin filament like myosin V, it can move on a right-handed spiral (Sun et al. 2007).

Single-headed myosins are non-processive motors, which make only one interaction with actin, then detach. In general these myosins have low duty ratios. Clustering of single-headed myosins could make them act as processive motors. Curiously, although Myo9B is monomeric, it can take multiple steps along actin before dissociating; the mechanism is currently unknown (Bähler 2008).

Regulation

Myosins can be regulated by phosphorylation of either the heavy or light chain. Regulation of class I myosins is varied; however, phosphorylation of a single serine or threonine between the ATP- and actin-binding sites in the heavy chain of the *Acanthamoeba* and *Dictyostelium* myosins I regulates their actin-activated Mg^{2+}-ATPase activity and motor activity. Phosphorylation by the myosin I heavy chain kinase, MIHCK, which is a p21-activated kinase (PAK), enhances the ATPase activity 40- to 80-fold (Brzeska and Korn 1996). In vertebrate myosins I, negatively charged glutamate or aspartate is present at the corresponding site, known as the TEDS site after the one-letter codes for the amino acids found at the site, indicating that they are constitutively active (Bement and Mooseker 1995).

The actin-activated Mg^{2+}-ATPase activities of smooth and nonmuscle class II myosins are regulated by phosphorylation of Ser^{19} of the regulatory light chain, which causes unfolding of the molecule. Autophosphorylation of class III myosins leads to a decrease in the affinity for actin (Kambara et al. 2006).

Calcium plays a role in the regulation of some myosins. Although the actin-activated Mg^{2+}-ATPase activity is higher in calcium vs. EGTA, the rate of actin translocation by vertebrate myosins I is less in buffers containing calcium (Coluccio 2008). This inhibition is reversed in the presence of exogenous calmodulin, suggesting that calcium causes calmodulin dissociation from the LCBD thereby compromising its function as a lever arm. In the case of Myo1c, calmodulin dissociation might reveal sites that are then available to interact with receptors on hair cells on the sensory epithelia of the inner ear (Cyr et al. 2002). Calcium indirectly regulates myosin II activity in skeletal muscle by binding to the thin filament-associated protein, troponin, which causes a conformational change in a second thin filament-associated protein, tropomyosin, which shifts position thereby exposing sights on the thin filaments to which myosin heads can bind. The conformation of myosin V in vitro is affected by calcium with it adopting an extended form in calcium, and a folded form in buffers containing no calcium (Wang et al. 2004).

Function

The large number of myosin classes present in eukaryotes correlates with tremendous diversity in cellular function including maintenance of cortical tension, intracellular transport, the extension of actin-containing membrane structures, and cell movement. Class I myosins in single-cell organisms contribute to pseudopod formation, motility, pinocytosis, membrane ruffling, endocytosis, secretion, and cortical tension. In vertebrates, myosins I maintain cortical tension and play specialized roles such as supporting the structure and function of intestinal microvilli, supporting adaptation in hair cells of the inner ear, and mediating the transport of GLUT4-containing vesicles in adipocytes (Coluccio 2008). Membrane tension has been shown to increase in cells overexpressing class I myosins (Nambiar et al. 2009). In skeletal and smooth muscle, myosin II-containing thick filaments slide relative to actin-containing thin filaments to effect muscle contraction (Cremo and Hartshorne 2008, Reggiani and Bottinelli 2008). In *Dictyostelium* amoebae, myosin II is required for cytokinesis, and cells in which expression of myosin II is knocked out cease dividing and become multinucleate (Knecht and Loomis 1987). In mice, each of the three nonmuscle myosin II isoforms is critical to normal organ development (Conti et al. 2008). ▶ Myosin III is expressed primarily in sensory cells including the stereocilia of the hair cells of the inner ear. In hair cells, myosin III transports espin 1 to the tips of stereocilia and supports their elongation (Salles et al. 2009). Class V myosins, found in many different organisms, bind various types of cargo through their tail domains, including melanosomes, endoplasmic reticulum, and secretory vesicles and carry them long distances within cells (Sellers and Weisman 2008). Mutations in myosin V give rise to mutant coat colors in mice because the transport of melanosomes in melanocytes is compromised. In humans, the neurological disease, Griscelli's Syndrome, is associated with mutations in myosin V. The plant-specific class XI myosins, responsible for

intracellular transport in characean algae, are similar to that of class V myosins and translocate actin in vitro at speeds of ~40–60 μm/s (Ito et al. 2007). Class VI myosins are found in membrane ruffles, at the Golgi and in endocytic and exocytic vesicles (Buss and Kendrick-Jones 2008). Multiple binding partners for myosin VI have been identified suggesting that it plays a variety of cellular roles. Snell's Waltzer mice, which lack functional myosin VI, are deaf. In the hair cells of the sensory epithelium of the inner ear, myosin VI is located at the base of stereocilia where it may play a role in assembling and anchoring the stereocilia. Myosin VII is widely expressed, but most of what is known about its cellular role centers on its location in vertebrates in the hair cells of the inner ear, where it is found in both the cell body and the hair bundle; and in the retina, where it is found in the retinal pigment epithelium and rod and cone photoreceptors cells. In humans, mutations in the myosin VII gene cause Usher syndrome type 1, characterized by profound congenital deafness, vestibular dysfunction, and retinitis pigmentosis leading to blindness. Roles for myosin VII in both maintenance of tension and transportation of cargo have been suggested (El-Amraoui et al. 2008). The class IX myosins in vertebrates, Myo9a and Myo9b, are Rho GTPase-activating proteins, which are negative regulators of Rho signaling. In melanoma cells, Myo9b accumulates in lamellipodia, membrane ruffles, and filopodia. Myo9b might control actin polymerization induced by the Rho proteins, cdc42 and Rac, to prevent undesired cell extension (Bähler 2008). ▶ Myosin X, found at the tips of filopodia, binds and transports VASP, which mediates actin assembly along filopodia, and there is a correlation between the length of filopodia and the concentration of VASP and myoX at the tips (Tokuo and Ikebe 2004). Like myosins I, III, VI, and VII, the class XV myosin, myosin XVa, is important in hearing (Boger et al. 2008). In the shaker-2 mouse, a missense mutation in myosin XVa causes deafness and circling behavior. Myosin XVa binds and transfers the scaffolding protein, whirlin, to the tips of stereocilia and is responsible for elongation of hair bundles on the sensory epithelia of the inner ear.

Summary

Myosins are a large and diverse group of actin-associated molecular motor proteins that participate in cytoskeletal functions in nearly all cells.

References

Adelman MR, Taylor EW. Isolation of an actomyosin-like protein complex from slime mold plasmodium and the separation of the complex into actin- and myosin-like fractions. Biochemistry. 1969;8(12):4964–4975.

Bähler M. Class IX myosins. Protein Cell Regul. 2008;7:391–401.

Bahloul A, Chevreux G, Wells AL, Martin D, Nolt J, Yang Z, et al. The unique insert in myosin VI is a structural calcium-calmodulin binding site. Proc Natl Acad Sci USA. 2004;101(14):4787–4792.

Bement WM, Mooseker MS. TEDS rule: a molecular rationale for differential regulation of myosins by phosphorylation of the heavy chain head. Cell Motil Cytoskeleton. 1995;31(2):87–92.

Boger ET, Frolenkov GI, Friedman TB, Belyantseva IA. Myosin XVA. Protein Cell Regul. 2008;7:441–467.

Brzeska H, Korn ED. Regulation of class I and class II myosins by heavy chain phosphorylation. J Biol Chem. 1996;271(29):16983–16986.

Buss F, Kendrick-Jones J. Myosin VI: a multifunctional motor protein. Protein Cell Regul. 2008;7:325–352.

Coluccio LM. Myosin I. Protein Cell Regul. 2008;7:95–124.

Coluccio LM, Geeves MA. Transient kinetic analysis of the 130-kDa myosin I (myr 1 gene product) from rat liver: A myosin I designed for maintenance of tension? J Biol Chem. 1999;274:21575–21580.

Conti MA, Kawamoto S, Adelstein RS. Non-muscle myosin II. Protein Cell Regul. 2008;7:223–264.

Cremo CR, Hartshorne DJ. Smooth-muscle myosin II. Protein Cell Regul. 2008;7:171–222.

Cyr JL, Dumont RA, Gillespie PG. Myosin-1c interacts with hair-cell receptors through its calmodulin-binding IQ domains. J Neurosci. 2002;22(7):2487–2495.

El-Amraoui A, Bahloul A, Petit C. Myosin VII. Protein Cell Regul. 2008;7:353–373.

El-Mezgueldi M, Bagshaw CR. The myosin family: biochemical and kientic properties. Protein Cell Regul. 2008;7:55–93.

Holmes KC. Myosin structure. In: Coluccio LM, editor. Myosins: a superfamily of molecular motors. Dordrecht: Springer; 2008. pp. 35–54.

Ito K, Ikebe M, Kashiyama T, Mogami T, Kon T, Yamamoto K. Kinetic mechanism of the fastest motor protein, Chara myosin. J Biol Chem. 2007;282(27):19534–19545.

Kambara T, Komaba S, Ikebe M. Human myosin III is a motor having an extremely high affinity for actin. J Biol Chem. 2006;281(49):37291–37301.

Knecht DA, Loomis WF. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. Science. 1987;236(4805):1081–1086.

Laakso JM, Lewis JH, Shuman H, Ostap EM. Myosin I can act as a molecular force sensor. Science. 2008;321(5885):133–136.

Mooseker MS, Foth BJ. The structural and functional diversity of the myosin family. Protein Cell Regul. 2008;7:1–34 (Myosins: a superfamily of molecular motors [L.M. Coluccio, ed.]).

Nambiar R, McConnell RE, Tyska MJ. Control of cell membrane tension by myosin-I. Proc Natl Acad Sci USA. 2009;106(29):11972–11977.

Odronitz F, Kollmar M. Drawing the tree of eukaryotic life based on the analysis of 2,269 manually annotated myosins from 328 species. Genome Biol. 2007;8(9):R196.
Phichith D, Travaglia M, Yang Z, Liu X, Zong AB, Safer D, et al. Cargo binding induces dimerization of myosin VI. Proc Natl Acad Sci USA. 2009;106(41):17320–17324.
Pollard TD, Korn ED. *Acanthamoeba* myosin. I. Isolation from *Acanthamoeba castellanii* of an enzyme similar to muscle myosin. J Biol Chem. 1973;248(13):4682–4690.
Reggiani C, Bottinelli R. Myosin II: sarcomeric myosins, the motors of contraction in cardiac and skeletal muscles. Protein Cell Regul. 2008;7:125–170 (Myosins: a superfamily of molecular motors [L.M. Coluccio, ed.]).
Salles FT, Merritt JRC, Manor U, Dougherty GW, Sousa AD, Moore JE, et al. Myosin IIIa boosts elongation of stereocilia by transporting espin 1 to the plus ends of actin filaments. Nat Cell Biol. 2009;11:443–450.
Sellers JR, Weisman LS. Myosin V. Protein Cell Regul. 2008;7:289–323.
Sun Y, Schroeder 3rd HW, Beausang JF, Homma K, Ikebe M, Goldman YE. Myosin VI walks "wiggly" on actin with large and variable tilting. Mol Cell. 2007;28(6):954–964.
Sweeney HL, Houdusse A. Structural and functional insights into the myosin motor mechanism. Annu Rev Biophys. 2010;39:539–557.
Szent-Györgyi AG. The early history of the biochemistry of muscle contraction. J Gen Physiol. 2004;123:631–641.
Tokuo H, Ikebe M. Myosin X transports Mena/VASP to the tip of filopodia. Biochem Biophys Res Commun. 2004;319(1):214–220.
Veigel C, Coluccio LM, Jontes JD, Sparrow JC, Milligan RA, Molloy JE. The motor protein myosin-I produces its working stroke in two steps [see comments]. Nature. 1999;398(6727):530–533.
Wang F, Thirumurugan K, Stafford WF, Hammer 3rd JA, Knight PJ, Sellers JR. Regulated conformation of myosin V. J Biol Chem. 2004;279(4):2333–2336.
Yang Y, Baboolal TG, Siththanandan V, Chen M, Walker ML, Knight PJ, et al. A FERM domain autoregulates *Drosophila* myosin 7a activity. Proc Natl Acad Sci USA. 2009;106(11):4189–41894.

Myristoyl CoA:Protein N-Myristoyltransferase

► NMT (N-Myristoyltransferase)

MYST3

► MOZ and MORF Lysine Acetyltransferases

MYST4

► MOZ and MORF Lysine Acetyltransferases

N

N8 (Human)

▶ TPD52 (Tumor Protein D52)

Natural Cytotoxicity Receptors (NCR)

▶ NK Receptor

Natural Killer Cell Group (NKG)

▶ NK Receptor

NCAM

▶ NCAM1

N-CAM

▶ NCAM1

NCAM1

Rüdiger Horstkorte, Bettina Büttner and Kaya Bork
Institute for Physiological Chemistry, Martin-Luther-University Halle-Wittenberg, Halle(Saale), Germany

Synonyms

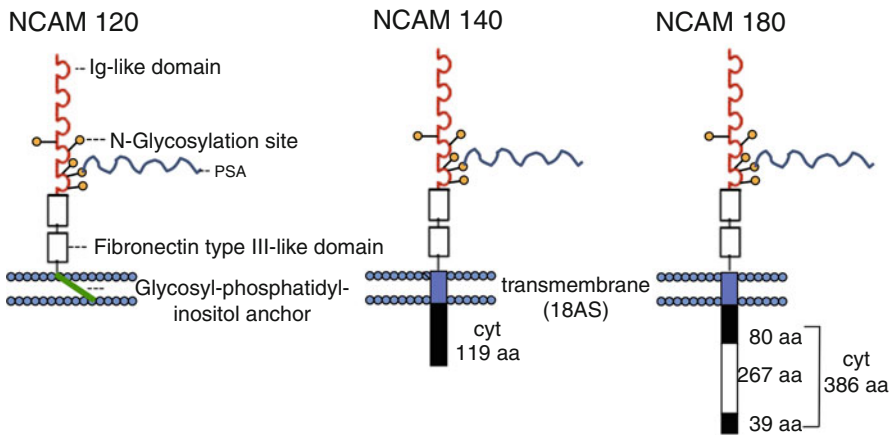
CD56; D2; NCAM; N-CAM

Historical Background

Cell–cell interactions via cell adhesion are the basis for the evolution of all multicellular organisms. The first experiments to understand cell adhesion were performed at the beginning of the last century by Wilson (1907), who dissociated sponges and allowed them to fuse and to reconstitute. Fifty years later, Townes and Holtfreter (1955) demonstrated that dissociated cells from amphibian embryos adhere to form random aggregates of their origin, with ectoderm forming an outer surface layer, endoderm forming a compact central ball, and mesoderm producing a loose array of cells. In 1963, Roger Sperry (1963) proposed that different cells bear distinct cell surface proteins that serve as markers or tags. One of these markers, the Neural Cell Adhesion Molecule (NCAM) was first described in 1974 by Elizabeth Bock (Jacque et al. 1974) and designated as D2 antigen. Three years later, the group of Edelman (Thiery et al. 1977) approved that NCAM mediates cell–cell adhesion and established it as one of the first cell adhesion molecules. When NCAM was sequenced and cloned during the mid-1980s, it became obvious that NCAM belongs to the immunoglobulin superfamily

Please note that there exist a close relative to NCAM, the Neural Cell Adhesion Molecule 2; also called OCAM or RNCAM. The overall similarity to NCAM is 45%. However, this entry is a summary only on the Neural Cell Adhesion Molecule.

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



NCAM1, Fig. 1 Schematic representation of the three major isoforms of NCAM

(Barthels et al. 1987) and that NCAM is not only responsible for static adhesion, but also transmits signals across the cell membrane. With the help of transgenic knockout techniques, Cremer and colleagues could demonstrate 1994 (Cremer et al. 1994) that NCAM is involved in learning and memory.

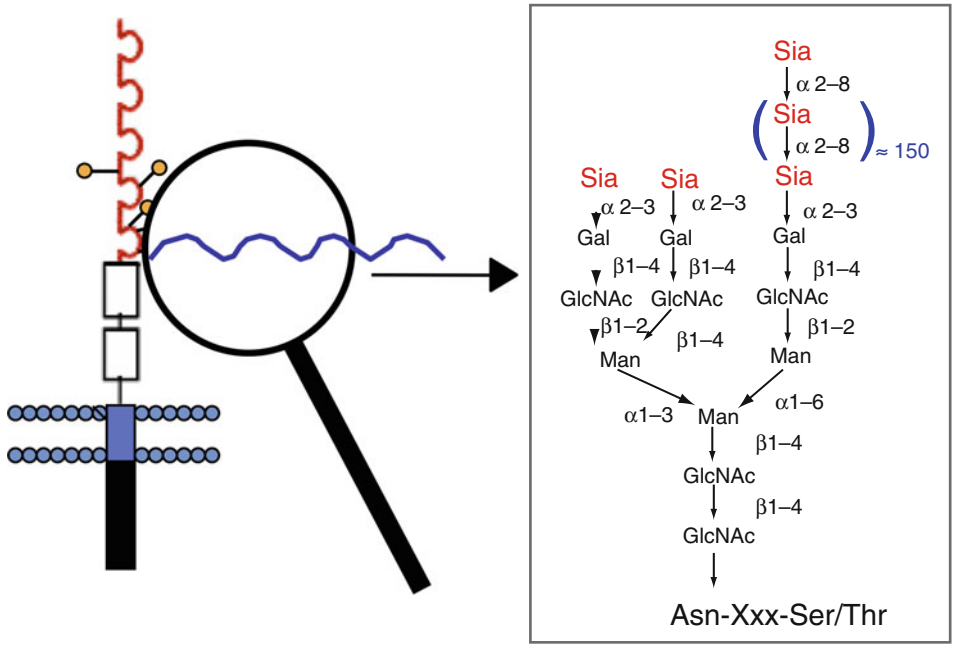
Structure and Function

NCAM is a glycoprotein belonging to the immunoglobulin superfamily. All members of this protein superfamily have in common the presence of at least one immunoglobulin-like domain, which received their name from immunoglobulins (antibodies). There are more than 750 proteins known with immunoglobulin-like domains. An immunoglobulin-like domain usually consists of 70–110 amino acids with a defined secondary structure including one disulfide bridge. NCAM consists of five immunoglobulin domains in its extracellular domain. Furthermore NCAM has two membrane proximal fibronectin type III (F3) homologous repeats. There exist three major isoforms of NCAM, which are generated by alternative splicing from one single gene (Barbas et al. 1988). The isoforms are named according their apparent molecular weight as NCAM120 (gylcosylphosphatidyl inositol anchor = GPI-anchored) or NCAM140 and NCAM180 (transmembrane-anchored). NCAM140 and NCAM180 differ in the intracellular domain in that NCAM180 has an additional insert of 267 amino acids (see Fig. 1). Although identified in the nervous system, NCAM is expressed in many other cell types or tissues, such as muscle cells or immune cells. This is underlined by the fact that NCAM corresponds to CD56 and is a marker of natural killer cells.

NCAM mediates preferentially homophilic NCAM–NCAM interactions, which are Ca^{2+}-independent. The homophilic NCAM–NCAM interactions mediate adhesion between neurons and neurons, neurons and glia cells, and between glia cells and glia cells. NCAM140 is known to be responsible for the fasciculation of axons or neurites and for promotion of axonal regeneration after injury. NCAM-deficient mice have defects in learning and memory and have a smaller brain compared to wild-type animals. Furthermore, the nervous system–specific NCAM180 is thought to stabilize cell–cell contacts at synapses. A recent model of NCAM–NCAM interaction suggests that two NCAMs form dimers on the cell curface (cis-interaction) and that these dimers bind to existing dimers on opposing cells (trans-interaction) via their Ig-domains 2 and 3 in antiparallel orientation ("zipper"-like) (Soroka et al. 2003).

Posttranslational Modification

NCAM is notable for several posttranslational modifications. Like many other proteins involved in signal transduction, NCAM was demonstrated to be phosphorylated. NCAM contains numerous (depending on its isoform) potential phosphorylation sites on Serine or Threonine residues but only one on a Thyrosine residue. In addition, NCAM has five N-glycosylation sites and nearly 30% of its apparent molecular weight is represented by glycans. It is of special interest that NCAM bears a very unique type of glycosylation, namely, polysialylation (Finne et al. 1983).



NCAM1, Fig. 2 Polysialylation of NCAM. Note that polysialylation of NCAM results in the introduction of more than 100 negatively charged sialic acids on a classical Aspargine-linked glycan structure

Polysialylation is characterized by the presence of up to 150 monomers of sialic acid on the outer chains of the N-glycans of the fifth immunoglobulin-like domain (see Fig. 2). Polysialylation on NCAM represents more than 95% of all polysialylation of a specific organism. The addition of polysialic acids introduce one negative charge per sialic acid and alter dramatically the function of NCAM. Polysialylated NCAM is much less adhesive compared to non-polysialylated NCAM. The expression of polysialic acid is strongly regulated. It is high during development and drops during lifetime. Adult mammals (including humans) express high levels of polysialylated NCAM only at sites of plasticity, such as hippocampus, the place of learning and memory.

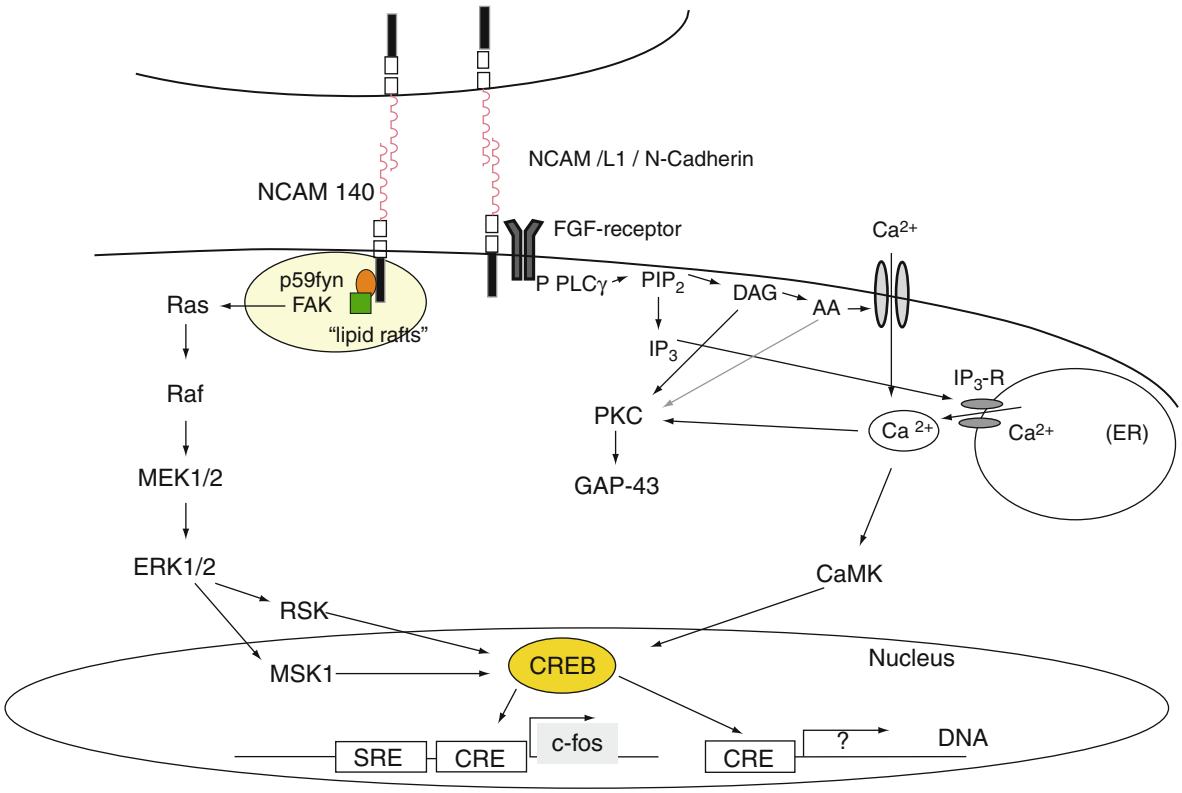
Please note that there are several and sometimes confusing abbreviations for polysialic acid or polysialylated NCAM used in the literature (PSA = polysialic acid; E-NCAM = embryonic NCAM; polySia = polysialic acid).

Signaling

The last two decades of research on NCAM-mediated signal transduction focuses mainly on NCAM-mediated promotion of neurite outgrowth, which will be discussed in the following. One central pathway of the NCAM-mediated signal transduction is the mitogen-activated protein kinase pathway (MAPK pathway). The MAPK pathway can be activated by two mechanisms: First, via the Fibroblast Growth Factor Receptor (FGF-receptor) (Doherty and Walsh 1996) and second via non-receptor kinases such as FAK and Fyn (Beggs et al. 1997).

The Mitogen-Activated Protein Kinase Pathway

It has been demonstrated that NCAM interacts directly with the FGF-receptor on one cell membrane (cis-interaction) via the F3 modules 1 and 2 (Kiselyov et al. 2003). NCAM binding is capable of activating the FGF-receptor upon NCAM–NCAM interaction. The FGF-receptor activation leads to recruitment of the adaptor proteins Shc and Grb-2, which then activate Ras as key enzyme of the MAPK pathway. Final target of the MAPK pathway is the transcription factor ▶ CREB (Jessen et al. 2001), which activates transcription of genes, which are responsible for neurite outgrowth. The NCAM-mediated FGF-receptor activation and neurite outgrowth can be inhibited by ATP, which could be explained by an overlapping ATP and FGF-receptor binding sites in the second F3 module of



NCAM1, Fig. 3 Simplified scheme of the NCAM-mediated signal transduction (For details see text or for review see Maness and Schachner (2007))

NCAM (Skladchikova et al. 1999). However, the also close polysialylation at the Ig module 5 of NCAM is essential for NCAM-dependent neurite outgrowth (Doherty et al. 1990). Another possibility to activate the MAPK pathway is via the recruitment of the non-receptor kinases FAK and Fyn. It has been demonstrated in several studies that NCAM is also associated with the receptor protein tyrosine phosphatase-alpha (RPTP-alpha). NCAM–NACM interaction leads to the activation of RPTP-alpha and to recruitment of Fyn and FAK, which then are responsible for further activation of the MAPK pathway (see Fig. 3).

Phospholipase C and Calcium

NCAM-mediated signaling has been demonstrated to involve an increase in intracellular Ca^{2+}-concentration (Kolkova et al. 2000). Several studies suggested that phospholipase Cγ (PLCγ) is responsible for the NCAM-mediated increase in Ca^{2+}-concentration. Upon NCAM–NCAM interaction, PLCγ cleaves PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG). ▶ IP_3 Receptors binds to its intracellular receptor at the ER-membrane and releases Ca^{2+} from the ER store. Furthermore ▶ arachidonic acid (AA) might be released from DAG by the DAG lipase, which further activates Ca^{2+}-channels at the plasma membrane. All this leads to an increase of intracellular Ca^{2+}. The increased Ca^{2+} binds to calmodulin and this leads to the activation of the Ca^{2+}-calmodulin-dependent protein kinase II. Ca^{2+}-calmodulin-dependent protein kinase II phosphorylates several target proteins, which are involved in neurite outgrowth (see Fig. 3).

Cyclic Adenosine Monophosphate and Cyclic Guanosine Monophosphate

The involvement of the two second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in NCAM signaling has been demonstrated in several studies by the use of specific inhibitors (Shimomura et al. 1998). However, the exact role of the respective heterotrimeric G-proteins (in the case of cAMP) or nitric oxid synthases (in the case of cGMP) is not clear yet.

Summary

NCAM may transmit signals into the cell by several mechanisms. This is, like in many other cases, a complex network of signal transduction pathways. The MAPK pathway seems to be very crucial for NCAM-mediated signal transduction. However, little is known about relative roles of the individual players and further research is necessary to elucidate the total network of NCAM signaling.

References

Barbas JA, Chaix JC, Steinmetz M, Goridis C. Differential splicing and alternative polyadenylation generates distinct N-CAM transcripts and proteins in the mouse. EMBO J. 1988;7:625–32.

Barthels D, Santoni MJ, Wille W, Ruppert C, Chaix JC, Hirsch MR, Fontecilla-Camps JC, Goridis C. Isolation and nucleotide sequence of mouse NCAM cDNA that codes for a Mr 79,000 polypeptide without a membrane-spanning region. EMBO J. 1987;6:907–14.

Beggs HE, Baragona SC, Hemperly JJ, Maness PF. NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). J Biol Chem. 1997;272:8310–9.

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, Brown R, Baldwin S, Kraemer P, Scheff S, et al. Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature. 1994;367:455–9.

Doherty P, Walsh FS. CAM-FGF receptor interactions: a model for axonal growth. Mol Cell Neurosci. 1996;8:99–111.

Doherty P, Cohen J, Walsh FS. Neurite outgrowth in response to transfected N-CAM changes during development and is modulated by polysialic acid. Neuron. 1990;5(2):209–19.

Finne J, Finne U, Deagostini-Bazin H, Goridis C. Occurrence of alpha 2–8 linked polysialosyl units in a neural cell adhesion molecule. Biochem Biophys Res Commun. 1983;112:482–7.

Jacque CM, Jorgensen OS, Bock E. Quantitative studies on the brain specific antigens S-100, GFA, 14-3-2, D1, D2, D3 and C1 in Quaking mouse. FEBS Lett. 1974;49:264–6.

Jessen U, Novitskaya V, Pedersen N, Serup P, Berezin V, Bock E. The transcription factors CREB and c-Fos play key roles in NCAM-mediated neuritogenesis in PC12-E2 cells. J Neurochem. 2001;79:1149–60.

Kiselyov VV, Skladchikova G, Hinsby AM, Jensen PH, Kulahin N, Soroka V, Pedersen N, Tsetlin V, Poulsen FM, Berezin V, Bock E. Structural basis for a direct interaction between FGFR1 and NCAM and evidence for a regulatory role of ATP. Structure. 2003;11(6):691–701.

Kolkova K, Novitskaya V, Pedersen N, Berezin V, Bock E. Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. J Neurosci. 2000;20:2238–46.

Maness PF, Schachner M. Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. Nat Neurosci. 2007;10:19–26.

Shimomura A, Okamoto Y, Hirata Y, Kobayashi M, Kawakami K, Kiuchi K, Wakabayashi T, Hagiwara M. Dominant negative ATF1 blocks cyclic AMP-induced neurite outgrowth in PC12D cells. J Neurochem. 1998;70:1029–34.

Skladchikova G, Ronn LC, Berezin V, Bock E. Extracellular adenosine triphosphate affects neural cell adhesion molecule (NCAM)-mediated cell adhesion and neurite outgrowth. J Neurosci Res. 1999;57(2):207–18.

Soroka V, Kolkova K, Kastrup JS, Diederichs K, Breed J, Kiselyov VV, Poulsen FM, Larsen IK, Welte W, Berezin V, Bock E, Kasper C. Structure and interactions of NCAM Ig1-2-3 suggest a novel zipper mechanism for homophilic adhesion. Structure. 2003;11:1291–301.

Sperry RW. Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc Natl Acad Sci USA. 1963;50:703–10.

Thiery JP, Brackenbury R, Rutishauser U, Edelman GM. Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. J Biol Chem. 1977;252:6841–5.

Townes PL, Holtfreter J. Directed movements and selective adhesion of embryonic amphibian cells. J Exp Zool. 1955;128:53–120.

Wilson HVP. On some phenomena of coalescence and regeneration in sponges. J Exper Zool. 1907;5:245–58.

(Nck-Associated Protein-4 = SOCS7)

► SOCS

NcoA-1

► Steroid Receptor Coactivator Family

NCoA-2

► Steroid Receptor Coactivator Family

Nebula

► Regulator of Calcineurin 1 (RCAN1)

Nek1

▶ NEKs, NIMA-Related Kinases

Nek10

▶ NEKs, NIMA-Related Kinases

Nek11

▶ NEKs, NIMA-Related Kinases

Nek2

▶ NEKs, NIMA-Related Kinases

Nek3

▶ NEKs, NIMA-Related Kinases

Nek4

▶ NEKs, NIMA-Related Kinases

Nek5

▶ NEKs, NIMA-Related Kinases

Nek6

▶ NEKs, NIMA-Related Kinases

Nek7

▶ NEKs, NIMA-Related Kinases

Nek8

▶ NEKs, NIMA-Related Kinases

Nek9/Nercc1

▶ NEKs, NIMA-Related Kinases

NEKs, NIMA-Related Kinases

Navdeep Sahota, Sarah Sabir, Laura O'Regan, Joelle Blot, Detina Zalli, Joanne Baxter, Giancarlo Barone and Andrew Fry
Department of Biochemistry, University of Leicester, Leicester, UK

Synonyms

Nek1; Nek2; Nek3; Nek4; Nek5; Nek6; Nek7; Nek8; Nek9/Nercc1; Nek10; Nek11

Historical Background

The NIMA-related kinase, or "Nek," family constitutes approximately 2% of all human kinases. They are related in sequence, as their name suggests, to NIMA (699 residues, 80 kDa), a serine/threonine protein kinase present in the filamentous fungus, *Aspergillus nidulans*. Ron Morris identified the gene, nimA, through analysis of a temperature-sensitive loss-of-function mutant that was never in mitosis (nim) when cells were incubated at the restrictive temperature (Morris 1975). Loss of NIMA activity led to G2 arrest, while overexpression of NIMA drove cells into a premature mitosis from any point in the cell cycle (Oakley and Morris 1983; Osmani et al. 1988, 1991). Mechanistically, NIMA is likely to regulate multiple aspects of mitotic entry, with evidence that its activity is required for nuclear pore disassembly, relocalization of the master regulator, cdc2-cyclin B, to the nucleus and spindle pole body (SPB), and chromatin condensation. NIMA is subsequently degraded in

an anaphase-promoting complex/cyclosome (APC/C)-dependent manner and this is necessary for mitotic exit (reviewed in O'Connell et al. 2003; O'Regan et al. 2007).

Aspergillus cells are syncytial and undertake a semi-closed mitosis. It may therefore have evolved control mechanisms that are specific for this form of cell division. However, an early suggestion that NIMA-related kinases might be conserved in other eukaryotes came from expressing *Aspergillus* NIMA in cells from diverse species, including humans, and observing cell cycle defects (Lu and Hunter 1995; O'Connell et al. 1994). With the complete sequencing of many genomes, it is now clear that kinases related to NIMA by sequence are indeed present in most eukaryotes (Parker et al. 2007). However, so far, only the NIM-1 protein from the highly related filamentous fungus, *Neurospora crassa*, has been proven to be a functional homologue of NIMA capable of rescuing an *Aspergillus nimA* mutant suggesting the possibility of functional divergence. Indeed, the budding yeast, *Saccharomyces cerevisiae*, and fission yeast, *Schizosaccharomyces pombe*, each have one NIMA-related kinase in their genome, Kin3 and Fin1, respectively, but these are not essential for mitotic entry. However, careful studies revealed that Fin1 does contribute to the timing of mitotic onset through regulating the localization of the polo-like kinase, Plo1, to the SPB, which in turn promotes activation of cdc2-cyclin B (Grallert and Hagan 2002). Fin1 also contributes to mitotic spindle formation and mitotic exit (Grallert and Hagan 2002; Grallert et al. 2004). Thus, Neks from different species do play roles in mitotic progression.

Surprisingly, some lower eukaryotes have many genes encoding Nek kinases. For example, the unicellular organisms *Chlamydomonas* and *Tetrahymena* have 10 and 39 Nek genes, respectively. The key to this expansion of Nek genes appears to lie in an alternative non-mitotic function for Nek kinases, that is, in ciliogenesis. *Chlamydomonas* produce two elongated cilia, or flagella, that allow the organism to swim in response to environmental stimuli. To date, only two of the *Chlamydomonas* Nek genes have been studied in depth, Fa2p and Cnk2p, but loss of either protein affects both flagella disassembly and cell cycle progression (Bradley and Quarmby 2005; Mahjoub et al. 2002). Meanwhile, *Tetrahymena* has hundreds of cilia that fall into different classes depending on their location and length, and all of the Neks tested so far in this organism localize to cilia and regulate cilia length (Wloga et al. 2006).

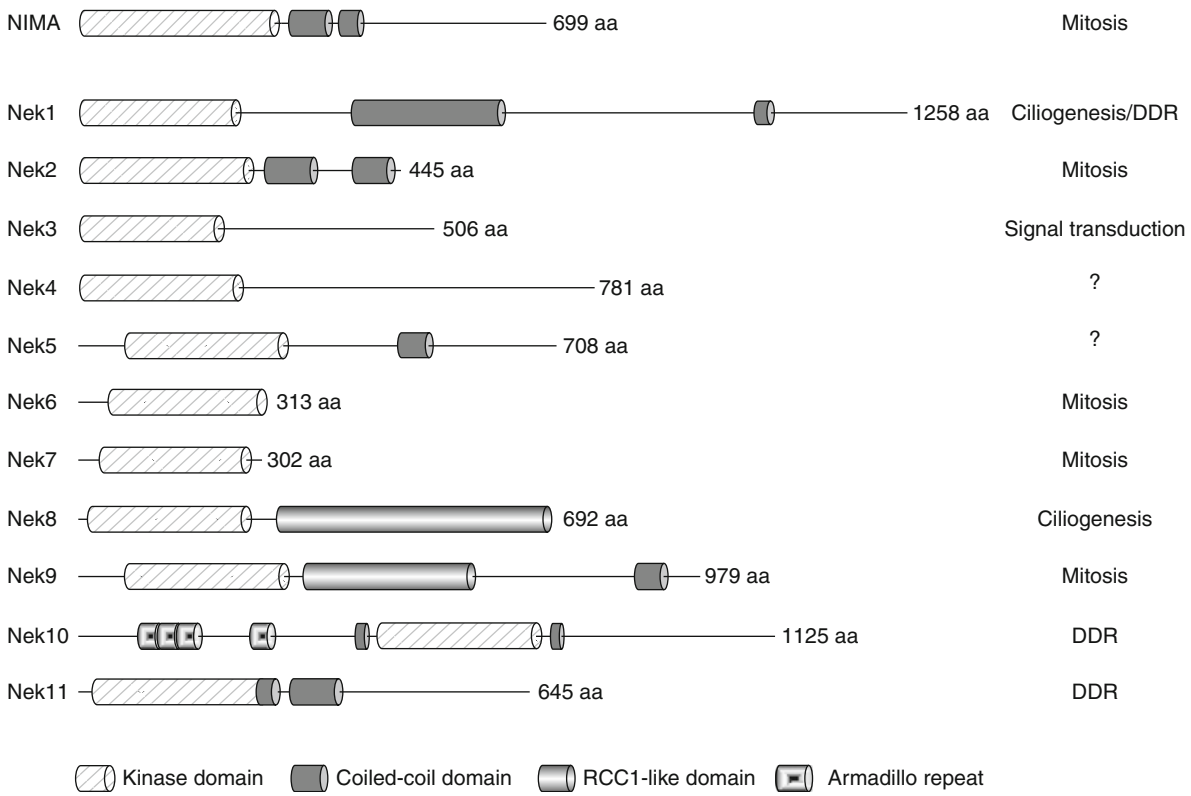
The human NIMA-related kinase family consists of 11 proteins, named Nek1 to Nek11, that are encoded by distinct genes (Fig. 1). Apart from Nek10, these share a common protein domain structure with an N-terminal catalytic kinase domain, containing all the signature motifs of a serine/threonine kinase, and a C-terminal regulatory domain that is highly variable in length and sequence. These differences in the non-catalytic regions contribute to the distinct patterns of expression, localization, activation, and regulation that are seen across this family. Functionally, though, studies performed across many systems including humans, support the hypothesis that the majority of Neks contribute in one way or another to cell cycle progression and/or ciliogenesis (O'Connell et al. 2003; O'Regan et al. 2007; Quarmby and Mahjoub 2005). Thus, altered expression or mutation of Neks can interfere with these key processes, implicating them in both human cancer and inherited ciliopathies.

Specifically, research on the human proteins has demonstrated a role for Nek2, Nek6, Nek7, and Nek9 in mitotic regulation, Nek10 and Nek11 in the DNA damage response, Nek1 and Nek8 in ciliogenesis, and Nek3 in signal transduction (Fig. 2). At the time of writing, little is known about the function of Nek4 and Nek5. A brief summary of findings to date on the regulation and function of each mammalian Nek is presented below.

Mitotic Neks: Nek2, Nek6, Nek7, and Nek9

Of all the human Nek kinases, **Nek2** is the most closely related by sequence to NIMA being 48% identical within the catalytic domain. For this reason, it is to date the most well-studied member of this family. It also shares a number of other important properties with NIMA, including cell cycle–dependent expression, APC/C-dependent degradation and localization to the centrosome, the higher eukaryotic microtubule organizing center and equivalent of the fungal SPB (Fry 2002; Hayward and Fry 2006). However, like the yeast Neks and unlike *Aspergillus* NIMA, human Nek2, as far as one can tell, is not essential for mitotic entry.

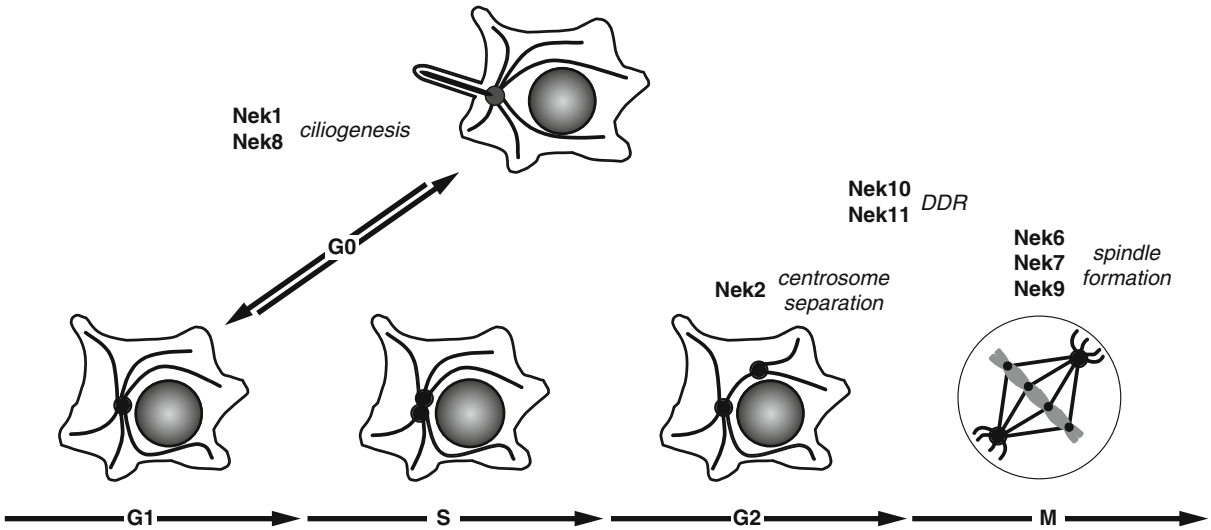
Nek2 is a ubiquitous protein with at least three splice variants, Nek2A (445 residues; 51 kDa), Nek2B (384 residues; 44 kDa), and Nek2C (437



NEKs, NIMA-Related Kinases, Fig. 1 *The human NIMA-related kinase family*. The schematic diagram shows the domain organization of the 11 human NIMA-related kinases, Nek1 to Nek11, below that of the *Aspergillus* NIMA kinase. These generally have an N-terminal catalytic domain followed by a C-terminal non-catalytic region containing potential regulatory motifs. Several Neks have putative coiled-coil sequences; in the case of Nek2 the first of these is an atypical leucine zipper that promotes dimerization, whereas the second has been identified as a SARAH domain that mediates interaction with Hippo pathway components. The length of each kinase is indicated (amino acid number) together with its best understood function

residues; 50 kDa; also called Nek2A-T). These all share the N-terminal kinase domain followed by a leucine zipper coiled-coil motif that promotes dimerization and activation. Nek2A then contains a second coiled-coil motif at its C-terminus. Nek2B is somewhat shorter than Nek2A and lacks the second coiled-coil, whereas Nek2C is identical to Nek2A apart from missing an 8 residue sequence that lies between the leucine zipper and C-terminal coiled-coil. The relative expression of the three variants differs depending on cell type and developmental stage, although generally Nek2A appears to be the predominant isoform. The major function of Nek2A is to regulate centrosome organization through the cell cycle, as described below. Similarly, Nek2B is required for assembly and maintenance of centrosome structure and this may be particularly important during early embryonic development as, in *Xenopus*, it is the only variant expressed at this stage (Uto and Sagata 2000). Interestingly, a role for Nek2B has been proposed in mitotic exit in human cells where its depletion leads to cytokinesis failure (Fletcher et al. 2005). The 8 residue deletion that leads to Nek2C creates a functional nuclear localization signal not present in Nek2A or Nek2B, raising the possibility of nuclear-specific functions for this variant (Wu et al. 2007).

Overall, Nek2 expression and activity are regulated in a cell cycle–dependent manner (Fry et al. 1995; Schultz et al. 1994). The protein is almost undetectable during G1 but accumulates abruptly at the G1/S transition and remains high until late G2. This is a combined result of transcriptional repression and



NEKs, NIMA-Related Kinases, Fig. 2 *Functions of NIMA-related kinases in the cell cycle*. The majority of human Neks play roles in cell cycle progression. A schematic view of cells progressing through the cell cycle with the point at which the Neks act and their best understood function is indicated. However, it should be stressed that each of these Neks is likely to have multiple functions beyond those indicated, for example, Nek6 and Nek7 are also implicated in nuclear pore complex disassembly and cytokinesis, while Nek1 may have functions in the DDR as well as ciliogenesis. The roles of Nek4 and Nek5 remain unknown at the time of writing, while Nek3 is involved in prolactin-mediated cell migration

APC/C-mediated degradation in G1 and transcriptional upregulation in S/G2 (Hayward and Fry 2006). However, Nek2A activity is further regulated by a number of key structural and sequence-specific features of the kinase. The N-terminal kinase domain contains a number of sites which undergo autophosphorylation upon dimerization through the leucine zipper (Rellos et al. 2007). It is likely that phosphorylation at least at some of these sites, particularly those in the activation loop, is required for kinase activation. The C-terminal non-catalytic domain also contains autophosphorylation sites, although the functions of these remain unclear. The C-terminal coiled-coil motif has recently been defined as a putative SARAH domain, which enables interaction with the Hippo pathway proteins, hSav1 and the Mst2 kinase (Mardin et al. 2010). Mst2 acts as an upstream activator of Nek2, phosphorylating sites in the C-terminal region that regulate localization of Nek2 to the centrosome. The C-terminal domain also contains a site that mediates direct interaction of Nek2 with the phosphatase, PP1 (Helps et al. 2000). PP1 negatively regulates Nek2 through dephosphorylation, while Nek2 may be able to inhibit PP1 by phosphorylation. This creates a very sensitive bistable switch that allows rapid Nek2 activation, once PP1 activity starts to decrease at the onset of mitosis (Eto et al. 2002). Nek2 is also negatively regulated by the focal adhesion scaffolding protein, HEF1, although the mechanism is not known (Pugacheva and Golemis 2005). Finally, the C-terminal domain of Nek2 contains two destruction motifs that target it for APC/C-mediated degradation in early mitosis (Hayes et al. 2006).

Nek2 localizes to the centrosome throughout the cell cycle (Fry et al. 1998b). An additional fraction is present in the cytoplasm where it colocalizes with and is trafficked along microtubules (Hames et al. 2005). This localization is dependent on the region between the leucine zipper and SARAH domain that encompasses sites phosphorylated by Mst2 (Hames et al. 2005; Mardin et al. 2010). The primary role of Nek2 at the centrosome is as a regulator of centrosome cohesion. Overexpression of active Nek2 induces premature centrosome splitting during interphase (Fry et al. 1998b), while expression of inactive Nek2 or depletion by RNAi inhibits centrosome separation and promotes monopolar spindle formation (Faragher and Fry 2003; Fletcher et al. 2005; Mardin et al. 2010). Nek2 promotes loss of cohesion or "disjunction" of centrosomes through phosphorylation of C-Nap1 and,

possibly rootletin and ▶ β-catenin (Bahmanyar et al. 2008; Fry et al. 1998a). These proteins, together with Cep68, form a flexible linker structure that extends between the proximal ends of the parental centrioles and which must be dismantled at the onset of mitosis to allow centrosome separation and spindle formation to occur. In addition to the role of Nek2 in centrosome separation, Nek2 has been implicated in regulating the microtubule organizing capacity of the centrosome through phosphorylation of Nlp and centrobin, chromatin condensation through phosphorylation of HMGA2, spindle checkpoint signaling through phosphorylation of Hec1, and nuclear pore complex disassembly through phosphorylation of Nup98 (Laurell et al. 2011; O'Regan et al. 2007).

Nek9 is a 979 residue protein of 113 kDa that, following its N-terminal catalytic domain, has a C-terminal regulatory region comprising a central Regulator of Chromosome Condensation 1 (RCC1)-like domain and a C-terminal coiled-coil motif. It is expressed throughout the cell cycle, but becomes phosphorylated and activated specifically in mitosis (Roig et al. 2002). Nek9 is subject to complex but, as yet, poorly understood regulation. It is thought that during interphase Nek9 adopts an autoinhibited conformation with the RCC1-like domain blocking access to the catalytic site. However, at the G2/M transition, a number of events lead to release of this conformation and activation of the kinase. These are likely to include dimerization, autophosphorylation (especially on T210 in the activation loop), and, potentially, activation by upstream kinases such as Cdk1 and Plk1 (Roig et al. 2002; Bertran et al. 2011). In terms of localization, Nek9 is mainly cytoplasmic, although it can also be found in the nucleus. However, its activated form appears to be specifically concentrated on spindle poles during mitosis (Roig et al. 2005).

Early functional studies on Nek9 by overexpression of wild-type or mutant constructs, or by inhibition through antibody microinjection, led to the hypothesis that Nek9 activity contributes to mitotic spindle formation (Roig et al. 2002). How exactly it does this remains unclear, but Nek9 can interact with the γ-tubulin ring complex (γ-TuRC) that nucleates microtubules both from the centrosome and within the spindle (Roig et al. 2005). Hence, one is tempted to speculate that Nek9 directly contributes to microtubule nucleation in mitosis. However, depletion of Nek9 from *Xenopus* egg extracts prevents the formation of spindles via either the centrosome- or chromatin-mediated pathways (Roig et al. 2005). This suggests that Nek9 function is unlikely to be restricted to regulating the microtubule nucleating activity of γ-tubulin. Nek9 also interacts with BICD2, a protein associated with microtubule-dependent motor proteins, raising the possibility that BICD2 might target Nek9 to microtubules, as well as potentially being a substrate (Holland et al. 2002). Furthermore, the presence of the RCC1-like domain, together with the demonstration that the ▶ Ran GTPase can bind to Nek9, points to a potential role in Ran-mediated spindle formation (Roig et al. 2002). Some reports suggest that Nek9 could have functions outside of mitosis. For example, Nek9 was reported to associate with the FACT complex, a chromatin modifying complex involved in replication and transcription (Tan and Lee 2004). This could explain the presence of a nuclear fraction of Nek9 in interphase and, although not consistent with the timing of bulk Nek9 activation, it is possible that the FACT complex activates a restricted pool of Nek9 in the nucleus, which in turn might be inhibited by the adenovirus E1A protein.

One major route through which Nek9 almost certainly regulates mitotic spindle organization is through interaction, phosphorylation, and activation of two other NIMA-related kinases, Nek6 and Nek7. Indeed, Nek9 was first identified through its association with Nek6 (Roig et al. 2002). Subsequently, Nek9 was shown to phosphorylate sites within the activation loop of Nek6 and this, together with the high degree of similarity between Nek6 and Nek7, led to the proposal that these three Neks form a mitotic cascade in which Nek9 acts upstream of Nek6 and Nek7 (Belham et al. 2003). Lately, it has been demonstrated that Nek9 may also activate Nek7, and by analogy Nek6, through an allosteric mechanism independent of phosphorylation (Richards et al. 2009).

Nek6 and **Nek7** are the smallest family members being only 313 (36 kDa) and 302 (35 kDa) residues, respectively. As a result they comprise little more than a catalytic domain with only a short (30–40 residue) N-terminal extension. By sequence, they are highly related to each other, sharing 86% amino acid identity within the catalytic domain, although the N-termini of the two proteins are not conserved. Often considered as a pair because of their similarity, both Nek6 and Nek7 are, like their upstream activator Nek9, cell cycle

regulated with maximal activity in mitosis. Moreover, while they give slightly different localization patterns, with Nek6 weakly associated to spindle fibers and Nek7 more concentrated on spindle poles, RNAi depletion experiments demonstrate that they are both essential not only for assembly of a robust mitotic spindle, but also potentially for completion of cytokinesis and cell abscission (Kim et al. 2007; O'Regan and Fry 2009; Yin et al. 2003; Yissachar et al. 2006). In support of a late mitotic role for these kinases, Nek6 activation, judged with a phosphospecific antibody against a key activation loop residue, peaks at the time of cytokinesis (Rapley et al. 2008), while mouse embryonic fibroblasts derived from Nek7$^{-/-}$ embryos show defects indicative of cytokinesis failure (Salem et al. 2010).

How these kinases regulate spindle formation and cytokinesis remains to be defined. However, despite their sequence similarity and requirement for both events, it is likely that their respective roles and substrates differ. Systems approaches have identified a large number of putative interacting partners and substrates for Nek6 (Ewing et al. 2007; Vaz Meirelles et al. 2010). These include proteins involved in chromatin condensation, microtubule binding, and nuclear pore complex organization. On this basis, Nek6 has been proposed to be a high confidence hub kinase with an expansive network of substrates involved in diverse cellular processes. Moreover, a number of these interactions depend on the N-terminal extension of Nek6 explaining why Nek6 and Nek7 may target different proteins (Vaz Meirelles et al. 2010). However, to date, the only substrates that have been studied in any detail for Nek6 and Nek7 are the kinesin motor, Eg5, which is phosphorylated by Nek6 promoting spindle pole separation (Rapley et al. 2008), and the nuclear pore complex component, Nup98, whose combined phosphorylation by Cdk1 and potentially multiple Neks, including Nek2, Nek6, and Nek7, promotes nuclear pore complex disassembly upon mitotic entry (Laurell et al. 2011).

Checkpoint Neks: Nek10 and Nek11

Nek10 is a protein of 1,125 residues in length (129 kDa) that is unique in this family, in that unlike the other Neks, Nek10 has its catalytic domain in the center of the protein with long N- and C-terminal non-catalytic regions. The catalytic domain is flanked by two coiled-coil motifs, while in addition the N-terminal region contains four armadillo repeats. Nek10 is one of the least characterized members of the family at the present time with, for example, no data yet on its localization. However, the first report into its function has placed Nek10 within the G2/M DNA damage checkpoint, suggesting that Nek10 is required for Erk1/2 activation in response to UV-induced damage (Moniz and Stambolic 2011). In unperturbed cells, Nek10 forms a trimeric complex, interacting with Mek1 via ▶ Raf-1. In response to UV treatment, Mek1 undergoes autophosphorylation and activation in a Nek10-dependent manner; this leads to phosphorylation of Erk1/2 and G2/M arrest. Importantly, though, UV treatment increases neither association of Nek10 with Raf-1 and Mek1, nor Nek10 activity, and there is no evidence that Nek10 directly phosphorylates Raf-1 or Mek1. Hence, it seems likely that other factors or events contribute to the mechanism by which Nek10 modulates Mek1 signaling.

Nek11 exists as at least four splice variants: Nek11 Long (Nek11L; 645 residues, 74 kDa), Nek11 Short (Nek11S; 470 residues, 54 kDa), and two isoforms that are present in databases but have yet to be reported, Nek11C (482 residues, 55 kDa) and Nek11D (599 residues, 69 kDa). Expression of at least the Nek11L isoform is cell cycle regulated being highest from S through to the G2/M phases of the cell cycle (Noguchi et al. 2002). Localization studies revealed that Nek11 localizes to the nucleus, and possibly nucleolus, in interphase cells, as well as spindle microtubules in prometaphase and metaphase cells (Noguchi et al. 2002, 2004). It was also suggested that Nek11 might be phosphorylated by the Nek2 kinase, converting Nek11 into an active conformation (Noguchi et al. 2004); however, this has yet to be verified. More convincingly, Nek11 is implicated in the DNA damage response (DDR). Nek11 activity is increased in response to stalled DNA replication and genotoxic stresses, such as ionizing radiation (IR), and this activation is blocked by caffeine, an inhibitor of the DDR kinases, ATM and ATR (Melixetian et al. 2009; Noguchi et al. 2002). The role of Nek11 in the G2/M DNA damage checkpoint appears to be a central one. Upon DNA damage, ATM and ATR phosphorylate and activate Chk1; this in turn activates Nek11 by phosphorylation of Ser273 (Melixetian et al. 2009).

Both Chk1 and Nek11 then phosphorylate Cdc25A on residues that promote binding of the β-TrCP E3 ubiquitin ligase. Ultimately, this results in the proteasomal degradation of Cdc25A arresting the cell in G2. While this model is highly attractive, it has been argued that casein kinase 1, and not Nek11, is the major kinase that regulates the phospho-dependent recruitment of β-TrCP. It is also worth noting that, in *Xenopus*, Erk1/2 can target Cdc25A for degradation through the β-TrCP pathway following genotoxic stress. Clearly, further work is required to determine the relative importance of these kinases in mediating G2/M arrest in response to different forms of DNA damage.

Ciliary Neks: Nek1 and Nek8

Nek1 was the first Nek to be identified in mammals and is also the largest member of the family, being composed of 1,258 residues (145 kDa). It was initially reported to have dual serine-threonine and tyrosine kinase activity in vitro, although this is unlikely to be the case in vivo (Letwin et al. 1992). The first clue to its function came almost a decade later when, completely unexpectedly, mutations of the gene encoding Nek1 were found to be causative in two mouse models for polycystic kidney disease (PKD), named *kat* (for kidney, anemia, testis) and kat^{2J} (Upadhya et al. 2000; Vogler et al. 1999). Cystic kidney diseases are now known to be common hallmarks of ciliopathies, whereby the underlying defect is in the formation or function of the primary cilium. Indeed, this was the first indication that Nek kinases may have some role in ciliogenesis. Since then, Nek1 has been localized to the primary cilia in a number of different cell types (Mahjoub et al. 2005; Shalom et al. 2008; White and Quarmby 2008). Here, it is proposed to negatively regulate ciliogenesis, as overexpression of wild-type and certain truncated forms of Nek1 inhibit ciliogenesis, whereas overexpression of mutants predicted to be catalytically inactive do not (White and Quarmby 2008). Interestingly, Nek1 may also be involved in cell cycle control and, specifically, the DDR. Like Nek11, Nek1 activity is elevated in response to IR and Nek1-deficient cells are sensitive to DNA damage (Polci et al. 2004). Furthermore, although to date no *bona fide* substrates have been identified for Nek1, a number of proteins involved in DNA double-strand break repair were found as Nek1 binding partners (Surpili et al. 2003).

Nek8 is a smaller protein than Nek1, being 692 residues in length (80 kDa). Interestingly, Nek8 shares a very similar domain organization to Nek9, with Nek8 also having an RCC1-like domain. On this basis, one might expect that Nek8 would have a role in mitosis. However, subsequent to identification of the mouse Nek1 PKD model, a missense mutation in the RCC1-like domain of Nek8 was identified in the *jck* mouse model of autosomal recessive juvenile PKD (Liu et al. 2002). Consistent with this, Nek8 was also found to localize to the primary cilia (Quarmby and Mahjoub 2005; Sohara et al. 2008). In fact, Nek8 concentrates in the proximal region of the cilia, known as the inversin compartment, a localization that is dependent on the inversin protein (Shiba et al. 2010). Inversin is a protein encoded by a gene that is mutated in the human childhood kidney disorder, nephronophthisis (NPHP). This disease is a typical ciliopathy with at least nine candidate Nphp genes identified so far. Importantly, Nek8 turns out to be Nphp9, thus implicating the Neks in human inherited disease for the first time (Otto et al. 2008). Mutations in Nek8 found in NPHP patients lead to loss of Nek8 localization from the cilia (Trapp et al. 2008). Nek8 has also been found to interact with ▶ polycystin-2 (PC-2), a causative gene for human autosomal dominant PKD (ADPKD), with abnormal phosphorylation of PC-2 detected in cells from the *jck* mouse (Sohara et al. 2008). To date, though, much still remains to be learnt about the cellular basis of renal cyst formation. Thus, it remains unclear whether defects in Nek1 and Nek8 cause cystic kidney disease directly, by interfering with the structure of the primary cilium itself, or more indirectly, by abrogating cilia-dependent signaling. It is also formally possible that these Neks somehow relay signals between the cilium, at the time when the cell is quiescent, and the mitotic spindle, once it reenters the cell cycle.

Signal Transduction Neks: Nek3

In contrast to most other Neks, there is no clear indication that **Nek3** (506 residues, 58 kDa) is a cell cycle–dependent kinase with conflicting reports over whether Nek3 expression in elevated in dividing or quiescent cells. Localization studies indicate that Nek3 is

predominantly cytoplasmic, with no evidence to date that Nek3 associates with centrosomes (Tanaka and Nigg 1999). Unexpectedly, however, yeast two-hybrid and co-immunoprecipitation studies found a direct interaction between Nek3 and members of the ► Vav family of guanine nucleotide exchange factors that was enhanced in response to signaling from the prolactin receptor (Miller et al. 2005). These studies suggested that prolactin receptor stimulation induces Nek3 kinase activity causing Vav2 to interact with the kinase domain of Nek3 and become phosphorylated. Phosphorylated Vav proteins then activate downstream signaling targets involved in tumor progression. Indeed, overexpression of Nek3 potentiated prolactin-mediated cytoskeletal reorganization of cells; however, if Nek3 was depleted then cytoskeletal reorganization was attenuated, as was cell migration and invasion. This is therefore the first report of a Nek kinase being involved in growth-related signaling events. However, Nek3 has also been reported to more directly regulate cytoskeletal dynamics in neurons by altering levels of acetylated tubulin, thus raising the possibility of a role for Nek3 in neuronal disorders (Chang et al. 2009).

Nek4 and Nek5

Little research has been carried out on **Nek4** (781 residues, 90 kDa). However, a recent study implies that this kinase might also play a role in microtubule regulation, as changes in Nek4 expression led to altered sensitivity of cells to microtubule poisons (Doles and Hemann 2010). There is no specific published data yet on **Nek5** (708 residues, 81 kDa). Intriguingly, though, Nek5 was identified in a microarray analysis of FOXJ1 target genes. This transcription factor governs motile cilia assembly by regulating genes involved in cilia biogenesis and function, suggesting that Nek5 may contribute to ciliogenesis in multiciliated cells.

Nek Kinases and Cancer

There is increasing evidence implicating Nek kinases in cancer. Most commonly, this involves upregulated expression, although a few rare mutations have been identified in cancer genome screens. However, whether elevated expression or mutation contributes to the transformed phenotype remains an important but currently unanswered question. The current data on Nek kinases and cancer can be summarized as follows. Nek1 may be required to protect genome stability as cells deficient in Nek1 form tumors in mice (Chen et al. 2011); this would be consistent with it having a role in the DNA damage checkpoint. Nek2 expression levels are frequently upregulated in a wide range of cancer cell lines and primary tumors, and the Nek2 gene has been reported to be amplified in breast and gastric cancers (Hayward and Fry 2006; Kokuryo et al. 2007; Suzuki et al. 2010; Tsunoda et al. 2009). Nek3 is enriched in breast carcinomas (Miller et al. 2005, 2007), while the Nek3 gene is located in a chromosomal region that is frequently deleted in several types of human cancer and a polymorphism in the Nek3 gene is linked to prostate cancer. Nek4 appears to be frequently deleted in lung cancer, although interestingly this might make tumors more sensitive to particular microtubule poisons (Doles and Hemann 2010). The Nek6 gene is found at a locus for which loss of heterozygosity is associated with several cancers, while, like Nek2, the expression of Nek6 is widely elevated in cancer cell lines and tumors (Nassirpour et al. 2010). Nek7 is also overexpressed in breast, colon, and larynx cancers (Capra et al. 2006), and mutations in Nek7 have been identified in lung and ovarian cancers. Nek8, although primarily associated with ciliogenesis, is upregulated in some breast tumors. Although this could suggest that Nek8 has alternative functions in cell cycle control, a link between cilia-dependent signaling and tumorigenesis is beginning to emerge. A few point mutations have been identified in Nek9, although their relevance remains unclear, and a link between Nek9 expression and human cancers is not yet well established. On the other hand, Nek10 has been identified as a candidate breast cancer susceptibility gene and mutations of this kinase have been reported in several human cancers, including lung. Finally, Nek11 expression is increased during colorectal cancer development suggesting that this kinase too is implicated in cancer progression (Sørensen et al. 2010).

Summary

In summary, of the 11 human Nek kinases, Nek2, Nek6, Nek7, and Nek9 function in mitotic regulation,

Nek10 and Nek11 in the DDR, Nek3 in signal transduction, and Nek1 and Nek8 in ciliogenesis. Currently, Nek4 and Nek5 have no assigned function. Hence, although these kinases are related to each other in sequence, it is clear that they are regulated in quite different manners and function in diverse processes. However, based on studies in both lower and higher eukaryotes, the common underlying theme is that many, albeit perhaps not all, Neks contribute to microtubule organization during cell cycle progression and/or ciliogenesis. Importantly, advances in understanding how this family contributes to cell cycle events make these kinases attractive targets for therapeutic interventions in human cancer. RNAi-mediated depletion of Nek2 and Nek6, for example, has been found to inhibit proliferation of cancer cell lines and tumor xenografts, and selective pharmacological inhibitors are beginning to be generated (Hayward et al. 2010; Jeon et al. 2010; Jin et al. 2006; Kokuryo et al. 2007; Nassirpour et al. 2010; Qiu et al. 2009; Suzuki et al. 2010; Tsunoda et al. 2009; Whelligan et al. 2010; Wu et al. 2008). Hence, although a better understanding of the basic biology of NIMA-related kinases is still required, there is growing evidence that Neks could serve as important targets for the management of cancers. Finally, the identification of mutations in Nek8 as causative for an inherited human disease emphasizes the need for further research into the processes and pathways in which this family of kinases operate.

References

Bahmanyar S, Kaplan DD, Deluca JG, Giddings Jr TH, O'Toole ET, Winey M, Salmon ED, Casey PJ, Nelson WJ, Barth AI. Beta-catenin is a Nek2 substrate involved in centrosome separation. Genes Dev. 2008;22:91–105.

Belham C, Roig J, Caldwell JA, Aoyama Y, Kemp BE, Comb M, Avruch J. A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. J Biol Chem. 2003;278:34897–909.

Bertran MT, Sdelci S, Regué L, Avruch J, Caelles C, Roig J. Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. EMBO J. 2011;30:2634–47.

Bradley BA, Quarmby LM. A NIMA-related kinase, Cnk2p, regulates both flagellar length and cell size in Chlamydomonas. J Cell Sci. 2005;118:3317–26.

Capra M, Nuciforo PG, Confalonieri S, Quarto M, Bianchi M, Nebuloni M, Boldorini R, Pallotti F, Viale G, Gishizky ML, Draetta GF, Di Fiore PP. Frequent alterations in the expression of serine/threonine kinases in human cancers. Cancer Res. 2006;66:8147–54.

Chang J, Baloh RH, Milbrandt J. The NIMA-family kinase Nek3 regulates microtubule acetylation in neurons. J Cell Sci. 2009;122:2274–82.

Chen Y, Chen CF, Chiang HC, Pena M, Polci R, Wei RL, Edwards RA, Hansel DE, Chen PL, Riley DJ. Mutation of NIMA-related kinase 1 (NEK1) leads to chromosome instability. Mol Cancer. 2011;10:5.

Doles J, Hemann MT. Nek4 status differentially alters sensitivity to distinct microtubule poisons. Cancer Res. 2010;70:1033–41.

Eto M, Elliott E, Prickett TD, Brautigan DL. Inhibitor-2 regulates protein phosphatase-1 complexed with NimA-related kinase to induce centrosome separation. J Biol Chem. 2002;277:44013–20.

Ewing RM, Chu P, Elisma F, Li H, Taylor P, Climie S, McBroom-Cerajewski L, Robinson MD, O'Connor L, Li M, Taylor R, Dharsee M, Ho Y, Heilbut A, Moore L, Zhang S, Ornatsky O, Bukhman YV, Ethier M, Sheng Y, Vasilescu J, Abu-Farha M, Lambert JP, Duewel HS, Stewart II B, Kuehl K, Hogue K, Colwill K, Gladwish B, Muskat R, Kinach SL, Adams MF, Moran GB, Morin TT, Figeys D. Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol Syst Biol. 2007;3:89.

Faragher AJ, Fry AM. Nek2A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. Mol Biol Cell. 2003;14:2876–89.

Fletcher L, Cerniglia GJ, Yen TJ, Muschel RJ. Live cell imaging reveals distinct roles in cell cycle regulation for Nek2A and Nek2B. Biochim Biophys Acta. 2005;1744:89–92.

Fry AM. The Nek2 protein kinase: a novel regulator of centrosome structure. Oncogene. 2002;21:6184–94.

Fry AM, Schultz SJ, Bartek J, Nigg EA. Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of Aspergillus nidulans. J Biol Chem. 1995;270:12899–905.

Fry AM, Mayor T, Meraldi P, Stierhof YD, Tanaka K, Nigg EA. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. J Cell Biol. 1998a;141:1563–74.

Fry AM, Meraldi P, Nigg EA. A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. EMBO J. 1998b;17:470–81.

Grallert A, Hagan IM. Schizosaccharomyces pombe NIMA-related kinase, Fin1, regulates spindle formation and an affinity of Polo for the SPB. EMBO J. 2002;21:3096–107.

Grallert A, Krapp A, Bagley S, Simanis V, Hagan IM. Recruitment of NIMA kinase shows that maturation of the S. pombe spindle-pole body occurs over consecutive cell cycles and reveals a role for NIMA in modulating SIN activity. Genes Dev. 2004;18:1007–21.

Hames RS, Crookes RE, Straatman KR, Merdes A, Hayes MJ, Faragher AJ, Fry AM. Dynamic recruitment of Nek2 kinase to the centrosome involves microtubules, PCM-1, and localized proteasomal degradation. Mol Biol Cell. 2005;16:1711–24.

Hayes MJ, Kimata Y, Wattam SL, Lindon C, Mao G, Yamano H, Fry AM. Early mitotic degradation of Nek2A depends on Cdc20-independent interaction with the APC/C. Nat Cell Biol. 2006;8:607–14.

Hayward DG, Fry AM. Nek2 kinase in chromosome instability and cancer. Cancer Lett. 2006;237:155–66.

Hayward DG, Newbatt Y, Pickard L, Byrne E, Mao G, Burns S, Sahota NK, Workman P, Collins I, Aherne W, Fry AM. Identification by high-throughput screening of viridin analogs as biochemical and cell-based inhibitors of the cell cycle-regulated Nek2 kinase. J Biomol Screen. 2010;15:918–27.

Helps NR, Luo X, Barker HM, Cohen PT. NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. Biochem J. 2000;349:509–18.

Holland PM, Milne A, Garka K, Johnson RS, Willis C, Sims JE, Rauch CT, Bird TA, Virca GD. Purification, cloning, and characterization of Nek8, a novel NIMA-related kinase, and its candidate substrate Bicd2. J Biol Chem. 2002;277:16229–40.

Jeon YJ, Lee KY, Cho YY, Pugliese A, Kim HG, Jeong CH, Bode AM, Dong Z. Role of NEK6 in tumor promoter-induced transformation in JB6 C141 mouse skin epidermal cells. J Biol Chem. 2010;285:28126–33.

Jin G, Aulabaugh A, Pocas J, Liu H, Kriz R, Sampath D. Development and comparison of nonradioactive in vitro kinase assays for NIMA-related kinase 2. Anal Biochem. 2006;358:59–69.

Kim S, Lee K, Rhee K. NEK7 is a centrosomal kinase critical for microtubule nucleation. Biochem Biophys Res Commun. 2007;360:56–62.

Kokuryo T, Senga T, Yokoyama Y, Nagino M, Nimura Y, Hamaguchi M. Nek2 as an effective target for inhibition of tumorigenic growth and peritoneal dissemination of cholangiocarcinoma. Cancer Res. 2007;67:9637–42.

Laurell E, Beck K, Krupina K, Theerthagiri G, Bodenmiller B, Horvath P, Aebersold R, Antonin W, Kutay U. Phosphorylation of Nup98 by multiple kinases Is crucial for NPC disassembly during mitotic entry. Cell. 2011;144:539–50.

Letwin K, Mizzen L, Motro B, Ben-David Y, Bernstein A, Pawson T. A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. EMBO J. 1992;11:3521–31.

Liu S, Lu W, Obara T, Kuida S, Lehoczky J, Dewar K, Drummond IA, Beier DR. A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in zebrafish. Development. 2002;129:5839–46.

Lu KP, Hunter T. Evidence for a NIMA-like mitotic pathway in vertebrate cells. Cell. 1995;81:413–24.

Mahjoub MR, Montpetit B, Zhao L, Finst RJ, Goh B, Kim AC, Quarmby LM. The FA2 gene of Chlamydomonas encodes a NIMA family kinase with roles in cell cycle progression and microtubule severing during deflagellation. J Cell Sci. 2002;115:1759–68.

Mahjoub MR, Trapp ML, Quarmby LM. NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. J Am Soc Nephrol. 2005;16:3485–9.

Mardin BR, Lange C, Baxter JE, Hardy T, Scholz SR, Fry AM, Schiebel E. Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. Nat Cell Biol. 2010;12:1166–76.

Melixetian M, Klein DK, Sørensen CS, Helin K. NEK11 regulates CDC25A degradation and the IR-induced G2/M checkpoint. Nat Cell Biol. 2009;11:1247–53.

Miller SL, DeMaria JE, Freier DO, Riegel AM, Clevenger CV. Novel association of Vav2 and Nek3 modulates signaling through the human prolactin receptor. Mol Endocrinol. 2005;19:939–49.

Miller SL, Antico G, Raghunath PN, Tomaszewski JE, Clevenger CV. Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. Oncogene. 2007;26:4668–78.

Moniz LS, Stambolic V. Nek10 mediates G2/M cell cycle arrest and MEK autoactivation in response to UV irradiation. Mol Cell Biol. 2011;31:30–42.

Morris N. Mitotic mutants of Aspergillus nidulans. Genet Res. 1975;26:237–54.

Nassirpour R, Shao L, Flanagan P, Abrams T, Jallal B, Smeal T, Yin MJ. Nek6 mediates human cancer cell transformation and is a potential cancer therapeutic target. Mol Cancer Res. 2010;8:717–28.

Noguchi K, Fukazawa H, Murakami Y, Uehara Y. Nek11, a new member of the NIMA family of kinases, involved in DNA replication and genotoxic stress responses. J Biol Chem. 2002;277:39655–65.

Noguchi K, Fukazawa H, Murakami Y, Uehara Y. Nucleolar Nek11 is a novel target of Nek2A in G1/S-arrested cells. J Biol Chem. 2004;279:32716–27.

O'Connell MJ, Norbury C, Nurse P. Premature chromatin condensation upon accumulation of NIMA. EMBO J. 1994;13:4926–37.

O'Connell MJ, Krien MJ, Hunter T. Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol. 2003;13:221–8.

O'Regan L, Blot J, Fry AM. Mitotic regulation by NIMA-related kinases. Cell Div. 2007;2:25.

O'Regan L, Fry AM. The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. Mol Cell Biol. 2009;29:3975–90.

Oakley BR, Morris NR. A mutation in Aspergillus nidulans that blocks the transition from interphase to prophase. J Cell Biol. 1983;96:1155–8.

Osmani SA, Pu RT, Morris NR. Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. Cell. 1988;53:237–44.

Osmani AH, O'Donnell K, Pu RT, Osmani SA. Activation of the nimA protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the bimE checkpoint. EMBO J. 1991;10:2669–79.

Otto EA, Trapp ML, Schultheiss UT, Helou J, Quarmby LM, Hildebrandt F. NEK8 mutations affect ciliary and centrosomal localization and may cause nephronophthisis. J Am Soc Nephrol. 2008;19:587–92.

Parker JD, Bradley BA, Mooers AO, Quarmby LM. Phylogenetic analysis of the Neks reveals early diversification of ciliary-cell cycle kinases. PLoS One. 2007;2:e1076.

Polci R, Peng A, Chen PL, Riley DJ, Chen Y. NIMA-related protein kinase 1 is involved early in the ionizing radiation-induced DNA damage response. Cancer Res. 2004;64:8800–3.

Pugacheva EN, Golemis EA. The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. Nat Cell Biol. 2005;7:937–46.

Qiu XL, Li G, Wu G, Zhu J, Zhou L, Chen PL, Chamberlin AR, Lee WH. Synthesis and biological evaluation of a series of

novel inhibitor of Nek2/Hec1 analogues. J Med Chem. 2009;52:1757–67.
Quarmby LM, Mahjoub MR. Caught Nek-ing: cilia and centrioles. J Cell Sci. 2005;118:5161–9.
Rapley J, Nicolas M, Groen A, Regue L, Bertran MT, Caelles C, Avruch J, Roig J. The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation. J Cell Sci. 2008;121:3912–21.
Rellos P, Ivins FJ, Baxter JE, Pike A, Nott TJ, Parkinson DM, Das S, Howell S, Fedorov O, Shen QY, Fry AM, Knapp S, Smerdon SJ. Structure and regulation of the human Nek2 centrosomal kinase. J Biol Chem. 2007;282:6833–42.
Richards MW, O'Regan L, Mas-Droux C, Blot JM, Cheung J, Hoelder S, Fry AM, Bayliss R. An autoinhibitory tyrosine motif in the cell-cycle-regulated Nek7 kinase is released through binding of Nek9. Mol Cell. 2009;36:560–70.
Roig J, Mikhailov A, Belham C, Avruch J. Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. Genes Dev. 2002;16:1640–58.
Roig J, Groen A, Caldwell J, Avruch J. Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. Mol Biol Cell. 2005;16:4827–40.
Salem H, Rachmin I, Yissachar N, Cohen S, Amiel A, Haffner R, Lavi L, Motro B. Nek7 kinase targeting leads to early mortality, cytokinesis disturbance and polyploidy. Oncogene. 2010;29:4046–57.
Schultz SJ, Fry AM, Sutterlin C, Ried T, Nigg EA. Cell cycle-dependent expression of Nek2, a novel human protein kinase related to the NIMA mitotic regulator of Aspergillus nidulans. Cell Growth Differ. 1994;5:625–35.
Shalom O, Shalva N, Altschuler Y, Motro B. The mammalian Nek1 kinase is involved in primary cilium formation. FEBS Lett. 2008;582:1465–70.
Shiba D, Manning DK, Koga H, Beier DR, Yokoyama T. Inv acts as a molecular anchor for Nphp3 and Nek8 in the proximal segment of primary cilia. Cytoskeleton (Hoboken). 2010;67:112–9.
Sohara E, Luo Y, Zhang J, Manning DK, Beier DR, Zhou J. Nek8 regulates the expression and localization of polycystin-1 and polycystin-2. J Am Soc Nephrol. 2008;19:469–76.
Sørensen CS, Melixetian M, Klein DK, Helin K. NEK11: linking CHK1 and CDC25A in DNA damage checkpoint signaling. Cell Cycle. 2010;9:450–5.
Surpili MJ, Delben TM, Kobarg J. Identification of proteins that interact with the central coiled-coil region of the human protein kinase NEK1. Biochemistry. 2003;42:15369–76.
Suzuki K, Kokuryo T, Senga T, Yokoyama Y, Nagino M, Hamaguchi M. Novel combination treatment for colorectal cancer using Nek2 siRNA and cisplatin. Cancer Sci. 2010;101:1163–9.
Tan BC, Lee SC. Nek9, a novel FACT-associated protein, modulates interphase progression. J Biol Chem. 2004;279:9321–30.
Tanaka K, Nigg EA. Cloning and characterization of the murine Nek3 protein kinase, a novel member of the NIMA family of putative cell cycle regulators. J Biol Chem. 1999;274:13491–7.
Trapp ML, Galtseva A, Manning DK, Beier DR, Rosenblum ND, Quarmby LM. Defects in ciliary localization of Nek8 is associated with cystogenesis. Pediatr Nephrol. 2008;23:377–87.
Tsunoda N, Kokuryo T, Oda K, Senga T, Yokoyama Y, Nagino M, Nimura Y, Hamaguchi M. Nek2 as a novel molecular target for the treatment of breast carcinoma. Cancer Sci. 2009;100:111–6.
Upadhya P, Birkenmeier EH, Birkenmeier CS, Barker JE. Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. Proc Natl Acad Sci USA. 2000;97:217–21.
Uto K, Sagata N. Nek2B, a novel maternal form of Nek2 kinase, is essential for the assembly or maintenance of centrosomes in early Xenopus embryos. EMBO J. 2000;19:1816–26.
Vaz Meirelles G, Ferreira Lanza DC, da Silva JC, Santana Bernachi J, Paes Leme AF, Kobarg J. Characterization of hNek6 interactome reveals an important role for its short N-terminal domain and colocalization with proteins at the centrosome. J Proteome Res. 2010;9:6298–316.
Vogler C, Homan S, Pung A, Thorpe C, Barker J, Birkenmeier EH, Upadhya P. Clinical and pathologic findings in two new allelic murine models of polycystic kidney disease. J Am Soc Nephrol. 1999;10:2534–9.
Whelligan DK, Solanki S, Taylor D, Thomson DW, Cheung K-M, Boxall K, Mas-Droux C, Barillari C, Burns S, Grummitt CG, Collins I, van Montfort RLM, Aherne GW, Bayliss R, Hoelder S. Aminopyrazine inhibitors binding to an unusual inactive conformation of the mitotic kinase Nek2: SAR and structural characterization. J Med Chem. 2010;53(21):7682–98.
White MC, Quarmby LM. The NIMA-family kinase, Nek1 affects the stability of centrosomes and ciliogenesis. BMC Cell Biol. 2008;9:29.
Wloga D, Camba A, Rogowski K, Manning G, Jerka-Dziadosz M, Gaertig J. Members of the NIMA-related kinase family promote disassembly of cilia by multiple mechanisms. Mol Biol Cell. 2006;17:2799–810.
Wu W, Baxter JE, Wattam SL, Hayward DG, Fardilha M, Knebel A, Ford EM, da Cruz e Silva EF, Fry AM. Alternative splicing controls nuclear translocation of the cell cycle-regulated Nek2 kinase. J Biol Chem. 2007;282:26431–40.
Wu G, Qiu XL, Zhou L, Zhu J, Chamberlin R, Lau J, Chen PL, Lee WH. Small molecule targeting the Hec1/Nek2 mitotic pathway suppresses tumor cell growth in culture and in animal. Cancer Res. 2008;68:8393–9.
Yin MJ, Shao L, Voehringer D, Smeal T, Jallal B. The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis. J Biol Chem. 2003;278:52454–60.
Yissachar N, Salem H, Tennenbaum T, Motro B. Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. FEBS Lett. 2006;580:6489–95.

NES (Nuclear Export Sequence)

► p38 MAPK Family of Signal Transduction Proteins

Net1 (Neuroepithelial Cell Transforming Gene 1 Protein)

Jeffrey A. Frost
Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, Houston, TX, USA

Synonyms

ARHGEF8

Historical Background

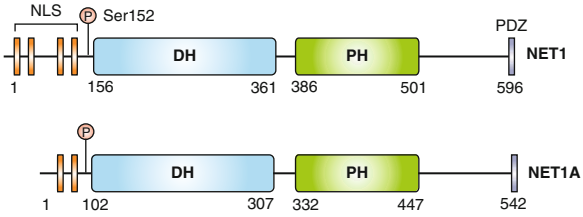
Rho family small GTPases control multiple cell functions, including organization of the actin cytoskeletal, cell motility and invasion, and cell cycle progression. They act as molecular switches, cycling between their active GTP-bound and inactive GDP-bound states. When bound to GTP, Rho GTPases initiate intracellular signaling by binding to downstream proteins known as effectors. The activation state of Rho GTPases is controlled by three families of proteins, known as GDP exchange factors (Rho GEFs), GTPase activating proteins (Rho GAPs), and guanine nucleotide dissociation inhibitors (Rho GDIs). Rho GEFs activate Rho proteins by stimulating the release of GDP, thereby allowing the binding of GTP. Rho GAPs accelerate the intrinsic GTPase activity of Rho proteins to hydrolyze GTP to GDP. Rho GDIs sequester inactive, GDP-bound Rho GTPases in the cytosol, thereby maintaining and stabilizing a ready pool of Rho proteins for subsequent activation. Within this regulatory network, it is the Rho GEFs that are primarily responsible for translating upstream signaling events into Rho protein activation.

During the mid-1990s a number of groups used NIH 3T3 cell focus formation assays to identify candidate oncogenes. In this type of screen, 3T3 cells are made to express a cDNA expression library derived from cancer cells, and then tested for subsequent loss of contact inhibition. This can be observed through the formation of cell foci, which are essentially masses of transformed cells. The neuroepithelial transforming gene 1 (Net1) was discovered in this way using a cDNA library derived from neuroepithelioma cells (Chan et al. 1996). The Net1 cDNA initially cloned from this screen lacked the coding sequence for the first 145 amino acids of full length Net1, which was subsequently found to be critical to its transforming activity. NIH 3T3 cells expressing truncated Net1 were found to exhibit anchorage-independent growth in soft agar assays, and were tumorigenic when injected into nude mice. Thus, Net1 fit the requirements for a putative oncogene.

Since that time it has become appreciated that Net1 belongs to a large family of Rho GEFs containing tandem Diffuse B cell lymphoma (Dbl) and Pleckstrin homology (PH) domains. There are nearly 70 members within the Rho GEF family that exhibit different specificities for Rho proteins, distinct regulatory mechanisms, and unique tissue distributions. Net1 was originally described as being ubiquitously expressed, with lower levels of expression in the heart, brain, and pancreas (Chan et al. 1996). However, a detailed study of its expression in different tissues has not been published. Two isoforms of Net1 exist, Net1 and Net1A, which are identical except for their N-terminal regulatory domains (Fig. 1). The unique portion of the N-terminus corresponding to the Net1 isoform consists of an 85 amino acid span that contains two nuclear localization signal (NLS) sequences (Schmidt and Hall 2002), while the unique 31 amino acid portion of Net1A has no identified function. The rest of the N-terminus is shared between Net1 isoforms, and this region contains two additional predicted NLS sequences. The DH domain of Net1 proteins binds to RhoA and in conjunction with the PH domain mediates RhoA activation. Although PH domains were originally characterized as phosphoinositide binding domains, the PH domain of Net1 has not been shown to bind phospholipids. The shared C-terminus of Net1 isoforms is 93 amino acids in length. Its only characterized function to date is mediated by its C-terminal PDZ domain–binding site.

Regulation of Net1 Activity

Net1 activity in the cell is regulated at the levels of transcription, subcellular localization, and posttranslational modification. Transcription of Net1 isoforms is controlled by alternate promoters which allows for



Net1 (Neuroepithelial Cell Transforming Gene 1 Protein), Fig. 1 Domain organization of Net1 proteins. Nuclear localization signal (*NLS*) sequences are shown in *orange*. The catalytic Dbl homology (*DH*) domain is shown in *light blue*. The Pleckstrin Homology (*PH*) domain is shown in *light green*. The C-terminal PDZ domain–binding site (*PDZ*) is shown in *dark blue*. Serine 152 is the negative regulatory Pak1 phosphorylation site. Numbers refer to amino acids within human Net1 and Net1A

their differential expression under certain circumstances (Dutertre et al. 2010). For example, in MCF7 breast cancer cells estrogen potently stimulates Net1 transcription and weakly downregulates Net1A transcription. On the other hand, progesterone stimulates the transcription of both Net1 and Net1A in MCF7 and T47D breast cancer cells (Dutertre et al. 2010). Most studies to date have not differentiated between Net1 isoforms when studying Net1 expression. Thus, TGFβ has been reported to stimulate Net1 transcription in human keratinocytes (Shen et al. 2001). IL-2 stimulation increases Net1 expression in Kit 225 human lymphocytes (Mzali et al. 2005), and both ▶ TNFα and LPA stimulate Net1 expression in AGS gastric cancer cells (Leyden et al. 2006; Murray et al. 2008).

Apart from transcriptional regulation, the cellular activity of Net1 proteins is also highly regulated. One important mode of regulation is by control of subcellular localization. Both Net1 isoforms contain multiple NLS sequences in their amino-termini, and when overexpressed in cells they accumulate in the nucleus (Schmidt and Hall 2002; Qin et al. 2005). Since RhoA activation is thought to occur only at the plasma membrane, this means that nuclear sequestration of Net1 isoforms is a mechanism to negatively regulate their activities. Regulatory signals that control the nuclear export of either Net1 isoforms are unknown at present. Both Net1 isoforms contain possible nuclear export signal (NES) sequences in their common PH domain, and treatment of cells with the CRM1 inhibitor leptomycin B has been shown to cause an increased accumulation of the N-terminal truncation mutant Net1ΔN in the nucleus (Schmidt and Hall 2002). However, since this was only a partial effect, it is possible that there are additional, leptomycin B–resistant exportins that control the nuclear exit of Net1 proteins.

The catalytic activity of Net1 is also regulated within the cell. Specifically, phosphorylation by the Rac and Cdc42 regulated kinase ▶ Pak1 negatively regulates Net1 catalytic activity (Alberts et al. 2005). Pak1 has been shown to phosphorylate Net1 on serines 152, 153, and 538 in vitro, and on serine 152 in cells. Serines 152 and 153 are contained within an amino-terminal extension of the DH domain that is restricted to a subset of RhoA-specific GEFs (Alberts and Treisman 1998). Substitution of serines 152 and 153 with the phosphorylation mimetic glutamate inhibits the ability of Net1 to catalyze GDP exchange on RhoA in vitro, and also blocks the ability of expressed Net1ΔN to cause actin stress fiber formation in cells. Similarly, overexpression of constitutively active Pak1 blocks stress fiber formation caused by co-expression of wild type Net1, but not by a Net1 mutant containing alanine substitutions at serines 152 and 153 (Alberts et al. 2005).

The cellular activity of Net1A is also tightly regulated by ubiquitylation (Carr et al. 2009). Net1 isoforms contain a C-terminal PDZ domain–binding site that mediates interaction with proteins within the Dlg1 family. Interaction of Net1A with Dlg1 prevents its ubiquitylation in MCF7 breast cancer cells, and increases the half-life of Net1A from 25 min to 10^5 h. This effect was specific for Dlg1, since co-expression of the Net1 interacting, PDZ domain containing protein Magi-1b did not significantly stabilize Net1A (Carr et al. 2009). Interestingly, the interaction of endogenous Net1A with Dlg1 in these cells is dependent on the formation of E-cadherin-mediated cell contacts, since disruption of these contacts causes a rapid and dramatic increase in Net1A ubiquitylation. In this same study, it was determined that Net1ΔN is stable when expressed in MCF7 cells, indicating that the amino-terminus of Net1A is important for regulating its degradation (Carr et al. 2009). The enhanced stability of Net1ΔN may also account for its strong transforming ability relative to full length Net1 isoforms.

Regulation of Actin Cytoskeletal Organization by Net1

Net1 has been shown to act as a GEF for RhoA, but not Rac1 or Cdc42 (Alberts et al. 1998). By analogy with

other RhoA-specific GEFs, Net1 may also have activity toward the RhoA subfamily members RhoB and RhoC, although this has not been tested. When overexpressed in mouse fibroblasts, Net1 stimulates the formation of actin stress fibers that are a hallmark of RhoA activation (Alberts et al. 1998). In this regard, the N-terminal truncation mutant Net1ΔN is far more efficient at stimulating stress fiber formation than full length Net1, which is most likely due to the enhanced localization of Net1ΔN outside the nucleus. Net1 has also been reported to mediate actin stress fiber formation in human keratinocytes following stimulation with TGFβ (Shen et al. 2001). Similar to other RhoA-specific GEFs, the ability of Net1ΔN to stimulate actin stress fiber formation is dependent on downstream activation of the RhoA effector kinases ▶ ROCK1 and ▶ ROCK2 (Tran et al. 2000).

Additional Physiological Roles of Net1

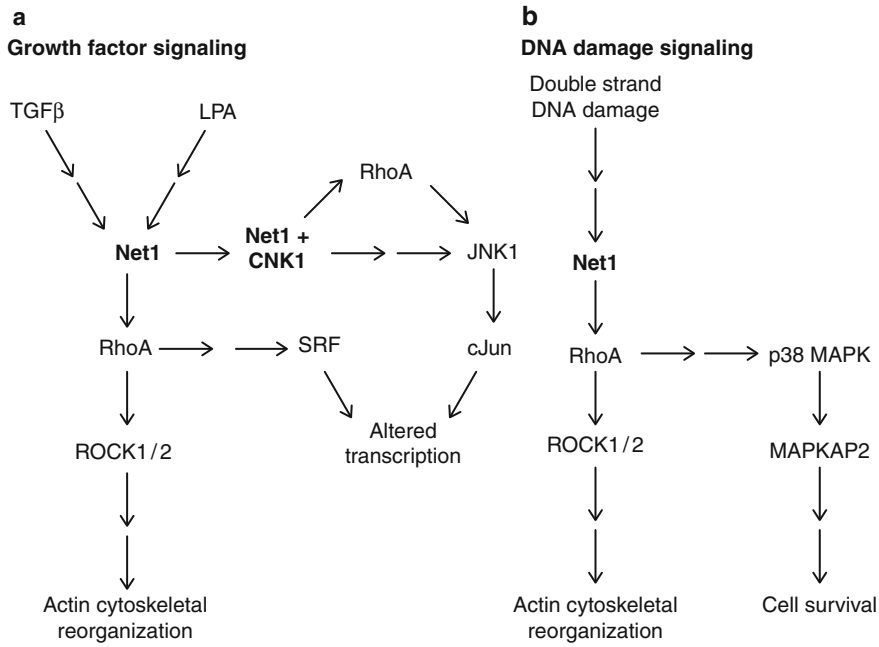
Net1 likely plays important roles during development and differentiation. For example, in chicken epiblasts Net1A expression is required for RhoA activation at the basal surface and maintenance of the integrity of the basement membrane (Nakaya et al. 2008). During gastrulation, epiblasts undergo an epithelial to mesenchymal transition (EMT) that is accompanied by loss of Net1A expression on the basal membrane, and enforced expression of RhoA or Net1A in these cells prevents basement membrane breakdown and EMT (Nakaya et al. 2008). Similarly, overexpression of xNet1A in *Xenopus* embryos inhibits gastrulation movements (Miyakoshi et al. 2004). Net1A expression is also strongly upregulated during mouse osteoclast differentiation in vitro, and inhibition of Net1A expression in these cells prevents cell fusion that is required for osteoclast formation (Brazier et al. 2006). All of these processes are associated with dramatic changes in actin cytoskeletal organization and altered cell motility, both of which are RhoA-regulated events.

By regulating the activation of RhoA and ▶ MAP Kinases, Net1 also impacts transcription factor activation. For example, overexpression of the N-terminal deletion mutant Net1ΔN in NIH 3T3 cells stimulates serum response factor (SRF) activation. This requires activation of both RhoA and the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) pathway (Alberts et al. 1998). In Hela cells, Net1ΔN expression stimulates SAPK/JNK activation through interaction with the scaffold protein Connector Enhancer of KSR 1 (CNK1), which results in efficient activation of the transcription factor c-Jun (Jaffe et al. 2005). Stimulation of c-Jun activity by Net1ΔN requires both RhoA and SAPK/JNK activation, and stimulation of c-Jun activity by the extracellular ligand LPA requires endogenous CNK1 expression. However, it is unknown whether LPA-stimulated c-Jun activity also requires endogenous Net1. Taken as a whole, these data suggest that Net1 controls RhoA and SAPK/JNK activation to regulate transcription factors such as SRF and c-Jun, which are associated with cell proliferation and transformation (Fig. 2a).

Net1 may also play a role in protecting cells from death following DNA damage (Guerra et al. 2008). It has recently been shown that Net1 is dephosphorylated on the negative regulatory phosphorylation site serine 152 following exposure of Hela cells to ionizing radiation (IR), suggesting that it is activated by IR treatment. In addition, RNAi-mediated knockdown of Net1 expression sensitizes these cells to IR-induced apoptosis. Knockdown of Net1 expression also prevents RhoA activation following IR, which has previously been shown to be required for protection from IR. Similarly, inhibition of Net1 expression in these cells blocks the activation of p38 MAPK and its downstream substrate MAPKAP2, both of which are required for cell survival following IR exposure (Fig. 2b). Thus, these data indicate that Net1 plays an important role in the survival response of cells to IR, which may have important consequences for cancer therapy approaches.

Net1 and Cancer

Net1 was originally identified as a potential oncogene in mouse fibroblasts, and subsequent work suggests that it may play an important role in human cancer. For example, using differential display and real time PCR, Net1 transcripts have been shown to be overexpressed in human gastric cancers. In addition, transfection of siRNA targeting both Net1 isoforms into AGS gastric cancer cells inhibited both their proliferation and their ability to invade a Matrigel extracellular matrix (Leyden et al. 2006). In a separate study, RNAi-mediated knockdown of both Net1 isoforms in AGS gastric cancer cells dramatically attenuated basal levels of RhoA activation and prevented their migration



Net1 (Neuroepithelial Cell Transforming Gene 1 Protein), Fig. 2 Regulation of cell signaling by Net1. Net1 proteins respond to diverse stimuli to contribute to intracellular signaling following exposure to (**a**) extracellular growth factors or (**b**) double-strand DNA damage

in wound healing and transwell assays. In this study Net1 was also shown to be required for migration and invasion of cells in response to the extracellular ligand LPA (Murray et al. 2008).

Unfortunately, few studies have focused on whether individual Net1 isoforms are differentially regulated, or control different aspects of cancer cell behavior. Thus, it is difficult to predict whether one or both Net1 isoforms contribute to human cancer. However, in a recent study evidence was presented suggesting that Net1 isoforms do play different roles in the cell and may uniquely contribute to cell transformation. Specifically, it was shown in MCF7 breast cancer cells that estrogen treatment strongly upregulated Net1 transcription and weakly repressed Net1A transcription (Dutertre et al. 2010). This was likely to be important for the ability of these cells to proliferate in response to estrogen, since transfection with siRNA specific for the Net1 isoform inhibited their proliferation. On the other hand, transfection of these cells with Net1A-specific siRNA did not significantly block proliferation, but adversely affected cell adherence. Thus, these data are the first to report divergent roles for Net1 isoforms in any cell type, and suggest that Net1 isoforms may make distinct contributions to cell cycle progression and cytoskeletal organization.

Evidence is also accumulating in translational studies to indicate that Net1 expression may be an important factor in predicting cancer outcome. For example, Net1 has been reported to be overexpressed in human gliomas (Tu et al. 2010). Importantly, in this study elevated Net1 expression correlated with higher grade cancers and was prognostic of lower overall survival. Similarly, in a separate study of breast cancer patients, co-expression of Net1 with alpha6beta4 integrin was predictive of decreased distant metastasis-free survival (Gilcrease et al. 2009). Because antibodies specific for individual Net1 isoforms are not available, neither of these studies differentiated between Net1 isoforms. However, when expression of transcripts for each Net1 isoform was examined in breast cancers, high expression of Net1 mRNA, but not Net1A, was prognostic for decreased metastasis-free survival (Dutertre et al. 2010). Clearly, more detailed studies will be required in the future to determine whether a particular Net1 isoform is more closely associated with oncogenesis. However, taken together these data seem to indicate that increased expression of Net1 as a whole is associated with more aggressive cancers and poor patient prognosis.

Summary

Net1 is a Rho GEF that is specific for the RhoA subfamily of small G proteins. Two isoforms exist in

most cells that exhibit differential subcellular distributions and that may play distinct roles in the cell. The activities of Net1 proteins are tightly regulated by phosphorylation and ubiquitylation. Net1 proteins play important roles in gastrulation and also contribute to cellular differentiation. Net1 proteins may also be aberrantly expressed in human cancers and contribute to cancer initiation and/or progression.

References

Alberts AS, Treisman R. Activation of RhoA and SAPK/JNK signaling pathways by the RhoA-specific exchange factor mNET1. EMBO J. 1998;17(14):4075–85.

Alberts AS, Qin H, Carr HS, Frost JA. PAK1 negatively regulates the activity of the Rho exchange factor NET1. J Biol Chem. 2005;280(13):12152–61.

Brazier H, Stephens S, Ory S, Fort P, Morrison N, Blangy A. Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts. J Bone Miner Res. 2006;21(9):1387–98.

Carr HS, Cai C, Keinanen K, Frost JA. Interaction of the rhoa exchange factor net1 with discs large homolog 1 protects it from proteasome mediated degradation and potentiates net1 activity. J Biol Chem. 2009;284(36):24269–80.

Chan AM, Takai S, Yamada K, Miki T. Isolation of a novel oncogene, NET1, from neuroepithelioma cells by expression cDNA cloning. Oncogene. 1996;12:1259–66.

Dutertre M, Gratadou L, Dardenne E, Germann S, Samaan S, Lidereau R, et al. Estrogen regulation and physiopathologic significance of alternative promoters in breast cancer. Cancer Res. 2010;70(9):3760–70.

Gilcrease MZ, Kilpatrick SK, Woodward WA, Zhou X, Nicolas MM, Corley LJ, et al. Coexpression of alpha6beta4 integrin and guanine nucleotide exchange factor Net1 identifies node-positive breast cancer patients at high risk for distant metastasis. Cancer Epidemiol Biomarkers Prev. 2009;18(1):80–6.

Guerra L, Carr HS, Richter-Dahlfors A, Masucci MG, Thelestam M, Frost JA, et al. A bacterial cytotoxin identifies the RhoA exchange factor Net1 as a key effector in the response to DNA damage. PLoS ONE. 2008;3(5):e2254.

Jaffe AB, Hall A, Schmidt A. Association of CNK1 with Rho guanine nucleotide exchange factors controls signaling specificity downstream of Rho. Curr Biol. 2005;15(5):405–12.

Leyden J, Murray D, Moss A, Arumuguma M, Doyle E, McEntee G, et al. Net1 and Myeov: computationally identified mediators of gastric cancer. Br J Cancer. 2006;94(8):1204–12.

Miyakoshi A, Ueno N, Kinoshita N. Rho guanine nucleotide exchange factor xNET1 implicated in gastrulation movements during Xenopus development. Differentiation. 2004;72(1):48–55.

Murray D, Horgan G, MacMathuna P, Doran P. NET1-mediated RhoA activation facilitates lysophosphatidic acid-induced cell migration and invasion in gastric cancer. Br J Cancer. 2008;99(8):1322–9.

Mzali R, Seguin L, Liot C, Auger A, Pacaud P, Loirand G, et al. Regulation of Rho signaling pathways in interleukin-2-stimulated human T-lymphocytes. FASEB J. 2005;19(13):1911–3.

Nakaya Y, Sukowati EW, Wu Y, Sheng G. RhoA and microtubule dynamics control cell-basement membrane interaction in EMT during gastrulation. Nat Cell Biol. 2008;10(7):765–75.

Qin H, Carr HS, Wu X, Muallem D, Tran NH, Frost JA. Characterization of the biochemical and transforming properties of the neuroepithelial transforming protein 1. J Biol Chem. 2005;280(9):7603–13.

Schmidt A, Hall A. The Rho exchange factor Net1 is regulated by nuclear sequestration. J Biol Chem. 2002;277(17):14581–8.

Shen X, Li J, Hu PP, Waddell D, Zhang J, Wang XF. The activity of guanine exchange factor NET1 is essential for transforming growth factor-beta-mediated stress fiber formation. J Biol Chem. 2001;276(18):15362–8.

Tran QC, Gautreau A, Arpin M, Treisman R. Ezrin function is required for ROCK-mediated fibroblast transformation by the Net and Dbl oncogenes. EMBO J. 2000;19(17):4565–76.

Tu Y, Lu J, Fu J, Cao Y, Fu G, Kang R, et al. Over-expression of neuroepithelial-transforming protein 1 confers poor prognosis of patients with gliomas. Jpn J Clin Oncol. 2010;40(5):388–94.

Neural Wiskott–Aldrich Syndrome Protein

► N-WASP

Neurotactin

► CX3CL1

Neurotensin Receptor (NTSR)

Tooru Mizuno
University of Manitoba, Winnipeg, MB, Canada

Synonyms

NTR; NTS

Historical Background

Neurotensin was originally isolated from bovine hypothalamus by Carraway and Leeman in 1973 and was identified as a 13 amino acid peptide (Carraway and Leeman 1973). It was named "neurotensin" in view of its hypotensive activity. Neurotensin is produced by neurons in the central nervous system (CNS) and N cells of the gastrointestinal tract. It was later discovered that neurotensin is also present in several types of tumors. Neurotensin acts as a hormone and a neurotransmitter or neuromodulator in the periphery and in the CNS, respectively. Neurotensin is involved in the regulation of a number of physiological functions such as analgesia, neurodegeneration, thermal regulation, metabolic regulation, pituitary hormone secretion, and gastrointestinal motility. Thus, signaling pathways downstream of neurotensin could be a potential drug target for the treatment of a variety of diseases. However, we had to wait 17 years to see the first receptor for neurotensin (neurotensin receptor 1, NTSR1). Subsequently, two additional subtypes of the neurotensin receptor (NTSR2 and NTSR3) and a candidate for NTSR4 have been identified. Development of subtype-selective neurotensin analogs facilitated investigations of the role of neurotensin receptors in mediating the effects of neurotensin.

Neurotensin Receptor Subtypes

Neurotensin exerts its effects primarily through two neurotensin receptor subtypes, NTSR1 and NTSR2. NTSR1 is a high-affinity (0.1–0.3 nM) neurotensin receptor which is not sensitive to levocabastine, a nonpeptide H1 histamine antagonist. NTSR2 has a low affinity (Kd = 3–5 nM) for neurotensin and is sensitive to levocabastine. Both NTSR1 and NTSR2 are 7-transmembrane G-protein-coupled receptors (GPCRs). The human *NTSR1* gene, encoding a 418 amino acid protein, is located on the long arm of chromosome 20 (20q13). The human *NTSR2* gene encodes a 410 amino acid protein and is located on chromosome 2 (2p25.1). Separate NTSR subtypes display distinctive tissue distribution patterns (Table 1). NTSR1 is distributed widely throughout the CNS and is also found in the small and large intestines as well as the liver. NTSR2 is located more diffusely in the CNS than NTSR1. Two additional receptors have been shown to bind neurotensin among other ligands. Both receptors are single transmembrane domain receptors of the type I family. The NTSR3 was originally identified as the intracellular sorting protein sortilin. The human *SORT1* gene, encoding an 831 amino acid protein, is located on the short arm of chromosome 1 (1p21.3-p13.1). NTSR3/sortilin, like NTSR1, binds neurotensin with high affinity once converted to its mature form upon cleavage. Sorting protein-related receptor (SorLA, also known as LR11) has been proposed as a fourth neurotensin receptor. The human *SorLA* gene, encoding a 2214 amino acid peptide, was mapped to chromosome 11 (11q 23.3-24). NTSR3/sortilin and SorLA/LR11 are located primarily in the CNS, but also in nonneuronal tissues (Table 1) (Vincent et al. 1999; Dobner 2005; St-Gelais et al. 2006).

Signal Transduction of NTSR Receptors

Stimulation of NTSR1 leads to the activation of multiple signaling pathways. The major signal transduction event associated with the activation of NTSR1 is the stimulation of phospholipase C (PLC), which is responsible for the production of inositol 1,4,5-triphosphate, Ca^{2+} mobilization, and activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a member of ▶ mitogen activating protein kinase (MAPK) in a variety of tissues and cells. Neurotensin-induced ERK1/2 activation is also mediated via the activation of small GTPase Ras protein and epidermal growth factor receptor (EGFR) in human colonial epithelial cells. NTSR1 activation stimulates ▶ nuclear factor kappaB (NF-κB) which is dependent on intracellular calcium release. Neurotensin activates the Rho family proteins RhoA, Rac1, and Cdc42 and neurotensin-induced ▶ NF-κB activation, but not ERK1/2 activation, is mediated through the activation of RhoA, Rac1, and Cdc42. Little is known about the signaling pathways activated by the other three receptor subtypes. When NTSR2 is challenged with neurotensin, internalization of receptor–ligand complexes occurs through the activation of ERK1/2. Stimulation of NTSR3/sortilin activates ERK1/2 and ▶ phosphoinositide 3-kinase (PI3K)-dependent pathways in microglial cell lines (Martin et al. 2005; Zhao and Pothoulakis 2006; Ferraro et al. 2009). Homodimer and/or heterodimer of GPCRs play

Neurotensin Receptor (NTSR), Table 1 Tissue distribution of neurotensin receptors

NTSR1	NTSR2	NTSR3/sortilin	SorLA/LR11 (NTSR4)
CNS	CNS	CNS	CNS
Anterior pituitary	Anterior pituitary	Thyroid gland	Kidney
Autonomic nervous system	Upper gastrointestinal tract	Heart	Ovary
Enteric nervous system	Heart	Adrenal gland	Testis
Gastrointestinal tract	Pancreas	Pancreas	Lymph node
Carotid body		Adipose tissue	
Liver		Skeletal muscle	
		Placenta	
		Testis	

important roles in receptor-trafficking, agonist-binding, and signal transduction. Heterodimerization/oligomerization alters receptor functions by forming new receptor complexes that exhibit ligand-binding properties distinct from monomeric receptors. NTSR1 and NTSR2 can form heterodimers, and NTSR2 suppresses neurotensin-induced NTSR1 activity (Hwang et al. 2009). The formation of NTSR1/NTSR2 heterodimers may provide additional functional resources to the cells.

Physiological Relevance of CNS NTSR Signaling

Neurotensin-containing neurons are neuroanatomically associated with the brain dopamine system, and neurotensin acts as a neuromodulator of dopamine transmission in several areas of the brain, including the nigrostriatal and mesolimbic pathways. Neurotensin increases the activity of dopaminergic neurons and dopamine release by antagonizing dopamine D_2 receptor function through NTSR1-mediated increases in intracellular Ca^{2+} and an interaction between NTSR1 and D_2 receptors. Neurotensin levels are increased and NTSR1 mRNA levels are lower in the substantia nigra of Parkinson's disease patients than in controls. Treatment with neurotensin analogs reduces tremor and muscle rigidity in animal models of Parkinson's disease. These findings argue for the possibility that enhanced signaling through substantia nigra NTSR1 supplements dopaminergic agonists to augment the function of the remaining dopaminergic neurons in Parkinson's disease. However, activation of NTSR1 by neurotensin enhances N-methyl-D-aspartate (NMDA)-induced increase in extracellular glutamate levels. The neurotensin-mediated potentiation of NMDA receptor signaling may be mediated by phosphorylation of the NMDA receptors by protein kinase C. Glutamate is the major excitatory neurotransmitter, and the excessive activation of glutamate receptors, especially NMDA receptors, has been postulated to contribute to the neuronal injury. In cultured dopaminergic neurons, neurotensin enhances the neurotoxic effects of glutamate on dopaminergic neurons via NTSR1 activation. Thus, treatment with selective NTSR1 antagonists may be beneficial in improving the symptoms of Parkinson's disease (Antonelli et al. 2007; Mustain et al. 2011). Abnormal neurotensin-NTSR1 signaling has been also found in the brain of Alzheimer's disease patients. Regardless of whether CNS neurotensin-NTSR1 signaling is beneficial or deleterious in neurodegenerative processes, this signaling pathway is likely to be involved in the etiology of neurodegenerative diseases.

Centrally administered neurotensin causes a variety of effects similar to those exhibited by antipsychotic drugs. Reduced signaling capacity through NTSR1 contributes to psychotic symptom in schizophrenia. Cerebrospinal fluid levels of neurotensin are lower in schizophrenic patients than in control subjects, whereas increased levels of neurotensin are associated with improvement in symptoms during treatment. The density of neurotensin receptors (primarily NTSR1) is decreased in the intermediate entorhinal cortex of schizophrenia patients. Both NTSR1-deficient mice and NTSR2-deficient mice show schizophrenia-like signs, such as amphetamine-induced hyperlocomotion and lower basal glutamate levels. Lack of neurotensin causes diminished prepulse inhibition (PPI), a schizophrenia-like sign, while NTSR1 deficiency does not

alter PPI and NTSR2-deficient mice have elevated PPI (Mustain et al. 2011). These findings support the idea that enhanced neurotensin-NTSR signaling is beneficial in improving schizophrenia symptoms. It is presently unclear what NTSR subtype is involved in this process. Brain-region-specific alterations in neurotensin-NTSR signaling may play a role in the etiology of schizophrenia.

Neurotensin has analgesic effects that are μ-opioid independent. Although NTSR2 was initially considered as a main receptor subtype mediating the antinociceptive effect of neurotensin, NTSR1 is also involved in neurotensin-induced analgesia. Both NTSR1 and NTSR2 are required for different aspects of neurotensin-induced analgesia and these two receptors modulate the pain-induced behavior responses by suppressing activity of distinct spinal and/or supraspinal neural circuits (Dobner 2005; St-Gelais et al. 2006; Roussy et al. 2009).

CNS neurotensin-NTSR signaling plays a role in neuroendocrine function via modulation of the activity of the hypothalamus-pituitary axis. Neurotensin stimulates secretion of gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH), and inhibits thyroid-stimulating hormone (TSH) secretion. Hypothalamic action of neurotensin increases prolactin secretion, while neurotensin action on the adenohypophysis causes a reduction in prolactin secretion. The direct hypothalamic effect of neurotensin in reducing prolactin secretion may be mediated by dopamine release into the portal system. It is unclear whether these effects of neurotensin are mediated via NTSR1 and/or NTSR2. Both receptors are expressed in the hypothalamus and anterior pituitary. Neurotensin has been implicated in the mediation of the pre-ovulatory positive feedback of estradiol on GnRH release via NTSR2 activation. NTSR2 also mediates neurotensin-induced increase in CRH and corticosterone secretion in response to stress (Lafrance et al. 2010; Stolakis et al. 2010; Mustain et al. 2011).

Neurotensin participates in the regulation of metabolism. Central administration of neurotensin reduces food intake via NTSR1. NTSR1 also mediates effects of other anorexigenic hormones, leptin and xenin. Genetic deletion of NTSR2 did not cause any metabolic abnormalities in mice, while NTSR1-deficient mice have increased body weight, food intake, and adiposity, implicating NTSR1 in metabolic regulation (Mustain et al. 2011).

CNS action of neurotensin reduces body temperature. NTSR1-deficient mice have increased body temperature and do not respond to the hypothermic effect of neurotensin, implicating neurotensin-NTSR1 signaling in thermal control.

Physiological Relevance of Peripheral NTSR Signaling

The effects of neurotensin in the gastrointestinal tract are mediated by both neural and hormonal mechanisms. Neurotensin elicits a relaxing effect on duodenal longitudinal muscle and induces contraction of the ileal muscle and the proximal colon. These neurotensin-induced alterations in gastrointestinal motilities were blocked by NTSR1 antagonist and were absent in NTSR1-deficient mice. Neurotensin-related peptide, xenin, also affects gastrointestinal motility partly via activation of NTSR1. These findings indicate that neurotensin regulates gastrointestinal motility via NTSR1 (Zhao and Pothoulakis 2006).

Neurotensin-NTSR1 signaling is associated with the progression and differentiation of tumors. NTSR1 is expressed in tumors of the ovary, pancreas, colon, prostate, and breast. High NTSR1 expression is associated with a larger tumor size, and the size of tumor becomes smaller in the presence of NTSR1 antagonist. In human breast cancer cell lines, functionally expressed NTSR1 receptor coordinated a series of transforming events, including cellular migration and invasion. The NTSR1 gene is a target of oncogenic pathways known to activate genes involved in cancer cell proliferation and transformation. When NTSR1 is challenged with neurotensin, a variety of signaling pathways are activated. For example, activation of NTSR1 promotes growth of colon tumors through NF-κB activation. Neurotensin also increased the activity of PI3K signaling by increasing specific microRNA species via NTSR1 in colon epithelial cells. During prolonged neurotensin agonist stimulation, NTSR1 receptors are recycled to the cell surface, leading to the long-term activation of ERK1/2, an oncogenic signaling pathway. These findings place NTSR1 at a major nexus between neurotensin and tumorigenesis. Although NTSR3 is also present in several types of cancer cells, the role of NTSR3 in cancer is presently unknown (St-Gelais et al. 2006; Bakirtzi et al. 2011; Mustain et al. 2011).

Physiological Relevance of Non-GPCR NTSRs

Whereas both NTSR3/sortilin and SorLA/LR11 are capable of binding neurotensin, the physiological relevance of these receptors is largely unclear. NTSR3/sortilin is predominantly expressed in regions of the CNS that neither synthesize nor respond to neurotensin. It binds a variety of ligands such as the receptor-associated protein (RAP) and lipoprotein lipase (LPL), and nerve growth factor precursor (proNGF) (St-Gelais et al. 2006). It is thus possible that NTSR3/sortilin subserves non-neurotensin-related functions in the CNS. NTSR3/sortilin may be involved in the neurotensin-induced migration of human microglial cells via the activation of both MAPK and ▶ PI3K signaling pathways (Martin et al. 2005). NTSR3/sortilin may also play a role in the regulation of metabolism. Levels of NTSR3/sortilin mRNA and protein were reduced in adipose tissue and skeletal muscle of obese mice and patients. NTSR3/sortilin promotes the formation of insulin-responsive glucose transporter 4 (GLUT4) storage vesicles in adipocyte cell line. Palmitate reduces the expression of NTSR3/sortilin in adipocytes and skeletal muscle cells (Tsuchiya et al. 2010). These recent findings suggest that NTSR3/sortilin plays a role in glucose homeostasis by modulating GLUT4 trafficking in adipose tissue and skeletal muscle and mediates FFAs-induced insulin resistance in these tissues.

It has been suggested that SorLA/LR11 regulates the processing of the amyloid precursor protein and levels of SorLA/LR11 are reduced in the brains of Alzheimer disease patients, suggesting a possible role of SorLA/LR11 in the pathogenesis of this disease (Andersen et al. 2005).

Summary

Neurotensin exerts diverse actions through NTSR receptors in the CNS and in the periphery. Alterations in NTSR signaling have been implicated in a wide range of pathologic conditions such as schizophrenia, Parkinson's disease, metabolic disorders, and cancer. Signaling pathways involving NTSR receptors and their downstream mediators could be potential drug targets for the treatment of these impairments. Pharmacological tools and genetically engineered animal models enabled us to manipulate NTSR signaling and we have begun to elucidate specific role for each NTSR subtype. Creation of animal models with cell type–specific ablation of specific NTSR subtype should help clarify the role of NTSR subtypes in the mediation of neurotensin action. Development of additional subtype-selective agonistic and antagonistic neurotensin analogs that cross the blood-brain barrier may offer new avenues for the treatment of disorders associated with altered NTSR receptor signaling.

References

Andersen OM, Reiche J, Schmidt V, Gotthardt M, Spoelgen R, Behlke J, et al. Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. Proc Natl Acad Sci USA. 2005;102(38):13461–6.

Antonelli T, Fuxe K, Tomasini MC, Mazzoni E, Agnati LF, Tanganelli S, et al. Neurotensin receptor mechanisms and its modulation of glutamate transmission in the brain: relevance for neurodegenerative diseases and their treatment. Prog Neurobiol. 2007;83(2):92–109.

Bakirtzi K, Hatziapostolou M, Karagiannides I, Polytarchou C, Jaeger S, Iliopoulos D, et al. Neurotensin signaling activates microRNAs-21 and -155 and Akt, promotes tumor growth in mice, and Is Increased in human colon tumors. Gastroenterology 2011. doi:10.1053/j.gastro.2011.07.038.

Carraway R, Leeman SE. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. J Biol Chem. 1973;248(19):6854–61.

Dobner PR. Multitasking with neurotensin in the central nervous system. Cell Mol Life Sci. 2005;62(17):1946–63.

Ferraro L, Tomasini MC, Beggiato S, Guerrini R, Salvadori S, Fuxe K, et al. Emerging evidence for neurotensin receptor 1 antagonists as novel pharmaceutics in neurodegenerative disorders. Mini Rev Med Chem. 2009;9(12):1429–38.

Hwang JI, Kim DK, Kwon HB, Vaudry H, Seong JY. Phylogenetic history, pharmacological features, and signal transduction of neurotensin receptors in vertebrates. Ann NY Acad Sci. 2009;1163:169–78.

Lafrance M, Roussy G, Belleville K, Maeno H, Beaudet N, Wada K, et al. Involvement of NTS2 receptors in stress-induced analgesia. Neuroscience. 2010;166(2):639–52.

Martin S, Dicou E, Vincent JP, Mazella J. Neurotensin and the neurotensin receptor-3 in microglial cells. J Neurosci Res. 2005;81(3):322–6.

Mustain WC, Rychahou PG, Evers BM. The role of neurotensin in physiologic and pathologic processes. Curr Opin Endocrinol Diabetes Obes. 2011;18(1):75–82.

Roussy G, Dansereau MA, Baudisson S, Ezzoubaa F, Belleville K, Beaudet N, et al. Evidence for a role of NTS2 receptors in the modulation of tonic pain sensitivity. Mol Pain. 2009;5:38.

St-Gelais F, Jomphe C, Trudeau LE. The role of neurotensin in central nervous system pathophysiology: what is the evidence? J Psychiatry Neurosci. 2006;31(4):229–45.

Stolakis V, Kalafatakis K, Botis J, Zarros A, Liapi C. The regulatory role of neurotensin on the hypothalamic-anterior

pituitary axons: emphasis on the control of thyroid-related functions. Neuropeptides. 2010;44(1):1–7.
Tsuchiya Y, Hatakeyama H, Emoto N, Wagatsuma F, Matsushita S, Kanzaki M. Palmitate-induced down-regulation of sortilin and impaired GLUT4 trafficking in C2C12 myotubes. J Biol Chem. 2010;285(45):34371–81.
Vincent JP, Mazella J, Kitabgi P. Neurotensin and neurotensin receptors. Trends Pharmacol Sci. 1999;20(7):302–9.
Zhao D, Pothoulakis C. Effects of NT on gastrointestinal motility and secretion, and role in intestinal inflammation. Peptides. 2006;27(10):2434–44.

NFAT

Rut Valdor, Brian T. Abe and Fernando Macian
Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA

Synonyms

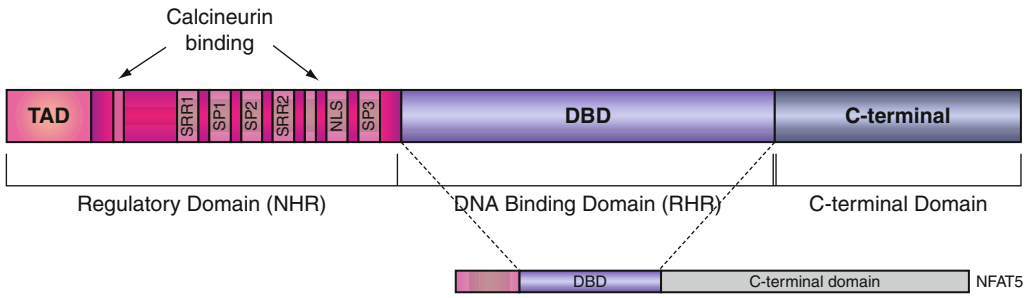
NFAT1 (Nuclear factor of activated T-cells 1, NFATp, NFAT preexisting, NFATc2, NFAT cytosolic 2); NFAT2 (Nuclear factor of activated T-cells 2, NFATc, NFAT cytosolic, NFATc1, NFAT cytosolic 1); NFAT3 (Nuclear factor of activated T-cells 3, NFATc4, NFAT cytosolic 4); NFAT4 (Nuclear factor of activated T-cells 4, NFATx, NFATc3, NFAT cytosolic 3); NFAT5 (Nuclear factor of activated T-cells 5, TonEBP, Tonicity-responsive enhancer binding protein, NFATz, OREBP, Osmotic response element-binding protein, NFATz, NFAT-L1, NFAT-related protein 1)

Historical Background

Initially defined as a factor that bound the interleukin (IL)-2 promoter in activated T cells, the first Nuclear Factor of Activated T cells (NFAT) protein was isolated almost 20 years ago and termed NFATp, as it was shown to be "preexistent" in the cytosol of resting cells. In activated T cells, NFATp interacted in the nucleus with Fos and Jun proteins to induce the expression of IL-2 (Jain et al. 1993; McCaffrey et al. 1993). Soon after that, new members of the NFAT family were also identified and the characterization of the calcineurin-mediated dephosphorylation and activation of NFAT proteins allowed establishing them as the targets of the immunosuppressive effects of cyclosporine A. Although initial efforts were focused in studying the role of NFAT proteins in the regulation of T cell activation, it soon became clear that this ubiquitously expressed family of transcription factors was also involved in the regulation of a multitude of programs of development and differentiation in many other cell types. This chapter reviews the current understanding on the mechanisms that regulate the activity of NFAT proteins and the functions that these proteins have in different cells and tissues.

Family and Structure

The NFAT family of transcription factors comprises five different members: NFAT1 (also known as NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx or NFATc3), and NFAT5. The activation of NFAT1, NFAT2, NFAT3, and NFAT4 is regulated by the calcium/calmodulin-activated phosphatase calcineurin, whereas NFAT5 is the only NFAT protein that is not regulated by calcium (Macian 2005). NFAT5, which was also identified as the Tonicity-responsive Enhancer-Binding Protein, has been shown to regulate the expression of osmoprotective genes in mammalian cells in response to osmotic stress (Lopez-Rodriguez et al. 2001). All NFAT family members share a conserved DNA-binding domain, which in turn shares structural homology with the Rel domain found in the NF-κB family of transcription factors. The DNA-binding domain confers specific DNA binding and mediates interactions with many transcriptional partners, including Fos and Jun proteins (Chen et al. 1998). With the exception of NFAT5, NFAT proteins have also an N-terminal regulatory domain, known as the NFAT homology region (NHR). The NHR contains transactivation and regulatory domains, which include interaction sites for calcineurin and several kinases that regulate NFAT activation by modifying the phosphorylation status of multiple serine-containing motifs (Fig. 1) (Hogan et al. 2003). NFAT5 also contains a Rel domain as a conserved DNA-binding and dimerization domain but it lacks a NHR (Lopez-Rodriguez et al. 2001). The C-terminal region is not conserved among the different NFAT proteins and has been shown to contain sites that may allow interactions with transcriptional co-activators.



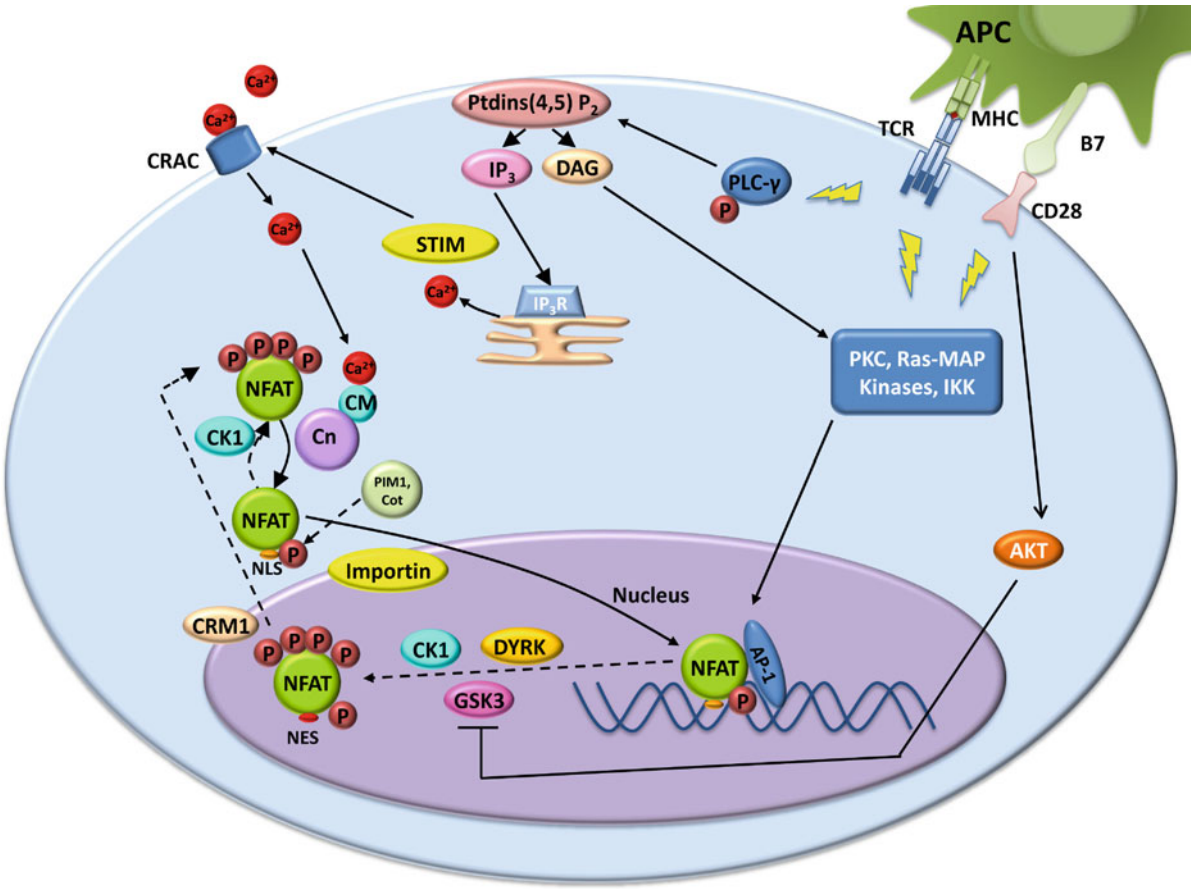
NFAT, Fig. 1 NFAT1, 2, 3, and 4 contain a regulatory domain (NHR or NFAT-homology domain), which comprises a transactivation domain (TAD), several target phosphorylation motifs for NFAT-kinases (SRR1, SRR2, SP1, SP2, and SP3), calcineurin-binding sites, and the nuclear localization signal (NLS); and a DNA-binding domain (DBD or Rel-homology region, RHR), which also contains residues required to interact with Fos and Jun proteins. NFAT5 shares a conserved DNA-binding domain but differs in the rest of its structure for the other NFAT family members

Regulation

NFAT activation is mainly regulated by its subcellular localization. The net result of the rate of nuclear import and export of NFAT proteins, which is controlled by their phosphorylation status, determines the overall level of activation of NFAT. Moreover, several other mechanisms have also been described to contribute to the fine regulation of the transcriptional activity of NFAT.

Regulation by calcium and calcineurin: Engagement of calcium-coupled receptors, such as the T cell receptor (TCR), induces the activation of the calcium/calmodulin-dependent phosphatase calcineurin, which binds NFAT proteins and directly dephosphorylates them, inducing their translocation into the nucleus. In T cells, where this complex regulation has been better characterized, engagement of the TCR induces activation of the phospholipase Cγ, which hydrolyzes phosphatidylinositol 4,5-biphosphate into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds IP_3 receptors in the endoplasmic reticulum and induces calcium-release from intracellular calcium stores. STIM proteins sense this depletion and activate calcium entry through interaction with ORA1, an integral component of the calcium-release activated calcium (CRAC) channels in the plasma membrane, causing a further increase in the intracellular calcium levels (Feske et al. 2006; Oh-hora and Rao 2008). In response to the increase in the intracellular calcium concentration, calcineurin is activated and binds to NFAT and dephosphorylates NFAT proteins, which are heavily phosphorylated and localized in the cytosol in resting cells (Fig. 2). At least 13 different phosphorylation sites located in serine-rich motifs and SPxx repeat motifs in the regulatory domain are dephosphorylated by calcineurin. Dephosphorylation of these residues causes a conformational change in NFAT that exposes a nuclear localization signal, allowing NFAT import into the nucleus, where it binds specific sites and cooperates with other transcription factors to activate the expression of distinct sets of genes (Hogan et al. 2003).

NFAT-kinases: Phosphorylation of NFAT proteins is required to promote nuclear export and to maintain cytosolic localization in resting conditions. Several kinases have been reported to be responsible for the phosphorylation of different serine-containing motifs in NFAT. Casein kinase1 (CK1) binds the N-terminal region of NFAT and regulates its nuclear export and cytosolic retention through phosphorylation of a serine-rich motif. Glycogen synthase kinase 3 (GSK3) phosphorylates serine-proline motives in NFAT1 and NFAT2, promoting NFAT nuclear export. These phosphorylation sites appear to be created by previous priming by cyclic-AMP dependent Protein Kinase-mediated phosphorylation of NFAT. Activation of the AKT kinase negatively regulates GSK3 and prolongs NFAT residence time in the nucleus. The dual-specificity tyrosine-(Y)-phosphorylation-regulated kinases DYRK1A and DYRK2 also regulate NFAT nuclear export through phosphorylation of an SP motif that primes for subsequent phosphorylation by CK1 or GSK3. There is evidence that other kinases, such as p38 and JNK, PIM1, or Cot, may also phosphorylate NFAT proteins and contribute to their regulation (Macian 2005; Muller and Rao 2010).



NFAT, Fig. 2 *NFAT regulation by calcium/calcineurin and NFAT-kinases in activated T cells*. Pathways involved in the activation of NFAT by nuclear import and modulation of its transcriptional activity are represented with solid arrows, whereas the pathways involved in NFAT nuclear export or cytosolic retention are depicted with dashed arrows. *APC* antigen presenting cell, *TCR* T cell receptor, *MHC* major histocompatibility complex, *PLC-γ* phospholipase Cγ, *P* phosphate, Ptdins(4,5)P_2, phosphatidylinositol 4,5-biphosphate, *IP3* inositol-1,4,5-triphosphate, *DAG* diacylglycerol, *IP3R* inositol-1,4,5-triphosphate receptor, Ca^{2+} calcium, *CRAC* calcium release activated calcium channel, *STIM* stromal interaction molecule, *NFAT* nuclear factor of activated T cells, *CM* calmodulin, *Cn* calcineurin, *CK1* casein kinase1, *NLS* nuclear localization signal, *NES* nuclear export signal, *PIM1* proto-oncogene serine/threonine-protein kinase 1, *Cot* cancer Osaka thyroid oncogene 1, *DYRK* dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1, 2, *GSK3* glycogen synthase kinase 3, *CRM1* exportin 1, *JNK* C-Jun N-terminal kinase, *AP-1* activator protein 1, *PKC* protein kinase C, *IKK* inhibitor of kappa B kinase

Transcriptional regulation of NFAT: NFAT2A is an isoform of NFAT2 that is regulated at a transcriptional level by an auto-regulatory loop under the control of an inducible NFAT-dependent promoter. In T cells, it has been shown that the activation of constitutively expressed isoforms of NFAT2 and NFAT1, induces the expression and accumulation of this inducible NFAT2A isoform (Serfling et al. 2006).

Posttranslational regulation: Several posttranslational mechanisms that also contribute to the regulation of NFAT activity have recently been described. Sumoylation of NFAT has been characterized as a NFAT nuclear retention mechanism, which might also regulate NFAT transcriptional activity. Recently, evidence has also been presented that poly(ADP-ribose) polymerase-1 (Parp-1) interacts with NFAT1 and NFAT2, regulating NFAT nuclear export and its transcriptional activity. NFAT1 has also been characterized to be ubiquitinated by the E3 ubiquitin ligase MDM2 in breast cancer cells, which leads to its proteasome-mediated degradation (Mancini and Toker 2009; Muller and Rao 2010).

Transcriptional Activity

Numerous reports have shown that NFAT proteins form transcriptional complexes cooperating with many other transcription factors to activate or repress the expression of specific genes. This allows cells to integrate calcium signaling with other signaling pathways to regulate the expression of specific programs of gene expression. Initially identified as the nuclear component of the NFAT activity that was responsible for the expression of IL-2 in activated T cells, Activator protein 1 (AP-1) complexes are the best characterized NFAT partners, and one of the main families of transcription factors that interacts with NFAT during T cell activation. In response to the engagement of the TCR and co-stimulatory receptors, calcium signaling and the Ras-MAPK pathway converge in the activation of NFAT and Fos and Jun proteins, which form the AP-1 complex. The DNA-binding domain of NFAT interacts with AP-1, forming a quaternary complex on DNA that activates the expression of activation-induced genes, including numerous cytokines. The number of transcription factors that have been identified to cooperate with NFAT has grown in the last few years. In many cases these interactions occur in specific cells of tissues and are responsible for the regulation of different programs of activation, differentiation, or development. A list of transcription factors that cooperate with NFAT to activate or inhibit the expression of particular genes is shown in Table 1. NFAT proteins are also able to form homodimers. These complexes bind to κB-like sites that contain two tandem NFAT-binding sites separated by one or two bases. NFAT dimers have been reported to bind the promoter of several genes to positively regulate their expression. Recently, dimers formed by NFAT1 have been implicated in regulating the expression of genes that are responsible for the induction of an unresponsive state in anergic T helper cells (Soto-Nieves et al. 2009). In addition, NFAT5 is a constitutive dimer and needs to form homodimers to bind to DNA and exert its transcriptional activity (Lopez-Rodriguez et al. 2001).

Functions

The innate immune system is our first line of defense against invading pathogens and, therefore, it must react quickly and efficiently. Cell types involved in this process include eosinophils, neutrophils, mast cells, and macrophages. NFAT proteins have been shown to be expressed in all these cell types and serve a pivotal role in transforming molecular signals to expression of genes. For instance, fungal infections are commonly seen in cyclosporine A treated individuals and appear to respond to impaired killing of fungi caused by inhibition of NFAT-mediated gene expression in neutrophils (Greenblatt et al. 2010). NFAT expression also plays an integral part of the mast cell response, as ligation of the FcεR with IgE results in intracellular calcium fluxes, causing mast cells to release histamine containing granules and to produce cytokines (Turner and Kinet 1999). Calcineurin has also been shown to regulate the toll-like receptor pathway and, therefore, blocking calcineurin activity and NFAT activation leads to altered innate and inflammatory responses.

The adaptive immune response is highly specific and efficient at targeting pathogens and infected cells for elimination. This arm of the immune system involves the uptake and processing of foreign material by antigen presenting cells and the presentation of this material to effector cells. The activation of effector cells is governed by the recognition of an antigen by the B cell Receptor (BCR) or its presentation in the context of an MHC molecule to cognate TCRs on T cells. NFAT has been most extensively studied in T cells, although roles in the regulation of B cell have also been characterized. NFAT proteins are activated in B cells in response to BCR engagement and have been shown to be involved in the induction of the programs of gene expression that regulate B cell activation and differentiation. As described in detail above, in T cells, TCR triggering by the recognition of antigen: MHC complexes induces the calcineurin-mediated dephosphorylation of NFAT proteins, which causes nuclear localization of these transcription factors and leads to the expression of genes involved in a productive immune response (Wu et al. 2007; Muller and Rao 2010). Coordinated engagement of the TCR and the co-stimulatory receptor CD28 leads to the nuclear presence of the NFAT transcriptional partners Fos and Jun, which are necessary for IL-2 production and proper T cell activation and proliferation. In the absence of co-stimulation, TCR triggering still leads to NFAT nuclear localization, however, without the opportunity to cooperate with Fos and Jun, NFAT proteins form homodimers that regulate the expression of

NFAT, Table 1 NFAT transcriptional partners

NFAT transcriptional partners	Interaction site	Effect of cooperation with NFAT
AP-1(Fos, Jun)	Cytokine promoters, *Rorgt*	Activating
ICER	*Il2* promoter	Inhibiting
$P21^{SNFT}$	*Il2* promoter	Inhibiting
Foxp3/Runx1	*Il2* promoter or *Gitr, Cd25, Ctla4* promoters	Inhibiting or activating, respectively
PPAR-γ	Cytokine promoters	Inhibiting
Mina	*Il4* promoter	Inhibiting
t-bet	*Ifng* enhancer	Activating
c-MAF/IRF4	*Il4* promoter	Activating
IRF4	Cytokine promoters and *Rorgt*	Activating
BRG1	*Il4* promoter	Activating
GATA3	*Il4 3'* enhancer	Activating
MEF2	*Nur77* promoter	Activating
Smad3	*Foxp3 promoter*	Activating
EGR1 and EGR4	*Tnf* promoter	Activating
C/EBP	*Pparg* promoter	Activating
OCT	*Il3* enhancer	Activating
MITF	*Cathepsin K* promoter	Activating
Osterix	*Col1a1 promoter*	Activating
NIP45	*Il4 promoter*	Activating
Pu.1	*Beta3 integrin* promoter	Activating

Abbreviations: *AP-1* activator protein 1, *ICER* inducible cyclic AMP early repressor, $p21^{SNFT}$ 21-kDa small nuclear factor isolated from T cells, *Foxp3* forkhead box P3, *Runx1* Runt-related transcription factor 1, *PPAR-γ* peroxisome proliferator-activated receptor-γ, *Mina* Myc induced nuclear antigen, *T-bet* Th1-specific T box transcription factor, *IRF4* interferon-regulatory factor 4, *BRG1* Brahma-related gene1, *GATA3* GATA-binding protein 3, *MEF2* myocyte-enhancer factor 2, *EGR* early growth response, *C/EBP* CCAAT/enhancer-binding protein, *OCT* octamer-binding transcription factor, *MITF* Microphthalmia associated transcription factor, *NIP45* NFAT interacting protein, *Gitr* Glucocorticoid-induced tumor necrosis factor receptor family-related gene, *Ctla4* cytotoxic T lymphocyte-associated protein 4, *Rorgt* Retinoid-related orphan receptor gamma thymus-specific isoform, *Ifng* interferon-g, *Col1a1* encoding type I collagen, *Il* interleukin, *NUR77* orphan nuclear receptor 77, *Tnf* tumor-necrosis factor

genes involved in keeping T cells in a nonresponsive state termed anergy. Several genes, such as Grail, Caspase3, Ikaros, Deltex1, and Egr2, are required for the induction of anergy and their expression depends on the activation of NFAT in T cells that receive tolerizing stimuli (Macian et al. 2002; Baine et al. 2009). The involvement of NFAT proteins in the regulation of immune tolerance is also mediated through their role in the development of regulatory T cells (Treg), a distinct population of T cells that express the transcription factor FoxP3 and have the capacity to suppress the activation of other T cell populations. NFAT proteins not only regulate the expression of FoxP3 but also cooperate with this transcription factor to activate (CTLA-4, CD25, and GITR) or inhibit (IL-2) the expression of genes in Tregs and, therefore, regulate the differentiation and function of these suppressor cells (Wu et al. 2006).

T cell development is also dependent on NFAT activity. The development of thymocytes in the thymus involves their maturation into CD4+ and CD8+ T cells that can interact with MHC molecules. In the thymus, immature T cells rearrange both the α and β chains of the TCR and mature from a double negative (CD4-CD8-) thymocyte into a double positive thymocyte that expresses both CD4 and CD8 co-receptors. From here, T cells undergo a rigorous process that involves being positively selected for the presence of a TCR that can interact with MHC molecules, but negatively selected for self-reactive TCR that can recognize self-antigens. The lack of calcineurin activity in immature thymocytes leads to a block in positive selection, suggesting a role for NFAT proteins in thymocyte development. Recent evidence has shown that NFAT1 and NFAT4 are responsible for regulating the thresholds for the transduction of signals that are

necessary to induce positive selection in the thymus (Gallo et al. 2007).

CD4+ T cells can differentiate into many different effector subsets, and NFAT proteins are involved in these processes. Naïve T helper cells can differentiate into Th1, Th2, and Th17 cells, depending on the cytokine profile of the extracellular environment. In all these differentiation events, NFAT regulates the transcription of genes that help define the particular T cell subtype, which depends on the choice of transcriptional partners. For instance, T-bet is an integral co-transcriptional partner for NFAT in the development of Th1 cells and the secretion of their signature cytokine IFN-γ. Likewise, GATA3–NFAT interactions are necessary for IL-4 expression and the maturation into Th2 cells. Similarly, the presence of IL-6 in the extracellular milieu induces the expression of Rorgt, which synergizes with NFAT in the expression of IL-17, IL-21, and IL-22 to allow Th17 differentiation (Muller and Rao 2010).

Outside of the immune system, NFAT expression has been reported in almost all tissues, although the expression of any individual NFAT protein is often limited to specific tissues. For instance, whereas NFAT1, NFAT2, and NFAT4 are expressed in cells of the immune system and in several nonlymphoid cells and tissues, NFAT3 expression has not been reported in immune cells. During the last few years an increasing number of reports have characterized the role that different NFAT proteins have as regulators of development, differentiation, and function in many cells and tissues, including, skeletal muscle differentiation, myocardial hypertrophy, control of heart valve formation, vascular development, cartilage formation, neuronal development, and the regulation of stem cell quiescence (Wu et al. 2007). The expression of different NFAT proteins is developmentally regulated in skeletal muscle and these proteins control progression from immature precursors to mature myocytes and contribute to the specification of muscle fiber type. NFAT proteins also regulate cartilage growth and bone remodeling. The role that NFAT proteins play in the regulation of osteoclast differentiation has been amply documented. In these cells, RANKL-mediated activation of NFAT2 directs the expression of a set of genes required for osteoclast differentiation. Initial characterization of a mouse model that lacked expression of NFAT2 unequivocally showed that NFAT2 played a key role in the formation of the heart's valves. In the adult heart, NFAT proteins partner with members of the GATA and MEF2 families of transcription factors to regulate myocardial hypertrophy. NFAT3 and NFAT4 are expressed in perivascular mesenchymal cells, which regulate the assembly of blood vessels during embryogenesis, and mice that lack those NFAT proteins present an abnormal vascular development. Vascular endothelial growth factor is a major activator of NFAT proteins in endothelial cells, and engagement of its receptor leads to the activation of NFAT-dependent genes such as COX2. NFAT proteins also regulate neuronal axon growth and are essential for neuronal development and the differentiation of Schwann cells. NFAT has also been shown to control beta cell growth in the endocrine pancreas and regulate insulin-signaling pathways and adipogenesis. Recently, NFAT2 has also been implicated in the maintenance of stem cell quiescence in the skin follicle by repressing the expression of the cell cycle kinase CDK4 (Wu et al. 2007; Aliprantis and Glimcher 2010).

As discussed above, NFAT proteins are key regulators that control cell development and differentiation in part by modulating proliferation and cell death. As such, altered NFAT signaling has been described associated with cancer. As expected due to their pivotal role in lymphocyte development, altered NFAT activity have been associated with several forms of B and T cell lymphoma and leukemia. Furthermore, different NFAT family members have been shown to be involved in the regulation of different properties of many cancer cell types. For instance, NFAT1 and NFAT5 appear to positively regulate migration and invasion of breast cancer cells. Given their role in angiogenesis, NFAT proteins have also been proposed to regulate tumor-associated angiogenesis. Furthermore, NFAT may also directly activate the expression of oncogenes, such as *Myc* (Mancini and Toker 2009; Muller and Rao 2010).

Pharmacological Modulation of NFAT Activity

Pharmacological agents currently used to modulate NFAT activity have found tremendous benefit in the clinic. These agents, which inhibit the activation of T cells and act as immunosuppressants, are widely used in therapies ranging from treatment of autoimmune disease to prevention of organ transplant rejection. However, their widespread use is limited by their

costly side effects, such as nephrotoxicity. The activation of NFAT is dependent on its dephosphorylation, and, therefore, the phosphatase calcineurin has been an important area of focus for inhibitor development. Nevertheless, the specificity of NFAT inhibition is limited by the fact that NFAT is not the only target of calcineurin. Cyclosporine A and FK506 are the most widely used and studied suppressors of NFAT activity. Both are calcineurin inhibitors whose mechanism of action is quite similar. They bind separate intracellular peptidyl prolyl isomerases (cyclosporine A-cyclophilin; FK506-FKBP12). These complexes then bind to distinct regions of calcineurin and inhibit its phosphatase activity. The fact that these compounds not only affect calcineurin but also the activity of their endogenous targets also limits their use as specific NFAT inhibitors. The search for more specific inhibitors has centered on targeting specifically the interaction of NFAT and calcineurin. Calcineurin-binding sites on NFAT have been mapped to the N-terminal regulatory domain and include the amino acid sequence SPRIET. A closer analysis of this sequence among NFAT family members revealed the consensus-binding sequence PxIxIT, which laid the groundwork for the discovery using combinatorial libraries of the highly potent VIVIT peptide (Hogan et al. 2003). This peptide has been successfully used in mouse models of graft rejection and tumor progression. The major benefit of the VIVIT peptide is in its higher specificity for the inhibition of NFAT function; however, this sequence is still conserved in other proteins, such as AKAP79, Cabin1, or MCIP1, that have calcineurin-binding activity. Due to the long term side effects of cyclosporine A and FK506, finding inhibitors with more specificity for inhibiting NFAT functions and less on calcineurin has been also pursued. A number of natural and synthetic compounds have been discovered and developed over the last years. Some small compounds have been shown to covalently bind to calcineurin and disrupt more specifically the calcineurin–NFAT interaction. Similarly, disrupting the ability of NFAT to bind DNA or enhancing the nuclear export of NFAT has also been explored. As binding partners and gene targets of NFAT are being discovered, further inhibitors with higher specificity could be developed. This should lead to a higher degree of precision to specifically alter NFAT activity.

Summary

Members of the NFAT family of transcription factors have been established as crucial regulators of numerous programs of development, differentiation, and activation in many cell types and tissues. NFAT activation is induced by the engagement of calcium-coupled receptors that eventually leads to the translocation of this transcription factor into the nucleus, where it cooperates with several transcriptional partners so that signals that emanate from different inputs can be integrated to ensure specific regulation of distinct program of gene expression. Circuits of regulation that fine-tune NFAT activation have also been proposed and are likely to play important roles in the regulation of NFAT activity. Given the wide range of tissue expression of NFAT proteins, it is clear that novel functions and targets of these transcription factors still remain to be discovered. The identification of specific functions for individual NFAT family members and the characterization of the differential spatial and temporal expression of specific NFAT proteins during unique programs of development should also enhance our understanding on how NFAT-regulated programs of development are orchestrated. The development of new more specific inhibitors of NFAT activity is also a pending issue that should resolve the inherent toxic effect associated with the use of calcineurin inhibitors. Given the increasing amount of evidence that clearly involves NFAT signaling in oncogenesis and cancer progression, these new therapeutic approaches should prove of great value not only to suppress NFAT-regulated immune responses, but also to design new interventions for the treatment of certain types of cancer and other pathologies controlled by this family of transcription factors.

References

Aliprantis AO, Glimcher LH. NFATc1 In inflammatory and musculoskeletal conditions. Adv Exp Med Biol. 2010; 658:69–75.

Baine I, Abe BT, Macian F. Regulation of T-cell tolerance by calcium/NFAT signaling. Immunol Rev. 2009;231:225–40.

Chen L, Glover JN, Hogan PG, Rao A, Harrison SC. Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. Nature. 1998;392:42–8.

Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, et al. A mutation in Orai1 causes immune deficiency by

abrogating CRAC channel function. Nature. 2006;441:179–85.
Gallo EM, Winslow MM, Cante-Barrett K, Radermacher AN, Ho L, McGinnis L, et al. Calcineurin sets the bandwidth for discrimination of signals during thymocyte development. Nature. 2007;450:731–5.
Greenblatt MB, Aliprantis A, Hu B, Glimcher LH. Calcineurin regulates innate antifungal immunity in neutrophils. J Exp Med. 2010;207:923–31.
Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev. 2003;17:2205–32.
Jain J, McCaffrey PG, Miner Z, Kerppola TK, Lambert JN, Verdine GL, et al. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature. 1993;365:352–5.
Lopez-Rodriguez C, Aramburu J, Jin L, Rakeman AS, Michino M, Rao A. Bridging the NFAT and NF-kappaB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress. Immunity. 2001;15:47–58.
Macian F. NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol. 2005;5:472–84.
Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. Cell. 2002;109:719–31.
Mancini M, Toker A. NFAT proteins: emerging roles in cancer progression. Nat Rev Cancer. 2009;9:810–20.
McCaffrey PG, Luo C, Kerppola TK, Jain J, Badalian TM, Ho AM, et al. Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. Science. 1993;262:750–4.
Muller MR, Rao A. NFAT, immunity and cancer: a transcription factor comes of age. Nat Rev Immunol. 2010;10:645–56.
Oh-hora M, Rao A. Calcium signaling in lymphocytes. Curr Opin Immunol. 2008;20:250–8.
Serfling E, Chuvpilo S, Liu J, Hofer T, Palmetshofer A. NFATc1 autoregulation: a crucial step for cell-fate determination. Trends Immunol. 2006;27:461–9.
Soto-Nieves N, Puga I, Abe BT, Bandyopadhyay S, Baine I, Rao A, et al. Transcriptional complexes formed by NFAT dimers regulate the induction of T cell tolerance. J Exp Med. 2009;206:867–76.
Turner H, Kinet JP. Signalling through the high-affinity IgE receptor Fc epsilonRI. Nature. 1999;402:B24–30.
Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell. 2006;126:375–87.
Wu H, Peisley A, Graef IA, Crabtree GR. NFAT signaling and the invention of vertebrates. Trends Cell Biol. 2007;17:251–60.

NFAT1 (Nuclear Factor of Activated T-Cells 1, NFATp, NFAT Preexisting, NFATc2, NFAT Cytosolic 2)

► NFAT

NFAT2 (Nuclear Factor of Activated T-Cells 2, NFATc, NFAT Cytosolic, NFATc1, NFAT Cytosolic 1)

► NFAT

NFAT3 (Nuclear Factor of Activated T-Cells 3, NFATc4, NFAT Cytosolic 4)

► NFAT

NFAT4 (Nuclear Factor of Activated T-Cells 4, NFATx, NFATc3, NFAT Cytosolic 3)

► NFAT

NFAT5 (Nuclear Factor of Activated T-Cells 5, TonEBP, Tonicity-Responsive Enhancer Binding Protein, NFATz, OREBP, Osmotic Response Element-Binding Protein, NFATz, NFAT-L1, NFAT-Related Protein 1)

► NFAT

NFE2L2

► Nrf2 (NF-E2-Related Factor2)

NF-E2-Related Factor 2

► Nrf2 (NF-E2-Related Factor2)

NF-Kappa-B Inhibitor Zeta

► IkBz

NFKBIZ

▶ IkBz

NFPR

▶ Formyl Peptide Receptor

NF-κB Family

Maria D. Sanchez-Niño[1], Ana B. Sanz[1], Maria C. Izquierdo[3], Jonay Poveda[2] and Alberto Ortiz[2]
[1]IdI-Paz, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain
[2]Unidad de Dialisis, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid, Madrid, Spain
[3]IIS-Fundacion Jimenez Diaz and Universidad Autonoma de Madrid, Madrid, Spain

Synonyms

Nuclear factor-kappa-B; Nuclear factor of kappa light polypeptide gene enhancer in B-cells

List of Discussed NF-κB Family Members and Regulatory Molecules

- NFκB1, p105, p50, KBF1, EBP-1
- RelA, p65, NFKB3
- RelB, I-REL
- REL, c-Rel
- NFκB2, p100, p52; LYT10
- Bcl-3, BCL4, D19S37
- NFKBIA, IκBα, IKBA, MAD-3, NFKBI
- NFKBIB, IκBβ, IKBB, TRIP9
- NFKBIE, IκBε, IKBE
- CHUK, IKK-α, IKBKA, IKK1, IKKA, NFKBIKA, TCF16
- IKBKB, IKK-β, IKK2, IKKB, NFKBIKB
- IKBKG, IKK-γ, NEMO, AMCBX1, FIP-3, FIP3, Fip3p, IP, IP1, IP2, IPD2, IKKAP1, IKKG
- IKBKE, IKK-ε, IKKE, IKKI
- MAP3K14, NIK, HS, HSNIK
- TNFAIP3, A20, OTUD7C, TNFA1P2
- OTUD7B, CEZANNE
- CYLD, HSPC057, CDMT, CYLD1, CYLDI, EAC, MFT1, SBS, TEM, USPL2

Historical Background: Discovery and Structure

NF-κB (nuclear factor-kappa-light-chain-enhancer of activated B-cells) is a collective term for a family of transcription factors. NFκB has a complex regulation, modulates the expression of a wide set of genes and biological responses and is involved in a variety of diseases. The dysfunction of NF-κB is associated with inflammatory disease, cardiovascular injury, cancer, diabetes, kidney injury, viral infections, and human genetic disorders, among others (Kumar et al. 2004).

NF-κB was identified as a protein that bound to a specific decameric DNA sequence (ggg ACT TTC C), within the intronic enhancer of the immunoglobulin kappa light chain in mature B- and plasma cells but not pre B-cells (Sen and Baltimore 1986). Later, it was demonstrated that NF-κB DNA-binding activity is induced by a variety of exogenous stimuli, that this activation is independent from de novo protein synthesis and that it binds to several DNA sequences.

NF-κB family members share structural homology with the retroviral oncoprotein v-Rel, resulting in their classification as NF-κB/Rel proteins (Gilmore 2006). There are five proteins in the mammalian NF-κB family: RelA (p65), RelB, c-Rel, p50 (NFκB1, generated from p105), and p52 (NFκB2, generated from p100). All of them may form homo- and heterodimeric complexes. The most common and best characterized active forms the RelA/p50 heterodimer (Hayden and Ghosh 2004).

Each member of the NF-κB family has a conserved N-terminal region termed the Rel homology domain (RHD). The Rel homology domain mediates the DNA binding, dimerization, and nuclear transport of the NF-κB proteins (Li and Verma 2002). However, the transcription activator domain (TAD) necessary for target gene expression is present only in the carboxyl terminus of RelA, c-Rel, and RelB subunits. Large precursors, p105 and p100, undergo processing to generate mature p50 and p52, respectively. The p50 and p52 NF-κB subunits do not contain transactivation

domains. However, they participate in target gene transactivation by forming heterodimers with RelA, RelB, or c-Rel (Li and Verma 2002). The p50 and p52 homodimers also bind to the nuclear protein Bcl-3, and such complexes can function as transcriptional activators.

NF-κB Activation

Activation of NF-κB requires a number of discrete steps (Fig. 1). There is a long list of known inducers of NF-κB activity, including many inflammatory cytokines such as TNF-α superfamily cytokines and IL-1, T-cell activation signals, growth factors, reactive oxygen species (ROS), bacterial lipopolysaccharide (LPS), and other stress inducers (http://people.bu.edu/gilmore/nf-kb/inducers/index.html).

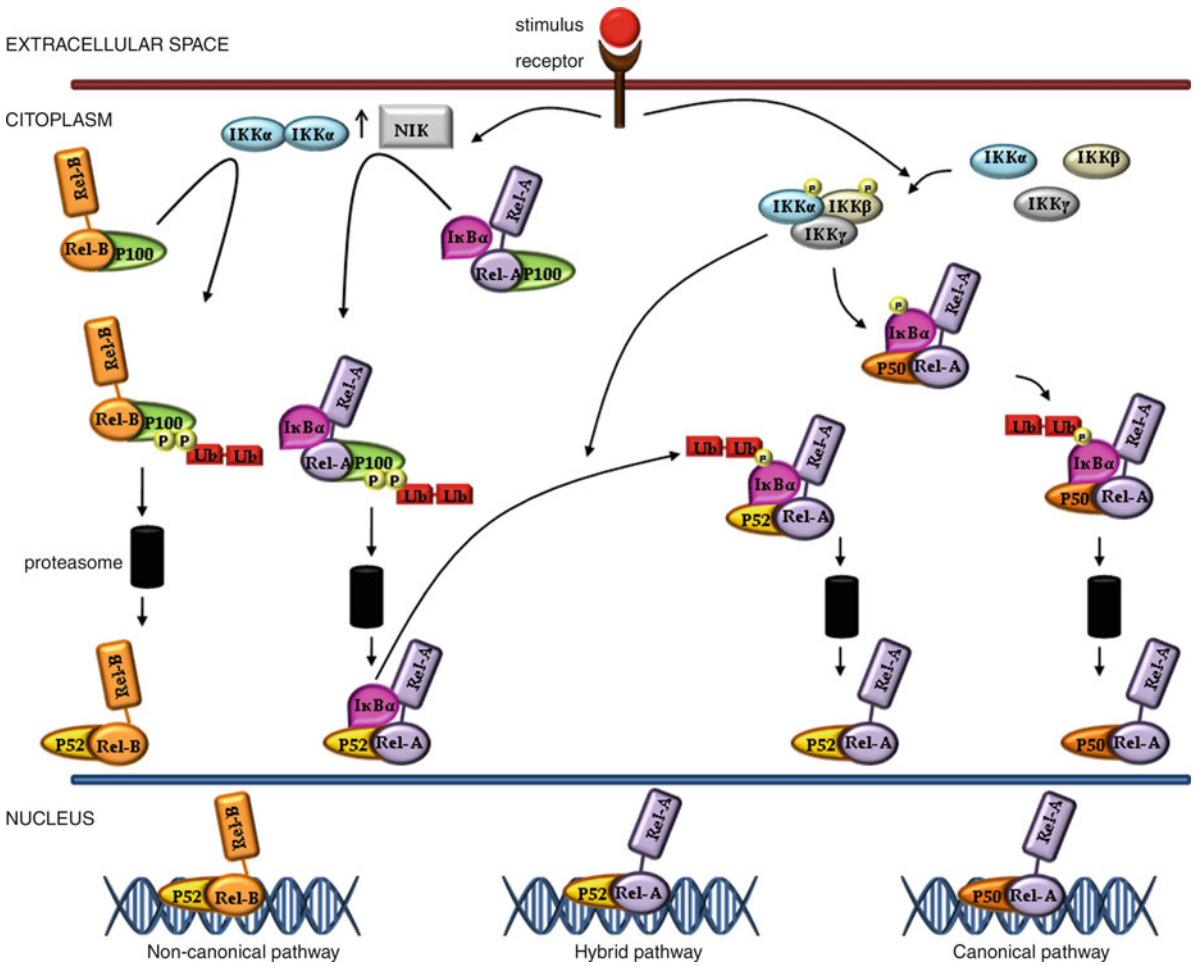
In the cytoplasm of almost all cell types inactive NF-κB is associated with inhibitory κB proteins (IκBs) that regulate NF-κB nuclear translocation and DNA binding (Sanz et al. 2010a). IκBs, are a class of inhibitor proteins that contain an N-terminal regulatory domain, followed by multiple copies of a sequence called ankyrin repeats and a COOH-terminal PEST domain that is important in regulating IκB turnover. The ankyrin repeats mediate the association between IκBs and NF-κB dimers and mask the nuclear localization signals (NLS) of NF-κB proteins, thus preventing nuclear translocation and keeping them in an inactive state in the cytoplasm. The most important IκBs are IκBα (associated with transient NF-κB activation), IκBβ (involved in sustained activation), and IκBε. Additional IκBs are Bcl-3, p100, and p105. Bcl-3 is the only that does not induce cytoplasmic retention of NF-κB, but regulates gene expression as a transcriptional co-activator for p50 and p52 homodimers in the nucleus. The phosphorylation, ubiquitination, and subsequent degradation by the 26S proteasome of IκB proteins is a key step in NF-κB activation that release active NF-κB (Karin and Delhase 2000). NF-κB activation is initiated by a signal-induced phosphorylation of IκBs, mediated by a high molecular weight complex that contains a serine-specific IκB kinase (IKK). IKKs have 52% amino acid identity and a similar structural organization, which includes kinase, leucine zipper, and helix-loop-helix domains. IKKs form both homo- and heterodimers. Gene disruption studies of the IKK genes in mice indicate that IKKβ is the critical kinase involved in activating the NF-κB pathway, while IKKα likely plays an accessory role (Yamamoto and Gaynor 2001). The IKK complex consists of three subunits, including the kinases IKK-α and IKK-β (catalytic subunits also called IKK-1 and IKK-2, respectively) (Sanz et al. 2010a), and the regulatory nonenzymatic subunit IKK-γ (NEMO, NF-κB essential modulator) (Sanz et al. 2010a). IKKs are key convergence steps for multiple signaling pathways that lead to NF-κB activation.

There is a temporal and selective control of NF-κB target gene activation. Some genes are transcribed following a short stimulation of NF-κB. These include negative regulators of NF-κB activity such as IκBα and inflammatory cytokines such as IL-6 and MCP-1. Other genes are transcribed only when NF-κB activation occurs for at least 1 h such as cell-surface receptors, adhesion molecules, and some chemokines such as RANTES/CCL5 (Hoffmann et al. 2002). DNA accessibility contributes in part to these temporal patterns.

NF-κB activation and nuclear translocation can proceed either through the classical/canonical pathway, that is a rapid and transient response of stimuli involving mainly RelA/p50, or the alternative/noncanonical NF-κB pathway, that involves slow activation of the RelB/p52 heterodimer leading to prolonged activation of NF-κB target genes (Sanz et al. 2010a) (Figs. 1 and 2). These pathways are activated by often distinct stimuli, require different IKK complexes, activate different NF-κB complexes and may have different target genes.

Canonical pathway: The classical NF-κB pathway is triggered by most of the stimuli shown to activate NF-κB. In this pathway, the activated IKK complex IKKα, IKKβ, and NEMO phosphorylates two specific serines near the N terminus of IκBα to trigger its ubiquitin-dependent degradation by the 26S proteasome, allowing nuclear migration of RelA/p50 and other NF-κB dimers (Haas 2009).

Noncanonical pathway: The alternative pathway results in nuclear translocation of the heterodimer RelB/p52 leading to prolonged activation of NF-κB target genes (Senftleben et al. 2001). Only a small number of stimuli are known to activate NF-κB via this pathway, including TNF superfamily members such as TWEAK, but also lymphotoxin-α, BAFF or RANKL (Sanz et al. 2010b). This pathway requires IKKα phosphorylation by NF-κB-inducing kinase



NF-κB Family, Fig. 1 *Schematic representation of canonical and noncanonical NF-κB pathway activation*. In the canonical pathway IKK activation leads to proteasomal IκB degradation, which allows nuclear migration of RelA/p50 and other complexes. In the noncanonical pathway NIK and IKKα recruitment lead to proteasomal processing of p100 to p52, allowing nuclear migration of RelB/p52 complexes. p100 may also retain in the cytoplasm RelA, c-Rel, and p50. Thus, proteasomal processing of p100 to p52 also generates RelA/p52, c-Rel/p52, or p52/p50 complexes. IκB molecules weakly sequester RelB/p52 complexes and they are free for nuclear translocation upon p100 processing. In the hybrid pathway noncanonical p100 processing generates RelA/p52 and c-Rel/p52 complexes that are retained in the cytosol by IκB proteins. Classical pathway degradation of IκB proteins allows nuclear migration of these complexes. *P* phosphorylation, *Ub* Ubiquitin

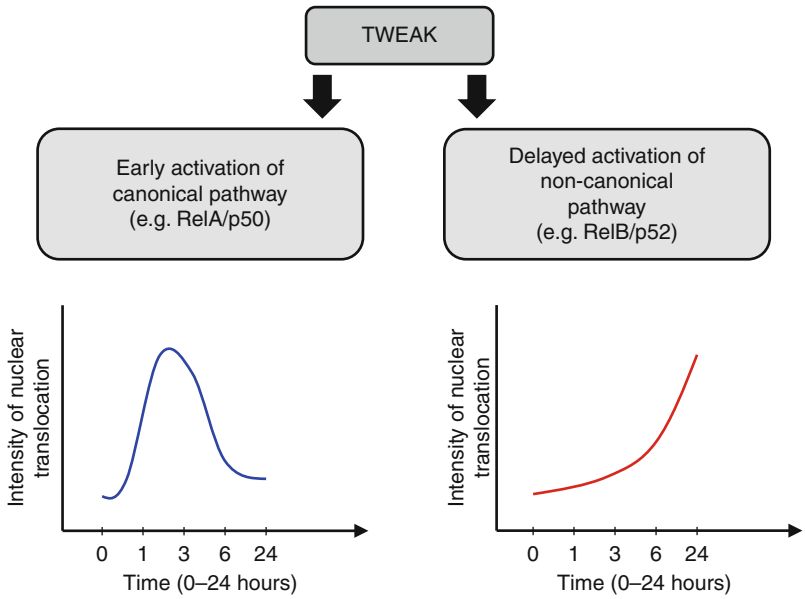
(NIK). IKKα phosphorylates p100 leading its polyubiquitination and promoting the proteasomal processing to p52, freeing the active p52/RelB dimers that migrate to the nucleus (Senftleben et al. 2001).

The noncanonical IKKε (IKKi) is a serine/threonine kinase inducible by inflammatory mediators that activates IRF-7, phosphorylates IκBα, NF-κB p65, and c-Rel and is required for activation of an NF-κB complex containing p52 and p65 (Wietek et al. 2006; Harris et al. 2006). However, mice bearing a deletion of the IKKε gene activate NF-κB normally in response to lipopolysaccharide.

Hybrid pathway: The hybrid pathway of NF-κB activation requires the contribution of both pathways: the NF-κB complex is generated by the alternative pathway and activated by the classical pathway (Sanz et al. 2010a).

Finalization of NF-κB Activation

NF-κB activation is regulated by negative feedback loops which control the duration of NF-κB nuclear localization in response to a stimulus. Negative



NF-κB Family, Fig. 2 *Temporal pattern profile of TWEAK-induced canonical and noncanonical NF-κB pathway activation in renal proximal tubular epithelial cells.* TWEAK is one of a handful of TNF superfamily cytokines that activates both the canonical NF-κB pathway, leading to early transient nuclear translocation of RelA/p50 and the noncanonical NF-κB pathway, leading to delayed nuclear translocation of RelB/p52

feedback mechanisms include NF-κB-dependent induction of IκBα, A20, and cezanne. Newly formed IκBα sequesters NF-κB subunits and terminates transcriptional activity unless a persistent activation signal is present. Deubiquitinating enzymes CYLD (cylindromatosis gene), A20, and cezanne induced by proinflammatory signaling can block IKK activation by removing polyubiquitin chains (Van der Heiden et al. 2010).

Target Genes

NF-κB complexes regulate the transcription of multiple genes related to inflammation, immunity, apoptosis, cell proliferation, and differentiation. At http://people.bu.edu/gilmore/nf-kb/ the reader can find updated lists of NF-κB gene targets classified by function as cytokines/chemokines and their modulators, immunoreceptors proteins involved in antigen presentation, cell adhesion molecules, acute phase proteins, stress response genes, cell-surface receptors, regulators of apoptosis, growth factors, ligands and their modulators, early response genes, transcription factors and regulators, viruses, enzymes, and others.

While NF-κB frequently promotes gene transcription, it may also function as a repressor of gene expression. Thus, anti-inflammatory cytokines may induce the synthesis of nuclear-located atypical IκB proteins, which bind to DNA-bound NF-κB dimers and repress transcription of inflammatory genes (Ghosh and Hayden 2008). Additional mechanism involve competition of RelA with co-activators of transcription, posttranslational modifications of RelA, and posttranslational modification of histones near the NF-κB target genes (Sanz et al. 2010a).

No clearcut differences in DNA-binding sequences have been observed for the different NF-κB complexes and considerable promiscuity is thought to occur.

The Role of NF-κB Signaling in Disease

Studies in knockout mice revealed specific functions of each NF-κB family proteins in the regulation of disease. Deletion of RelA or IKK-β genes in mice causes embryonic lethality due to high apoptosis in the liver indicating that their function is indispensable during development (Beg et al. 1995; Li et al. 1999). On the other hand, mice lacking RelB are immunodeficient but develop normally to adulthood (Sha et al. 1995). Mice lacking c-Rel or p52 have defective immune functions (Caamano et al. 1998).

Dysregulated activation of the NF-κB pathway is involved in the pathogenesis of a number of human diseases. The NF-κB family controls multiple processes, including immunity, inflammation, cell survival, differentiation and proliferation, and regulates

cellular responses to stress, hypoxia, stretch, and ischemia. Activation of the NF-κB pathway is involved in the pathogenesis of chronic inflammatory disease, kidney disease, atherosclerosis, viral infections, neurological diseases, cancer and aging, among others. Evidence for the involvement of NF-κB in disease often comes from functional studies in experimental cell and animal models as well as descriptive data from animal models and human samples. We will provide some examples of NF-κB involvement in disease processes.

Inflammation

NF-κB is involved in the pathophysiology of autoimmune and inflammatory disorders, such as rheumatoid arthritis, asthma, and others.

NF-κB is activated in the inflamed synovium of rheumatoid arthritis patients as well as in the synovium of animal models in this disease.. Intra-articular gene transfer of IKK-β into the joints of normal rats resulted in synovial inflammation, while arthritis dominant-negative adenoviral IKK-β construct ameliorated the severity of adjuvant. NF-κB "decoy" or RelA antisense oligodeoxynucleotides prevented the development of arthritis in rats (Neurath et al. 1996).

Asthma is characterized by the lung infiltration of inflammatory cells. Increased NF-κB and is evident in biopsies from asthmatic patients (Kumar et al. 2004). Treatment with steroids decreases NF-κB activity in mice, cultured cells (Kumar et al. 2004), and in asthmatic patients as well as reducing the symptoms of the disease.

Kidney Disease and Aging

The role and regulation of NF-κB in kidney disease was recently reviewed and includes functional studies showing improvement of kidney disease outcomes when targeting NF-κB in experimental models (Sanz et al. 2010a). Many drugs used in kidney disease target NF-κB, including steroids, calcineurin inhibitors, drugs targeting the renin-angiotensin system and statins, but no functional studies of therapies specifically targeting NF-κB are available in humans. The recent observation that inflammation downregulates klotho mRNA expression and protein via NF-κB activation provides a link between inflammation, kidney disease, NF-κB, and accelerated aging (Moreno et al. 2011 accepted). Klotho is a kidney-secreted hormone with antiaging properties and Klotho knockout mice die prematurely from accelerated aging.

Cardiovascular Disease

NF-κB has been linked to both cardiovascular health and disease. NF-κB can protect cardiovascular tissues from injury or contribute to pathogenesis depending on the cellular and physiological context.

Atherosclerosis is a chronic lipid-driven inflammatory disease characterized by accumulation of lipids in arterial walls, which can lead to a heart attack or stroke. Recruitment of monocytes and their extravasation into the subendothelial space, a key event in atherogenesis, is regulated by NF-κB. Activated NF-κB has been identified in situ in human atherosclerotic plaques (Collins and Cybulsky 2001). Interestingly, dietary compounds that block the initiation of atherogenesis are known to inhibit NF-κB activation.

One consequence of atherosclerosis is tissue ischemia. Ischemia-reperfusion promotes alterations in oxygen availability leading to NF-κB activation through proinflammatory cytokines and endogenous ligands for toll-like receptors. Blocking NF-κB using pharmacological inhibitors or decoy oligonucleotides can reduce myocardial infarction in animal models. However in a murine myocardial infarction model NF-κB activation was essential for the protection of cardiomyocytes from apoptosis via induction of cytoprotective genes (Van Der Heiden et al. 2010). This illustrates the "good and evil" aspects of NF-κB.

Human Immunodeficiency Virus (HIV) and Other Viral Infections

Most viruses encode proteins that are capable of activating NF-κB. HIV infection induces NF-κB activation that allows evading the immune response. HIV has two NF-κB binding sites called long terminal repeat (LTR) that are involved in viral transcription. NF-κB activation by viral infection is required for viruses to induce proliferative responses, like expression of cyclin D1, replicate their genetic material, and induce pathogenic responses.

Neurological Diseases

NF-κB is associated with antiapoptotic as well as proapoptotic mechanisms. Consistent with its role in regulating apoptosis, NF-κB serves a cell survival role in stressed neurons through the upregulation of antiapoptotic and antioxidant genes. NF-κB activation may also be involved in the initiation of neuritic plaques and neuronal apoptosis during the early phases of Alzheimer's disease, whereas mature plaque types

show mainly reduced NF-κB activity (Kumar et al. 2004).

Cancer

Constitutive NF-κB activation is involved in some forms of cancer and the inhibition of NF-κB abrogates cell proliferation in these tumors (Kumar et al. 2004). Genes encoding RelA, c-Rel, p105/p50, and p100/p52 proteins are located within regions of the genome involved in oncogenic rearrangements or amplifications. Mutations that can lead to tumors include those that inactivate IκB proteins as well as amplifications of genes encoding NF-κB. In tumor models, NF-κB is activated in tumor cells in response to chemotherapy, and inhibition of NF-κB by viral expression of IκB leads to enhancement in the apoptotic response of the chemotherapy.

Therapeutic Targeting of NF-κB

The identification of NF-κB as a key player in the pathogenesis of the disease suggests that NF-κB-targeting drugs aimed at blocking NF-κB activity might be effective in the clinic. Suppression of NF-κB activation has potential therapeutic applications. In fact, some well known commercially available drugs, such as glucocorticoids, nonsteroidal anti-inflammatory drugs, and calcineurin inhibitors, modulate NF-κB activity.

Repression of NF-κB-dependent gene expression is one of the major elements of immunosuppression and anti-inflammation by glucocorticoids. Glucocorticoids induce IκBα synthesis and enhance the cytosolic retention of NF-κB in monocytes and lymphocytes (Yamamoto and Gaynor 2001). However glucocorticoids block NF-κB activation by different mechanisms in different cell types.

Nonsteroidal anti-inflammatory drugs are used in the treatment of chronic inflammatory disease. Most of them also target NF-κB. Both aspirin and salicylate inhibit NF-κB activation in patients with chronic inflammatory conditions by inhibiting ATP binding to IKKβ.

Cyclosporin A (CsA) and tacrolimus (FK-506) are calcineurin inhibitor immunosuppressive agents used in organ transplantation to prevent rejection. Both inhibit the NF-κB pathway in lymphocytes by distinct mechanisms: preventing IκBα degradation and translocation of c-Rel from the cytoplasm to the nucleus, respectively (Yamamoto and Gaynor 2001).

Proteasome inhibitors may prevent NF-κB activation function by reducing IκB degradation. There are a variety of proteasome inhibitors, some in clinical use, like bortezomib.

Since oxygen radical species promote NF-κB activation, drugs with antioxidant properties may inhibit NF-κB activation.

A variety of other approaches has been used to inhibit NF-κB activation in cell culture or experimental animal models (http://people.bu.edu/gilmore/nf-kb/inhibitors/index.html). These include small molecules, siRNA, oligodeoxynucleotides, degradation-resistant IκBs, and other specific NF-κB inhibitors, whose efficacy has been shown in animal models of inflammatory disease. Since NF-κB may have dualistic roles in disease, specific NF-κB inhibition might result in unintended side effects. As an example, NF-κB may promote both cell survival and inflammation in cells stimulated with certain cytokines, such as TNF and NF-κB inhibition in these circumstances may result both in less secretion of inflammatory mediators and cell death.

Summary

NF-κB is a term used for a family of transcription factors composed of homo- or heterodimeric DNA-binding protein complexes that may be activated in response to a wide variety of stimulus inducing cell stress. NF-κB, in turn, promotes the transcription or repression of a wide array of genes involved in many key cell biology processes. As a result, NF-κB contributes to the pathogenesis of many diseases. Specific NF-κB inhibition is promising in experimental animal models, but experience in humans is limited. By contrast, many commonly used drugs may target NF-κB directly or indirectly as one of several mechanisms of action.

References

Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature. 1995;376:167–70.

Caamaño JH, Rizzo CA, Durham SK, Barton DS, Raventós-Suárez C, Snapper CM, Bravo R. Nuclear factor

(NF)-kappa B2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. J Exp Med. 1998;187:185–96.
Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J Clin Invest. 2001; 107:255–64.
Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. Nat Rev Immunol. 2008;8:837–48.
Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 2006;25:6680–4.
Haas AL. Linear polyubiquitylation: the missing link in NF-kappaB signalling. Nat Cell Biol. 2009;11:116–8.
Harris J, Olière S, Sharma S, Sun Q, Lin R, Hiscott J, Grandvaux N. Nuclear accumulation of cRel following C-terminal phosphorylation by TBK1/IKKepsilon. J Immunol. 2006; 177:2527–35.
Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev. 2004;18:2195–224.
Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science. 2002;298:1241–5.
Karin M, Delhase M. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin Immunol. 2000;12:85–98.
Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-kappaB: its role in health and disease. J Mol Med. 2004;82:434–48.
Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol. 2002;2:725–34.
Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, Johnson R, Karin M. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J Exp Med. 1999;189:1839–45.
Moreno JA, Izquierdo MC, Sanchez-Niño MD, Suárez-Alvarez B, Lopez-Larrea C, Jakubowski A, Blanco J, Ramirez R, Selgas R, Ruiz-Ortega M, Egido J, Ortiz A, Sanz AB. The inflammatory cytokines TWEAK and TNF{alpha} reduce renal klotho expression through NF{kappa}B. J Am Soc Nephrol. 2011;22:1315–25.
Neurath MF, Pettersson S, Meyer zum Büschenfelde KH, Strober W. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. Nat Med. 1996;2:998–1004.
Sanz AB, Sanchez-Niño MD, Izquierdo MC, Jakubowski A, Justo P, Blanco-Colio LM, Ruiz-Ortega M, Selgas R, Egido J, Ortiz A. TWEAK activates the non-canonical NFkappaB pathway in murine renal tubular cells: modulation of CCL21. PLoS One. 2010a;5:e8955.
Sanz AB, Sanchez-Niño MD, Ramos AM, Moreno JA, Santamaria B, Ruiz-Ortega M, Egido J, Ortiz A. NF-kappaB in renal inflammation. J Am Soc Nephrol. 2010b;21:1254–62.
Sen R, Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell. 1986;47:921–8.
Senftleben U, Cao Y, Xiao G, Greten FR, Krähn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC, Karin M. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science. 2001;293:1495–9.
Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell. 1995;80:321–30.
Van der Heiden K, Cuhlmann S, le Luong A, Zakkar M, Evans PC. Role of nuclear factor kappaB in cardiovascular health and disease. Clin Sci (Lond). 2010;118:593–605.
Wietek C, Cleaver CS, Ludbrook V, Wilde J, White J, Bell DJ, Lee M, Dickson M, Ray KP, O'Neill LA. IkappaB kinase epsilon interacts with p52 and promotes transactivation via p65. J Biol Chem. 2006;281:34973–81.
Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest. 2001;107:135–42.

NHERF

Byong Kwon Yoo and C. Chris Yun
Emory University School of Medicine, Division of Digestive Diseases, Department of Medicine, Atlanta, GA, USA

Synonyms

NHERF (N^+/H^+ Exchange Regulating Factor); SLC9A3R1 (Solute carrier family 9 member 3 regulator 1); EBP50 (Ezrin-radixin-moesin-Binding phosphoprotein 50 kDa)

Historical Background

The functional presence of NHERF-1 was implicated in the late 1980s from a series of studies of inhibition of Na^+/H^+ exchange in rabbit kidney brush border membrane by cyclic AMP-dependent protein kinase (PKA). It took almost another decade before the cloning and molecular identification of NHERF-1 (Weinman et al. 1995). Shortly after, NHERF-2 was cloned from a yeast two-hybrid screen as an NHE3 interacting protein and was initially named E3KARP based on its ability to mediate PKA-dependent inhibition of Na^+/H^+ exchanger 3 (NHE3) in PS120 fibroblasts (Yun et al. 1997). NHERF-3 was initially identified as a protein that is upregulated in low dietary phosphate and was later shown to interact with the type II Na/P_i cotransporter (Npt2a) (Gisler et al. 2001). NHERF-4 was shown to interact with the intestinal receptor guanylyl cyclase C to inhibit the catalytic

activity of the receptor in response to heat-stable enterotoxin (Scott et al. 2002).

Introduction

The NHERF family consists of four related proteins that are present in the brush border membrane of the mammalian intestine, colon, and renal proximal tubules. These proteins contain 2 or 4 PSD-95/Dlg/ZO-1 (PDZ) domains (Fig. 1) (Weinman et al. 1995; Yun et al. 1997; Gisler et al. 2001; Scott et al. 2002). NHERF-1 and NHERF-2 contain two PDZ domains as well as an ezrin-radixin-moesin-merlin (ERM) binding domain at the carboxyl terminus (Lamprecht et al. 1998). NHERF-3 and NHERF-4 have four PDZ domains without ERM binding domain (Gisler et al. 2001; Scott et al. 2002). PDZ domain interaction with their interacting ligands or proteins generally occurs at the ligand COOH terminus although non-canonical interaction with an internal motif has been reported. NHERFs are able to interact with multiple proteins through their PDZ domains, including transporters, channels, transmembrane receptors, and other cytoskeleton proteins localized at or below the plasma membrane (Table 1). NHERFs play significant roles in maintenance and regulation of a broad range of cellular functions in a variety of tissues through the interactions with multiple target proteins.

NHERF Basics

Localization

NHERF-1 and NHERF-2 are expressed in a broad range of tissues and organs (Yun et al. 1997). NHERF-3 and NHERF-4 show highest expression in the kidney and gastrointestinal tract (Gisler et al. 2001; Scott et al. 2002). Immunofluorescent confocal microscopic analysis of NHERF proteins shows that NHERF proteins have different subcellular localization in polarized epithelial cells. NHERF-1 and NHERF-3 are located in the brush border membrane under basal conditions. The brush border localization of NHERF-2 was shown, but it is predominantly in the intermicrovillar clefts just below the brush border membrane. NHERF-4 is primarily distributed in the cytosol as well as in the subapical region, but not in the brush border membrane (Donowitz et al. 2005). NHERF expression in non-epithelial cells is less well documented, but the expression of NHERF-1 and NHERF-2 in neurons and astrocytes, where these proteins show a membranous expression, has been reported.

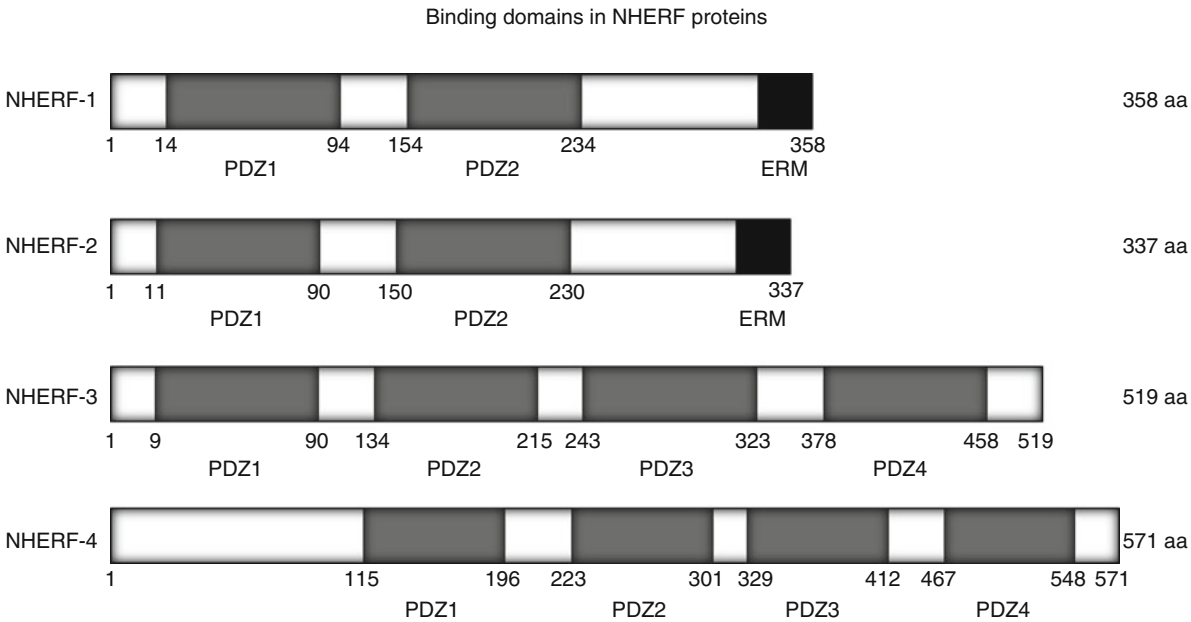
Regulation of NHERF

NHERF-1 is regulated by phosphorylation. NHERF-1 is constitutively phosphorylated on Ser 289 by G protein–coupled receptor kinase 6 (GRK 6), which enhances oligomerization of NHERF-1 (He and Yun 2010). Furthermore, NHERF-1 is phosphorylated by the cyclin-dependent kinase Cdc2 at Ser279 and Ser301, which impair oligomerization, and at S77 by protein kinase C, which interferes with parathyroid hormone (PTH)-induced signaling (Weinman et al. 2007). In contrast to NHERF-1, NHERF-2 does not appear to be regulated by phosphorylation (Lamprecht et al. 1998). Both NHERF-1 and NHERF-2 have been shown to form homotypic as well as heterotypic dimers. Dimerization of NHERF is thought to affect their interaction with other proteins. NHERF-3 mRNA expression is regulated by peroxisome proliferator-activated receptor alpha (PPARα), a ligand-activated transcription factor that plays an important role in the regulation of lipid homeostasis (Tachibana et al. 2008). Regulatory mechanism of NHERF-4 has not yet been studied.

Association with Cell Surface Proteins

Transporters and Channels

The majority of functional characterization of the NHERF family came from heterologous expression of these proteins. In addition to the importance of NHERF-1 and NHERF-2 in regulation of NHE3 by PKA, these studies have provided evidence for the importance of NHERFs for trafficking, membrane retention, and dimerization of the cystic fibrosis transmembrane regulator (CFTR) (Singh et al. 2009). Although initial studies suggested redundancy in the functions of the NHERF family, studies of rodents that are genetically targeted to delete one or more of the NHERF family members have helped to reveal distinct physiological roles of NHERF proteins. NHERF-1 is essential for cAMP- and parathyroid hormone (PTH)-induced inhibition of renal NHE3, but not intestinal



NHERF, Fig.1 Binding domains in NHERF proteins. Four members of NHERF family (NHERF1-4) are shown with PDZ domains and ERM binding regions. Numerical numbers denote lengths of amino acids

NHE3 (Weinman et al. 2005). Ablation of NHERF-1 decreases forskolin-induced secretion of bicarbonate by CFTR (Singh et al. 2009). NHERF-1 is also essential for the recruitment of Npt2a to the brush border membrane of renal proximal tubule cells (Weinman et al. 2005). NHERF-2 appears to play a dual role in regulation of NHE3. Glucocorticoid- or lysophosphatidic acid (LPA)-mediated stimulation of NHE3 is dependent on the presence of NHERF-2 (He and Yun 2010). Similarly, NHERF-2 is necessary for inhibition of NHE4 by cyclic GMP kinase II (cGKII), protein kinase C (PKC), and Ca^{2+}-mediated signaling (He and Yun 2010). NHERF-3 ablation in mouse colon abolishes cAMP- and Ca^{2+}-induced inhibition of NHE3 (Donowitz et al. 2005). NHERF-3 is also involved in the localization of organic cation/cartinin transporter (OCTN2, Slc22a5) and H^+/dipeptide transporter (PepT1, Slc15a1) in the brush border membrane (Sugiura et al. 2008). NHERF-4 activates NHE3 via a Ca^{2+}-dependent mechanism (Zachos et al. 2008).

G Protein–Coupled Receptors

NHERF proteins interact with several G protein–coupled receptors (GPCRs), including the β_2-adrenergic receptor (β_2-AR), κ-opioid receptor, PTH type 1 receptor (PTH1R), P2Y receptor, and lysophosphatidic acid receptor (Ritter and Hall 2009). The first insight into the role of NHERF in GPCR-mediated signaling came from the finding that agonist-promoted association of NHERF-1 with the carboxyl terminus of β_2-AR displays NHERF-1 from NHE3 blocking the inhibition of NHE3 by PKA (Ritter and Hall 2009). Evidence shows that NHERF-1 regulates β_2-AR trafficking by regulating agonist-promoted recycling of receptor proteins, which can be perturbed by interruption of NHERF-1 binding, and hence directing the receptor to lysosome (Ritter and Hall 2009). In addition, the NHERF proteins regulate GPCR-mediated signaling through selective recruitment of signaling proteins, including phospholipase C and G proteins, which could potentiate or redirect G protein–mediated signaling (Mahon et al. 2002).

Receptor Tyrosine Kinases

In addition to GPCRs, NHERFs associate with receptor tyrosine kinases, including platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR). The binding of NHERF-1 to the carboxyl terminus of PDGFR potentiates receptor activity only when NHERF-1 is allowed to oligomerize (Maudsley et al. 2000). Evidence shows that the interaction between PDGFR and NHERF-1

NHERF, Table 1. Interactions of NHERF proteins with ligands and other proteins

NHERF proteins	Domains	Binding partners	Authors
NHERF-1	PDZ1	β2-adrenergic receptor (β2AR)	Hall et al. (1998)
		Platelet-derived growth factor receptor (PDGFR)	Maudsley et al. (2000)
		κ opioid receptor	Li et al. (2002)
		Parathyroid hormone receptor	Sneddon et al. (2003)
		5-HT4a serotonin receptor	Joubert et al. (2004)
		Epidermal growth factor receptor (EGFR)	Lazar et al. (2004)
		Cystic fibrosis transmembrane conductance regulator (CFTR)	Short et al. (1998)
		Trp 4 & 5 calcium channels	Tang et al. (2000)
		Na^+/phosphate cotransporter (Npt2a)	Gisler et al. (2001)
		Phosphatase and tensin homologue (PTEN)	Takahashi et al. (2006)
		G protein-coupled receptor kinase 6A	Hall et al. (1999)
		Phospholipase C β-1,2 & 3	Tang et al. (2000)
	PDZ2	NHE3	Weinman et al. (1995)
		H^+ ATPase	Breton et al. (2000)
		Yes-associated protein (Yap65)	Mohler et al. (1999)
		β-catenin	Shibata et al. (2003)
NHERF-2	PDZ1	PDGFR	Maudsley et al. (2000)
		CFTR	Sun et al. (2000)
		Trp 5 calcium channel	Embark et al. (2004)
	PDZ2	β2AR	Hall et al. (1998)
		PDGFR	Takahashi et al. (2006)
		Lysophosphatidic acid 2 receptor (LPA_2)	Oh et al. (2004)
		Lysophosphatidic acid 5 receptor (LPA_5)	Lin et al. (2010)
		NHE3	Yun et al. (1997)
		PTEN	Takahashi et al. (2006)
		Cyclic GMP kinase II	Cha et al. (2005)
		PLCβ-3	Hwang et al. (2000)
		Protein kinase C (PKC)	Lee-Kwon et al. (2003)
		Serum glucocorticoid regulated kinase (SGK) 1 & 3	Yun et al. (2002)
		Podocalyxin	Takeda et al. (2001)
NHERF-3	PDZ1	CFTR	Wang et al. (2000)
		NHE3	Gisler et al. (2003)
		Renal urate anion exchanger (URAT1)	Anzai et al. (2004)
	PDZ2	Proton-coupled peptide transporter (PEPT2)	Kato et al. (2004)
		Intestinal anion exchanger down-regulated in adenoma (DRA)	Gisler et al. (2003)
	PDZ3	NPT 1 & 2	Gisler et al. (2003)
		PEPT2	Kato et al. (2004)
		CFTR	Wang et al. (2000)
	PDZ4	Organic cation transporter, novel (OCTN) 1 & 2	Kato et al. (2005)
		NHERF 1 & 2	Gisler et al. (2003)
NHERF-4	PDZ1	OCTN 1 & 2	Kato et al. (2005)
	PDZ2	OCTN 1 & 2	Watanabe et al. (2006)
	PDZ3	Guanylyl cyclase C	Scott et al. (2002)
	PDZ4	Epithelial Ca2+ channel, transient receptor potential cation channel, subfamily V, member 6 (TRPV6)	Kim et al. (2007)
NHERF-1 NHERF-2	ERM	Ezrin, Radixin, Moesin, and Merlin	Murthy et al. (1998)

can be disrupted by phosphorylation of the carboxyl terminus of PDGFR by GRK2 (Hildreth et al. 2004). Recent study showed that NHERF-1 facilitates actin cytoskeletal reorganization mediated by PDGFR (Theisen et al. 2007). Unlike PDGFR, EGFR lacks the carboxyl terminal PDZ binding sequence, but yet it was shown that EGFR interacts with NHERF-1 involving a non-canonical internal PDZ binding motif (Lazar et al. 2004). This interaction appears to stabilize EGFR at the cell surface by restricting EGF-induced receptor degradation, which causes EGFR to remain longer at the cell surface.

NHERF as a Signaling Molecule

Cellular Signals

PDGFR is activated through dimerization and autophosphorylation upon ligand binding. NHERF-1 dimers enhance dimerization of PDGFR to potentiate mitogenic signals transduced by extracellular signal-regulated kinase (Erk) 1/2. Similarly, transient receptor potential 4 (TRP4) calcium channel associates with phospholipase C (PLC) β isoforms to activate protein kinase C signals by binding to NHERF PDZ1 domain. NHERF-1 has significant importance in PTH-mediated signaling as evidenced by the defective PTH signaling in NHERF-1-deficient mice (Weinman et al. 2005). NHERF-1 modulates PTH signaling by affecting PTH receptor recycling, membrane retention, and desensitization.

In addition to the regulation of the membrane receptors and channels, NHERF-1 interacts with Akt and inhibits PKA-mediated Erk1/2 activation by decreasing the stimulatory effect of 14-3-3 binding to B-Raf (Wang et al. 2008).

Cancer

Overexpression of NHERF-1 in breast cancer cells and the transcriptional regulation of NHERF-1 by estrogen suggested a potential role of NHERF-1 in cancer. In addition, unpublished data in our lab show the elevated expression of NHERF-2 in colon adenocarcinoma. However, the mechanism and effects of NHERFs in tumorigenesis are unclear. The interaction of NHERF-1 with PDGFR and EGFR appears to suggest an oncogenic role of NHERF-1. In addition, NHERF-1 expression is elevated in hepatocellular carcinoma where NHERF-1 complexes with β-catenin to promote Wnt signaling (Shibata et al. 2003). On the other hand, NHERF-1 or NHERF-2 recruits phosphatase and tensin homolog (▶ PTEN) tumor suppressor to restrict the activation of the PI3K (Georgescu et al. 2008). Therefore, NHERFs appear to play a dual role in tumorigenesis and their role in cancer requires additional studies.

Summary

From the uncertain identity as a cofactor of cAMP-induced regulation of NHE3, NHERF proteins have firmly rooted their identity as the major molecular scaffolds. The role of the NHERF proteins extends beyond the regulation of ion transporters. Growing evidence links NHERF to cancer, inflammatory diseases, hypertension, and neurological disorder. However, the functional roles and the underlying mechanisms of NHERF-mediated regulation are incompletely understood. A combination of biochemical and cellular approaches along with physiological studies using animal models lacking one or more of the NHERF proteins should advance the understanding of the physiological and pathophysiological functions of the NHERF proteins.

Acknowledgment This work was supported by the grant from the National Institutes of Health (DK071597 and DK061418).

References

Anzai N, Miyazaki H, Noshiro R, Khamdang S, Chairoungdua A, Shin HJ, et al. The multivalent PDZ domain-containing protein PDZK1 regulates transport activity of renal urate-anion exchanger URAT1 via its C terminus. J Biol Chem. 2004;279(44):45942–50.

Breton S, Wiederhold T, Marshansky V, Nsumu NN, Ramesh V, Brown D. The B1 subunit of the H+ATPase is a PDZ domain-binding protein. Colocalization with NHE-RF in renal B-intercalated cells. J Biol Chem. 2000;275(24):18219–24.

Cha B, Kim JH, Hut H, Hogema BM, Nadarja J, Zizak M, et al. cGMP inhibition of Na+/H+ antiporter 3 (NHE3) requires PDZ domain adapter NHERF2, a broad specificity protein kinase G-anchoring protein. J Biol Chem. 2005;280(17):16642–50.

Donowitz M, Cha B, Zachos NC, Brett CL, Sharma A, Tse CM, et al. NHERF family and NHE3 regulation. J Physiol. 2005;567(Pt 1):3–11.

Embark HM, Setiawan I, Poppendieck S, van de Graaf SF, Boehmer C, Palmada M, et al. Regulation of the epithelial

Ca2+ channel TRPV5 by the NHE regulating factor NHERF2 and the serum and glucocorticoid inducible kinase isoforms SGK1 and SGK3 expressed in Xenopus oocytes. Cell Physiol Biochem. 2004;14(4–6):203–12.

Georgescu MM, Morales FC, Molina JR, Hayashi Y. Roles of NHERF1/EBP50 in cancer. Curr Mol Med. 2008;8(6): 459–68.

Gisler SM, Stagljar I, Traebert M, Bacic D, Biber J, Murer H. Interaction of the type IIa Na/Pi cotransporter with PDZ proteins. J Biol Chem. 2001;276(12):9206–13.

Gisler SM, Pribanic S, Bacic D, Forrer P, Gantenbein A, Sabourin LA, et al. PDZK1: I. a major scaffolder in brush borders of proximal tubular cells. Kidney Int. 2003;64(5): 1733–45.

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, et al. The beta2-adrenergic receptor interacts with the Na+/H+-exchanger regulatory factor to control Na+/H+ exchange. Nature. 1998;392(6676):626–30.

Hall RA, Spurney RF, Premont RT, Rahman N, Blitzer JT, Pitcher JA, et al. G protein-coupled receptor kinase 6A phosphorylates the Na(+)/H(+) exchanger regulatory factor via a PDZ domain-mediated interaction. J Biol Chem. 1999;274(34):24328–34.

He P, Yun CC. Mechanisms of the regulation of the intestinal Na+/H+ exchanger NHE3. J Biomed Biotechnol. 2010: 238080.

Hildreth KL, Wu JH, Barak LS, Exum ST, Kim LK, Peppel K, et al. Phosphorylation of the platelet-derived growth factor receptor-beta by G protein-coupled receptor kinase-2 reduces receptor signaling and interaction with the Na(+)/H(+) exchanger regulatory factor. J Biol Chem. 2004;279(40):41775–82.

Hwang JI, Heo K, Shin KJ, Kim E, Yun C, Ryu SH, et al. Regulation of phospholipase C-beta 3 activity by Na+/H+ exchanger regulatory factor 2. J Biol Chem. 2000;275(22): 16632–7.

Joubert L, Hanson B, Barthet G, Sebben M, Claeysen S, Hong W, et al. New sorting nexin (SNX27) and NHERF specifically interact with the 5-HT4a receptor splice variant: roles in receptor targeting. J Cell Sci. 2004;117(Pt 22): 5367–79.

Kato Y, Yoshida K, Watanabe C, Sai Y, Tsuji A. Screening of the interaction between xenobiotic transporters and PDZ proteins. Pharm Res. 2004;21(10):1886–94.

Kato Y, Sai Y, Yoshida K, Watanabe C, Hirata T, Tsuji A. PDZK1 directly regulates the function of organic cation/carnitine transporter OCTN2. Mol Pharmacol. 2005;67(3): 734–43.

Kim HJ, Yang DK, So I. PDZ domain-containing protein as a physiological modulator of TRPV6. Biochem Biophys Res Commun. 2007;361(2):433–8.

Lamprecht G, Weinman EJ, Yun CH. The role of NHERF and E3KARP in the cAMP-mediated inhibition of NHE3. J Biol Chem. 1998;273(45):29972–8.

Lazar CS, Cresson CM, Lauffenburger DA, Gill GN. The Na+/H+ exchanger regulatory factor stabilizes epidermal growth factor receptors at the cell surface. Mol Biol Cell. 2004;15(12):5470–80.

Lee-Kwon W, Kim JH, Choi JW, Kawano K, Cha B, Dartt DA, et al. Ca2+-dependent inhibition of NHE3 requires PKC alpha which binds to E3KARP to decrease surface NHE3 containing plasma membrane complexes. Am J Physiol Cell Physiol. 2003;285(6):C1527–36.

Li JG, Chen CG, Liu-Chen LY. Ezrin-radixin-moesin-binding phosphoprotein-50/Na+/H+ exchanger regulatory factor (EBP50/NHERF) blocks U50,488H-induced down-regulation of the human kappa opioid receptor by enhancing its recycling rate. J Biol Chem. 2002;277(30):27545–52.

Lin S, Yeruva S, He P, Singh AK, Zhang H, Chen M, et al. Lysophosphatidic acid stimulates the intestinal brush border Na(+)/H(+) exchanger 3 and fluid absorption via LPA(5) and NHERF2. Gastroenterology. 2010;138(2):649–58.

Mahon MJ, Donowitz M, Yun CC, Segre GV. Na(+)/H(+) exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. Nature. 2002;417(6891):858–61.

Maudsley S, Zamah AM, Rahman N, Blitzer JT, Luttrell LM, Lefkowitz RJ, et al. Platelet-derived growth factor receptor association with Na(+)/H(+) exchanger regulatory factor potentiates receptor activity. Mol Cell Biol. 2000;20(22): 8352–63.

Mohler PJ, Kreda SM, Boucher RC, Sudol M, Stutts MJ, Milgram SL. Yes-associated protein 65 localizes p62 (c-Yes) to the apical compartment of airway epithelia by association with EBP50. J Cell Biol. 1999;147(4):879–90.

Murthy A, Gonzalez-Agosti C, Cordero E, Pinney D, Candia C, Solomon F, et al. NHE-RF, a regulatory cofactor for Na(+)-H+ exchange, is a common interactor for merlin and ERM (MERM) proteins. J Biol Chem. 1998;273(3):1273–6.

Oh YS, Jo NW, Choi JW, Kim HS, Seo SW, Kang KO, et al. NHERF2 specifically interacts with LPA2 receptor and defines the specificity and efficiency of receptor-mediated phospholipase C-beta3 activation. Mol Cell Biol. 2004;24(11):5069–79.

Ritter SL, Hall RA. Fine-tuning of GPCR activity by receptor-interacting proteins. Nat Rev Mol Cell Biol. 2009;10(12): 819–30.

Scott RO, Thelin WR, Milgram SL. A novel PDZ protein regulates the activity of guanylyl cyclase C, the heat-stable enterotoxin receptor. J Biol Chem. 2002;277(25):22934–41.

Shibata T, Chuma M, Kokubu A, Sakamoto M, Hirohashi S. EBP50, a beta-catenin-associating protein, enhances Wnt signaling and is over-expressed in hepatocellular carcinoma. Hepatology. 2003;38(1):178–86.

Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, et al. An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. J Biol Chem. 1998;273(31):19797–801.

Singh AK, Riederer B, Krabbenhoft A, Rausch B, Bonhagen J, Lehmann U, et al. Differential roles of NHERF1, NHERF2, and PDZK1 in regulating CFTR-mediated intestinal anion secretion in mice. J Clin Invest. 2009;119(3):540–50.

Sneddon WB, Syme CA, Bisello A, Magyar CE, Rochdi MD, Parent JL, et al. Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50). J Biol Chem. 2003;278(44):43787–96.

Sugiura T, Kato Y, Wakayama T, Silver DL, Kubo Y, Iseki S, et al. PDZK1 regulates two intestinal solute carriers (Slc15a1 and Slc22a5) in mice. Drug Metab Dispos. 2008;36(6): 1181–8.

Sun F, Hug MJ, Lewarchik CM, Yun CH, Bradbury NA, Frizzell RA. E3KARP mediates the association of ezrin and protein kinase A with the cystic fibrosis transmembrane

conductance regulator in airway cells. J Biol Chem. 2000;275(38):29539–46.
Tachibana K, Anzai N, Ueda C, Katayama T, Yamasaki D, Kirino T, et al. Regulation of the human PDZK1 expression by peroxisome proliferator-activated receptor alpha. FEBS Lett. 2008;582(28):3884–8.
Takahashi Y, Morales FC, Kreimann EL, Georgescu MM. PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. EMBO J. 2006;25(4):910–20.
Takeda T, McQuistan T, Orlando RA, Farquhar MG. Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton. J Clin Invest. 2001;108(2):289–301.
Tang Y, Tang J, Chen Z, Trost C, Flockerzi V, Li M, et al. Association of mammalian trp4 and phospholipase C isozymes with a PDZ domain-containing protein, NHERF. J Biol Chem. 2000;275(48):37559–64.
Theisen CS, Wahl 3rd JK, Johnson KR, Wheelock MJ. NHERF links the N-cadherin/catenin complex to the platelet-derived growth factor receptor to modulate the actin cytoskeleton and regulate cell motility. Mol Biol Cell. 2007;18(4):1220–32.
Wang S, Yue H, Derin RB, Guggino WB, Li M. Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. Cell. 2000;103(1):169–79.
Wang B, Yang Y, Friedman PA. Na/H exchange regulatory factor 1, a novel AKT-associating protein, regulates extracellular signal-regulated kinase signaling through a B-Raf-mediated pathway. Mol Biol Cell. 2008;19(4):1637–45.
Watanabe C, Kato Y, Sugiura T, Kubo Y, Wakayama T, Iseki S, et al. PDZ adaptor protein PDZK2 stimulates transport activity of organic cation/carnitine transporter OCTN2 by modulating cell surface expression. Drug Metab Dispos. 2006;34(11):1927–34.
Weinman EJ, Steplock D, Wang Y, Shenolikar S. Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border membrane Na(+)-H+ exchanger. J Clin Invest. 1995;95(5):2143–9.
Weinman EJ, Cunningham R, Wade JB, Shenolikar S. The role of NHERF-1 in the regulation of renal proximal tubule sodium-hydrogen exchanger 3 and sodium-dependent phosphate cotransporter 2a. J Physiol. 2005;567(Pt 1):27–32.
Weinman EJ, Biswas RS, Peng G, Shen L, Turner CL EX, et al. Parathyroid hormone inhibits renal phosphate transport by phosphorylation of serine 77 of sodium-hydrogen exchanger regulatory factor-1. J Clin Invest. 2007;117(11):3412–20.
Yun CHC, Oh S, Zizak M, Steplock D, Tsao S, Tse CM, et al. cAMP-mediated inhibition of the epithelial brush border Na+/H+ exchanger, NHE3, requires an associated regulatory protein. Proc Natl Acad Sci USA. 1997;94(7):3010–5.
Yun CC, Chen Y, Lang F. Glucocorticoid activation of Na(+)/H(+) exchanger isoform 3 revisited. The roles of SGK1 and NHERF2. J Biol Chem. 2002;277(10):7676–83.
Zachos NC, Hodson C, Kovbasnjuk O, Li X, Thelin WR, Cha B, et al. Elevated intracellular calcium stimulates NHE3 activity by an IKEPP (NHERF4) dependent mechanism. Cell Physiol Biochem. 2008;22(5–6):693–704.

NHERF (N^+/H^+ Exchange Regulating Factor)

► NHERF

NimO (*Aspergillus*)

► Dbf4

NINAC (p174, p132)

► Myosin III

NIPK

► Tribbles

NK Receptor

Bin Xu, Mesfin Gewe, Kathryn Finton and Roland K. Strong
Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Synonyms

Killer immunoglobulin-like receptors (KIR); Killer lectin-like receptors (KLR); Natural cytotoxicity receptors (NCR); Natural killer cell group (NKG)

Historical Background

Natural Killer (NK) cells are a major component of the innate immune system, providing surveillance against infected or transformed cells without the requirement of prior host sensitization. NK cells were first discovered by their cytotoxic potency against tumor cells (Kiessling et al. 1975), and their importance was

demonstrated early on in herpes viral infections, usually mild or localized, which become severe and life-threatening in NK-deficient patients. NK cell effector functions are governed by the balance of potentially opposing signals from a diverse array of both activating and inhibitory receptors on the cell surface that are not rearranged from germ-line receptor segments, unlike B and T cell receptors (BCRs, TCRs) on lymphocytes. While NK cells are now well characterized in terms of their origin, differentiation, and receptor repertoire, advances are still being made in NK cell receptor modulation, ligand recognition, and signaling and activation mechanisms which greatly enrich our understanding of NK cell biology: an important component of a complex immune system that remains incompletely understood.

Function and Signaling

Because of their potent cytolytic activity, the function of NK cells needs to be tightly regulated to limit potential autoreactivity. Molecular events driving activation occur at the NK immune synapse (NKIS), the surface of contact between NK and potential target cells. The NKIS comprises an array of molecular interactions including receptor–ligand pairs, adhesion molecules, signaling adaptors, signaling effectors, membrane lipid rafts, and cytoskeletal molecules (MacFarlane and Campbell 2006). NK cells kill sensitive target cells by release of cytotoxic granules that contain perforin and granzymes; perforin polymerizes and forms a transmembrane pore that allows the delivery of granzymes and associated molecules into the cytosol where they initiate various apoptotic death pathways. NK cells express a variety of receptors that serve either to activate or to suppress their cytolytic activity (Lanier 2005). NK cell surface receptors are commonly divided into activating and inhibitory receptors, but they can also be structurally classified into C-type lectin-like receptors and immunoglobulin (Ig)-type receptors.

The functional response of an NK cell is the result of the integration of signals transduced by the set of activating and inhibitory receptors engaged upon target cell interrogation (Bryceson and Long 2008). The majority of known activating receptors transduce signals through their association with one or two of four transmembrane-spanning accessory proteins: the DNAX-activating proteins with molecular weights of 10 kDa (DAP10) or 12 kDa (DAP12), ▶ CD3ζ, or the Fcε-receptor γ-chain (FcεRIγ; Table 1). All four accessory adaptor proteins exist as disulfide-linked homodimers, but CD3ζ and FcεRIγ can also form disulfide-linked heterodimers. Electrostatic interactions link activating receptors and accessory adaptor molecules through either an arginine or lysine residue in the receptor or an aspartic or glutamic acid residue on the accessory protein. The cytoplasmic domains of DAP12, CD3ζ, and FcεRIγ all contain one or more immunoreceptor tyrosine-based activation motifs (ITAMs) containing a sequence signature: YxxL/I (x denotes any amino acid). Two of these motifs are typically separated by six to eight amino acids in the tail of the molecule [YxxL/Ix(6–8)YxxL/I]. Receptor–ligand binding leads to phosphorylation of tyrosine residues within these motifs by membrane proximal Src kinase family molecules. This in turn leads to recruitment of protein tyrosine kinases (PTKs) of the spleen tyrosine kinase (Syk) family, such as Syk and ζ-associated protein of 70 kDa (▶ ZAP-70). Subsequent downstream signaling pathways can involve phosphatidylinositol 3 kinase (▶ PI3K), Vav, phospholipase Cγ (PLCγ), protein kinase C (PKC), and extracellular signal-regulated kinase (ERK). PLCγ activation leads to intracellular calcium flux. Activation of PLCγ, PKC, Vav, and PI3K, as well as elevation of cytosolic calcium concentrations is an important event for initiation of the cytolytic functions by NK cells. Activation of ERK is important for granule-mediated cytotoxicity and ▶ IFN-γ production.

Alternatively, the cytoplasmic signaling domain of DAP10 contains a YINM motif, which may lead to the association of DAP10 with PI3K or the adaptor molecule Grb2, as opposed to Syk or ZAP70. Significant differences have emerged that distinguish DAP10-mediated activation signals from those downstream of ITAMs (Billadeau et al. 2003). This may lead to divergent effector functions: DAP10 can stimulate cytotoxicity, but not IFN-γ production by NK cells, whereas DAP12 can trigger both responses. Multiplicity of activating signals results in complex functional cross talk, leading to the integration of different receptor–ligand interactions directing NK-mediated cell killing.

NK cells are also activated in response to interferons or macrophage-derived cytokines, including IL-2, IL-12, and IL-15. Activated NK cells in turn release cytokines such as ▶ TNF-α and IFN-γ, which

NK Receptor, Table 1 Ectodomain structure, ligand information and endodomain signaling motifs are catalogued for a series of NKRs

Activating	Structure	Ligand	Signaling
NKG2D	C-type lectin homodimer	MICA/B, ULBP1-6	DAP10
NKG2C/E-CD94	C-type lectin heterodimer	HLA-E	DAP12
CD16	Ig monomer	IgG	FcεRIγ, CD3ζ
NKp46 (NCR1)	Ig monomer	Influenza hemagglutinin	FcεRIγ, CD3ζ
NKp44 (NCR2)	Ig monomer	Influenza hemagglutinin	DAP12
NKp30 (NCR3)	Ig monomer	B7H6, BAT3, viral pp65	FcεRIγ, CD3ζ
KIR2DS1	Ig monomer	HLA-Cw2,4,5,6 (C2 epitope)	DAP12
KIR2DS4	Ig monomer	HLA-A, C	DAP12
KIR3DS1	Ig monomer	HLA-Bw4?	DAP12
KIR2DL4	Ig monomer	HLA-G?	FcεRIγ, 1 ITIM
2B4	Ig monomer	CD48	ITSM, SAP
NTB-A	Ig monomer	NTB-A	ITSM, SAP
CRACC	Ig monomer	CRACC	ITSM, SAP
DNAM-1	Ig monomer	CD112, CD155	Protein kinase C
NKp80	C-type lectin homodimer	AICL	Undefined
Inhibitory	Structure	Ligand	Signaling
NKG2A/B-CD94	C-type lectin heterodimer	HLA-E	1 ITIM
KIR2DL1	Ig monomer	HLA-Cw2,4,5,6 (C2 epitope)	2 ITIM
KIR2DL2	Ig monomer	HLA-Cw1,3,7,8 (C1 epitope)	2 ITIM
KIR2DL3	Ig monomer	HLA-Cw1,3,7,8 (C1 epitope)	2 ITIM
KIR3DL1	Ig monomer	HLA-A, B (Bw4 epitope)	2 ITIM
KIR3DL2	Ig homodimer	HLA-A3, A11	2 ITIM
LIR1 (ILT2)	Ig monomer	HLA class I, HLA-G, UL18	4 ITIM
LIR2 (ILT4)	Ig monomer	HLA-G	3 ITIM
NKR-P1A	C-type lectin homodimer	LLT1	1 ITIM
LAIR1(CD305)	Ig monomer	Collagen	2 ITIM
CEACAM1	Ig monomer	CEACAM1, CEA	2 ITIM
SIGLEC7	Ig monomer	α2,8 disialic acid	1 ITIM
SIGLEC9	Ig monomer	α(2,3)- & α(2,6)-disialic acid	1 ITIM + 1 ITSM
IRp60	Ig monomer	Undefined	4 ITIM

can promote cellular resistance to infection and influence adaptive immunity resulting in infection clearance. NK cells also express the Fc receptor (FcR) molecule (CD16), an activating receptor that binds the Fc portion of antibodies. This allows NK cells to target cells against which a humoral response has been mobilized and to lyse cells through antibody-dependent cellular cytotoxicity (ADCC).

Although the extracellular domains of NK cell inhibitory receptors are diverse, the cytoplasmic signaling motifs of these transmembrane receptors are remarkably similar. The cytoplasmic tails of inhibitory receptors have one or more copies of conserved sequence motifs (V/IxYxxL/V) known as immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Binding of ligands to inhibitory receptors activates ▶ Src family kinases that phosphorylate ITIM tyrosine residues, leading to the recruitment of other enzymes, such as SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2, or the inositol phosphatase, ▶ SHIP. These tyrosine phosphatases are able to dephosphorylate protein substrates of tyrosine kinases linked to activating NK cell receptors. Recruitment and activation of SHP-1 by some inhibitory receptors has been functionally implicated in delivery of the dominant inhibitory signal in human NK cells. NK cells tolerate normal, autologous cells by sensing appropriate levels of surface-expressed self-MHC class I molecules that induce an inhibitory signal. Infection or transformation often alters MHC class I cell surface expression, typically as part of a strategy to evade T cell responses, which is then detected by NK cells. This "missing-self" hypothesis

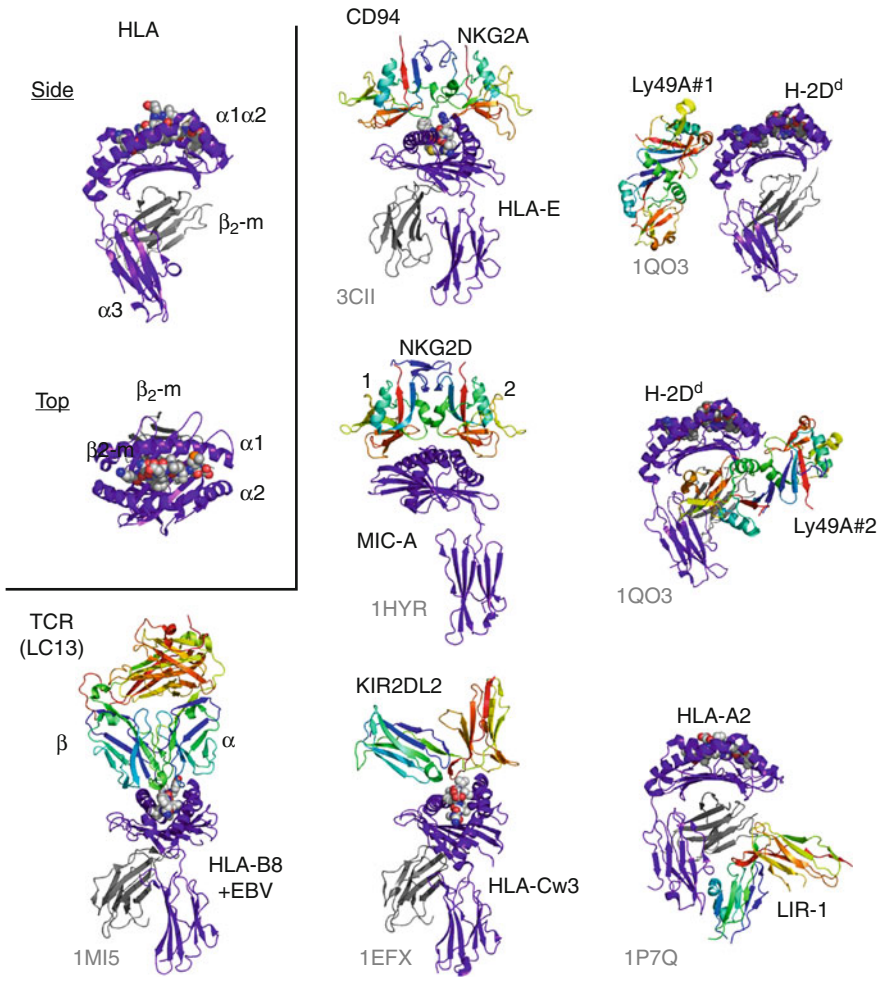
was first proposed by Karre and colleagues (Karre et al. 1986) and subsequently demonstrated by Yokoyama and colleagues (Karlhofer et al. 1992). MHC class I–mediated inhibition is therefore crucial to the role played by NK cells.

Specificities and Recognition Mechanisms: Inhibitory Receptors

Major inhibitory NK receptors in humans include inhibitory isoforms of the Killer Immunoglobulin Receptors (KIRs, also CD158), the lectin-like receptor CD94/NKG2A, and the Leukocyte Immunoglobulin-like Receptors (LIRs, also CD85), which all recognize classical or nonclassical MHC class I molecules, and some less well-characterized inhibitory receptors that bind non-MHC class I molecules, such as the lectin-like receptor Natural Killer Receptor P1 (NKR-P1, also KLRB1 or CD161). In mice, the KIRs are replaced with the structurally distinct, C-type lectin-like Ly49 family of NK receptors. Inhibitory receptors that recognize classical and nonclassical MHC class I proteins limit signals through activating receptors. In this way, recognition of normal MHC class I expression dominantly suppresses NK attack of normal cells, whereas a lack of self-MHC class I shifts the balance toward targeted cell killing. Structural and functional studies of inhibitory Ly49 and H-2 complexes provided among the first detailed insights into NK cell recognition (Tormo et al. 1999). The homodimeric Ly49A receptor binds to H-2D^d at two distinct sites, one of which involves the α1 and α2 domains of MHC class I, whereas the second interaction site spans the underside of the platform domain (α1 and α2), α3 domain and β_2-microglobulin (Figs. 1 and 2). The second site is considerably more extensive than the first site and also overlaps the CD8 binding site on MHC class I molecules. The first binding site is consistent with *trans* interaction between NK and target cells where the second, more extensive contact site is likely a regulatory *cis* interaction between Ly49 and class I molecules on the same NK cell. Both binding sites are distinct from that of the TCR.

The highly polymorphic KIR family of human NK receptors consists of at least fifteen active genes and two pseudogenes. KIRs are either activating or inhibitory and contain either two or three extracellular Ig-like domains (named D0, D1, and D2). KIR nomenclature is based on the number of extracellular Ig domains (2D versus 3D) and the size of their cytoplasmic tails. Receptors possessing an ITIM-containing long cytoplasmic tail are designated by an L, whereas activating receptors with a short cytoplasmic tail are designated by an S. The cytoplasmic region of activating receptors does not contain an ITIM, but instead contains a positively charged residue that can interact with adaptor molecules such as DAP12. The KIRs are known to show allotypic specificity toward different HLA alleles (Table 1). The best characterized KIR–ligand interactions come from two complex crystal structures of two-domain inhibitory KIRs: KIR2DL2/HLA-Cw3 and KIR2DL1/HLA-Cw4 (Boyington et al. 2000; Fan et al. 2001). KIR2DL1/2 binds to HLA-C with a similar docking orientation as TCRs, with the D1/D2 domains contacting the α1/α2 helices of the MHC peptide–binding cleft and positions P7 and P8 of the bound peptide (Figs. 1 and 2). The KIR2DL1/2/HLA-C interface is dominated by charge complementarity and is mediated by six loops; three loops from D1 contact the α1 helix and bound peptide while one hinge loop and two loops from D2 contact the α2 helix. Allotypic specificity of KIR2D toward HLA-C alleles is determined by a dimorphism at residues 77 and 80 of HLA and a reciprocal dimorphism in KIR2D at position 44. For example, KIR2DL1 (M44) will recognize the C2 epitope (N77/K80) of HLA-C, whereas KIR2DL2 (K44) will recognize the C1 epitope (S77/N80). In the absence of any KIR3D structures, a KIR3DL1/ligand complex model has been proposed, but remains to be confirmed. Analogous to the KIR2D complex structure, the D1/D2 domains contact the α1/α2 helices of HLA and position 8 of the bound peptide. The D0 domain contacts the α1 helix, stabilized by hydrophobic contacts with D1 and D2, consistent with the finding that D0 contributes to avidity. KIR3DL1 is known to recognize HLA-A and -B molecules with the Bw4 epitope (residues 77–83). Several lines of evidence also point to the importance of residue 80 of the Bw4 epitope for KIR interaction.

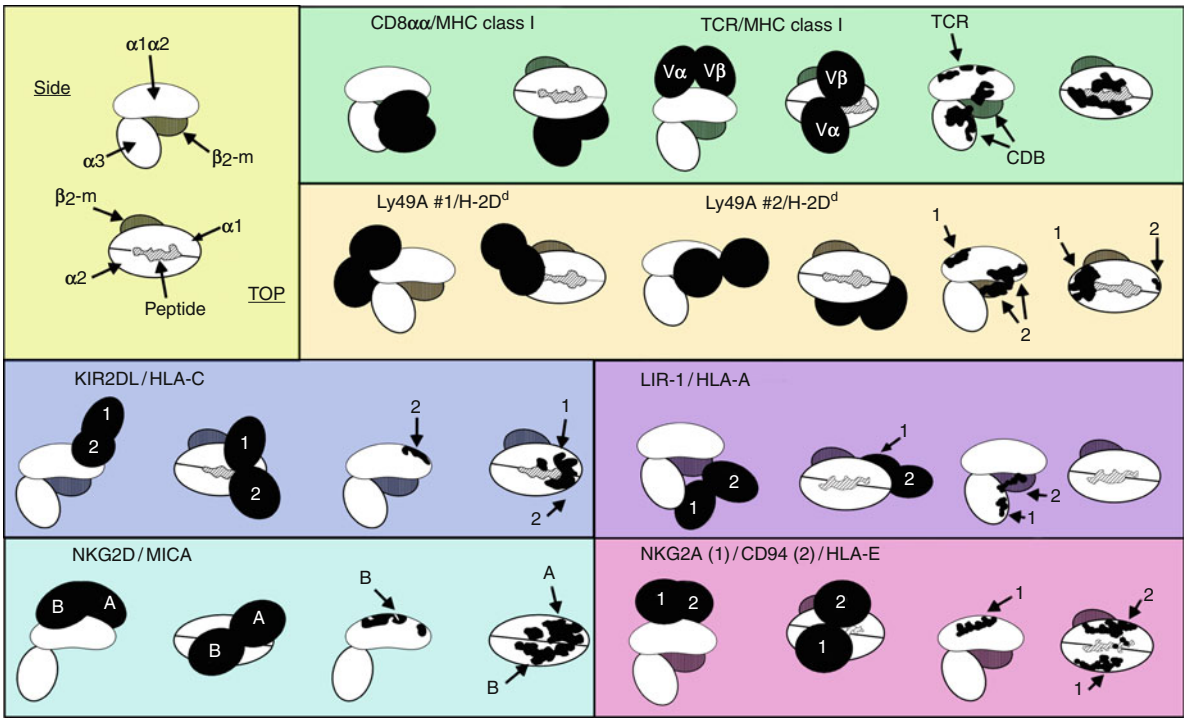
Besides receptors for classical MHC class I molecules, NK cells can indirectly gauge MHC class I expression on target cells through the NKG2x/CD94 receptor complexes. The NKG2x/CD94 receptors, which include both the inhibitory NKG2A/CD94 and activating NKG2C/CD94 receptors, contain C-type lectin-like ectodomains and recognize HLA-E, a nonclassical MHC class I molecule that presents



NK Receptor, Fig. 1 NK receptor–ligand complex structures. Ribbon representations of an αβ TCR/MHC class I complex and several NK receptor–ligand structures. MHC class I molecules are shown in *purple*. Receptors are colored in *rainbow spectrum*; β_2m is shown in *gray*. Class I–bound peptides are shown in *solid spheres* and colored by element. PDB accession codes for the coordinate files used to generate the figure are indicated in *gray*

MHC class I leader peptides. As with the KIR and Ly49 receptors, the inhibitory NKG2A/CD94 receptor binds the ligand with a higher affinity than the activating NKG2C/CD94 receptor. There are few conformational changes in either CD94-NKG2A or HLA-E upon binding, which reflects a typical "lock and key" recognition in innate receptor–ligand interactions (Petrie et al. 2008). A patch of amino acids (residues 167–170) of NKG2A account for the approximately sixfold-higher affinity of the inhibitory NKG2A/CD94 receptor compared to its activating counterpart. These residues do not contact HLA-E or peptide directly but instead form part of the heterodimer interface with CD94. An evolutionary analysis reveals that residues at the CD94 interface have evolved under positive selection, suggesting that the evolution of these genes is driven by an interaction with pathogen-derived ligands. Consistent with this possibility, data show that NKG2C/CD94, but not NKG2A/CD94, weakly but specifically binds to the CMV MHC-homologue UL18 (Kaiser et al. 2008).

NK cells can also be inhibited by members of the LIR (or immunoglobulin-like transcript (ILT)) family. Some members of LIRs (including LIR1-3) are inhibitory receptors with intracellular ITIM motifs, while others (including LIR6-7) are activating receptors associated with adaptor proteins for signaling. LIR-1 and LIR-2 are receptors for a broad range of class I molecules, including nonclassical molecule HLA-G. HLA-G is highly expressed in the trophoblast, and the inhibitory function of LIR-HLA-G interaction has been implicated in immunological tolerance of the fetus. The LIRs show a distinct recognition mode with regard to its class I HLA-A2 or nonclassical



NK Receptor, Fig. 2 NK receptor–ligand complex schematics. Schematic representations of an αβ TCR/MHC class I complex and several NK receptor–ligand structures to highlight interaction surfaces. Each row shows two views of a receptor–ligand complex, first showing the organization of domains in the complex (receptor domains in *black*, labeled where a distinction between domains is significant; MHC class I ligand heavy chains in *white* and β_2m in *dark gray*). The arrangement of domains in the ligands is detailed in the inset; the approximate solvent-accessible surface area of the bound peptide, if present, is shown as a *cross-hatched* area. The two *right*-most columns show approximate footprints of receptors and co-receptors on the ligands as *black hatches*, labeled by receptor component, subsite or domain as appropriate

MHC HLA-G ligand (Figs. 1 and 2). Both complex structures of HLA-A2 bound to LIR-1 and HLA-G bound to LIR-2 (Willcox et al. 2003; Shiroishi et al. 2006) showed the LIR binds at a site bridging the α3 and β_2m domains. The overall orientation of the MHC molecules with regard to LIR receptors resembles somewhat the *cis* binding site of Ly49A on H-2D^d, though none of the LIR domains are in contact with the underside of the platform domain of the MHC molecules (Figs. 1 and 2). The LIR-1 binding site on HLA-A2 overlaps with the CMV UL18 binding site. Binding sites on LIR-1 and LIR-2 similarly comprise residues at the interdomain hinge and a patch at the tip of domain 1. The overall geometry of the LIR receptor–MHC ligand complex is most consistent with a *trans* interaction involving recognition of an MHC class I protein on a target cell by a LIR receptor on an opposing NK cell.

Although the binding of an inhibitory receptor to MHC class I represents the major mechanism for inhibition and prevention of NK cell autoreactivity, there are other inhibitory receptors which bind to non-MHC class I ligands. NKR-P1 binds to lectin-like LLT1 molecules in humans. More information on the regulation of NKR-P1 ligands expression is required to determine the functional implications of this receptor–ligand interaction. Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) is an inhibitory receptor that binds to collagen and is widely expressed on immune cells. Several members of the sialic acid binding Ig-like lectin (Siglec) family of receptors, which bind sialyl groups with various specificities, carry ITIMs in their cytoplasmic tail. Presumably, Siglec molecules function as inhibitory receptors; yet, the biological significance remains elusive.

Specificities and Recognition Mechanisms: Activating Receptors

Activating receptors include ▶ NKG2D, CD16, natural cytotoxicity receptors (NCRs), activating KIRs, the signaling lymphocyte-activation molecules (SLAM) family of receptors, and DNAX accessory molecule-1 (DNAM-1). One of the best characterized NK-activating receptors is NKG2D (Gonzalez et al. 2006). It is a type II integral membrane protein associated with the adaptor protein DAP10. NKG2D binds several ligands, including MHC class I chain–related A/B (MICA/B) and the UL16-binding proteins, 1-6 (ULBP1-6). Expression of these ligands is upregulated in a variety of epithelially derived tumors and virally infected cells. Currently, the mechanisms that upregulate NKG2D ligand expression on cancer cells are not well understood. While NKG2D provides an important defense mechanism against tumors and viral infection, it can also contribute to autoimmunity. For example, MICA and MICB are dramatically upregulated in rheumatoid arthritis synoviocytes and are capable of activating autoreactive autologous T cells in an NKG2D-dependent manner. MICA/B and ULBP1-6 are class I homologs that are not encoded by genes in the MHC complex and do not function as peptide-binding structures to present antigens to T cells. Multiple crystal structures of the receptor alone and three complexes (human NKG2D/MICA, NKG2D/ULBP3, and murine NKG2D/Rae-1β) show that NKG2D interacts with its MHC class I homologs in a manner very similar to how TCRs interact with classical MHC class I molecules (Strong and McFarland 2004; Figs. 1 and 2). Unlike KIR and Ly49A interactions at the first binding site, the NKG2D binding sites are much less dominated by charge–charge interactions. Both homodimer-related binding sites on NKG2D contribute approximately equally to the interactions in both complexes, reflecting a binding site that has evolved to bind multiple target sites without side-chain rearrangements. The considerable recognition degeneracy of NKG2D, accommodating structurally divergent, polymorphic families of ligands, is enabled not by a conformationally plastic binding site ("induced fit" or "conformer selection"), but rather by a "rigid adaptation" mechanism.

Another NK-activating receptor is the low-affinity receptor for IgG, CD16, which mediates ADCC and signals through adaptors containing cytoplasmic ITAMs. Several receptors, which activate antibody-independent, natural cytotoxicity are also associated with ITAM-containing signaling adaptors (Table 1). These receptors include NKp30, NKp44, and ▶ NKp46, which are referred to as NCRs (Biassoni 2009). CD16, NKp30, and NKp46, all use both FcεRIγ and CD3ζ as signaling adaptors. NKp44, on the other hand, partners with DAP12. Both NKp46 and NKp44 have been reported to bind viral hemagglutinin on infected cells while their cellular ligands have not been identified. NKp46 also contributes to enhanced killing of mitotic cells by NK cells, suggesting a role of NK cells in controlling the expansion of rapidly dividing cells. A recent study identified a tumor cell surface molecule that belongs to the B7 family that triggers NKp30-mediated activation of human NK cells (Brandt et al. 2009). This molecule, designated as B7H6, was not detected in normal human tissues but was expressed on human tumor cells. The expression of stress-induced self-molecules such as MICA/B and B7H6 associated with cell transformation serves as a mode of cell recognition in innate immunity. NKp30 also mediates killing of immature dendritic cells by NK cells. A nuclear factor, human leukocyte antigen-B-associated transcript 3 (BAT3), was also described as a ligand for NKp30. This ligand is released from tumor cells and plays an important role in tumor rejection in a multiple myeloma model via NKp30-mediated cytotoxicity. In addition, the main tegument protein (pp65) of human cytomegalovirus (HCMV) was proposed as an exogenous viral ligand as it inhibits NKp30-mediated cytotoxicity.

The specificity of the activating KIR family members has not been extensively characterized as compared with those of the inhibitory ones (Lanier 2005). KIR2DS1 and KIR2DS4 recognize HLA-C or HLA-A molecules weakly and KIR3DS1 is thought to recognize HLA-B with the Bw4 epitope, while ligands for other activating KIR receptors are undefined. The high sequence similarity between several pairs of activating and inhibitory KIR suggests they arose by gene duplication. However, in general, the activating KIRs either do not bind the cognate HLA class I, or bind with an affinity much weaker than that of the paired inhibitory KIR. The activating KIR receptors all have short cytoplasmic tails, and they transduce signals and trigger function through the ITAM-bearing adaptor DAP12. The exception is KIR2DL4. It has a functional ITIM in its cytoplasmic tail, yet it also associates with the

FcεRIγ adaptor protein, a feature for an activating receptor. Despite the presence of an ITIM, ligation of KIR2DL4 with its specific monoclonal antibody (mAb) leads to cytotoxicity and IFN-γ production in IL-2-activated NK cells. Soluble HLA-G has been described as its ligand.

Other activating receptors signal through motifs in their own cytoplasmic tail, or through pathways that have not been well characterized. 2B4, NK-T-B-antigen (NTB-A), and CD2-like receptor-activating cytotoxic cells (CRACC), all belong to the SLAM family of receptors (Veillette 2006). They are predominantly expressed on hematopoietic cells and regulate both innate and adaptive immunity. They are members of the Ig superfamily that consist of multiple Ig-like domains in the extracellular region, a transmembrane segment, and a cytoplasmic domain containing multiple tyrosine-based motifs. Comparing with other ITAM-based activating receptors, SLAM receptors have distinct activation pathways. In humans, activation through these receptors is accompanied by the phosphorylation of unique TxYxxV/L/I sequence–based immunoreceptor tyrosine-based switch motifs (ITSMs) in their cytoplasmic tails and the recruitment of SLAM-associated protein (SAP) as adaptors. SAP couples SLAM family receptors to FYNT, a Src-related PTK that is expressed by hematopoietic cells. In mice, however, 2B4 apparently functions as an inhibitory receptor. It is unclear if 2B4 itself can independently trigger effector functions or rather serves as a costimulatory receptor. The strongest evidence supporting an activating role in human NK cells comes from the demonstration that transfection of CD48, its ligand, into certain NK-resistant target cells renders them susceptible to NK-mediated cytotoxicity and triggers the production of IFN-γ by the human NK cells. Such action can be blocked by anti-CD48 mAb and anti-human 2B4 mAb. Adaptor protein SAPs appears to play important roles in SLAM receptor functions. In patients with certain mutations in SAP, NK cells can no longer be activated through the 2B4 receptor. Furthermore, immature human NK cells express 2B4 receptors that mediate inhibitory function, presumably because these immature NK cells lack SAP. 2B4 may, therefore, be a multifunctional receptor in both human and mice, displaying different functions depending on factors such as NK cell differentiation and activation. The complex crystal structure between the N-terminal domains of mouse 2B4 and CD48 reveals that they interact in a mode related to, yet distinct from, that of the homophilic NTB-A dimer of the same SLAM family. Binding is accompanied by the rigidification of flexible 2B4 regions containing most of the polymorphic residues across different species and receptor isoforms (Velikovsky et al. 2007).

Other activating NK receptors, such as DNAM-1 and NKp80, have been implicated in various immune functions. DNAM-1 receptor is a member of the Ig superfamily that is expressed in a variety of human lymphocytes including NK cells. It has been implicated in cell adhesion and in triggering NK- and T-cell-mediated cytolysis. CD112 and CD155, which are components of cellular adherens junctions, have been identified as ligands for DNAM-1. These ligands are frequently upregulated, and interaction between DNAM-1 on NK cells and CD112 and CD155 on tumor cells augments NK cell–mediated cytotoxicity and cytokine production. The interaction of NK cell receptors with members of the nectin-like molecules indicates the importance of adhesion in the triggering of NK cell function. Physical association of DNAM-1 with LFA-1 is necessary for NK and T cell triggering. NKp80 is another NK-activating receptor with unknown signaling properties. The cellular ligand of NKp80 was recently identified as activation-induced C-type lectin (AICL). AICL is expressed on myeloid cells, and is upregulated by inflammatory stimuli. Thus, NKp80–AICL interactions may be important for NK cell-myeloid cell cross talk during inflammation.

Tumor and Viral Evasion Mechanisms

Tumors use several mechanisms to evade the immune system, including NK-mediated responses. Aberrant loss of the activating receptor NKG2D in cancer is a key mechanism of immune evasion (Groh et al. 2002). Production of soluble, secreted NKG2D ligands or growth factors, such as TGF-β released from tumors, is a mechanism for downregulating NKG2D expression. TGF-β may also play an important role in the conversion of normal T cells into regulatory T cells (T_{reg}), a population of T cells that is found in high levels in the tumor microenvironment. T_{reg} cells reduce NKG2D expression, suppress NKG2D-mediated NK cell cytotoxicity, and accelerate the progression of tumors.

Although the expression of MICA/B may result in tumor elimination, MICA shedding, through directed proteolysis from the surface of tumor cells, into the

plasma is a common characteristic of many epithelially derived tumors. The process of shedding is mediated by interactions between MICA and a protein disulphide isomerase, endoplasmic reticulum protein 5 (ERp5 or PDI-P5). ERp5 forms a transitory complex with MICA and reduces an inaccessible disulfide bond in the α3 domain, which must induce a conformational change that is essential for the proteolytic cleavage of MICA (Kaiser et al. 2007).

Epigenetic repression of NKG2D ligand transcription, such as limiting the accessibility of promoter sequences to transcription factors or micro-RNA-induced repression of gene expression, is another tumor evasion mechanism. Transcription of MICA/B and ULBP1 is mainly regulated by ubiquitous transcription factors including Sp1, Sp3, and NFY. However, the binding of these transcription factors to their promoters may be regulated by the histone deacetylases (HDACs) and the structure of chromatin. HDACs remove the acetyl groups of histone to form compacted chromatin, which restrains the accessibility of promoter sequences to transcription factors. HDACs are overexpressed in many cancer types, and they participate in the repression of numerous proteins with tumor-suppression activities (Lopez-Soto et al. 2009). Micro-RNAs may also target the MICA/B 3'-untranslated region to repress expression (Stern-Ginossar et al. 2007).

Viral immune modulatory mechanisms also manipulate NK receptor and ligand expression (Wilkinson et al. 2008), and virus-derived decoy ligands for inhibitory receptors are used as viral evasion mechanisms. HCMV encodes an inhibitory decoy glycoprotein ligand, UL18, which is structurally similar to MHC class I proteins, including a peptide-binding groove and association with host β_2-microglobulin. UL18 is used to evade NK detection by binding to the inhibitory NK receptor LIR-1 with high affinity. Selective upregulation of inhibitory MHC class I antigens is another viral evasion mechanism; viral proteins that bind MHC class Ib proteins and upregulate their expression, while differentially downregulating CD8 + T cell activating MHC class Ia antigens, include HCMV-encoded UL40, US2, US11, and HIV-encoded Nef. A nine amino acid sequence (VAMPRTLIL) in HCMV UL40, identical to HLA-C leader sequences, binds to HLA-E and allows its surface expression when MHC class I expression is repressed by other viral mechanisms. On the other hand, HCMV-encoded US2 and US11 have been shown to selectively downregulate HLA-A surface expression without interfering with HLA-E expression. Similar evasion mechanisms are also observed with the HIV-encoded Nef protein, which directs HLA-A and B for lysosomal degradation while sparing HLA-C and HLA-E.

Viral proteins that directly interact with NK-activating ligands are also deployed by viruses to evade NK surveillance by sequestering and retaining ligands intracellularly. HCMV UL16 sequesters the NKG2D ligands MICB, ULBP1, and ULBP2 and downregulates their surface expression. Another HCMV-encoded protein, UL142, uses similar mechanism to downregulate MICA. Various serotypes of human adenovirus also encode proteins that are used to modulate surface expression of NK-activating receptors: The E3/19 K protein sequesters MICA/B proteins in the ER, downregulating surface expression. Expression of NK-activating ligands is also modulated by virus-derived micro-RNAs. The HCMV-encoded miRNA, hcmv-miR-UL112, selectively downregulates the NKG2D ligand MICB (Stern-Ginossar et al. 2007). Viruses also manipulate components of the host ubiquitin pathway to protect infected cells from NK responses. The KSHV-encoded immune evasion proteins, K3 and K5, have E3 ubiquitin ligase activity, possess a variant RING domain, and downregulate surface expression of NKG2D ligands MICA/B by ubiquitination of conserved lysine residues in the cytoplasmic tail of MICA/B which result in its trafficking from the cell surface to an intracellular compartment.

Summary

NK cells are capable of recognizing and destroying multiple pathogenic cell targets. This broad specificity is mediated by multiple activating and inhibitory surface receptors and intracellular signal transduction molecules. NK receptors are regulated in a spatial and temporal fashion at the NK synapse where adhesion molecules and NK receptors interact with their ligands on the target cell. Healthy cells expressing MHC class I will engage NK inhibitory receptors and thus be spared, whereas infected or transformed cells, that have an aberrant expression of MHC class I and cell stress markers, become susceptible to attack. Significant progress has been made in the identification of many activating and inhibitory receptors, their ligands,

and signaling pathways. However, many receptors, such as NCRs and activating KIRs, have poorly defined or unknown ligands. The signaling mechanisms of receptors such as NKp80 and many of the mechanisms governing the regulation of receptor expression also remain unknown. Furthermore, the interplay between the complex array of activating and inhibitory receptors leading to NK cell function needs to be defined. Future studies aimed toward identifying unknown ligands and understanding the dynamic interactions between receptor signaling and its effect on cellular function will help in the understanding of NK cell biology and the role that NK cells play in the immune system.

References

Biassoni R. Human natural killer receptors, co-receptors, and their ligands. Current Protocols in Immunology, vol. 84. New York: Wiley; 2009. p. 14.10.1–40.

Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. Nat Immunol. 2003;4:557–64.

Boyington JC, Motyka SA, Schuck P, Brook AG, Sun PD. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. Nature. 2000;405:537–43.

Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in human. J Exp Med. 2009;206:1495–503.

Bryceson YT, Long EO. Line of attack: NK cell specificity and integration of signals. Curr Opin Immunol. 2008;20:344–52.

Fan QR, Long EO, Wiley DC. Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex. Nat Immunol. 2001;2:452–60.

Gonzalez S, Groh V, Spies T. Immunobiology of human NKG2D and its ligands. Curr Top Microbiol Immunol. 2006;298:121–38.

Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature. 2002;419:734–8.

Kaiser BK, Yim D, Chow IT, Gonzalez S, Dai Z, Mann HH, et al. Disulfide-isomerase-enabled shedding of tumour-associated NKG2D ligands. Nature. 2007;447:482–6.

Kaiser BK, Pizarro JC, Kerns J, Strong RK. Structural basis of NKG2A/CD94 recognition of HLA-E. Proc Natl Acad Sci USA. 2008;105:6696–701.

Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. Nature. 1992;358:66–70.

Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defense strategy. Nature. 1986;319:675–8.

Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol. 1975;5:112–7.

Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225–74.

Lopez-Soto A, Folgueras AR, Seto E, Gonzalez S. HDAC3 represses the expression of NKG2D ligands ULBPs in epithelial tumour cells: potential implications for the immunosurveillance of cancer. Oncogene. 2009;28:2370–82.

MacFarlane AW, Campbell KS. Signal transduction in natural killer cells. Curr Top Microbiol Immunol. 2006;298:23–57.

Petrie EJ, Clements CS, Lin J, Sullivan LC, Johnson D, Huyton T, et al. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. J Exp Med. 2008;205:725–35.

Shiroishi M, Kuroki K, Rasubala L, Tsumoto K, Kumagai I, Kurimoto E, et al. Structural basis for recognition of the nonclassical MHC molecule HLA-G by the leukocyte Ig-like receptor B2 (LILRB2/LIR2/ILT4/CD85d). Proc Natl Acad Sci USA. 2006;103:16412–7.

Stern-Ginossar N, Elefant N, Zimmermann A, Wolf DG, Saleh N, Biton M. Host immune system gene targeting by a viral miRNA. Science. 2007;317:376–81.

Strong RK, McFarland BJ. NKG2D and related immunoreceptors. Adv Protein Chem. 2004;68:281–312.

Tormo J, Natarajan K, Natarajan K, Margulies DH, Mariuzza RA. Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. Nature. 1999;402:623–31.

Veillette A. Immune regulation by SLAM family receptors and SAP-related adaptors. Nat Rev Immunol. 2006;6:56–66.

Velikovsky CA, Deng L, Chlewicki LK, Fernandez MM, Kumar V, Mariuzza RA. Structure of natural killer receptor 2B4 bound to CD48 reveals basis for heterophilic recognition in signaling lymphocyte activation molecule family. Immunity. 2007;27:572–84.

Wilkinson GW, Tomasec P, Stanton RJ, Armstrong M, Prod'homme V, Aicheler R, et al. Modulation of natural killer cells by human cytomegalovirus. J Clin Virol. 2008;41:206–12.

Willcox BE, Thomas LM, Bjorkman PJ. Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. Nat Immunol. 2003;4:913–9.

NKG2D

Michael T. McCarthy and Christopher A. O'Callaghan
Centre for Cellular and Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Headington, Oxford, UK

Synonyms

CD314; Killer cell lectin-like receptor subfamily K; Member 1 (KLRK1)

Historical Background

Natural killer (NK) cells are key components of the innate immune response and can mediate cellular cytotoxicity without previous antigen exposure. The mechanisms underlying NK cell activation have been uncovered in a stepwise fashion from the early 1980s (for a detailed review of the field and its development see (Lanier 2008)). The role of the CD16 FcγRIII cell surface receptor in antibody-dependent NK cell activation was described in 1983. In 1986, Karre and colleagues proposed the "missing self hypothesis" hypothesis, and demonstrated the presence of inhibitory NK cell surface receptors responsive to the self-MHC (major histocompatibility complex) molecules on normal host cells. The search for antibody-independent NK cell activating receptors led to the identification of the NKG2 family of NK cell receptors in 1991. Among these was NKG2D, a molecule that shared limited homology with other NKG2 family members and was expressed in both NK and T cells as a predicted type 2 transmembrane C-type lectin receptor. In 1994, Bahram et al. identified two polymorphic genes encoding molecules related to classical MHC class I molecules which they named major histocompatibility complex I chain-related genes A and B (MICA, MICB) (Bahram et al. 1994). In 1999, NKG2D was identified as the activating receptor for MICA and MICB (Bauer et al. 1999).

NKG2D is recognized as a dominant activating NK cell receptor, capable of activating NK cells (Bauer et al. 1999), and influencing T cell activation. It is expressed constitutively in human NK cells, γδ T cells, and $CD8^+$ T cells, and may also be found in some invariant natural killer T (iNKT) cell and $CD4^+$ T cell populations. In mice, NKG2D is constitutively expressed in NK cells, but is only expressed in $CD8^+$ T cells when they have been activated. NKG2D has been identified in a wide range of mammals, including nonhuman primates, pigs, cattle, and rats.

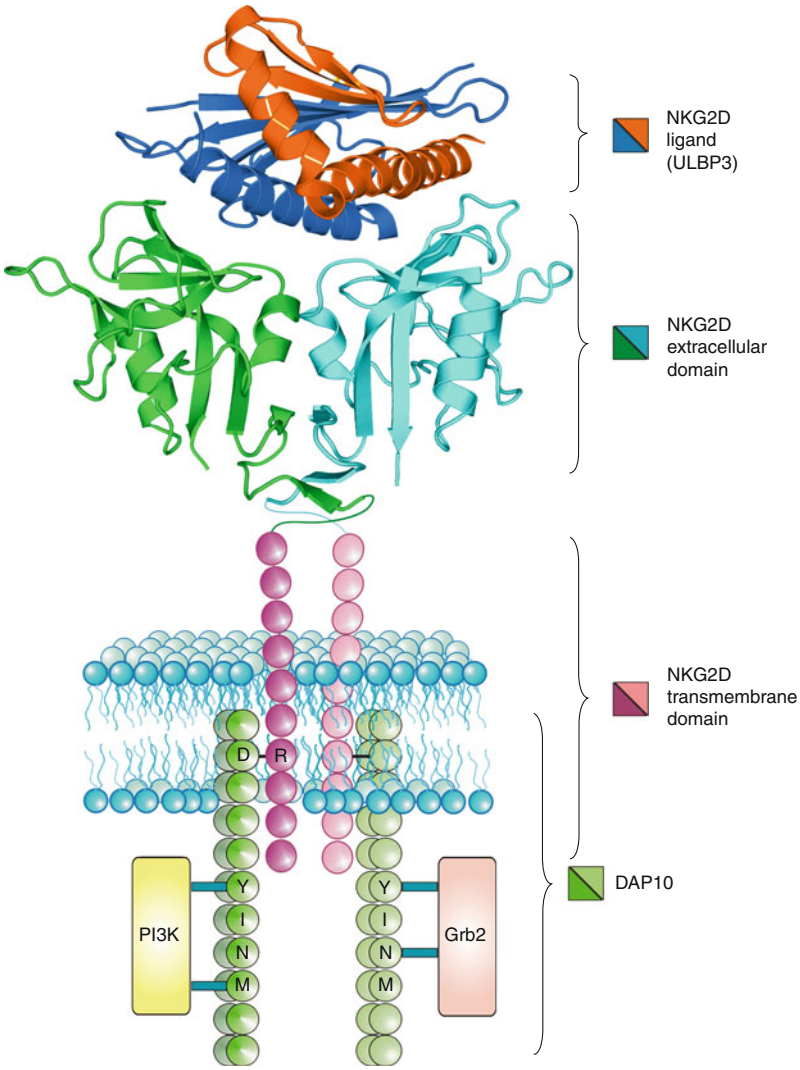
NKG2D Structure

Human NKG2D is a 316 amino acid type 2 transmembrane C-type lectin-like protein encoded by the KLRK1 gene (Killer cell Lectin-like Receptor subfamily K-1) within the NK gene complex at chromosome 12p13.2-12.3. Structural studies show that human NKG2D exists as a dimer and lacks functional calcium coordinating carbohydrate binding domains (Fig. 1) (Li et al. 2001). Each NKG2D monomer contains two β sheets and two helical elements, with four intrachain disulphide bonds. Murine NKG2D is structurally similar to human NKG2D (Wolan et al. 2001), but unlike human NKG2D, it exists in two isoforms: a long isoform of 232 amino acids (NKG2D-L, isoform A), and a short 219 amino acid isoform (NKG2D-S, isoform B) (Diefenbach et al. 2002).

The cytoplasmic portion of human NKG2D does not possess intrinsic signaling capability. Instead, a charged amino acid (arginine) in the transmembrane portion of NKG2D interacts with an aspartate residue in the adaptor molecule DAP10 (DNAX-activation protein 10). Each NKG2D molecule binds to a DAP10 dimer in this fashion to form a hexameric structure capable of signal transduction. The YINM motif in the cytoplasmic region of DAP10 recruits downstream signaling molecules. Murine NKG2D interacts with both DAP10 and DAP12, an ITAM (immunoreceptor tyrosine-based activation motifs)-containing adaptor molecule, through similar transmembrane region interactions. Both long and short NKG2D isoforms are capable of interacting with and signaling through either DAP10 or DAP12 in vitro, but in vivo the long form of NKG2D only interacts with DAP10 (O'Callaghan 2009). Human NKG2D does not interact with DAP12.

NKG2D Signaling

Signal transduction from NKG2D is complex (Fig. 1). A critical early event in the NKG2D-DAP10 signal cascade is tyrosine phosphorylation of the YINM motif in DAP10 by the src family tyrosine kinase, Lck and src kinase inhibitors abrogate downstream signal transduction. The phosphorylated YINM motif recruits further intermediates: the p85 subunit of ► PI3K (phosphatidylinositide-3 kinase) and the adaptor protein Grb-2 (growth factor receptor-bound protein 2). Both binding events are essential for signal transmission. The GNEF (guanine nucleotide exchange factor) ► Vav1, which plays an important role in actin reorganization, is recruited to Grb-2, where it is phosphorylated and activated. From here, signal transmission diverges, but it is known that Vav1 acts in part by binding the γ2 isoform of phospholipase C (PLCγ2), which amongst other downstream targets



NKG2D, Fig. 1 Human NKG2D ligand binding and signaling. The NKG2D extracellular domain and ULBP3 are shown in cartoon form based on the crystal structure of the complex. Each NKG2D molecule interacts with a DAP10 dimer through an association between an arginine residue (R) in the transmembrane region of NKG2D, and an aspartate residue (D) in the transmembrane domain of DAP10. Upon ligand binding, signaling from NKG2D is transmitted through DAP10. Key interactions with DAP10 include binding of the p85 subunit of PI3K to the YXXM motif in DAP10, while Grb2 recognizes YXNX, following the phosphorylation of DAP10 by src family tyrosine kinases

activates MAPK8 (mitogen-activated protein kinase 8, JNK1). In contrast, MAPK1 (ERK2) appears to be an important downstream target of PI3K. These signaling pathways are reviewed by Lanier (2008).

While human NKG2D does not interact with DAP12, NKG2D-DAP12 signaling remains an important consideration in murine NKG2D models. Unlike DAP10, NKG2D-DAP12 signaling is dependent on the ITAM domain of the cytoplasmic portion of DAP12. Engagement of ligand by NKG2D leads to the phosphorylation of two tyrosine residues in the DAP12 ITAM by src family tyrosine kinases. This enables recruitment of the syk tyrosine kinase, which is necessary for NKG2D-DAP12 function. It is likely that the downstream components of this pathway are similar to those in other ITAM-associated receptors, which have been studied in greater detail in different contexts.

NKG2D Ligands

A striking characteristic of NKG2D is the wide range of distinct ligands with which it can productively interact. Such ligand diversity indicates that NKG2D has evolved as an important receptor receiving input from the various stimuli which act through the upregulation of the different ligands. For further detailed reviews, see Mistry and O'Callaghan (2007) and Champsaur and Lanier (2010).

Humans express eight distinct functional NKG2D ligands: MICA and MICB (major histocompatibility complex I chain-related genes A and B), and the UL16 binding proteins (ULBP) 1–6. MICA and MICB are distant homologues of classical MHC class I molecules. Both are highly polymorphic, a feature of much interest, with 71 and 30 known alleles respectively identified to date. MICA and MICB are transmembrane molecules with MHC class I-like α1, α2, and α3 domains. However, in contrast to classical MHC class I molecules they do not associate with β2-microglobulin, and the cleft separating the α2 and α3 domains is closed and does not bind peptides.

In contrast, the ULBPs have limited polymorphism and lack an α3 domain. ULBP 1–3 were originally identified as proteins that were bound by the cytomegalovirus protein UL16 and that interacted with NKG2D to induce NK cell cytokine secretion and cytotoxicity. Seven related gene sequences were subsequently described in the same region of chromosome 6q. Three of these encode functional molecules, ULBP 4 (RAET1E, LETAL), ULBP 5 (RAET1G), and ULBP 6 (RAET1L). ULBPs 1–3 and ULBP 6 are GPI-linked cell surface proteins, while ULBP 4 and 5 exist principally in transmembrane forms. A GPI-linked splice variant of ULBP 5 has been described, while ULBP 4 has three functional splice variants, each encoding transmembrane receptors.

Mice do not have MICA or MICB genes. There are three families of murine NKG2D ligands: the retinoic acid early (RAE) 1 family including α, β, γ, δ and ε; H60 a, b and c; and murine ULBP-like transcript 1 (Mult1). RAE1 α, β, and γ were described in 1996 as glycophoshatidyl-inositol (GPI)-linked cell surface molecules of unknown function, isolated from retinoic acid–treated mouse embryonal carcinoma cells. The murine NKG2D ligands show limited homology with murine classical MHC class I molecules and have an α1, α2 domain structure which does not present peptide, and does not associate with β2 microglobulin. H60a, H60b, and Mult1 are transmembrane proteins, while H60c is a GPI-linked cell surface protein. The binding reactions between murine NKG2D and its ligands have been studied in detail, and binding affinities vary distinctly between NKG2D and its various ligands (O'Callaghan et al. 2001; Mistry and O'Callaghan 2007).

The ligands for NKG2D are expressed on some normal cells, including gastrointestinal epithelial cells, and activated T cells, but are primarily displayed in pathological settings, notably in response to viral infection, some intracellular bacterial infections, and in many cancers. While several stimuli, broadly described as physiological stressors, are known to increase cell surface expression of NKG2D ligands on target cells, the mechanisms underlying regulation of NKG2D ligand expression remains poorly understood (Mistry and O'Callaghan 2007).

NKG2D Function

NKG2D may play significant roles in viral immunity, tumor immunity, autoimmune diseases, and transplant rejection (Ogasawara and Lanier 2005). These roles are mediated through specific cellular processes, especially the induction of natural killer cell cytotoxicity and cytokine production. The role of NKG2D in T cell costimulation remains unclear (Lopez-Larrea et al. 2008; O'Callaghan 2009).

Natural cytotoxicity. Natural cytotoxicity involves the coordination of several complex cellular processes including cytoskeletal reorganization, immune synapse formation, and perforin translocation. Freshly isolated human NK cells are capable of mounting a cytotoxic response to a target expressing NKG2D ligands without further experimental manipulation. Signaling through NKG2D can override inhibitory signaling through the interaction of HLA-E with CD94/NKG2A (Bauer et al. 1999) although in vivo NK cell activation is likely to reflect a broad balance between activating and inhibitory signaling. NKG2D may mediate TCR-independent cytotoxicity in IL-2 treated $CD8^+$ T cells, γδ-TCR T cells, and some $CD4^+$ T cell populations, but the *in vivo* significance of this is less certain and remains to be firmly established.

Cytokine and chemokine production. Early studies suggested that isolated ligation of NKG2D on NK cells and γδ-TCR T cells is sufficient to induce secretion of cytokines, primarily IFNγ, TNFα and to a lesser extent, GMCSF and TNFβ (Fauriat et al. 2010). In one study, isolated human NKG2D ligation induced little cytokine secretion, but IFNγ and TNFα, prototypical NK cell cytokines, in addition to MIP-1α, MIP-1β, and RANTES, were produced when NKG2D was triggered together with CD16 (FCγRIII), natural cytotoxicity receptors, 2B4 or LFA-1 (Fauriat et al. 2010).

Costimulation. CD28 is the prototypical TCR costimulatory molecule, and signals through the p85

subunit of PI3K, which is recruited to a YMNM motif in the cytoplasmic domain of CD28. Given this shared signaling pathway with NKG2D, it was hypothesized that NKG2D might costimulate TCR-dependent T cell activation. Cytotoxicity, cytokine production (IFNγ, TNFα, IL-2, and IL-4) and proliferation was enhanced by the MICA-NKG2D interaction in cytomegalovirus-specific $CD8^+$ T cells (Groh et al. 2001). Similar findings have been demonstrated in freshly isolated naïve ($CD62L^+$, $CD45RO^-$) human αβ TCR $CD8^+$ T cells (Maasho et al. 2005). Both these studies used cytokine (IL-2 or IL-15) supported T cells in their experiments. When the T cell costimulatory capacity of NKG2D was compared to that of CD28, in the absence of cytokine support, NKG2D ligation did not mediate costimulation (Ehrlich et al. 2005).

Murine knockouts. NKG2D knockout mice have provided further insights into NKG2D function (Guerra et al. 2008; Zafirova et al. 2009). Crossbreeding of NKG2D deficient mice with mice which develop prostate cancer or lymphoma demonstrate earlier and more rapid progression of these cancers in NKG2D-deficient mice (Guerra et al. 2008). Furthermore, NK cells from NKG2D-deficient mice have a diminished ability to kill NKG2D ligand expressing target tumor cells (Zafirova et al. 2009). NK cell development also appears to be affected, with more rapid NK cell division, and enhanced cytokine responses to NK cell stimulation (Zafirova et al. 2009).

NKG2D Regulation

Several factors modify the cell surface expression of NKG2D (Mistry and O'Callaghan 2007; O'Callaghan 2009). Cytokines appear to be the principle regulators of NKG2D cell surface concentration on both NK cells and T cells. Altering NKG2D cell surface density may alter the influence of NKG2D on cellular activation. TGFβ decreases cell surface NKG2D expression in NK cells, while IL-21 decreases NKG2D cell surface expression in both NK cells and $CD8^+$ T cells. IL-15 increases NKG2D expression on $CD8^+$ T cells, $CD4^+$ T cells, and NK cells, while IL-2, IL-12, and IFNα have been shown to increase NKG2D expression on NK cells.

Excessive exposure to NKG2D ligands is reported to lower NKG2D cell surface concentrations in both physiological and pathological settings. In pregnancy, syncytiotrophoblast cells of the placenta produce exosomes containing soluble NKG2D ligands, which decrease NK cell NKG2D cell surface expression and reduce NK cell cytotoxicity (Hedlund et al. 2009). Tumor cells have been shown to downregulate NKG2D cell surface concentration in a similar fashion (Groh et al. 2002).

Summary

NKG2D is a homodimeric C-type lectin like molecule which acts as an activating receptor in NK cells, and can play an activating and potentially costimulating role in γδ T cells and $CD8^+$ T cells, and some $CD4^+$ T cell and iNKT cell populations. It is widely conserved in mammals suggesting an important immune role. It is a promiscuous receptor with multiple diverse ligands, which are now well characterized in humans and mice. Future work is needed to further elucidate NKG2D signaling mechanisms, define functional interactions between NKG2D and other immune signaling molecules, and to identify regulatory mechanisms that govern NKG2D ligand expression in target cells.

References

Bahram S, Bresnahan M, Geraghty DE, Spies T. A second lineage of mammalian major histocompatibility complex class I genes. Proc Natl Acad Sci USA. 1994;91(14):6259–63.

Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress- inducible MICA. Science. 1999;285(5428):727–9.

Champsaur M, Lanier LL. Effect of NKG2D ligand expression on host immune responses. Immunol Rev. 2010;235(1):267–85.

Diefenbach A, Tomasello E, Lucas M, Jamieson AM, Hsia JK, Vivier E, et al. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. Nat Immunol. 2002;3(12):1142–9.

Ehrlich LI, Ogasawara K, Hamerman JA, Takaki R, Zingoni A, Allison JP, et al. Engagement of NKG2D by cognate ligand or antibody alone is insufficient to mediate costimulation of human and mouse CD8+ T cells. J Immunol. 2005;174(4):1922–31.

Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood. 2010;115(11):2167–76.

Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol. 2001;2(3):255–60.

Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T- cell activation. Nature. 2002;419(6908):734–8.

Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity. 2008;28(4):571–80.
Hedlund M, Stenqvist AC, Nagaeva O, Kjellberg L, Wulff M, Baranov V, et al. Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. J Immunol. 2009;183(1):340–51.
Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol. 2008;9(5):495–502.
Li P, Morris DL, Willcox BE, Steinle A, Spies T, Strong RK. Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA. Nat Immunol. 2001;2(5):443–51.
Lopez-Larrea C, Suarez-Alvarez B, Lopez-Soto A, Lopez-Vazquez A, Gonzalez S. The NKG2D receptor: sensing stressed cells. Trends Mol Med. 2008;14(4):179–89.
Maasho K, Opoku-Anane J, Marusina AI, Coligan JE, Borrego F. NKG2D is a costimulatory receptor for human naive CD8+ T cells. J Immunol. 2005;174(8):4480–4.
Mistry AR, O'Callaghan CA. Regulation of ligands for the activating receptor NKG2D. Immunology. 2007;121(4):439–47.
O'Callaghan CA, Cerwenka A, Willcox BE, Lanier LL, Bjorkman PJ. Molecular competition for NKG2D: H60 and RAE1 compete unequally for NKG2D with dominance of H60. Immunity. 2001;15(2):201–11.
Ogasawara K, Lanier LL. NKG2D in NK and T cell-mediated immunity. J Clin Immunol. 2005;25(6):534–40.
O'Callaghan CA. UCSD-Nature Molecule Pages. Nature Publishing Group. 2009. http://www.signaling-gateway.org/molecule/query?afcsid=A001666. Accessed 15 Sept 2010.
Wolan DW, Teyton L, Rudolph MG, Villmow B, Bauer S, Busch DH, et al. Crystal structure of the murine NK cell-activating receptor NKG2D at 1.95 A. Nat Immunol. 2001;2(3):248–54.
Zafirova B, Mandaric S, Antulov R, Krmpotic A, Jonsson H, Yokoyama WM, et al. Altered NK cell development and enhanced NK cell-mediated resistance to mouse cytomegalovirus in NKG2D-deficient mice. Immunity. 2009;31(2):270–82.

NKp46

Simona Sivori
Dipartimento di Medicina Sperimentale (DI.ME.S.) and Centro di Eccellenza per la Ricerca Biomedica, Università di Genova, Genova, Italy

Synonyms

CD335

Historical Background

NK cell function is finely regulated by a series of inhibitory or activating receptors. The inhibitory receptors, specific for major histocompatibility complex (MHC) class I molecules, allow NK cells to discriminate between normal cells and cells that have lost the expression of MHC class I (e.g., tumor cells). In the absence of sufficient signaling by their HLA class I-specific inhibitory receptors, human natural killer (NK) cells become activated and display potent cytotoxicity against cells that are HLA class I negative. This indicates that the NK receptors responsible for the induction of cytotoxicity recognize ligands on target cells different from HLA class I molecules. These receptors have been termed natural cytotoxicity receptors (NCR) and include NKp46, together with NKp44 and NKp30 (Bottino et al. 2000). A direct correlation exists between the surface density of NCR and the ability of NK cells to kill various target cells (Sivori et al. 1999). Importantly, mAb-mediated blocking of these receptors has been shown to suppress cytotoxicity against most NK-susceptible target cells. However, the process of NK-cell triggering during target cell lysis may also depend on the concerted action of NCR and other triggering receptors, such as ▶ NKG2D, or surface molecules, including 2B4 and NKp80, that appear to function as co-receptors rather than as true receptors.

Distribution and Molecular/Biochemical Characterization of NKp46

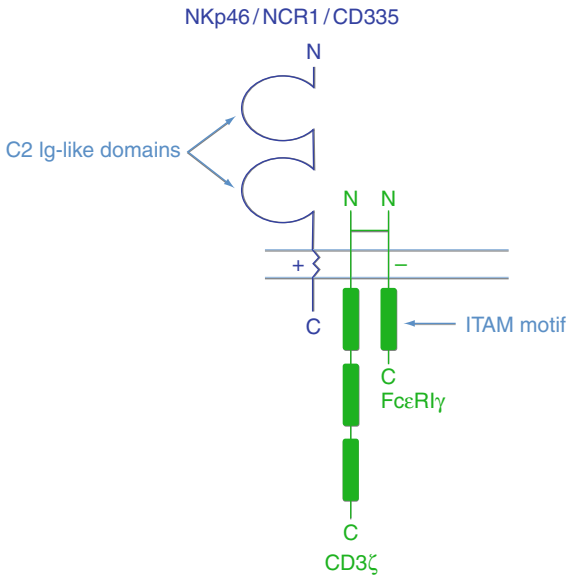
NKp46 is a surface molecule of 46 kDa expressed by all human NK cells (including the $CD56^{bright}$ $CD16^{-}$ subset) irrespective of their state of activation. It is mostly confined to NK cells and its engagement induces a strong activation of NK-mediated cytolysis, Ca^{++} mobilization, and cytokine release (Moretta et al. 2001; Sivori et al. 1997).

NKp46 is encoded by the NCR1 gene, which maps in the telomeric region of "leukocyte receptor complex" (LRC) on human chromosome 19q13.42 (Kelley et al. 2005; Pessino et al. 1998). The NCR1 gene is conserved during speciation, at least in rodents, bovine, and primates (Biassoni 2008).

NKp46 is a type I transmembrane glycoprotein belonging to the Ig superfamily. It is characterized by two extracellular Ig-like domains of the C2 type

(about 190 aa), connected by a short peptide (25 aa) to the transmembrane domain (about 20 aa) and by a small cytoplasmic domain (about 30 aa) (Fig.1). The amino-terminal portion of the transmembrane region of NKp46 contains the positively charged amino acid arginine, which likely forms a salt bridge with the aspartic acid residue located in a similar topological context of the transmembrane domains of CD3ζ or FcεRIγ. The cytoplasmic tail does not contain immunoreceptor tyrosine-based activation motifs (ITAM), typically involved in the activation of the signal cascade(s) (Moretta et al. 2001; Pessino et al. 1998). NKp46 can transduce positive signaling thanks to its association with CD3ζ or FcεRIγ, two small polypeptides characterized by short extracellular portions and by cytoplasmic tails containing three and one ITAM, respectively, that become tyrosine phosphorylated upon receptor engagement. In NK cells, CD3ζ and FcεRIγ are also involved in intracellular signaling via CD16 and have been shown to be phoshorylated by tyrosine kinases of the ▶ Src family, such as p56lck, followed by the recruitment of Syk and ▶ ZAP-70 tyrosine kinases (Moretta et al. 2001).

The crystal structure of NKp46 indicates a certain degree of structural conservation with KIR2DL2, CD89, glycoprotein VI platelet collagen receptor, LILRB1 (LIR-1/ILT2/CD85j), and LILRB2 (LIR2/ILT4/CD85d) molecules, which are encoded by genes that map close to each other on chromosome 19q13.42 (Ponassi et al. 2003).



NKp46, Fig. 1 *NKp46 structure*. NKp46 is a type I glycoprotein belonging to the Ig-superfamily. It contains, in its transmembrane portion, a positively charged amino acid involved in the association with signal-transducing molecules characterized by negatively charged residues in their transmembrane portion and tyrosine-based motifs in their cytoplasmic tails

NKp46 Ligands

The identification of the NKp46 ligand(s) on cancer cells is still being investigated. At this regard, it has been suggested that membrane-associated heparan sulfate proteoglycans (HSPGs) might be involved in the recognition of tumor cells by NKp46 and NKp30 (Bloushtain et al. 2004). These results have been not confirmed by other studies, at least for NKp30-mediated killing (Warren et al. 2005). Moreover, since heparan sulfate is also found on normal cells that are not killed by NK cells, it is likely that it does not represent a specific ligand of NKp46 or NKp30. Indeed, heparan sulfate is already known as a co-ligand for different growth factors, chemokines, lipid-binding proteins, and adhesion proteins (Capila and Linhardt 2002).

NKp46 is also involved in the killing of virus-infected cells and it has been described to recognize the hemagglutinin of influenza virus (IV-HA) and the hemagglutinin-neuraminidase of Sendai virus (SV-HN) (Arnon et al. 2004; Gazit et al. 2006; Mandelboim et al. 2001). This recognition seems to depend on the sialylation of NKp46 receptor, in particular on α2,6-linked sialic acid carried by Thr-225 residue (located into the membrane proximal domain of NKp46) (Arnon et al. 2004). The Thr-225 of NKp46 plays a critical role in interaction not only with viral hemagglutinins, but also with unknown tumor cellular ligands, probably via different mechanisms not involving its sialylation (Arnon et al. 2004).

Other authors have suggested that vimentin, expressed on the surface of *Mycobacterium tuberculosis*–infected monocyte cell lines, mediates NKp46 binding to these cells and contributes to their lysis (Garg et al. 2006).

It has been suggested that the positively charged amino acids K133, R136, H139, R142, and K146 present in the membrane proximal domain may be involved in ligand interaction. Indeed mutagenesis of these amino acids on NKp46 to noncharged amino

acids decreases the affinity of interaction with tumor cells at least 10–100 times, without affecting the interaction with viral ligands (Zilka et al. 2005).

NKp46 on CTL

Although the cell-surface expression of NKp46 is the most selective marker for human and mouse NK cells described so far, there are non-NK populations that also express NKp46, including discrete subsets of γδ T cells in mice and subsets of intraepithelial lymphocytes in patients with celiac disease. In various chronic infectious and inflammatory diseases (such as celiac disease or citomegalovirus infection), human CTLs can aberrantly express NKp46 as well as other activating NK receptors, such as NKG2C or NKp44. These activating NK receptors, thanks to their association with ITAM-bearing molecules (DAP12, CD3ζ or FcεRIγ), could interfere with normal T cell tolerance mechanisms by both inducing the expansion and unleashing the effector function of CTL, independently of TCR signaling (Meresse et al. 2006).

NKp46 on NK-22 Cells

NKp46 is also expressed on a cell subset found in the lamina propria of mouse small intestine and characterized by constitutive production of interleukin (IL)-22. This cell subpopulation seems to play a role in protecting mucosa and controlling inflammation (Satoh-Takayama et al. 2008). Constitutive IL-22 production by NKp46$^+$ cells is abolished in germ-free mice and infection of mice with enteric pathogens increases both NKp46$^+$ cell numbers and their IL-22 expression. These cells display greatly reduced cytotoxic activity and IFN-γ production, but produce large amounts of IL-22 in response to IL-23.

A similar subpopulation has been found also in tonsils and Peyer's patches in humans, although these cells displayed the NCR NKp44 rather than NKp46 (Cella et al. 2009). Mouse IL-22$^+$ NKp46$^+$ cells and human IL-22$^+$ NKp44$^+$ cells have been collectively called NK-22 cells (Cella et al. 2009). Human NK-22 cells also produce IL-26 and LIF (Cella et al. 2009).

More recently the phenotype of human NK-22 cells has been refined. In particular, the revised phenotype of the human IL-22-producing NKp44$^+$ cells is the following: RORγt$^+$, NKp46$^+$, NKp44$^+$, NKG2D$^+$, CD56$^+$, CD127$^+$ (IL-7 receptor α chain), CD117$^+$, KIR$^-$, perforin$^-$, and granzyme$^-$ (Vivier et al. 2009).

Summary

NKp46 is a surface molecule of 46 kDa expressed by all human NK cells irrespective of their state of activation. It is mostly confined to NK cells and its engagement induces a strong activation of NK function. In particular, NKp46 plays an important role in the killing of virus-infected and tumor cells. The identification of the NKp46 ligand(s) on cancer cells is still being investigated. Indeed it is important to understand whether NKp46 ligand(s) down-modulation or loss can be involved in tumor resistance to NK-mediated cytolysis. Moreover this identification will allow designing strategies to increase tumor susceptibility to NK-mediated killing by manipulating the NKp46 ligand(s) expression at the tumor cell surface. Although NKp46 is the most selective marker for human and mouse NK cells described so far, there are non-NK populations that also express NKp46, including discrete subsets of γδ T cells in mice, human CTLs in various chronic infectious and inflammatory diseases, and mucosal RORγt$^+$ IL-22-producing cells.

References

Arnon TI, Achdout H, Lieberman N, Gazit R, Gonen-Gross T, Katz G, et al. The mechanisms controlling the recognition of tumor- and virus-infected cells by NKp46. Blood. 2004;103:664–72. doi:10.1182/blood-2003-05-1716.

Biassoni R. Natural killer cell receptors. Adv Exp Med Biol. 2008;640:35–52. doi:10.1007/978-0-387-09789-3_4.

Bloushtain N, Qimron U, Bar-Ilan A, Hershkovitz O, Gazit R, Fima E, et al. Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. J Immunol. 2004;173:2392–401.

Bottino C, Biassoni R, Millo R, Moretta L, Moretta A. The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering. Hum Immunol. 2000;61:1–6.

Capila I, Linhardt RJ. Heparin-protein interactions. Angew Chem Int Ed Engl. 2002;41:391–412. doi:10.1002/1521-3773(20020201)41:3<390::AID-ANIE390>3.0.CO;2-B.

Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature. 2009;457:722–5. doi:10.1038/nature07537.

Garg A, Barnes PF, Porgador A, Roy S, Wu S, Nanda JS, et al. Vimentin expressed on *Mycobacterium tuberculosis*-infected human monocytes is involved in binding to the NKp46 receptor. J Immunol. 2006;177:6192–8.
Gazit R, Gruda R, Elboim M, Arnon TI, Katz G, Achdout H, et al. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. Nat Immunol. 2006;7:517–23. doi:10.1038/ni1322.
Kelley J, Walter L, Trowsdale J. Comparative genomics of natural killer cell receptor gene clusters. PLoS Genet. 2005;1:129–39. doi:10.1371/journal.pgen.0010027.
Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature. 2001;409:1055–60. doi:10.1038/35059110.
Meresse B, Curran SA, Ciszewski C, Orbelyan G, Setty M, Bhagat G, et al. Reprogramming of CTLs into natural killer-like cells in celiac disease. J Exp Med. 2006;203:1343–55. doi:10.1084/jem.20060028.
Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197–223. doi:10.1146/annurev.immunol.19.1.197.
Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, et al. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. J Exp Med. 1998;188:953–60.
Ponassi M, Cantoni C, Biassoni R, Conte R, Spallarossa A, Moretta A, et al. Expression and crystallographic characterization of the extracellular domain of human natural killer cell triggering receptor NKp46. Acta Crystallogr D Biol Crystallogr. 2003;59:2259–61. doi:10.1107/S090744490301895X.
Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity. 2008;29:958–70. doi:10.1016/j.immuni.2008.11.001.
Sivori S, Vitale M, Morelli L, Sanseverino L, Augugliaro R, Bottino C, et al. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. J Exp Med. 1997;186:1129–36.
Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, et al. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. Eur J Immunol. 1999;29:1656–66.
Vivier E, Spits H, Cupedo T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? Nat Rev Immunol. 2009;9:229–34. doi:10.1038/nri2522.
Warren HS, Jones AL, Freeman C, Bettadapura J, Parish CR. Evidence that the cellular ligand for the human NK cell activation receptor NKp30 is not a heparan sulfate glycosaminoglycan. J Immunol. 2005;175:207–12.
Zilka A, Landau G, Hershkovitz O, Bloushtain N, Bar-Ilan A, Benchetrit F, et al. Characterization of the heparin/heparan sulfate binding site of the natural cytotoxicity receptor NKp46. Biochemistry. 2005;44:14477–85. doi:10.1021/bi051241s.

NLS (Nuclear Localization Sequence)

► p38 MAPK Family of Signal Transduction Proteins

NMT

► NMT (N-Myristoyltransferase)

NMT (N-Myristoyltransferase)

Sujeet Kumar[1,2], Ponniah Selvakumar[1,2], Jonathan R. Dimmock[3] and Rajendra K. Sharma[1,2]
[1]Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada
[2]Cancer Research Unit, Saskatchewan Cancer Agency, Saskatoon, SK, Canada
[3]Drug Design and Discovery Research Group, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

Synonyms

Glycylpeptide N-tetradecanoyltransferase; Myristoyl CoA:protein N-myristoyltransferase; NMT; N-myristoyltransferase; Peptide N-myristoyltransferase.

Historical Background

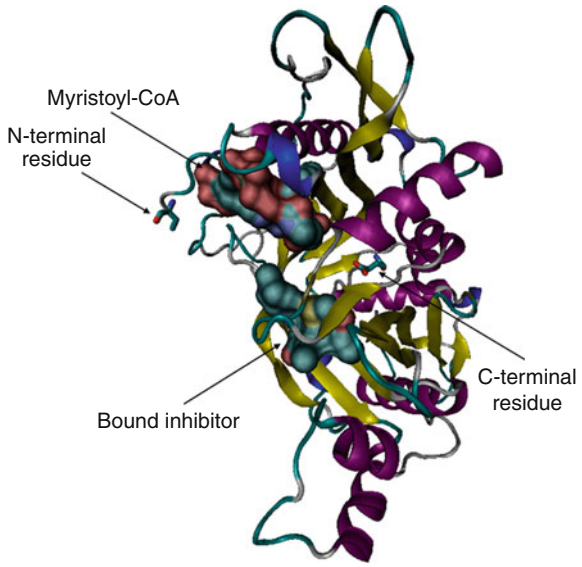
Protein functions are coordinated by a variety of secondary modifications which include the addition of chemical moieties of low molecular weight such as phosphate, acetate, and formate as well as of high molecular weight which includes carbohydrate and lipid molecules. In particular, the addition and removal of lipophilic moieties to proteins influences membrane targeting, signaling, and their functions. However, the current understanding of the roles and biological significance of fatty acid modifications in the regulation of protein functions is in a state of infancy. The three prevalent forms of lipid modification in eukaryotes are addition of glycosylphosphatidylinositols (GPIs),

isoprenoids, and fatty acids (myristoylation and palmitoylation) (Hannoush and Sun 2010). The protein modification by GPIs is a post-translational process which involves anchoring of a complex structure involving both lipids and carbohydrates to the carboxy terminus of protein molecules, whereas protein prenylation involves the post-translational irreversible attachment of a 15-carbon (farensyl) or a 20-carbon (geranylgeranyl) by a thioester bond to one or more cysteines located at or near to the carboxy terminus of the proteins (Hannoush and Sun 2010). Among the two fatty acid alteration processes, palmitoylation is a reversible change which occurs post-translationally and involves the addition of a 16-carbon chain palmitic acid to internal cysteine (S-acylation) residues via a thioester bond. However, sometimes palmitoylation can also occur via formation of an amide linkage (N-acylation) or an oxyester linkage (O-acylation) (Hannoush and Sun 2010). The protein myristoylation process on the other hand is an irreversible lipidic modification that refers to the covalent attachment of myristate, a 14-carbon saturated fatty acid, generally to the N-terminal glycine residue of proteins (Boutin 1997; Resh 1999; Farazi et al. 2001; Selvakumar et al. 2007; Wright et al. 2010; Hannoush and Sun 2010). The very first myristoylated protein to be discovered was catalytic subunit of cAMP-dependent protein kinase from bovine cardiac muscle which was found to be blocked at the N-terminus by n-tetradecanoic acid and a similar modification was very soon reported in the β-subunit of calcineurin (Boutin 1997; Selvakumar et al. 2007). Protein myristoylation was at very first thought to be a co-translational protein modification occurring after removal of the initiator methionine residue by methionine aminopeptidases (Wilcox et al. 1987); however, it is now reported to occur post-translationally as well (Farazi et al. 2001; Wright et al. 2010; Hannoush and Sun 2010). With subsequent studies, it has now been shown that myristoylation is not restricted to the N-terminal glycine but it can also occur on the internal lysine and cysteine residues (Boutin 1997). However, myristoylation other than at the N-terminal glycine is restricted to a very small fraction of the reported myristome. Myrisitic acid is considered a rare fatty acid in the cells and constitutes less than 1% of the total fatty acid pool (Boutin 1997). However, it is estimated that at least approximately 0.5% of the eukaryotic proteins are myristoylated (Hannoush and Sun 2010; Wright et al. 2010). This suggests a special role for myristoylation which cannot be substituted by other lipidic modifications of proteins. However, the myristoylation process so far has received less attention than the other related lipid modification processes, namely, prenylation and palmitoylation.

Myristoyl-CoA:Protein N-Myristoyltransferase (NMT) and Its Isoforms

The transfer of myristate to the N-terminal glycine of proteins is catalyzed by the enzyme myristoyl-CoA: protein N-myristoyltransferase (NMT) which is found ubiquitiously among lower and higher eukaryotes (Boutin 1997; Resh 1999; Selvakumar et al. 2007; Wright et al. 2010; Farazi et al. 2001). The enzyme follows an ordered Bi Bi reaction mechanism in which the apoenzyme binds myristoyl-CoA to form a NMT-myristoyl-CoA binary complex which subsequently binds to protein/peptide substrates. The catalytic conversion (N-myristoylation) is via direct nucleophilic addition–elimination reaction. The sequential release of CoA and myristoyl peptide follows the formation of enzyme–product complex from the enzyme–substrate complex (Resh 1999; Farazi et al. 2001; Wright et al. 2010). The enzyme is highly selective for myristoyl-CoA in vitro and in vivo (Farazi et al. 2001; Wright et al. 2010). The protein belongs to GNAT superfamily of enzymes and consists of saddle-shaped β-sheet flanked by α helices. There is pseudo twofold symmetry with regions corresponding to N- and C-terminal portions of the enzyme. The N-terminal half forms the myristoyl-CoA binding site whereas the C-terminal half forms the major portion of the peptide binding site (Fig. 1) (Farazi et al. 2001; Wright et al. 2010). NMTs have a very high common preference for myristoyl-CoA but have varying peptide substrate specificities (Farazi et al. 2001; Wright et al. 2010).

There is substantial biochemical evidence that suggests that these enzymes exist as isozymes in vivo, varying in either apparent molecular weight and/or subcellular distribution (Boutin 1997; Wright et al. 2010). The first report on the multiple isoforms of NMT came from studies on bovine brain NMT (King and Sharma 1992) and further isozymes varying in size and tissue distribution have been reported from



NMT (N-Myristoyltransferase), Fig. 1 Structural model of human NMT1 bound to myristoyl-CoA and an inhibitor (generated from PDB code 3IU2). The distinct myristoyl-CoA and the inhibitor binding sites are shown in proximity to the N-terminal and C-terminal portion of the molecule, respectively. This image was made with VMD (ver 1.8.7). VMD is developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign

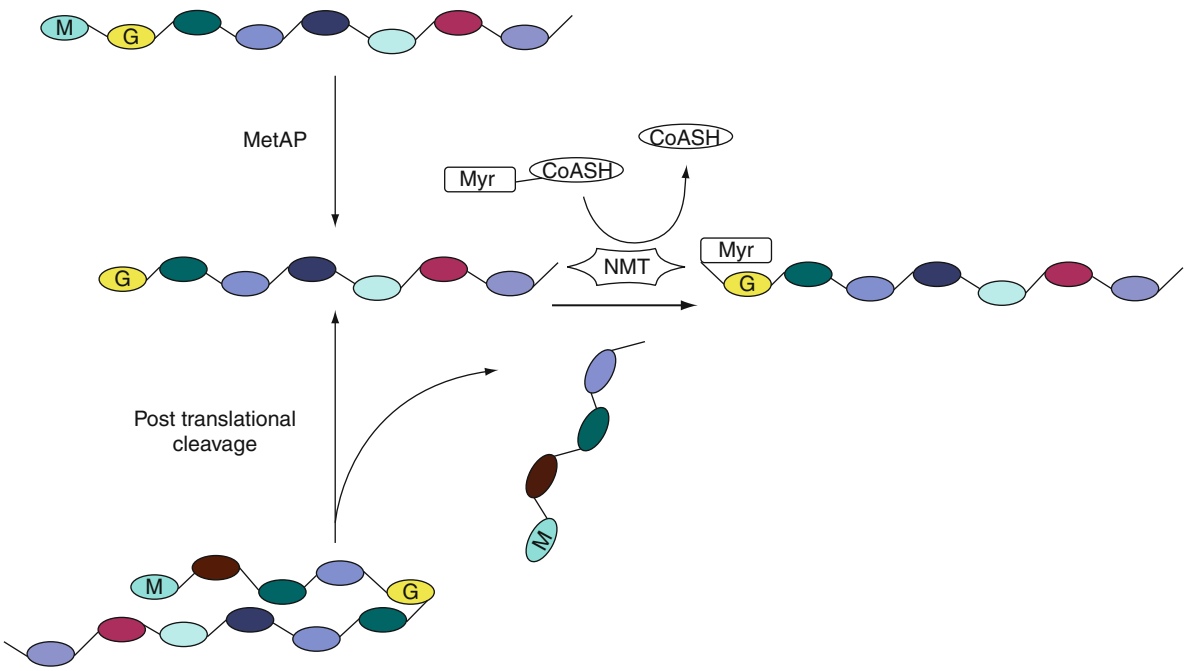
a variety of sources (Boutin 1997; Selvakumar et al. 2007; Wright et al. 2010). Five isoforms of NMT were shown to exist in the murine leukemia cell line L1210 whereas two isoforms were reported to exist in the bovine brain cortex (Boutin 1997; Selvakumar et al. 2007; Wright et al. 2010). Later it was shown that the bovine brain contains a heterogenous mixture of NMT subunits (Glover et al. 1997). Subsequently it has been established now that NMT exists as two isoforms generally termed as NMT1 and NMT2 (Giang and Cravatt 1998). While NMT2 appears as a single 65 kDa protein, NMT1 is processed to exist as four distinct isoforms ranging from 49–68 kDa in size (Giang and Cravatt 1998). The two isoforms NMT1 and NMT2 have an overall sequence identity of 76–77% with most divergence at their N termini indicating that these are two distinct families of NMTs. These isoforms are conserved well across higher eukaryotes (Giang and Cravatt 1998). However, to the best of our knowledge, among the lower eukaryotes only NMT1 has been reported so far. Out of the two NMT isoforms NMT1 has been studied in great detail from a variety of hosts and there is a great wealth of information regarding NMT1 as compared to NMT2 and hereafter we would be referring NMT1 as NMT unless otherwise stated.

Myristoylation and Its Biological Significance

Myristoylation catalyzed by NMT is linking of myristate via an amide bond to the glycine residue at the N-terminus of protein molecules (Boutin 1997; Resh 1999; Farazi et al. 2001; Selvakumar et al. 2007; Wright et al. 2010; Hannoush and Sun 2010). The availability of amino terminal glycine is an absolute requirement and the modification occurs on a general consensus motif of GXXXS/T (where X is any amino acid) (Boutin 1997; Resh 1999; Farazi et al. 2001; Wright et al. 2010; Hannoush and Sun 2010). N-Myristoylation can take place in either of the two ways (Fig. 2):

1. Co-translational protein myristoylation takes place while the protein chain is being synthesized by the ribosome (Wilcox et al. 1987). After the few initial amino acid residues (within 100) have been synthesized by the ribosome and the initiator methionine is removed by a methionine aminopeptidase to expose an available N-terminal glycine
2. Post-translational myristoylation occurs when an internal glycine within a polypeptide is exposed following a proteloyic cleavage (Farazi et al. 2001; Wright et al. 2010; Hannoush and Sun 2010)

N-myristoylation is reported in *Dictoystelium* amoeboid cells, in wheat germ, and among several eukaryotes and thus is a ubiquitious process (Boutin 1997; Farazi et al. 2001; Selvakumar et al. 2007; Wright et al. 2010). The N-terminal glycine myristoylation of various key proteins is inevitable for normal cell functioning and thus NMT is essential for survival and growth in a number of organisms (Boutin 1997; Farazi et al. 2001; Wright et al. 2010). Targeting the endogenous NMT functions of the individual organisms has therefore been a candidate choice for the treatment of many human pathogens including *Candida albicans, Cryptococcus neoformans, Leishmania major, Trypanosoma brucei, and Plasmodium falciparum* (Boutin 1997; Farazi et al. 2001; Wright et al. 2010; Frearson et al. 2010). Comparative analysis of the various NMTs has shown that the peptide binding pocket is more divergent than the myristoyl-CoA-binding site and thus the identification and



NMT (N-Myristoyltransferase), Fig. 2 Schematic representation of the co-translational and posttranslational N-myristoylation process. Abbreviations: *CoASH* coenzymeA, *Myr-CoASH* myristoyl coenzymeA, *Myr* myristate, *M* methionine, *G* glycine, *MetAP* methionine aminopeptidase

development of specific inhibitors focus on exploiting substrate specificities and targeting the peptide-binding pocket of NMT (Wright et al. 2010). A large number of moieties of diverse structures (generally grouped into four structural classes: analogs of myristate and myristoyl-CoA, myristoylpeptide derivatives, histidine analogs, and other synthetic compounds) have been shown to inhibit NMT activity and have been discussed elsewhere (Boutin 1997; Farazi et al. 2001; Selvakumar et al. 2007).

The substrates of myristoylation among higher eukaryotes including humans are the regular endogenous, physiological enzymes and proteins such as protein kinase A, protein kinase G, the NADH-cytochrome b5 reductase, nitric oxide synthase, recoverin, members of the Ras family of proteins, and most of the G protein alpha subunit. The detailed list of the substrate proteins is available in a number of reviews elsewhere (Boutin 1997; Resh 1999; Maurer-Stroh et al. 2004; Selvakumar et al. 2007). N-Myristoylation of proteins ensures their diverse functional roles such as specific protein–protein and protein–lipid interactions; ligand-induced conformational changes and subcellular targeting (Resh 1999; Farazi et al. 2001; Wright et al. 2010). On the other hand, N-myristoylation also controls the functional roles of several proteins involved in a disease condition and thus many pathogenic states are linked to undesired myristoylation activity (Boutin 1997; Resh 1999; Selvakumar et al. 2007; Wright et al. 2010). The enzymes involved in signaling process such as the non-receptor tyrosine kinases fyn, lyn, src are myristoylated and associated with the undesired states linked to myristoylation (Boutin 1997; Resh 1999; Selvakumar et al. 2007; Wright et al. 2010). Among the two human isozymes NMT1 and NMT2, the various undesired myristoylation processes linked to the disease condition can be broadly grouped into two categories:

1. The first category includes the myristoylation of viral proteins by the host NMT after the infection of proteins to the host (Maurer-Stroh and Eisenhower 2004). The myristoylation of structural and various other proteins of viruses is required for the maturation and infectivity of the viral particles (Boutin 1997; Resh 1999; Wright et al. 2010).

The inhibition of the myristoylation process targeting specific isoforms of the NMT can thus target the virus infectivity. The viruses requiring myristoylation of their proteins for infectivity include the dreaded human pathogens HIV, poliovirus, hepatitis B virus, and many more (Boutin 1997; Resh 1999; Maurer-Stroh and Eisenhower 2004). Many of the oncogenes from viruses are homologues of tyrosine kinases and require N-myristoylation for the infectivity of viral particles. Myristoylation is required for transformation by ▶ Src (a tyrosine kinase) and for its activation. Mutation of the N-terminal glycine of the viral oncogenes with tyrosine kinase activity blocks the transformation ability by interfering with the proper localization to plasma membrane and cell attachment (Boutin 1997; Wright et al. 2010). In the case of HIV infections, viral proteins Gag and Nef require myristoylation by host cell NMT to carry out their function properly (Wright et al. 2010). Gag is the precursor polyprotein for structural components of viral capsids and requires myristoylation for intracellular localization and its targeting to the lipid rafts in the plasma membrane during virus assembly. Nef on the other hand comprises many virulence factors to modify the cellular environment of infected cells to facilitate viral replication and evade detection by cells of the immune system. Among the two human NMT isozymes, NMT2 has increased affinity for the viral proteins and drugs, specifically targeting the distinct substrate profile of human NMT isozymes has been proposed to be developed as anti-HIV molecules (Wright et al. 2010). However, it has also been reported that NMT1 myristoylates Gag in vivo and inhibiting NMT1 but not NMT2 negatively affects HIV production (Wright et al. 2010).

2. The second group of pathogenic states attached to morbid myristoylation is linked to the upregulated NMT acitivity in hyperproliferative cells. It has been reported that in the mammary epithelial cells the proliferative capacity correlates with NMT activity (Wright et al. 2010). Elevated NMT activity has been observed in human colorectal cancer, adenocarcinoma, gallbladder cancer, and various other carcinomas (Boutin 1997; Selvakumar et al. 2007; Wright et al. 2010). Several protein kinases of the Src family have established roles in oncogenesis and a number of studies have shown that c-Src is a critical regulator of human cancers (Boutin 1997; Wright et al. 2010). Myristoylated tyrosine kinases, $pp60^{c\text{-}src}$ and $pp60^{c\text{-}yes}$ are several fold higher in colonic pre-neoplastic lesions and neoplasms compared with normal colon cells (Bolen et al. 1987; Wright et al. 2010). Differential expression of $pp60^{c\text{-}src}$ has been observed in colonic tumor-derived cell lines and colonic polyps prone to developing cancer (Cartwright et al. 1990). Higher levels of cytoskeletal-associated $pp60^{c\text{-}src}$ protein tyrosine kinase activity have been observed in intestinal crypt cells along with higher expression of $pp60^{c\text{-}yes}$ in the normal intestinal epithelium. Studies have revealed that $pp60^{c\text{-}src}$ is overexpressed in human colon carcinoma and it has enhanced kinase activity in progressive stages and metastases of human colorectal cancer (Bolen et al. 1987). Furthermore, it has been shown that Src kinase activity is positively regulated by myristoylation and the non-myristoylated c-Src exhibited has reduced kinase activity (Patwardhan and Resh 2010). The elevated NMT activity during carcinogenesis may be due to the higher demand for myristoylation of various proteins/oncoproteins (src, ras, etc.) which are overexpressed and activated during tumorogenesis. A direct relationship between elevations of NMT expression and activity in colon cancer progression has been reported (Magnuson et al. 1995). It has also been demonstrated that in colon cancer cell lines, elevated expression of NMT correlates with high levels of c-Src levels (Rajala et al. 2000). Blockage of N-myristoylation in colonic cell lines compromises colony formation and proliferation and also reduces localization of $pp60^{c\text{-}src}$ to plasma membrane (Rajala et al. 2000). The studies have been suggestive that NMT represents both a valuable clinical marker and therapeutic target for cancer (Boutin 1997; Selvakumar et al. 2007; Ducker et al. 2005; Wright et al. 2010). A several fold increase in NMT activity in polyps and stage B1 tumors compared to normal colonic mucosa have been proposed to be used as a diagnostic/prognostic tool for colorectal cancer (Shrivastav et al. 2007; Magnuson et al. 1995). siRNA-mediated NMT knockdown shows that among the two human NMT isoforms, silencing NMT1 strongly inhibits tumor growth in a mouse model mainly through loss of c-Src activation and its target FAK as well as reduction of various protein

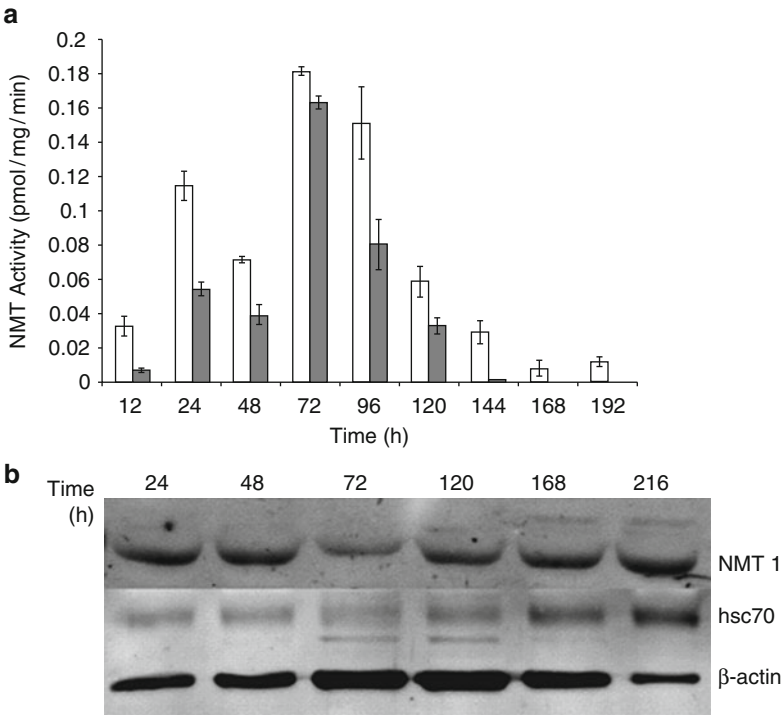
kinase–regulated pathways. It has been concluded that NMT1 and NMT2 have only partially overlapping functions and that NMT1 is critical for tumor cell proliferation further suggesting that isoform-specific inhibitors might be developed as potential anticancer agents (Ducker et al. 2005). Therefore, the specific inhibition or regulation of either NMT in vivo may in turn allow for the selective control of particular myristoylation-dependent cellular functions. It has been observed that NMT1 and NMT2 interact differentially with the apoptotic-related proteins. NMT2 has been found to interact with both Bcl2 and ▶ p53 while NMT1 interacts only with p53 (Selvakumar et al. 2007). Further it has also been noted that the proteases *m*-calpain and caspase-3 interact differently with NMT isoforms (Selvakumar et al. 2007).

Regulation of N-Myristoyltransferase

The process of N-myristoylation appears to be a tightly regulated reaction which involves the coordinated action of several different enzymes (e.g., N-methionylaminopeptidase, fatty acid synthetase, long chain acyl-CoA synthetase, acyl-CoA-binding proteins, etc.), access of the NMT to the limited pools of myristoyl-CoA, and the timely N-myristoylation of nascent polypeptide substrates to ensure proper functional roles and avoid potential interfering reactions (e.g., N-acetylation and polypeptide misfolding) (Wilcox et al. 1987; Boutin 1997; Glover et al. 1997; Resh 1999; Farazi et al. 2001; Wright et al. 2010). This implies the existence of mechanisms designed to ensure the regulated activity of NMT in a timely manner. From the available evidences to date, it is believed that NMT activity is regulated by at least three mechanisms in mammalian cells. The first one involves the targeting of the NMT to the ribosomes which involves N-terminal domain of NMT (Glover et al. 1997). Targeting to ribosomes appears to be consistent with its role as a co-translational protein modifier. It was earlier proposed that the N-terminal domain of NMT has no catalytic roles (Farazi et al. 2001). However, no information is available regarding the catalytic regulation of NMT in terms of the interaction of the N-terminus residues with other parts of the molecule from higher eukaryotes. The available crystal structure of NMT from *S. cerevisise* shows that the extended N-terminus is located close to the myristoyl-CoA binding site as well as the peptide binding site and it has been proposed that this region plays an important role in coordinated control of catalytic activity of NMT (Wu et al. 2007). Furthermore, the identification of two isoforms (NMT1 and NMT2) in higher eukaryotes with divergent N-terminus have raised the possibility that this region might be involved in regulating enzyme activity by modulating regulation/interaction with targets substrates or intracellular locations of NMT (Farazi et al. 2001). The establishment of the catalytic role of the N-terminus of NMT from mammalian counterparts is precluded unless further studies in this direction are carried out.

The second regulatory process controlling the functions of NMT in vivo is via the phosphorylation-mediated regulation of NMT activity. It has been observed that Akt/PKB-mediated phosphorylation of NMT in HepG2 cells attenuates NMT activity (Shrivastav et al. 2009). Further, the downregulation of NMT activity in several breast cancer cell lines has been correlated to the phosphorylation of NMT. Studies so far in this direction have been few and this is the only report showing in vivo phosphorylation of NMT in metastatic cell lines (Shrivastav et al. 2009). In earlier reports, it was noted that in cases of colorectal cancer both NMT expression levels and activity are significantly upregulated (Selvakumar et al. 2007; Magnuson et al. 1995; Rajala et al. 2000). Furthermore, in cases of colorectal cancer, calcineurin expression levels are also high compared to normal colonic epithelial tissue (Lakshmikuttyamma et al. 2005). In cases of colorectal cancer, the NMT activity is high presumably because of the dephosphorylation of NMT by the elevated levels of calcineurin, a ser/thr phosphatase. In other words, dephosphorylation of NMT upregulates its activity. This hypothesis is supported by earlier findings which show that in the presence of the phosphatase inhibitor sodium orthovanadate, NMT activity is reduced (King et al. 1995). It has also been identified in human phospho-proteome analysis studies that in normal cell lines NMT is phosphorylated in vivo at serine 47 (Olsen et al. 2006). However, detailed studies are required to discern the in vivo modulations of NMT by its phosphorylation.

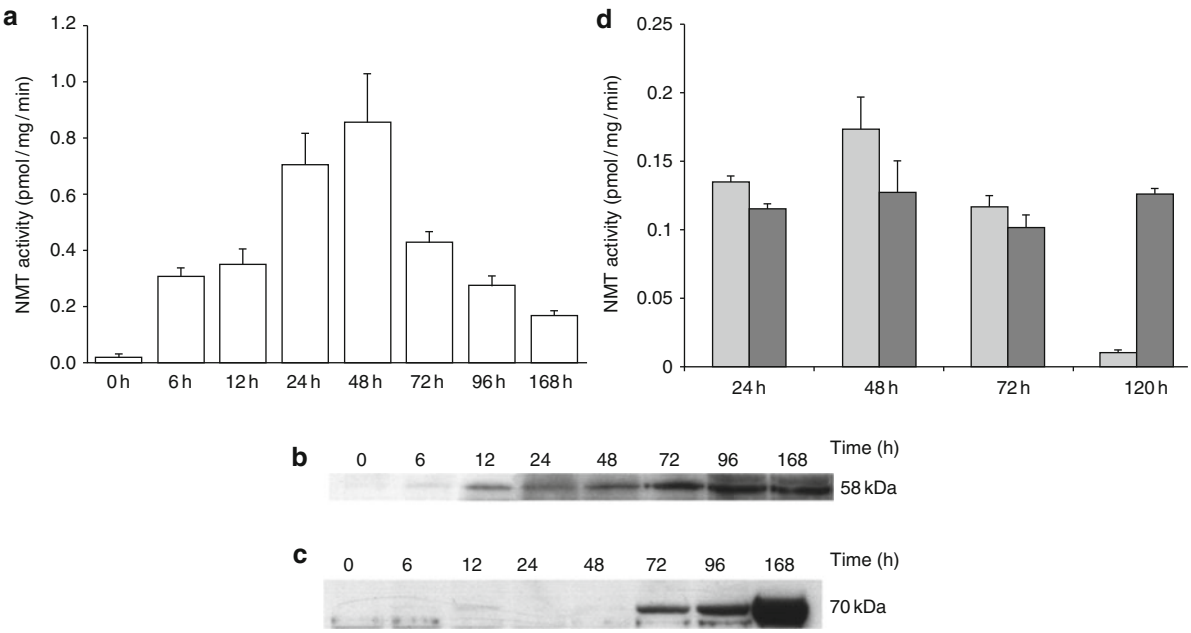
The most well-documented regulatory process controlling NMT functions is the modulation of NMT activity by its interaction with heat shock cognate protein 70. Initially it was identified as a monomeric



NMT (N-Myristoyltransferase), Fig. 3 NMT activity and hsc70 profile during the maturation of BMDM. (**a**) NMT activity in BMDM from the bone marrow of wild-type mice (□) or heterozygous (+/−) *Nmt1*-deficient mice (■) at indicated time points using $pp60^{c-Src}$ derived peptide substrate. (**b**) Expression of NMT and hsc70 during the differentiation of BMC (Adapted from Shrivastav et al. 2008. Copyright 2008. The American Association of Immunologist, Inc.)

NMT inhibitor protein of molecular mass 71 kDa (NIP71) (King and Sharma 1993). It was observed that during subcellular fractionation of the bovine brain tissue NMT activity resides both in the cytosolic as well as particulate fraction of the cells. However, the separation of the particulate fraction from the total cellular lysate enhanced NMT activity of the remaining soluble fraction. These findings indicated the existence of an inhibitor protein associated with the membranous fraction which was purified and characterized and was termed NIP71. In studies on colon cancer cell lines, it has been observed that the upregulation of NMT is accompanied by increase in c-src levels and a decrease in the expression levels of NMT inhibitor protein (NIP71) (Rajala et al. 2000). Later it was shown that the purified NIP71 and Hsc70 cross react with each other's specific antibody and have similar IC_{50} values for NMT inhibition in vitro (Selvakumar et al. 2007). Based on MS-MALDI and sequence similarity analysis, NIP71 was reported to be homologous to the Hsc70 (Selvakumar et al. 2007). NMT is essential for growth and development of many organisms (Farazi et al. 2001; Wright et al. 2010) and it has been shown that it is essential for the early development of the mouse and is the main enzyme in early embryonic development (Yang et al. 2005). Studies on NMT regulation in developmental processes using wild type and heterozygous (+/−) and homozygous (−/−) *Nmt1*-deficient mice have shown that NMT is essential for monocytic differentiation of bone marrow–derived macrophages (BMDM) (Shrivastav et al. 2008). It has been observed that during the stages of macrophagic differentiation of bone marrow cells from the wild type and heterozygous (+/−)*Nmt1*-deficient mice NMT activity rises in the early stages reaching a maximum at 72 h and then decreases for the remaining time exhibiting a parabolic activity profile for NMT (Fig. 3a). It was also noticed that in bone marrow cells, the expression levels of NMT remain constant over time; however there is an increase in the expression levels of Hsc70 which is concomitant with the time point for the decreased NMT activity (Fig. 3b).

The NMT activity follows a very similar parabolic activity profile during in vitro studies on macrophagic differentiation process using promonocytic cell lines. However, the maximum activity is achieved at 48 h which then dramatically falls over the remaining time



NMT (N-Myristoyltransferase), Fig. 4 NMT activity and protein expression in U937 cells treated with PMA for different time periods. (**a**) NMT activity as a function of time using $pp60^{c-Src}$ derived peptide substrate. (**b** and **c**) Expression profile of NMT and NIP71 as function of time during the differentiation process. The U937 cells treated with PMA for indicated time points analyzed by western blot using (*B*) monoclonal anti-NMT1 or (*C*) polyclonal anti-NIP71 antibody. (**d**) NMT activity in the lysates of PMA treated U937 cells at indicated time points after transfection with (*light gray*) scrambled or (*dark gray*) hsc70 siRNA (Adapted from Shrivastav et al. 2008. Copyright 2008. The American Association of Immunologist, Inc.)

course (Fig. 4a). The NMT expression levels do not decline over time; however there is an increase in the level of inhibitor protein NIP71 which accounts for the downregulation of NMT activity (Fig. 4b, c). The roles of Hsc70 in NMT regulation in vivo has been further established by siRNA knockdown of Hsc70 in the PMA-treated U937 promonocytic cell lines (Shrivastav et al. 2008). It has been observed that upon the knockdown of Hsc70, the NMT activity profile remains almost constant with time (Fig. 4d).

Taken together, the modulation of NMT activity seems to be regulated by a variety of mechanisms which involves its specific targeting to particular subcellular location, protein modification, and interaction with other cellular proteins. NMT activity is modulated in various situations including in mononuclear phagocytes by IFN gamma and tumor necrosis factor (TNF), in HL60 cells by retinoic acid and TPA, and in rat liver by 3-methylcholanthrene treatment (Boutin 1997). Myristoylation is a long-lasting process however not without exceptions (Hannoush and Sun 2010). There are reports on demyristoylation of the modified substrate proteins (Boutin 1997; Wright et al. 2010). Furthermore in vitro studies have suggested that NMT activity can be enhanced by a variety of organic solvents and various other solutes and molecules (Boutin 1997; Selvakumar et al. 2007). However, a mechanistic understanding of these processes is still lacking.

Summary

N-myristoylation is a key process in cellular pathways which regulates the functioning of many proteins often in conjugation with other protein modifications. The enzyme is an important target for the treatment of many infectious diseases. The roles played by NMT (both isoforms NMT1 and NMT2) in maintaining cellular homeostasis are diverse and many new proteins are now known to undergo this lipidic modification (Maurer-Stroh et al. 2004; Martin et al. 2008). The roles of myristoylation in controlling the functions of the newly identified proteins are not yet well understood. Many of these proteins are linked to pathological states and thus myristoylation may play a part in

pathogenesis. Currently the other lipid modifications (palmitoylation and prenylation) are of intense interest as targets of therapeutic interventions; however, despite the fact that NMT plays a key role in growth and developmental processes and is also associated with various pathological states, the regulation of this enzyme has not been studied in much detail. Further studies are definitely warranted not only to understand the regulatory mechanism of NMT but also on the roles of various myristoylated proteins implicated in signaling and other cellular pathways. Understanding the roles of myristoylated proteins and the regulation of myristoylation process will clarify the roles of myristoylation in modulating cellular function and thus help in establishing new modalities and novel targets for the therapeutic intervention of many health disorders.

References

Bolen JB, Veillette A, Schwartz AM, DeSeau V, Rosen N. Activation of pp 60c-src protein kinase activity in human colon carcinoma. Proc Natl Acad Sci USA. 1987;84:2251–5.

Boutin JA. Myristoylation. Cell Signal. 1997;9:15–35.

Cartwright CA, Meisler A, Eckhart W. Activation of the $pp60^{c\text{-}src}$ protein kinase is an early event in colonic carcinogenesis. Proc Natl Acad Sci USA. 1990;87:558–62.

Ducker CE, Upson JJ, French KJ, Smith CD. Two N-myristoyltransferase isozymes play unique roles in protein myristoylation, proliferation, and apoptosis. Mol Cancer Res. 2005;3:463–76.

Farazi TA, Waksman G, Gordon JI. The biology and enzymology of protein N-myristoylation. J Biol Chem. 2001;276:39501–4.

Frearson JA, Brand S, McElroy SP, Cleghorn LA, Smid O, Stojanovski L, Price HP, Guther ML, Torrie LS, Robinson DA, Hallyburton I, Mpamhanga CP, Brannigan JA, Wilkinson AJ, Hodgkinson M, Hui R, Qiu W, Raimi OG, van Aalten DM, Brenk R, Gilbert IH, Read KD, Fairlamb AH, Ferguson MA, Smith DF, Wyatt PG. N-myristoyltransferase inhibitors as new leads to treat sleeping sickness. Nature. 2010;464:728–32.

Giang DK, Cravatt BF. A second mammalian N-myristoyltransferase. J Biol Chem. 1998;273:6595–8.

Glover CJ, Hartman KD, Felsted RL. Human N-myristoyltransferase amino-terminal domain involved in targeting the enzyme to the ribosomal subcellular fraction. J Biol Chem. 1997;272:28680–9.

Hannoush RN, Sun J. The chemical tool box for monitoring protein fatty acylation and prenylation. Nat Chem Biol. 2010;6:498–506.

King MJ, Sharma RK. Demonstration of multiple forms of bovine brain myristoyl CoA:protein N-myristoyltransferase. Mol Cell Biochem. 1992;113:77–81.

King MJ, Sharma RK. Identification, purification and characterization of a membrane-associated N-myristoyltransferase inhibitor protein from bovine brain. Biochem J. 1993;291:635–9.

King MJ, Pugazhenthi S, Khandelwal RL, Sharma RK. In vivo modulation of N-myristoyltransferase activity by orthovanadate. Mol Cell Bicohem. 1995;153:151–5.

Lakshmikuttyamma A, Selvakumar P, Kanthan R, Kanthan SC, Sharma RK. Increased expression of calcineurin in human colorectal adenocarcinomas. J Cell Biochem. 2005;95:731–9.

Magnuson BA, Raju RV, Moyana TN, Sharma RK. Increased N-myristoyltransferase activity observed in rat and human colonic tumors. J Natl Cancer Inst. 1995;87:1630–5.

Martin DD, Vilas GL, Prescher JA, Rajaiah G, Falck JR, Bertozzi CR, Berthiaume LG. Rapid detection, discovery and identification of post-translationally myristoylated proteins during apoptosis using a bioorthogonal azidomyristate analogue. FASEB J. 2008;22:797–806.

Maurer-Stroh S, Eisenhower F. Myristoylation of viral and bacterial proteins. Trends Microbiol. 2004;12:178–85.

Maurer-Stroh S, Gouda M, Novatchkova M, Schleiffer A, Schneider G, Sirota FL, Wildpaner M, Hayashi N, Eisenhaber F. MYRbase: analysis of genome-wide glycine myristoylation enlarges the functional spectrum of eukaryotic myristoylated proteins. Genome Biol. 2004. doi:10.1186/gb-2004-5-3-r21.

Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell. 2006;127:635–48.

Patwardhan P, Resh MD. Myristoylation and membrane binding regulate c-Src stability and kinase activity. Mol Cell Biol. 2010;30:4094–107.

Rajala RV, Dehm S, Bi X, Bonham K, Sharma RK. Expression of N-myristoyltransferase inhibitor protein and its relationship to c-Src levels in human colon cancer cell lines. Biochem Biophys Res Commun. 2000;273:1116–20.

Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochem Biophys Acta. 1999;1451:1–16.

Selvakumar P, Shrivastav A, Dimmock JR, Sharma RK. N-myristoyltransferase: a novel molecular target for cancer. Prog Lipid Res. 2007;46:1–36.

Shrivastav A, Varma S, Saxena A, Decoteau J, Sharma RK. N-myristoyltransferase: a potential novel diagnostic marker for colon cancer. J Transl Med. 2007;5:58.

Shrivastav A, Varma S, Lawman Z, Yang SH, Ritchie SA, Bonham K, Singh SM, Saxena A, Sharma RK. Requirement of N-myristoyltransferase 1 in the development of monocytic lineage. J Immunol. 2008;180:1019–28.

Shrivastav A, Varma S, Senger A, Khandelwal R, Carlsen S, Sharma RK. Overexpression of Akt/PKB modulates N-myristoyltransferase activity in cancer cells. J Pathol. 2009;218:391–8.

Wilcox C, Hu JS, Olson EN. Acylation of proteins with myristic acid occurs cotranslationally. Science. 1987;238:1275–8.

Wright MH, Heal WP, Mann DJ, Tate EW. Protein myristoylation in health and disease. J Chem Biol. 2010;3:19–35.

Wu J, Tao Y, Zhang M, Howard MH, Gutteridge S, Ding J. Crystal structures of *Saccharomyces cerevisiae*

N-myristoyltransferase with bound myristoyl-CoA and inhibitors reveal the functional roles of the N-terminal region. J Biol Chem. 2007;282:22185–94.
Yang SH, Shrivastav A, Kosinski C, Sharma RK, Chen MH, Berthiaume LG, Peters LL, Chuang PT, Young SG, Bergo MO. N-myristoyltransferase 1 is essential in early mouse development. J Biol Chem. 2005;280:18990–5.

N-Myristoyltransferase

▶ NMT (N-Myristoyltransferase)

Nociceptin Opioid Receptor

▶ Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function

Non-Lysosomal Cysteine Protease

▶ Calpain

Nonmuscle Myosin Light Chain Kinase

▶ MYLK (Myosin Light Chain Kinase)

Notch (Notch1, Notch2, Notch3, Notch4)

Gibeom Park and Woong-Yang Park
Department of Biomedical Sciences,
Seoul National University College of Medicine,
Jongnogu, Seoul, South Korea

Synonyms

TAN1; Translocation-associated notch homolog

Historical Background

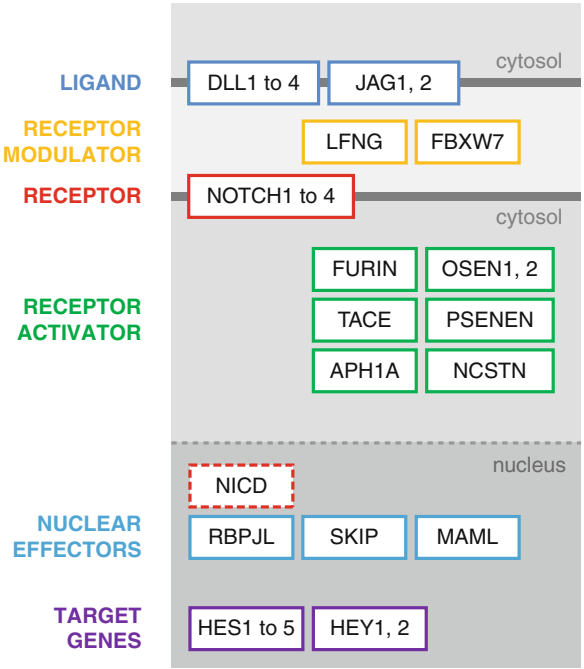
Notch mutants in *Drosophila melanogaster* were originally reported in 1919 (Morgan and Bridges 1919). Notch was named based on the *Drosophila melanogaster* mutants that exhibited irregular notches of missing tissue at the tips of their wing blades. This phenotype was caused by heterozygous loss-of-function mutations in a gene subsequently named "Notch" that was cloned in 1983 (Artavanis-Tsakonas et al. 1983).

Notch and Notch Signaling

Notch proteins are single-pass transmembrane receptors that mediate cell-to-cell communications for the regulation of cell fate decisions during developmental stages and also adult life. Notch receptors are highly conserved in throughout evolution and are found in a diverse range of organisms from worms to humans. The Notch family includes four receptors, NOTCH1, NOTCH2, NOTCH3, and NOTCH4. These receptors have an extracellular domain that contains multiple epidermal growth factor (EGF)-like repeats and an intracellular region that contains a RAM domain, ankyrin repeats, and a C-terminal PEST domain (Fig. 1).

The Notch receptor is synthesized as a 300-kDa precursor and is cleaved in the trans-Golgi compartment. The resulting extracellular and lumenal N-terminal fragment and the transmembrane domain and intracellular domain C-terminal fragment are assembled into the mature heterodimer receptor through a noncovalent linkage. The extracellular and lumenal portion of Notch undergoes extensive N- and O-linked glycosylation during synthesis and secretion, which is critical for proper folding of the receptor and its subsequent interactions with ligands (Fortini 2009).

Canonical Notch signaling is activated by binding of a ligand from the DSL family, that includes Delta and the Serrate/Jagged subfamily that is located on the adjacent cell's surface. Ligand binding activates proteolysis of Notch between gly1743 and val1744 (termed site 3 or S3) by γ-secretase. One fragment is the Notch intracellular domain (NICD) that is translocated to the nucleus. NICD functions in transcriptional regulation of the Hairy/E(spl) family (*Hes* genes in mammals, *her* genes in zebrafish), that codes for inhibitory basic helix-loop-helix (bHLH) transcriptional regulators that control many different secondary targets, including Notch ligand genes and



Notch (Notch1, Notch2, Notch3, Notch4), Fig. 1 Notch activation and signaling pathway

the *Hes*/*her* genes themselves. The other fragment is the Notch extracellular domain (NECD) that is endocytosed by the ligand-expressing cell.

Lateral inhibition is a feedback loop in which adjacent and developmentally equivalent cells assume completely different fates. Binding of Delta and Notch across the two cells in the opposite direction generates transcriptional feedback. Delta expression by the signal-sending cell is stronger than by the signal-receiving cell, therefore, Notch signaling by the signal-receiving cell is activated. This signaling results in transcription of *Hes*/*her* genes, thereby producing an achaete-scute complex (AS-C) to block expression of differentiation genes and Delta transcription. Decreased Delta expression by the signal-receiving cell diminishes the Delta-Notch binding from the signal-receiving to signal-sending cell, thus inactivating Notch signaling by the signal-sending cell. As a result, the *Hes*/*her* genes are not transcribed by the signal-sending cells, enhancing differentiation and upregulating Delta expression. The final outcome of this feedback loop is that the signal-receiving cell maintains strong expression of the Notch receptor Notch expression and Notch signaling through *Hes*/*her*-dependent transcriptional feedback, while the signal-sending cell maintains strong ligand expression and repressed Notch signaling through AS-C-dependent transcriptional feedback (Lewis et al. 2009).

Physiological Roles of Notch Signaling

Notch and Development and Differentiation

The Notch pathway, with TGF-β, Wnt, and Hedgehog, is a representative pathway that regulates developmental and differentiation gene expression programs, so that at the correct time and place, cells with the same propensity for a particular cell fate can give rise to daughters that exhibit differences in morphology and protein expression.

A variety of strategies are used to achieve developmental goals. One strategy is to form gradients of signaling proteins either on cell surfaces or in extracellular spaces that help determine cell fates when they activate cellular receptors. These signaling proteins are known as morphogens. Another strategy is to utilize hierarchical sequences of gene expression so that over time different progeny will become different types of cells. Notch signaling is involved with these strategies to achieve proper cell development and differentiation (Beckerman 2005).

Somitogenesis

Notch signaling coordinates and synchronizes the cell clocks of individual cells when somites form. Segmentation and antero-posterior polarity of somites are formed under the control of Notch signaling and its associated pathways.

Somites are musculoskeletal segments, derived from presomitic mesoderm (PSM). Wnt and FGF are produced at the tail end of the PSM and spread to the anterior portion, generating a morphogen gradient. Anteriorly, somites form in response to lowered Wnt and FGF levels, and at the tail PSM grows caudally, expanding the embryo. This growth is a rhythmic process that results in segmentation, and originates from cell-intrinsic oscillation. The oscillation is paced by autoregulation of *Hes*/*her* genes, which results in a negative feedback loop. Delays in transcription and translation within the feedback loop determine the period of oscillation.

Blocking of Notch signaling disrupts segmentation. However, Notch signaling does not produce oscillation itself, but rather functions in the coordination. Even

Notch-dissociated cells show oscillating expression, though in a less regular pattern (Lewis et al. 2009).

Mesp2 is a central mediator of Notch signaling in the somite mesoderm, generating antero-posterior polarity in the presumptive somite via a complex signaling network involving Dll1- and Dll3-Notch signaling and the Notch regulator presenilin 1 (Kuan et al. 2004).

Neural Development

Notch signaling controls neural cell fates, both the early-fate decisions between neural and epidermal and the late-fate decisions between different subtypes of neural cells (Cau and Blader 2009). Proneural clusters are developed in neural cells or epidermal cells. Following several iterations of the lateral inhibition feedback loop as cluster communication, only one cell of the cluster downregulates the Notch pathway and becomes a neural precursor. The remaining cells are blocked from reaching the neural fate and, are either reselected during a second wave of neurogenesis or secondarily adopt an epidermal fate.

Notch is also required during the specification between two different neural subtypes. These binary decisions can either involve sister cells, as during the formation of *Drosophila* sense organs, or cells that are not linearly related, such as the R3 and R4 photoreceptors of the *Drosophila* eye. Once specified as a neural progenitor, the sensory organ precursor (SOP or pI) divides to generate two cells, pIIa and pIIb, which communicate via Notch. Subsequent divisions generate the four cells of the sensory organs, as well as a glial cell that undergoes apoptosis.

Hematopoietic System

Canonical Notch signaling is essential for the generation of definitive embryonic hematopoietic stem cells, but is dispensable for their maintenance during adult life (Sandy and Maillard 2009). Notch controls several early steps of T-cell development, as well as specific cell fate and differentiation decisions in other hematopoietic lineages. In addition, emerging evidence indicates that Notch is a potent, context-specific regulator of T-cell immune responses, which includes several disease models relevant to patients.

Notch signaling intensity varies throughout T-cell development. The Notch target genes *Deltex1* and *Hes1* are expressed at very low levels in bone marrow hematopoietic stem cells (HSCs). Upon arrival in the thymic environment, early T progenitors (ETPs) strongly upregulate the expression of Notch target genes. Expression levels of Notch target genes gradually increase during development from the ETP stage to the double negative 3a (DN3a) stage. After the β-selection checkpoint, during which Notch signaling is significantly downregulated, the intensity of Notch signaling steadily decreases from the DN3b stage to the CD4+/CD8+ double positive (DP) stage. Thymic single positive (SP) CD4+ and CD8+ T cells, as well as naive peripheral T cells, express low amounts of Notch target genes. Upon T-cell activation in the periphery, Notch signaling increases sharply in a context-dependent manner.

Notch and Cancer

The highly conserved Notch signaling pathway plays pleiotropic roles during embryonic development and is important for the regulation of self-renewing tissues (Koch and Radtke 2007). The physiological functions of this signaling cascade range from stem cell maintenance and influencing cell fate decisions of slightly differentiated progenitor cells, to the induction of terminal differentiation processes, all of which are recapitulated in different forms of cancers. Although Notch signaling is mainly associated with oncogenic and growth-promoting roles, depending on the tissue type, it can also function as a tumor suppressor.

Oncogenic Function

The first data describing the oncogenic consequences of aberrant Notch signaling in solid tumors were derived from animal studies characterizing a frequent insertion site, named int3, of the mouse mammary tumor virus (MMTV). The int3 site was later identified as the Notch4 locus. MMTV insertions have also been found in the Notch1 locus, albeit with a lower frequency. Together, these result on aberrant Notch signaling and mouse mammary tumorigenesis lead to the question of how significant aberrant Notch signaling is for human breast cancer. To date, only correlative evidence for the involvement of Notch signaling in human breast cancer is available.

Medulloblastoma has primarily been associated with aberrant ▶ sonic hedgehog signaling (Shh), which induces N-▶ MYC expression. The primitive nature of medulloblastoma tumor cells and the fact

that Notch signaling is involved in the maintenance of neural stem and progenitor cells motivated several groups to investigate the potential role of Notch in medulloblastoma. Expression studies using primary medulloblastoma tumor samples showed increased mRNA expression of NOTCH2, but not of NOTCH1. Increased expression of the target gene *Hes1* correlated with a poor patient survival prognosis. Blocking of Notch signaling resulted in increased apoptosis and a reduction of viable cell in tumor cells.

While the causative role of aberrant Wnt signaling for the development of colorectal cancer is well established, it is currently less clear whether Notch signaling might have a similar oncogenic function within the gut. Since gene expression profiles of crypt cells and colorectal cancer cell lines appear to be very similar, colorectal cancer cells may represent the transformed counter part of crypt cells. Since Notch is a gate keeper of crypt cells, it is likely that Notch and Wnt signaling occur simultaneously in adenomas and crypt cells. Indeed, expression of the Notch target gene *Hes1* has been observed in adenomas of APC^{Min} mice, as well as in primary human colorectal tumors.

Notch signaling has been shown to play an important role during embryonic pancreas development by maintaining an undifferentiated precursor cell type. Notch receptors, ligands, and downstream targets, such as *Hes1*, were found to be upregulated in preneoplastic lesions, as well as in invasive pancreatic cancers in humans and mice. This suggests that Notch signaling in pancreatic cancers might be an early event leading to the accumulation of undifferentiated precursor cells.

Global gene expression profiling and immunohistochemistry have revealed the expression of multiple Notch receptors and ligands in primary lesions of human malignant melanomas, therefore, expanding the list of possible pathways involved in melanoma development. Subsequent studies using established melanoma cell lines showed that pharmacological blocking of Notch signaling can have growth suppressive effects. The oncogenic function of Notch signaling within these cell lines was linked to increased ► β-catenin-mediated signaling, as well as increased MAPK and AKT signaling.

Historically, human NOTCH was identified at the chromosomal breakpoint of a subset of T cell lymphoblastic leukemias/lymphomas containing a t(7;9)(q34; q34.3) chromosomal translocation. The translocation fuses the 3′ portion of NOTCH1 to the T-cell receptor Jb locus. This translocation results in a truncated NOTCH1 protein (N1ICD) that is constitutively active and aberrantly expressed. However, this seminal discovery did not reveal the full oncogenic potential of the truncated version of N1ICD. Less than 1% of all human T-cell leukemias or lymphomas contain this translocation. However, more importantly, aberrant Notch signaling was subsequently found in several human leukemias and lymphomas that lacked genomic rearrangements, signifying that upregulated Notch signaling might have a common role in human leukemogenesis.

Definitive proof for a central role of NOTCH1 in human T-cell acute lymphoblastic leukemia (T-ALL) cell lines came from a recent study that identified somatic activating mutations in the NOTCH1 receptor independent of the t(7;9) translocation. These mutations were detected in more than 50% of human T-ALL cases. Additionally, these mutations were found in all previously defined T-ALL subtypes.

Notch signaling was constitutively active in human clear cell renal cell carcinoma (CCRCC) cell lines. Blocking Notch signaling attenuated proliferation and restrained anchorage-independent growth of CCRCC cell lines and inhibited growth of xenotransplanted CCRCC cells in nude mice. Notch1 knockdown was accompanied by elevated levels of the negative cell-cycle regulators p21(Cip1) and/or p27(Kip1). Moreover, Notch1 and the Notch ligand Jagged1 were expressed at significantly higher levels in CCRCC tumors than in normal human renal tissues. Growth of primary CCRCC cells was attenuated upon inhibition of Notch signaling.

In adults, blood vessels in most organs are quiescent, but the growth of solid tumors requires specific embryonic signaling pathways to direct new blood vessels to grow around and into the tumor. VEGF is important in this process. Notch signaling has a strong effect on angiogenesis as seen in gain- or loss-of-function studies of Notch1, Notch1/4, Jagged1, Dll1, Dll4, Hey1/Hey2, and Presenilins (PS1 and PS2) (Iso et al. 2003; Li and Harris 2005).

By examining the whole-genome transcriptome profile of a xenograft model of breast cancer and its metastasis form to brain, activation of Notch signaling was found to be crucial in brain metastasis. Over 2,000 genes were differentially expressed in brain metastatic cells, which included various metastasis-related genes

and many genes related to angiogenesis, migration, tumorigenesis, and cell-cycle regulation. Interestingly, the Notch signaling pathway was activated in correlation with increased Jag2 mRNA expression, activated NICD, and NICD/CLS promoter-luciferase activity. Increased migration and invasion of brain metastatic cells as compared with primary breast cancer cells were inhibited by inactivation of Notch signaling using DAPT, a γ-secretase inhibitor, and RNAi-mediated knockdown of Jag2 and Notch1 (Nam et al. 2008; Jeon et al. 2008).

Tumor Suppressor Function

Instead of maintaining progenitor cells in an undifferentiated state, or influencing their cell fate decisions, in some tissues such as skin, prostatic epithelium, hepatocellular carcinoma, and small-cell lung cancer, Notch can also induce differentiation that is associated with growth suppression (Koch and Radtke 2007). However, the growth inhibitory role of Notch has mainly (with the exception of the prostate) been based on activated Notch1 overexpression studies. Thus, further experiments are needed in these tissues and cancer types to clarify whether Notch indeed has tumor suppressive functions.

Cancer Stem Cells (CSCs)

The strongest evidence to date for a role of Notch in CSCs is in breast cancer, embryonal brain tumors, and gliomas. γ-secretase inhibitors (GSIs) abolish the formation of secondary mammospheres from a variety of human breast cancer cell lines, as well as in primary patient specimens. In breast ductal carcinoma in situ (DCIS), the ability to form multilineage spheroids termed "mammospheres," indicators of stem-like cells, is dramatically decreased by GSIs, Notch-4 monoclonal antibodies, or Gefitinib. This finding suggests cooperation between epidermal growth factor receptor (EGFR) and Notch-4 in DCIS "stem cell" maintenance. There is evidence for a feedback loop between Her2/Neu and Notch, which may maintain CSCs in Her2/Neu-overexpressing tumors. Sansone and colleagues showed that in mammospheres from human breast cancers, IL-6 induces Notch-3 signaling, increases expression of Jagged-1, and, through Notch-3, promotes a hypoxia-resistant phenotype. The same group described the p66Shc-Notch-3 pathway as essential for maintaining the hypoxia-resistant phenotype of human breast cancer mammospheres. Fan and colleagues showed that Notch inhibition selectively depletes medulloblastoma CSCs as determined by CD133-high status or dye exclusion. The same group has described very similar findings in glioblastoma CSCs. Importantly, in gliomas, Notch confers radioresistance to CSCs. GSI treatment selectively enhanced radiation-induced death of glioma CSCs, but not bulk glioma cells. This effect was replicated by Notch-1 or Notch-2 knockdown, and was accompanied by AKT inhibition and reduced Mcl-1 expression. Other malignancies are being actively investigated. A role of Notch, STAT3, and TGF-β in hepatocellular carcinoma CSC maintenance has been suggested. In Gemcitabine-resistant pancreatic carcinoma cells, EMT (epithelial-mesenchymal transition) is associated with activation of Notch signaling, potentially linking Notch to the "Weinberg model" of stemness acquisition through EMT and to treatment resistance. Inhibition of Notch signaling through GSIs or Delta-4 monoclonal antibodies decreased the number of CSC and/or their tumorigenicity in some preclinical models (Pannuti et al. 2010).

Drug Development

The Notch signaling pathway in various cancers can be targeted at various levels, including receptor-ligand binding, release of NICD, as well as the coactivator complex. A promising strategy to block receptor-ligand binding employs inhibitory antibodies directed against Jagged1 or DLL4. Blocking DLL4 led to dysfunctional neovascularization and inhibition of tumor growth. The most promising results have been achieved using small-molecule inhibitors of the γ-secretase complex (GSI) that prevented the release of NICD. A phase I clinical trial using the GSI MK0752 inhibitor was initiated in 2005. The third protein component of the Notch signaling complex that may possibly provide a suitable drug target is the coactivator complex consisting of CSL, MAML, and CBP/p300. Small inhibitory peptides acting as dominant negative forms of MAML or CLS decrease the transcriptional activation of target genes (Koch and Radtke 2007).

Even targeting developmental pathways such as Notch, will most likely not give the elusive "magic bullet," and will require the development of rational drug combinations. Such cocktails will be made

possible only through a thorough understanding of cross-talk between Notch and other developmental and nondevelopmental pathways that may play roles in CSCs in specific malignancies. Our knowledge is rapidly evolving, but there is evidence to support some combinations of treatments. The following examples are not meant to be all-inclusive. However, these classes of agents are reasonable candidates for combination with Notch inhibitors: (1) Inhibitors of the PI3-kinase-AKT-mTOR pathway; (2) ▶ NF-κB inhibitors; and (3) Her2/Neu inhibitors, platinum compounds, EGFR inhibitors, and Hedgehog inhibitors. In breast cancer, a newly discovered feedback loop between Notch and ERα supports combining Notch inhibitors with antiestrogens. Antiestrogens plus GSI and Hedgehog-inhibitors plus GSI combinations are being investigated in ongoing clinical trials. In the case of the Hedgehog inhibitor-GSI combination, anti-CSC effects are being specifically measured.

Ultimately, the best use of Notch inhibitors and other CSC-targeted agents will be in the context of personalized medicine. To that end, it must be determined: (1) which cancers and specific cancer subtypes contain Notch-dependent CSCs; (2) what role specific components of Notch signaling play in these CSCs; (3) what pathways cross-talk with Notch in specific CSCs; and (4) how Notch activity can be measured in CSCs from individual patients (e.g., in biopsy material).

The design of clinical trials for CSC-targeted agents will have to consider that anti-CSC effects will not necessarily translate into rapid tumor volume changes. Disease-free or recurrence-free survival will be the most informative endpoints. For situations when this would require prohibitively long follow-ups, it will be important to develop accurate surrogate biomarkers that reflect anti-CSC effects. These may include spheroid formation assays, flow cytometry, and molecular tests, but posttreatment tumor tissue will be required in most cases. A question of potentially great interest is whether it is possible to assess CSC numbers or the relative "stemness" of individual tumors by studying CTCs. These cells can be isolated from patient blood by several methods, one of which is US Food and Drug Administration approved. Although these trials may be challenging, the payoff may be novel treatments that eliminate or greatly reduce treatment resistance in a broad range of malignancies (Pannuti et al. 2010).

Notch and Genetic Diseases

Loss of function of Notch pathway components can cause inherited genetic diseases such as Alagille syndrome, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and spondylocostal dysostosis (SCD) (Fiuza and Arias 2007).

Mutations in the *Jagged1* gene are responsible for Alagille syndrome, which is normally diagnosed in the first 2 years of life. This is an autosomal dominant mutation that causes defects in bile duct formation leading to liver problems, and is also responsible for kidney, eye, heart, and skeleton development problems. The great diversity of the disease presentation suggests that other factors may influence the outcome, such as genetic properties of Notch signaling regulators.

Mutations in human Notch 1 and 3 are responsible for CADASIL syndrome. These mutations lead to an autosomal vascular disorder resulting in the loss of the arteriolar vascular smooth muscle cells that are substituted by granular eosinophilic material. One specific feature of CADASIL syndrome is its late onset around the age of 45 years. This disease is linked with a variety of symptoms ranging from migraines and subcortical ischemic strokes to progressive dementia and premature death.

SCD is a family of diseases that results in vertebral defects. Essentially, SCD is caused by mutations in Dll3 resulting in rib defects that lead to abnormalities in vertebral segmentation and trunk size. Understanding the mechanisms of Notch signaling regulation is crucial in the development of therapeutic approaches for the treatment of these diseases.

Notch and Other Diseases

Alzheimer's Disease

Notch is expressed by neurons in the adult brain where it is present at particularly high levels in the hippocampus. The prospect that Notch is the substrate of γ-secretase/presenilin and plays a role in learning and memory suggests a potential link between Notch signaling and the pathogenesis of Alzheimer's disease (Woo et al. 2009). In post-mitotic neurons, Notch proteins interact with PSs (presenilins) and with APP (amyloid precursor protein), which have roles in the

memory deficits associated with Alzheimer's disease. In some cases, mutations in the genes encoding APP, PS1, and PS2 are responsible for early-onset Alzheimer's disease. The phenotype of PS1 deletion mice is similar to that observed in Notch knockout mice. The PS1/PS2 double knockout phenotype is even more similar, suggesting closely related functions for these proteins. Indeed, knockouts of any one of several c-secretase components cause developmental abnormalities that are similar to those caused by Notch 1 and Notch 2 knockouts.

Glomerular Disease

Albuminuria associated with sclerosis of the glomerulus affects millions of people and leads to a progressive decline in renal function. Activation of the Notch pathway, which is critical to glomerular patterning, contributes to the development of glomerular disease. Expression of the intracellular domain of Notch1 (ICN1) was increased in glomerular epithelial cells in diabetic nephropathy and in focal segmental glomerulosclerosis. Conditional in vivo re-expression of ICN1 exclusively in podocytes caused proteinuria and glomerulosclerosis. In vitro and in vivo studies showed that ICN1 induced apoptosis of podocytes through the activation of p53. Genetic deletion of a Notch transcriptional partner (Rbpj) specifically in podocytes or pharmacological inhibition of the Notch pathway (with a c-secretase inhibitor) protected rats with proteinuric kidney diseases (Niranjan et al. 2008).

Connection to Other Signaling Pathways

The *Drosophila* Disheveled gene, which encodes a component of the Wingless signaling pathway, interacts antagonistically with Notch and one of its ligands, Delta. Notch1 activation induced p21 in differentiating mouse keratinocytes. The induction was associated with the targeting of Rbpjk (RBPSUH) to the p21 promoter. Notch1 also activated p21 through a calcineurin-dependent mechanism acting on the p21 TATA box-proximal region. Notch signaling through the calcineurin/► NFAT pathway also involved calcipressin and Hes1.

Oncogenic Ras activates Notch signaling. Wild-type Notch1 is necessary to maintain the neoplastic phenotype in Ras-transformed human cells in vitro and in vivo. The oncogenic effect of NOTCH1 on primary melanoma cells was mediated by ► beta-catenin, which was upregulated following NOTCH1 activation. Inhibiting ► beta-catenin expression reversed NOTCH1-enhanced tumor growth and metastasis.

Microarray studies of the mouse presomitic mesoderm transcriptome demonstrated that the segmentation clock drives the periodic expression of a large network of cyclic genes involved in cell signaling. Mutually exclusive activation of the Notch-fibroblast growth factor (► FGF) and Wnt pathways during each cycle suggested that coordinated regulation of these 3 pathways underlies the clock oscillator. Another study identified two clusters, the first cluster contains the known cyclic genes of the Notch pathway: Hes1, Hes5, Hey1, Id1, and Nrarp, a direct target of Notch signaling. In the same cluster as the Notch pathway were members of the FGF-MAPK pathway, including Spry2 and Dusp6. The second cluster of periodic genes contained genes cycling in an opposite phase to the Notch-► FGF cluster. This cluster included a majority of the cyclic genes associated with Wnt signaling, including Dkk1, c► Myc, Axin2, Sp5, and ► Tnfrsf19.

NOTCH and MYC regulate two interconnected transcriptional programs containing common target genes that regulate cell growth in primary human T-cell lymphoblastic leukemias. In bone marrow progenitor cells and T-cell acute lymphoblastic leukemia (T-ALL) cell lines, constitutively active NOTCH1 transcriptionally activated the NFKB pathway via the IKK complex, thereby causing increased expression of NFKB target genes.

Expression of NOTCH1 in human keratinocytes was under the control of ► P53. NOTCH1 suppressed tumor formation through negative regulation of ROCK1/ROCK2 and MRCK-α (CDC42BPA), which are effectors of small RHO GTPases implicated in neoplastic progression.

Some T-ALL cells show resistance to γ-secretase inhibitors that act by blocking NOTCH1 activation. Using microarray analysis, ► PTEN was identified as the gene most consistently downregulated in γ-secretase inhibitor-resistant T-cell lines. Studies in normal mouse thymocytes indicated that Notch1 regulated ► Pten expression downstream.

Genes in the Notch pathway were expressed in mature podocytes in humans and in rodent models of diabetic nephropathy and focal segmental glomerulosclerosis.

Summary

Notch activity via cell–cell contacts generates molecular differences between adjacent cells. The Notch pathway can mediate both instructive and lateral signaling in neural differentiation and tumorigenesis. Many Notch regulatory processes have been identified, but are not yet truly characterized. Notch activity regulation by ligand inhibitory effects is well described, but its mechanism of action is still unclear. The role and mechanisms of Notch and ligand trafficking are not well understood. CSL-independent Notch signaling remains undefined, both as a molecular pathway and in its effects. Further work is necessary to understand Notch signaling in all its complexity, and to provide insight into how to tackle Notch signaling in a more specific way to better approach different clinical contexts.

Acknowledgment This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare and Family Affairs (A084022) and BRL (Basic Research Laboratory) program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2010–0001200).

References

Artavanis-Tsakonas S, Muskavitch MA, Yedvobnick B. Molecular cloning of Notch, a locus affecting neurogenesis in *Drosophila melanogaster*. Proc Natl Acad Sci USA. 1983;80:1977–81.

Beckerman M. Cell Fate and Polarity. In: Molecular and cellular signaling. New York: Springer; 2005. p. 305–329.

Cau E, Blader P. Notch activity in the nervous system: to switch or not switch? Neural Dev. 2009;4:36.

Fiuza UM, Arias AM. Cell and molecular biology of Notch. J Endocrinol. 2007;194:459–74.

Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. Dev Cell. 2009;16:633–47.

Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. Arterioscler Thromb Vasc Biol. 2003;23:543–553.

Jeon HM, Jin X, Lee JS, Oh SY, Sohn YW, Park HJ, Joo KM, Park WY, Nam DH, Depinho RA, Chin L, Kim H. Inhibitor of differentiation 4 drives brain tumor-initiating cell genesis through cyclin E and notch signaling. Genes Dev. 2008;22:2028–33.

Koch U, Radtke F. Notch and cancer: a double-edged sword. Cell Mol Life Sci. 2007;64:2746–62.

Kuan CY, Tannahill D, Cook GM, Keynes RJ. Somite polarity and segmental patterning of the peripheral nervous system. Mech Dev. 2004;121:1055–68.

Lewis J, Hanisch A, Holder M. Notch signaling, the segmentation clock, and the patterning of vertebrate somites. J Biol. 2009;8:44.

Li JL, Harris AL. Notch signaling from tumor cells: a new mechanism of angiogenesis. Cancer Cell. 2005;8:1–3.

Morgan TH, Bridges CB. The Inheritance of a Fluctuating Character. J Gen Physiol. 1919;1:639–43.

Nam DH, Jeon HM, Kim S, Kim MH, Lee YJ, Lee MS, Kim H, Joo KM, Lee DS, Price JE, Bang SI, Park WY. Activation of notch signaling in a xenograft model of brain metastasis. Clin Cancer Res. 2008;14:4059–66.

Niranjan T, Bielesz B, Gruenwald A, Ponda MP, Kopp JB, Thomas DB, Susztak K. The Notch pathway in podocytes plays a role in the development of glomerular disease. Nat Med. 2008;14:290–8.

Pannuti A, Foreman K, Rizzo P, Osipo C, Golde T, Osborne B, Miele L. Targeting Notch to target cancer stem cells. Clin Cancer Res. 2010;16:3141–52.

Sandy AR, Maillard I. Notch signaling in the hematopoietic system. Expert Opin Biol Ther. 2009;9:1383–98.

Woo HN, Park JS, Gwon AR, Arumugam TV, Jo DG. Alzheimer's disease and Notch signaling. Biochem Biophys Res Commun. 2009;390:1093–7.

NOXA, PMAIP (Phorbol-Myristate-Acetate-Induced Protein), APR (Adult T cell Leukemia-Derived PMA-Responsive)

► BCL-2 Family

Npn3

► Sulfiredoxin

NR1B1

► Retinoic Acid Receptors (RARA, RARB, and RARC)

NR1B2

► Retinoic Acid Receptors (RARA, RARB, and RARC)

NR1B3

▶ Retinoic Acid Receptors (RARA, RARB, and RARC)

NR3C4 (Nuclear Receptor Subfamily 3, Group C, Member 4)

▶ Androgen Receptor (AR)

Nrf2

▶ Nrf2 (NF-E2-Related Factor2)

Nrf2 (NF-E2-Related Factor2)

Sang Geon Kim, Woo Hyung Lee and Young Woo Kim
College of Pharmacy, Seoul National University, Seoul, South Korea

Synonyms

NFE2L2; NF-E2-related factor 2; Nrf2

Historical Background

Nrf2 was found as a member of nuclear factor erythroid 2 (NF-E2) transcription factor family in 1994 (Moi et al. 1994). Nrf2 is a basic Leucine Zipper (bZIP) transcription factor that belongs to the Cap'n'Collar (CNC) family (p45-NFE2, Nrf1, Nrf2, and Nrf3) and is expressed ubiquitously in various tissues (Moi et al. 1994). Yamamoto and his colleagues showed that Nrf2 forms a heterodimer with small Mafs and induces phase-II detoxifying enzymes through antioxidant response elements (AREs) in the promoter regions of the target genes (Tong et al. 2006). A myriad of studies have identified Nrf2 as a sensor that acts against oxidative stress or electrophilic chemicals. In spite of the similarity in nucleotide sequences between Nrf2 and NF-E2, it was not involved in erythropoiesis and development in a murine model.

Oxidative stress is featured by high levels of reactive oxygen species (ROS), which exerts a harmful effect on cellular components and induces defensive responses. ROS originates from hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and peroxynitrite ($ONOO^-$) form powerful oxidants in the cell. Thus, ROS generation is the fate of aerobic organisms as a natural by-product of oxygen metabolism. In order to avoid cellular damage inflicted by oxidative perturbation, aerobic organisms have developed novel antioxidant defense systems. Among these, Nrf2 and its cytoplasmic repressor kelch-like ECH-associated protein 1 (Keap1) serve sulfhydryl-containing sensors that respond to oxidative stress (Tong et al. 2006); oxidative stress modifies reactive cysteine residues in Keap1 and/or Nrf2. Under no oxidative stimuli, Keap1 binds to the amino-terminal Nrf2-ECH homology 2 (Neh2) domain of Nrf2 and provokes its ubiquitin/proteasomal degradation (Tong et al. 2006). In cells challenged with oxidative stimuli, Keap1 dissociates from Nrf2 and thereby ubiquitin/proteasomal degradation of Nrf2 is hampered. Hence, mice deficient in Nrf2 exacerbate sensitivity to carcinogens or tumorigens, which supports the concept that Nrf2-mediated gene transcription is necessary for the prevention of chemical carcinogenesis by cytoprotective agents (Kensler and Wakabayashi 2010).

An increasing number of studies have described a series of synthetic and phytochemical compounds that activate Nrf2 in cell or animal models (Eggler et al. 2008) (Table 1). Because most of these agents have beneficial effects in a variety of disease models, current pharmacological interventions that target the activity of Nrf2 are expected to advance into novel drug discovery for human diseases.

Regulation of Nrf2 Activity

Domain Structure of Nrf2

Nrf2-ECH homology (Neh) domains are highly conserved in mammalian cells; Nrf2 has six Neh domains (Fig. 1). The domain structure of Nrf2 has been extensively studied by Yamamoto group. Each Neh domains has a distinct role in regulating the activity of Nrf2. First, Neh1 domain contains CNC-bZIP domain that

Nrf2 (NF-E2-Related Factor2), Table 1 Nrf2-activating compounds

Categories	Nrf2 activators
Aromatic organic compounds	BHT (butylated hydroxytoluene)
	tBHQ(tert-butylhydroquinone)
	BHA (butylated hydroxyanisole)
Dithiolethiones	oltipraz, D3T (1,2-dithiole-3-thione)
Isothiocyantes	sulforaphane
Oleanoic triterpenoids	CDDO-Im (2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide)
Flavonoids	genistein, isoliquiritigenin
Cyclopentenone prostaglandin	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
Polyphenols	EGCG ((−)-epigallocatechin-3-gallate), resveratrol

leads to the formation of heterodimer with small Mafs lacking a transactivation domain. Using yeast two hybrid analysis, it has been shown that the Neh2 domain of Nrf2 interacts with Kelch/DGR domain of Keap1, and which induces ubiquitin/proteasomal degradation of Nrf2 via Cul3 ubiquitin ligase (Tong et al. 2006). Whereas Neh2 degron is redox-sensitive, Neh6 degron is not and is required for maximal turnover of Nrf2. Studies have shown that Neh4 and Neh5 domains cooperatively bind with either CREB-binding protein (CBP) or silencing mediator of retinoid and thyroid receptors (SMRT)(Ki et al. 2005) and enhance target gene transactivation (Li and Kong 2009). In addition, the carboxy-terminal Neh3 domain of Nrf2 contributes to Nrf2 transactivation activity.

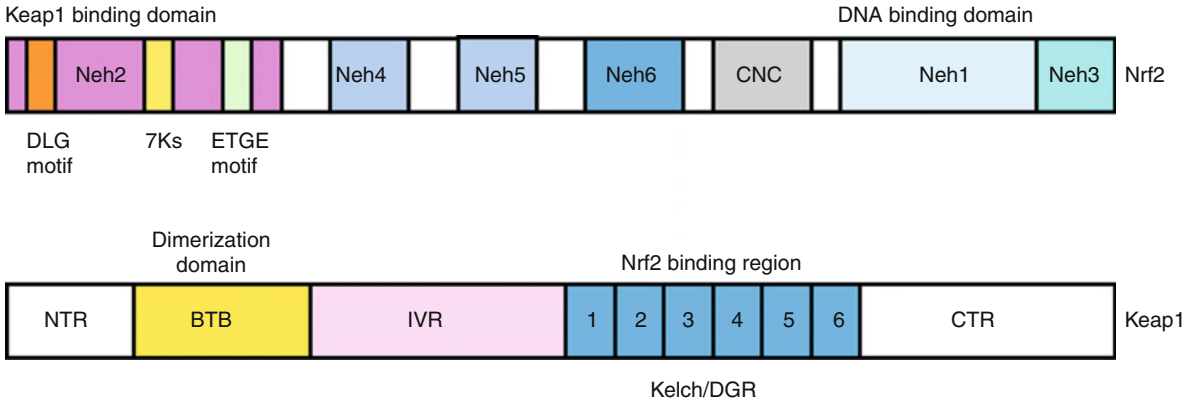
Degradation of Nrf2 Protein

In normal cells, Nrf2 is short-lived but is rapidly stabilized by oxidative stress. In this process, Keap1 has been recognized as a key repressor molecule that causes ubiquitination/proteasomal degradation. Keap1 is a cysteine-rich protein and serves an oxidative sensor molecule in response to free radical stress. The sulfhydryl modifications of cysteine residues of Keap1 affect Nrf2 degradation (Keap1 has nine reactive cysteine residues that respond to oxidative stress) (Fig. 1). Among them, C273 and C288 are located in the intervening region (IVR) of Keap1, and thus these mutations abrogated the basal inhibitory activity of Nrf2. So, oxidative stress supposedly causes the intermolecular disulfide bond formation of C273 and C288 and, thereby, downregulates Keap1 activity.

Keap1 has two functional domains called Kelch/DGR and broad complex, tramtrack, bric-a-brac (BTB) (Fig. 1) that are involved in Nrf2 degradation. Kelch/DGR domain interacts with Neh2 of Nrf2. BTB domain of Keap1 recruits ubiquitin-ligase complex components and also forms a homodimer of Keap1. Neh2 domain of Nrf2 contains two distinct binding motifs with Kelch/DGR domain of Keap1. One is ETGE motif (formulated as D/N-X-E-T/S-G-E) that has a high affinity ($K_a = 20 \times 10^7\ M^{-1}$) to Kelch/DGR domain of Keap1, whereas the other is DLG motif (formulated as L-X-X-Q-D-X-D-L-G) that has a low affinity ($K_a = 0.1 \times 10^7\ M^{-1}$). X-ray crystallography unraveled that Kelch/DGR domain is a shape of six-bladed β-propeller and each Kelch domain consists of four antiparallel β-strands. Kelch/DGR domain possesses arginine triad (R380, R415 and R483), and these amino acids explain why ETGE motif of Neh2 exerts higher binding affinity than that of DLG. Between these two motifs, 7 lysine residues (7Ks) are located and ubiquitinated by E3 ligase. In addition, the results of nuclear magnetic resonance analysis indicated that the binding ratio of Keap1/Neh2 domain is 2:1 (Li and Kong 2009), suggesting that Keap1 makes homodimer formation.

In addition to Keap1, the ubiquitin-proteasome system is responsible for Nrf2 degradation (Tong et al. 2006). Ubiquitin consists of 76 amino acids and is a highly conserved regulatory protein which designates certain proteins subjected to degradation. E1, an ubiquitin-activating enzyme, generates an ubiquitin-adenylate intermediate using ATP, and then transfers ubiquitin to the active cysteine residue of E1. E2, an ubiquitin-conjugating enzyme, and E3, ubiquitin ligase, cooperatively accomplish the ubiquitination of target proteins. Lysine residue of target protein and C-terminal glycine of ubiquitin forms isopeptide bond by E3 ubiquitin ligase complex. Nrf2 is ubiquitinated by Cul3-BTB^{keap1} E3 ligase which belongs to the members of RING domain E3 ligases (Tong et al. 2006). Cul3-BTB^{keap1} E3 ligase is composed of Keap1, Rbx1, Cullin3, and Ubc5 (E2 enzyme). As the member of E3 ligase complex, Kelch/DGR domain of Keap1 binds to Nrf2 and the BTB domain recruits the components of E3 ligase complex.

According to these results, Yamamoto's group proposed "hinge and latch model-two-sites binding mechanism" that describes Keap1-Nrf2 system (Tong et al. 2006). ETGE motif serves "hinge" since it forms a strong binding complex with the Kelch/DGR domain



Nrf2 (NF-E2-Related Factor2), Fig. 1 The domain structures of Nrf2 and Keap1

of Keap1 even under oxidative stress. In contrast, DLG motif works as a "latch" which allows Nrf2 to be disoriented under oxidative stress and impedes Nrf2 ubiquitination by Cul3-BTB^{keap1} E3 ligase. Reactive cysteines in the IVR domain of Keap1 are modified by oxidative stress. It is postulated that these sulfhydryl modifications may change the conformation of Nrf2 structure (Tong et al. 2006). "Hinge and latch model" successfully accounts for the mode of actions of various Nrf2 activators, including ROS, reactive nitrogen species (RNS), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, and sulforaphane. Recently, the direct interaction between ^{154}KRR motif in p21 and DLG/ETGE motifs in Nrf2 hampers ubiquitination of Nrf2 by competitively binding with Keap1 (Chen et al. 2009), which supports the model of Yamamoto's group. Collectively, "hinge and latch model" may account for redox-sensitive Nrf2 activity regulation in association with Keap1.

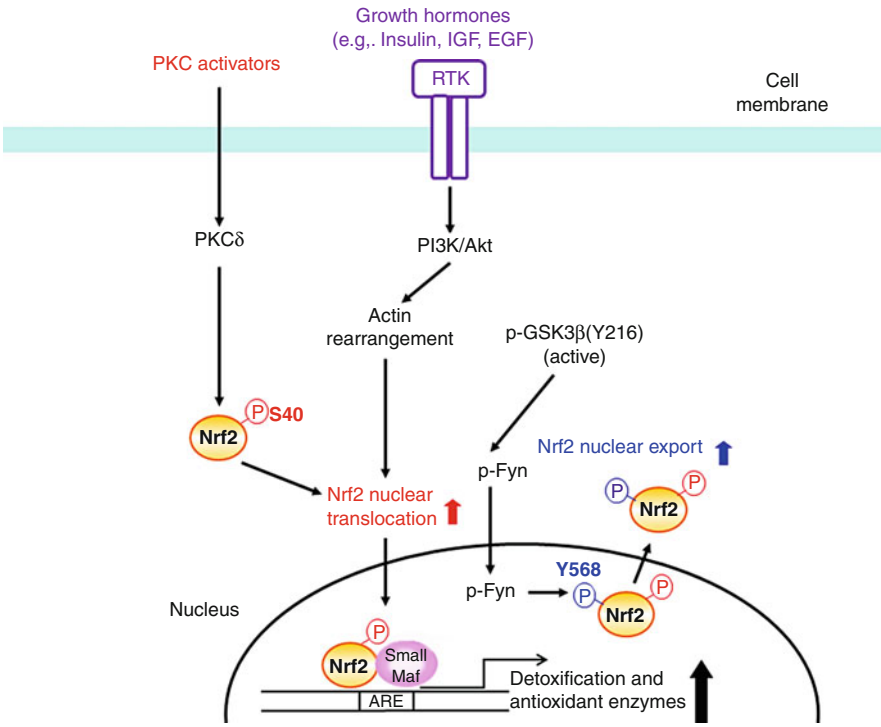
Nuclear Localization of Nrf2

Nrf2 contains three nuclear localization signal (NLS) motifs and two nuclear export signal (NES) motifs (Li et al. 2006; Li and Kong 2009). NLS_N was identified at the amino-terminus, whereas NLS_C was at the carboxy-terminus. bNLS is characterized at the basic region of Nrf2. NES_{TA} is localized at the Neh5 transactivation domain, whereas NES_{zip} is at the ZIP domain of Nrf2. Li et al. discovered NES_{TA} motif (^{175}LLSIPELQCLNI^{186}) of Neh5 domain based on the consensus leucine-rich NES motif that is formulated as $\Phi^4(X)_{2-3}\Phi^3(X)_{2-3}\Phi^2X\Phi^1$ (Φ represents hydrophobic amino acids and X represents any amino acids). EGFP-NES_{TA} chimeric protein (a truncated form of Nrf2) promoted cytoplasmic distribution of Nrf2. So, mutation of C183 of NES_{TA} abrogated oxidant-induced ARE activity. The fluorescence resonance energy transfer (FRET) assay failed to show direct interaction between NES_{TA} and Keap1, indicating that nuclear localization of Nrf2 might be modulated by NES_{TA} motif independently of Keap1. Li and Kong have proposed another Nrf2 signaling model in association with Keap1-independent Nrf2 regulation (Li and Kong 2009); in this model, the balance between NLSs and NESs determines the subcellular localization of Nrf2 in response to oxidative stress or antioxidants. NES_{TA} and bNLS motifs possess similar strong driving force for the translocation of Nrf2. Since the driving forces among the motifs are well balanced under normal condition, Nrf2 is expelled to cytoplasm. Nrf2 is translocated into the nucleus when redox-sensitive NES_{TA} is halted by oxidative stress (i.e., NES_{zip} is redox-insensitive).

The Signals of Nrf2 Regulation

Phosphatidylinositol 3-Kinase

Phosphatidylinositol 3-kinase (PI3K) phosphorylates phosphatidylinositol-4,5-trisphosphate [PtdIns(4,5)P2] into phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3], which is the secondary messenger for other kinases such as serine-threonine Akt kinase. ▶ PI3K regulates microfilaments and translocation of actin-associated proteins (Fig. 2). In response to oxidative stress, PI3K activation induces actin cytoskeleton rearrangement. Actin depolymerization promotes translocation of a complex of Nrf2 and actin into the nucleus and enables activating Nrf2 to bind to the ARE for phase II enzyme induction by oxidative stress (Kang et al. 2005).



Nrf2 (NF-E2-Related Factor2), Fig. 2 The signaling pathways for Nrf2 activity regulation

Protein Kinase C Delta

Protein kinase C (PKC) disseminates signals into target molecules in response to extracellular stimuli. PKC pathway may be an initial triggering step for recognizing cellular redox state transition. Pickett's group revealed that Nrf2 activation requires phosphorylation at S40 by PKCδ critical for nuclear translocation of Nrf2 (Fig. 2) (Kaspar et al. 2009). Recently, both phosphorylation of Nrf2 S40 by PKCδ and antioxidant-induced modification of Keap1 C151 contribute to stabilization and nuclear translocation of Nrf2 (Niture et al. 2009).

Glycogen Synthase Kinase-3β

It has been shown that tyrosine kinase Fyn phosphorylated Y568 of Nrf2 and regulates chromosomal region maintenance 1 (Crm1/exportin 1)-mediated nuclear export of Nrf2 (Fig. 2). Glycogen synthase kinase-3β (GSK-3β) was identified as an upstream kinase of Fyn (Kaspar et al. 2009). Inhibition of GSK-3β induces nuclear accumulation of Nrf2 and transcriptionally activates the induction of Nrf2 target genes (i.e., phase-II enzymes). Hydrogen peroxide directly phosphorylates Y216 of GSK-3β, which leads to GSK-3β activation, implying that ROS affects GSK-3β-mediated Nrf2 activity regulation (Kaspar et al. 2009).

Nrf2 Target Genes and Biological Functions

The Genes That Contain ARE(s)

The induction of phase-II detoxification enzymes and phase-III efflux transporters through ARE depends on the activity of Nrf2. Major antioxidant enzymes contain one or more functional ARE(s) in their promoter regions (Table 2). Once Nrf2 dissociates from its Keap1 binding in response to oxidative stress, the activating Nrf2 translocates into the nucleus and binds ARE comprised in the promoters of target genes. Unlike canonical bZIP proteins, Nrf2 has no ability to form homodimer (Li and Kong 2009). Instead, Nrf2 forms heterodimer with small Maf proteins such as MafF/G/K which lack canonical transactivation domain. In addition, Nrf2 is directly acetylated by p300/CREB-binding protein (CBP) under the condition of arsenite-induced stress (Sun et al. 2009). Eighteen lysine residues were identified as acetylation sites in Neh1 DNA-binding domain. Intriguingly, combined lysine-to-arginine mutations

Nrf2 (NF-E2-Related Factor2), Table 2 Nrf2 target genes

Functions	Target genes
Phase-I enzymes	None
Phase-II enzymes	GSTA2
	NQO1
	heme oxygenase-1
	UDP-glucuronosyltransferases (UGT) 1A6
	glutamate-cysteine ligase modifier (GCLM)
	glutamate-cysteine ligase catalytic subunits (GCLC)
Phase-III enzymes	Multidrug resistance protein (MRP) 2/3/4/5/6, Organic anion transporting polypeptide (Oatp) 1a1/2b1
Iron-binding protein	Ferritin H

on the acetylation sites unaffected the stability of Nrf2, but compromised its DNA-binding activity (Sun et al. 2009). Nrf2 activation and target gene transcription contribute to the detoxification and excretion of detrimental xenobiotics. Phytochemicals and synthetic compounds may have cytoprotective and chemopreventive effects through Nrf2 activation (Eggler et al. 2008). Hence, a deficiency of Nrf2 abrogates the abilities of these agents to protect cells against toxic chemicals or physical stresses, as shown in the experiments using animals or cells.

As the cores of energy metabolism, mitochondria regulate the balance between constitutive and excessive levels of cellular ROS. The mitochondrial respiratory chain not only produces ROS under a basal condition, but also serves a major ROS source under pathological situations. Oxidative stress causes mitochondrial permeability transition, mitochondrial dysfunction, and apoptosis. Hence, the maintenance of mitochondrial function is crucial in protecting cells or organs from toxicants. Compared to nuclear DNA, mitochondria DNA are vulnerable to oxidative stress because of two reasons: (1) mitochondria are the organelles that produce ROS via electron transport chain, and (2) mitochondria DNA repair mechanisms are insufficient. Therefore, the induction of phase-II detoxifying enzymes by Nrf2 might be closely associated with cytoprotective effect against toxicant-induced injury, which may result from not only a decrease in cellular ROS, but protection of mitochondria (Kensler et al. 2007). Collectively, it is hypothesized that the roles of Nrf2 in apoptosis include regulation of redox-homeostasis, increase in adaptive antioxidant capacity, activation of phase-II detoxifying enzymes, and mitochondrial protection, all of which contribute to cell viability.

Cancer

Exposure to toxic external stimuli such as xenobiotics and viral infections might cause genetic defects and thus increase cancer incidence. Carcinogenesis is induced by complex mechanisms which are characterized as multiple genetic defects and uncontrolled growth. These genetically defected genes often have effects on signal transduction pathways regarding cell survival, proliferation, and trans-differentiation. In particular, excess ROS provokes DNA damage such as point mutation, deletion-insertion, and microsatellite instability. Thus, it is a reasonable prediction that antioxidants and antioxidative enzymes contribute to preventing genetic defects of cells from radical stress. Various experimental models have shown that induction of antioxidative and cytoprotective enzymes by chemicals contributes to cancer chemoprevention, and which accompanies Nrf2 activation in most cases.

Many research groups have made a huge effort to develop Nrf2 activators as chemopreventive agents. However, it is now accepted that constitutive Nrf2 activation may also contribute to malignancy and radiation/drug resistance in cancer. The mutations in Keap1 and overwhelming expression of Nrf2 occur in the tissue of lung cancer patients (Lau et al. 2008). Mutated Keap1 possesses substantially impeded binding affinity with Nrf2, leading to augmented anti-apoptotic and antioxidative effects. Therefore, some cancer cells with Keap1 mutations acquire the capacity to survive from harsh tumor microenvironment through Nrf2 activation. Recently, Kensler and Wakabayashi proposed "U-Shaped model" that describes the modulation of cancer risk in terms of the Keap1-Nrf2 pathway (Kensler and Wakabayashi 2010). Nrf2 activation is clinically practical only between the biologically effective dose (BED) and a maximal-tolerated dose (MTD). Low level of Nrf2 expression makes cells susceptible to carcinogenesis or toxicity, whereas high level of Nrf2 expression in tumor might attribute to cancer malignancy.

Lately, epigenetic regulatory pathway of Nrf2-Keap1 has been underscored in cancer cells by several research groups. Hypermethylation of CpG islands of Keap1 was discovered in lung adenocarcinoma.

In addition, CpG island methylations in the promoter region of Nrf2 gene was identified in transgenic adenocarcinoma of mouse prostate, but not in normal tissue (Yu et al. 2010). Thus, Nrf2 level and its downstream target gene expression are substantially repressed in this prostate tumor model. These results suggest that the epigenetic approach is also necessary for the understanding of Nrf2 role in cancer.

Cardiovascular Diseases

ROS is involved in the pathologic processes of cardiovascular diseases such as atherosclerosis, hypertension, and coronary heart disease. In cardiovascular diseases, ROS production (H_2O_2 and O_2^-) is increased due to NAD(P)H oxidase, peroxidase, and cyclooxygenase. In particular, vascular smooth muscle cells (VSMCs) and endothelial cells are the major sources of ROS. Nrf2 is a potential target for the intervention of cardiovascular diseases. Studies have shown that the Nrf2/heme oxygenase-1 (HO-1) pathway is associated with the inhibition of VSMC proliferation and migration, and which helps provide a condition for obtaining anti-atherosclerotic activity (Li et al. 2009). After balloon angioplasty in rabbit aorta, local adenoviral transfer of Nrf2 contributes to reducing VSMC proliferation, oxidative stress, and inflammatory responses (Li et al. 2009). However, the lack of change in neointimal hyperplasia by ectopic Nrf2 expression implies that Nrf2 may induce anti-apoptosis of VSMCs. In human aortic endothelial cells, laminar flow, but not oscillatory flow, induces Nrf2 activation and its target gene transactivation (Li et al. 2009); the degree of Nrf2 activation differs between atherosclerosis-resistant and atherosclerosis-susceptible regions of the mouse aorta. Although Nrf2 regulates antioxidant defense system, it still remains elusive what the exact molecular mechanism of Nrf2 is in cardiovascular system.

Summary

Oxidative stress is critical in homeostasis and survival of aerobic organisms. Nrf2 target gene induction plays a role in antioxidant defense systems. In cells challenged with oxidative stimuli, Keap1 is not able to degrade Nrf2 so that antioxidative and cytoprotective enzymes are activated. So, Nrf2-mediated gene induction by pharmacological agents may account for cancer chemoprevention, and amelioration of hepatic and cardiovascular diseases. However, incremental Nrf2 activation was observed in cancer tissues, implying that it may also contribute to invoking cancer malignancy and chemoresistance. The exact role and mechanism of Nrf2 regulation and its functional consequences are still elusive, and further molecular and clinical investigation is requisite.

Acknowledgments This work was supported by the National Research Foundation of Korea grant funded by the Korea government (MEST) (No. 2009-0063233) and the World Class University project (which is also funded by Korea government) (R32-2008-000-10098-0).

References

Chen W, Sun Z, Wang XJ, Jiang T, Huang Z, Fang D, et al. Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. Mol Cell. 2009;34:663–73.

Eggler AL, Gay KA, Mesecar AD. Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2. Mol Nutr Food Res. 2008;52 Suppl 1:S84–94.

Kang KW, Lee SJ, Kim SG. Molecular mechanism of nrf2 activation by oxidative stress. Antioxid Redox Signal. 2005;7:1664–73.

Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med. 2009;47:1304–9.

Kensler TW, Wakabayashi N. Nrf2: friend or foe for chemoprevention? Carcinogenesis. 2010;31:90–9.

Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol. 2007;47:89–116.

Ki SH, Cho IJ, Choi DW, Kim SG. Glucocorticoid receptor (GR)-associated SMRT binding to C/EBPbeta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. Mol Cell Biol. 2005;25:4150–65.

Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. Pharmacol Res. 2008;58:262–70.

Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. Mol Carcinog. 2009;48:91–104.

Li W, Yu SW, Kong AN. Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain. J Biol Chem. 2006;281:27251–63.

Li J, Ichikawa T, Janicki JS, Cui T. Targeting the Nrf2 pathway against cardiovascular disease. Expert Opin Ther Targets. 2009;13:785–94.

Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci USA. 1994;91:9926–30.

Niture SK, Jain AK, Jaiswal AK. Antioxidant-induced modification of INrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. J Cell Sci. 2009;122:4452–64.
Sun Z, Chin YE, Zhang DD. Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. Mol Cell Biol. 2009;29:2658–72.
Tong KI, Kobayashi A, Katsuoka F, Yamamoto M. Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. Biol Chem. 2006; 387:1311–20.
Yu S, Khor TO, Cheung KL, Li W, Wu TY, Huang Y, et al. Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. PLoS ONE. 2010. doi:10.1371/journal.pone.0008579.

nrip1 (Nuclear Receptor-Interacting Protein 1)

Ping-Chih Ho and Li-Na Wei
Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN, USA

Synonyms

RIP140 (Receptor-Interacting Protein 140)

Historical Background

Determining the regulatory mechanisms of nuclear receptor action was one major focal topic of research in the 1990s. During this period, many nuclear receptor-associated proteins were identified as transcriptional coregulators, which were broadly categorized as coactivators and corepressors. Human receptor interacting protein 140 (RIP140) was identified as a ligand-dependent interacting protein of estrogen receptor α (ERα) by far-western blotting, and mouse RIP140 was isolated as a corepressor of orphan receptor TR2 from a yeast two-hybrid screening and later found to also interact with ▶ retinoic acid receptor (RAR) in a ligand-enhanced manner (Cavailles et al. 1995; Lee et al. 1998; Lee and Wei 1999). An official gene name *nrip1* was established for RIP140 by the HUGO gene nomenclature committee. The mouse gene is located on chromosome 16, in region C3.1; whereas the human gene is located on q11.2 of chromosome 21. DNA sequence analyses conclude that this gene is conserved in all vertebrate species examined. Experimental data have validated its interaction and gene coregulatory activity for all the nuclear receptors examined, as well as several transcription factors. The list includes AR, ER, GR, RARα/β, RXRα/β, PPARα/γ/δ, PXR, LXRα/β, ▶ VDR, AhR, ERRα/β/γ, RORβ, HNF4α, TR2, ▶ TR4, c-jun, SF-1, RelA, and ▶ GRIP1. Most experimental data in the past have demonstrated its ligand-enhanced gene repressive activity. Recent studies have begun to elucidate its other functions beyond the nucleus, such as modulation of signal transduction and biological processes in the cytoplasm.

Major sites of RIP140 expression

Sites	Detection method
Ovary	Western blotting and reporter
Muscle	Western blotting
White Adipose tissue	Western blotting
Heart	Western blotting and Northern blotting
Macrophage	Western blotting
Testis	Northern blotting and reporter
Brain	Northern blotting and reporter
Lung	Northern blotting
Stomach	Northern blotting
Kidney	Northern blotting
Spleen	Northern blotting
Placenta	Northern blotting
Uterus	Reporter
Pituitary	Reporter

nrip1 (Nuclear Receptor-Interacting Protein 1), Fig. 1 Expression of RIP140 in the mouse and detection methods

Expression of RIP140 and Its Regulation

RIP140 expression in the mouse can be detected as early as embryonic stage E12.5. In studies using promoter-driven reporters, Northern blotting, or Western blotting, RIP140 is detected in various organs and cell types (Fig. 1). Its expression level, based upon Western blot results, is higher in ovary and metabolic tissues such as adipose tissue, muscle, and liver. Regulation of its expression involves transcriptional regulation through several binding sites for hormone receptors

nrip1 (Nuclear Receptor-Interacting Protein 1), Fig. 2 Regulatory mechanisms of RIP140 expression, including the stimuli, underlying mechanisms, and cells/tissues in which stimuli are identified

Up-regulation of RIP140

Stimuli	Mechanism	Cell type
Androgen	Not clear	LNCaP
Estrogen	Transcriptional	MCF-7
Vitamin D	Not clear	SCC25
Retinoid	Transcriptional	MCF-7 and NT2/D1
pregnant mare serum gonadotrophin (PMSG)	Transcriptional?	Rat granulosa cell
Dioxin	Transcriptional	MCF-7
miR-346	Translational	P19

Down-regulation of RIP140

Stimuli		
Progestin	Not clear	T-47D
human chorionic gonadotropin (hCG)	Transcriptional?	Rat granulosa cell
HSL-null mice	Not clear	White adipocytes

such as ER (Thenot et al. 1999) and RAR (Kerley et al. 2001), translational regulation through the action of a microRNA miR-346 (Tsai et al. 2009), and ubiquitin-mediated protein degradation (Ho and Wei, unpublished) (Fig. 2). In hormone sensitive lipase-null mice, its expression is altered in adipose tissues. In mice fed a short-term high-fat diet, it is up-regulated in the epididymal adipose tissue (Strom et al. 2008; Ho et al. 2009). These findings suggest that the whole-body metabolic status can affect the regulatory machineries for RIP140 expression. In the brain, a novel form of RIP140 mRNA, which possesses an alternatively spliced 5'UTR, has been detected. Because miR-346 is generated from glutamate receptor ionotropic delta 1 gene that has been proposed to be involved in certain neurological diseases, translational regulation of RIP140 by miR-346 would suggest a potential link between RIP140 and brain disorders.

Posttranslational Modifications of RIP140

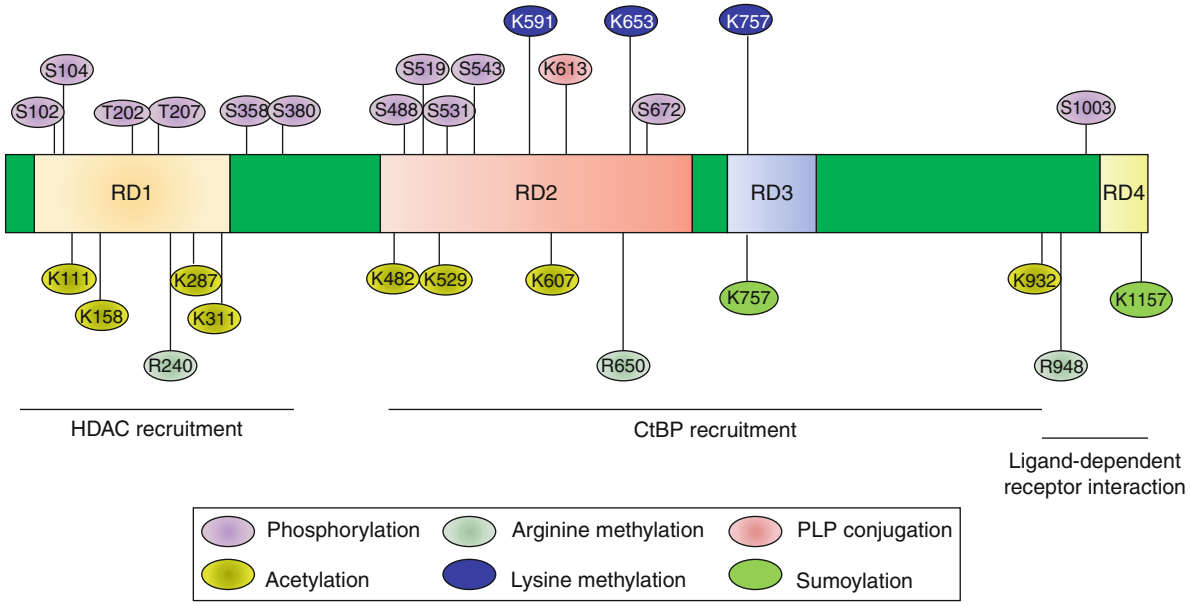
Posttranslational modifications (PTMs) provide an important regulatory mechanism to control or modulate the function, location, interaction, and stability of proteins in response to extracellular and/or intracellular stimuli. Using mass spectroscopy (MS) analyses, many PTMs have been found on RIP140, and most of these PTMs appear to affect RIP140's interaction with other proteins, which ultimately affects its stability, sub-cellular distribution, and biological activities. Figure 3 shows the established PTMs of RIP140.

Phosphorylation

RIP140 can be phosphorylated at nine serine and two threonine residues. Phosphorylation at Thr202 and Thr207 by ERK2 facilitates its recruiting p300 which in turn acetylates RIP140 at Lys158 and Lys287 (Ho et al. 2008). These sequential PTMs enhance RIP140's gene repressive activity by enhancing the recruitment of HDAC3. Phosphorylation at Ser102 and Ser1003 is catalyzed by Protein kinase C epsilon (PKCε), which promotes PRMT1-mediated methylation at Arg240, Arg650, and Arg948 (Gupta et al. 2008). These sequential PTMs lead to the recruitment of exportin 1, which would facilitate its nuclear export (see following). Interestingly, a high-fat diet can initiate this nuclear export pathway in adipocytes by activating nuclear PKCε activity.

Acetylation

Nine lysine residues on RIP140 can be modified by acetylation. Acetylation at Lys482, Lys 529, and Lys607 in repressive domain 2 (RD2)/RD3 region promotes nuclear export and reduces its gene repressive activity; whereas acetylation at Lys158 and Lys287 in RD1 region leads to a stronger gene repressive



nrip1 (Nuclear Receptor-Interacting Protein 1), Fig. 3 Posttranslational modifications (PTMs) of RIP140. The boxes show the full length protein with four repressive domains (RDs 1–4)

activity and nuclear retention (Huq et al. 2009). Acetylation at Lys158 and Lys287 is catalyzed by p300 following ERK2-mediated phosphorylation (see above). Interestingly, this is elevated in the adipocyte differentiation process of the 3 T3-L1 model indicating that RIP140's gene repressive activity is increasingly needed in later stages of adipocyte differentiation.

Methylation

Methylation can occur at either arginine or lysine residues of RIP140. Arginine methylation at Arg240, Arg650, and Arg946 is mediated by PRMT1, which promotes the interaction of RIP140 with the exportin 1 subunit CRM1, thereby stimulating RIP140's nuclear export and reducing its nuclear activity in transcriptional repression. On the other hand, lysine methylation at Lys591, Lys653, and Lys757 elevates its gene repressive activity by unknown mechanisms (Huq et al. 2009). Interestingly, demethylation at these three lysine residues of RIP140 is required for its methylation at the three arginine residues. This suggests a signal crosstalk among various protein methylation enzyme machineries in the nucleus in order to coordinate RIP140's PTMs and, as a result, its interacting partners, subcellular localization, and biological activity.

PLP Conjugation

PLP (pyridoxal 5′-phosphoate) conjugation is found at Lys613 of RIP140, which elevates its gene repressive activity (Huq et al. 2007). This modification is regulated by the cellular status of PLP level, the active form of vitamin B6. This finding suggests that RIP140 may sense PLP or other nutritional factors through PTMs, which then modulates its property and biological activity.

Sumoylation

Sumo-1 conjugation is found for the human protein at Lys 756 and Lys 1154 (Lys757 and Lys 1157 for the mouse RIP140), which may regulate its gene repressive activity and nuclear distribution. The sumoylation enzyme for RIP140 has not been identified. Interestingly, Lys757 on the mouse protein can be methylated, but its relationship with potential Lys757 sumoylation is unclear.

Functional Roles of RIP140

The ubiquitous expression profile of RIP140 suggests its role in many biological processes. Based on the phenotype of RIP140-null mice, this gene is essential for normal ovulation in female animals, and metabolism in general. At the molecular level, RIP140 is known,

mostly, for its corepressive activity in gene transcription through its four RDs which mediate its interaction with HDAC, CtBP, and other chromatin remodeling proteins. Recent studies indicate that RIP140 can also function as a coactivator for transcription of certain genes, including fatty acid synthase (FAS) in hepatocytes and several proinflammatory genes in macrophages. The demonstrated opposing activities, with regards to gene transcriptional control, of RIP140 would indicate that the coregulatory function of RIP140 in transcription might be gene-, transcription factor-, and/or cell context-specific. A hypothesis for its diverse functions was first proposed based upon its extensive PTMs (Huq et al. 2005). This hypothesis has been validated in several studies which examined the functional significance of specific PTMs of RIP140 as detailed above (Mostaqul et al. 2008). In addition, its tightly regulated nuclear export and cytoplasmic distribution strongly suggest other functional roles for RIP140 outside the nucleus. This has also begun to be established in more recent studies, including its activity in modulating insulin-stimulated glucose uptake in adipocytes (Ho et al. 2009) and adipokine secretion (Ho and Wei, unpublished). It is clear that its biological activity is intimately regulated by its PTMs, which are stimulated by certain extracellular/intracellular cues or stimuli. The functional roles of RIP140 in animals have been demonstrated using, primarily, genetically manipulated mice. However, animal phenotypes are the manifestation of multiple defects in various tissues/organs, and therefore should not be simply interpreted as the direct consequence of a single gene defect in a specific tissue, organ, or cell. Further, recent demonstration of cytoplasmic functions of RIP140 has complicated the interpretation of data generated from studying genetically altered animals. In the following, its functional roles deduced from studying whole animals or cell cultures are summarized.

Role in Ovulation

Depletion of RIP140 in mouse revealed its essential role in female fertility, particularly for the control of ovulation. The absence of RIP140 impacts many steps including follicular rupture, cumulus cell-oocyte complex expansion, and oocyte release (White et al. 2000). Further studies indicate that RIP140 is also critical for the expression of EGF-like factors which are essential in cumulus expansion and, possibly, follicular rupture.

Role in Adipocyte

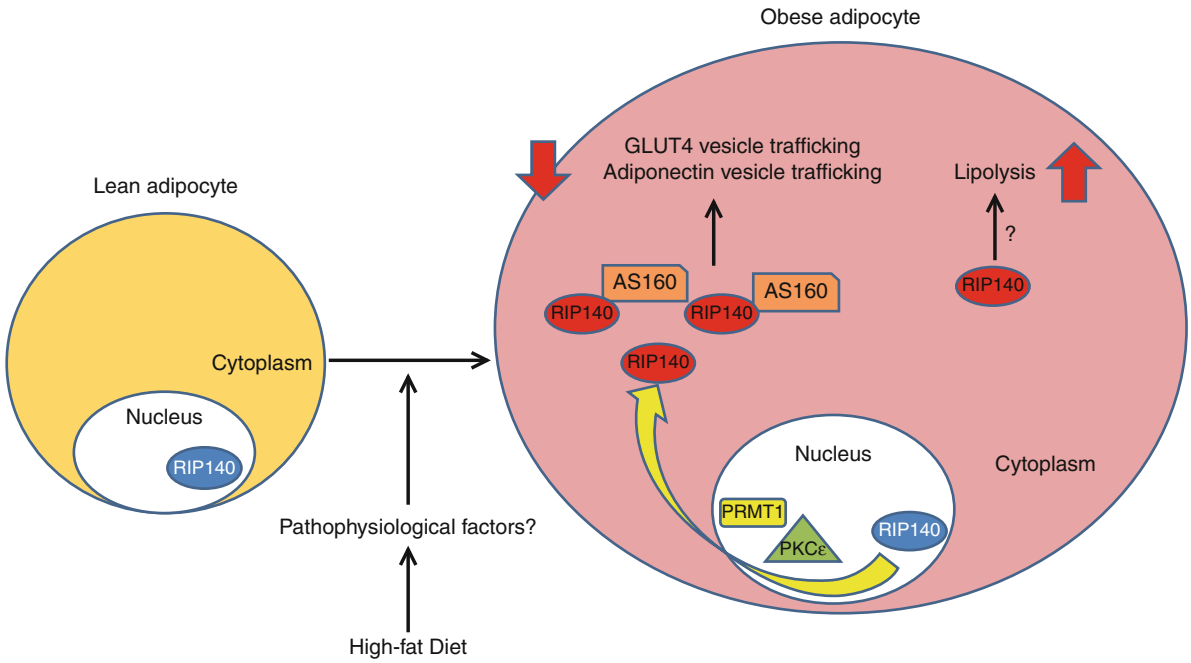
RIP140-null mice exhibit a lower fat content and are resistant to diet-induced metabolic disorders. In the 3T3-L1 adipocyte differentiation model, depletion of RIP140 can substantially change lipid accumulation, fatty acid oxidation, glycolysis, glucose uptake, and mitochondria biogenesis in these cells. These results demonstrate RIP140's activity in modulating metabolism in adipocytes. Among potential target genes of RIP140 in adipocytes, UCP-1 is important for energy metabolism. In brown adipocytes that express a higher level of coactivator PGC-1, UCP-1 is up-regulated to enhance thermogenesis. In white adipocytes where RIP140 is detected at a higher level, UCP-1 is down-regulated to reduce thermogenesis. Importantly, in RIP140-null animals, white adipose tissue gains certain brown adipocyte features, indicating potential antagonism between RIP140 and PGC-1 in adipocyte metabolism. Recently, RIP140 is also found to have specific functions in the cytoplasm of adipocytes (see later section).

Role in Hepatocytes

In liver cells, RIP140 can function as a corepressor or coactivator for LXR. As a corepressor of LXR, RIP140 suppresses PEPCK expression to reduce gluconeogenesis. As a coactivator of LXR, RIP140 enhances SREBP-1c and FAS expression to increase lipogenesis. Interestingly, RIP140-null mice are resistant to the development of hepatic steatosis under a high-fat diet (Fritah et al. 2010b). In studying these animals, RIP140 has also been identified as an important regulator for triglyceride storage in sepsis, starvation, and cancer cachexia (Diaz et al. 2008). These results indicate a potential role for RIP140 in liver lipogenesis and triglyceride storage.

Role in Muscle Cells

RIP140 is differentially expressed in muscle cells: glycolytic fibers express a higher level, but oxidative fibers express a lower level, of RIP140 (Fritah et al. 2010b; Seth et al. 2007). Microarray and metabolic analyses of muscle cells reveal that depleting RIP140 enhances the expression of genes involved in oxidative phosphorylation, fatty acid oxidation, and mitochondria biogenesis. This study demonstrates the role of RIP140 in controlling muscle metabolism and provides a clue for the defect of cardiac function in



nrip1 (Nuclear Receptor-Interacting Protein 1), Fig. 4 The activities of cytoplasmic RIP140 related to adipocyte dysfunctions. In a normal state, adipocytes are lean and RIP140 is mainly localized within nuclei. After a short-term high-fat diet, certain pathophysiological factors up-regulate nuclear PKCε activity and promote nuclear export of RIP140 by a PKCε-PRMT1-dependent pathway. Cytoplasmic RIP140 interacts with AS160 to reduce GLUT4 and adiponectin vesicle trafficking and promote lipolysis by unknown mechanisms

RIP140 overexpressed transgenic mice (see later sections).

Role in Macrophages

For macrophages, RIP140 can function by interacting with ▶ NF-κB and CBP (Zschiedrich et al. 2008). This is important for LPS-stimulated transcription of several inflammatory genes. Depleting RIP140 in macrophages impairs LPS-stimulated proinflammatory cytokine production, because NF-κB transcriptional activity is reduced. This study demonstrates a regulatory role for RIP140 in classic (M1) macrophage activation and suggests that RIP140 may be involved in inflammation and/or inflammation-related diseases. Interestingly, PGC-1 beta has been demonstrated as an important activator for anti-inflammatory (M2) activation of macrophages. This also suggests a potentially antagonistic relationship between PGC-1 and RIP140 in regulating macrophage activation.

Role in Cardiomyocytes

In studying whole-body RIP140-overexpressing transgenic mice, a role for RIP140 in cardiac hypertrophy and functions was identified (Fritah et al. 2010a). This finding suggests a need to control the expression level of RIP140 in cardiomyocytes. However, it remains unclear if the cardiac defects are results directly from the expression of RIP140 in cardiomyocytes, or that may be caused by systemic changes in whole body metabolism. However, since lipid usage in cardiomyocytes can significantly impact cardiac functions, it is important to evaluate the specific functions of RIP140 in cardiomyocytes.

Cytoplasmic RIP140: Player in Pathophysiology of Metabolic Diseases

The finding that PTMs dramatically alter RIP140's property and subcellular distribution (nuclear export) would suggest certain functions for RIP140 in the cytoplasm. Recently, studies have shown that a short-term high-fat diet can promote cytoplasmic accumulation of RIP140 in epididymal adipose tissue, accompanied by the up-regulation of nuclear PKCε activity which provides the initial trigger for

RIP140's nuclear export (Ho et al. 2009). Detailed molecular studies show that RIP140 interacts with AS160, which blocks AS160 inactivation by Akt/PKB and retards GLUT4 vesicle trafficking (Fig. 4). These studies establish the first cytoplasmic function of RIP140. In addition, cytoplasmic RIP140 can also retard adiponectin secretion through its action on AS160, and enhance lipolysis by an unknown mechanism (Fig. 4). These adipocyte defects could have severe pathological consequences. For instance, increased lipolysis in adipocytes causes high circulating fatty acid levels, and these fatty acids can accumulate in muscle cells, hepatocytes, and cardiomyocytes to trigger apoptosis. Adiponectin is the most abundant and protective adipokine that is known to modulate systemic glucose homeostasis and lipid metabolism. Reduction in adiponectin secretion is an important feature in diabetic mice and human patients.

The findings that cytoplasmic accumulation of RIP140 in adipocytes may contribute to their dysfunctions such as impaired glucose uptake, adipokine secretion, and lipolysis would strongly support its functional role in regulating systemic metabolism as demonstrated in whole-body knockout mice, which, in part, is attributable to the cytoplasmic RIP140. In animals, a high-fat diet promotes RIP140's nuclear export in adipocytes. From a clinical point of view, targeting cytoplasmic RIP140, or blocking signaling pathways that promote RIP140's cytoplasmic accumulation, may provide a more specific, beneficial/protective effect in the management of metabolic disorders.

Summary

RIP140 was first identified as an universal gene transcriptional corepressor that acts, primarily, on nuclear receptors in a ligand-enhanced manner. Its gene repressive activity is mediated by its four RDs that recruit various corepressive factors and histone modifying enzymes. RIP140 can be extensively modified by PTMs, which drastically affect its ability to recruit its interacting partners and alter its subcellular distribution, stability, and functions. Studies of whole-body gene knockout and over-expression transgenic mice provide insights into its physiological role, which is to modulate metabolic, inflammatory, and reproductive activities in animals. Studies of its PTMs reveal its cytoplasmic distribution and novel functions in the cytoplasm, which is to modulate insulin sensitivity, glucose homeostasis, and lipid metabolism through interacting with various signaling molecules and adaptors in the cytoplasm. In the nuclei, it acts, primarily, to antagonize coactivators' activity such as that of PGC-1. In the cytoplasm, it acts also to antagonize specific signaling pathways such as insulin signaling, glucose uptake, and lipolysis control. In the context of system biology, RIP140 seems to provide one counteracting mechanism to modulate most of the stimulating or activating signals including hormones and cytokines. It is tempting to speculate a Yin-Yang regulatory principle manifested through RIP140, both in the nucleus and the cytoplasm, to counteract most of the stimulating or activating including hormones and cytokines.

This would suggest that targeting RIP140 might be a promising strategy in treating metabolic disorders that are mostly due to disturbance in hormone or cytokine signals that disrupt normal biological processes. However, the promiscuous feature of RIP140's antagonistic activity toward a wide variety of hormonal inputs or cytokine signals may undermine the application of this strategy. To this end, a careful dissection of its extensive PTMs to understand the regulation of its specific biological activity can be very helpful. Additionally, studying its regulation specifically through degradation may provide new clues.

References

Berriel Diaz M, Krones-Herzig A, Metzger D, Ziegler A, Vegiopoulos A, Klingenspor M, et al. Nuclear receptor cofactor receptor interacting protein 140 controls hepatic triglyceride metabolism during wasting in mice. Hepatology. 2008;48(3):782–91.

Cavailles V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, et al. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J. 1995;14(15):3741–51.

Fritah A, Steel JH, Nichol D, Parker N, Williams S, Price A, et al. Elevated expression of the metabolic regulator receptor-interacting protein 140 results in cardiac hypertrophy and impaired cardiac function. Cardiovasc Res. 2010a;86(3):443–51.

Fritah A, Christian M, Parker MG. The metabolic coregulator RIP140: an update. Am J Physiol Endocrinol Metab. 2010b;299(3):E335–40.

Gupta P, Ho PC, Huq MD, Khan AA, Tsai NP, Wei LN. PKCepsilon stimulated arginine methylation of RIP140 for its nuclear-cytoplasmic export in adipocyte differentiation. PLoS ONE. 2008;3(7):e2658.

Ho PC, Gupta P, Tsui YC, Ha SG, Huq M, Wei LN. Modulation of lysine acetylation-stimulated repressive activity by

Erk2-mediated phosphorylation of RIP140 in adipocyte differentiation. Cell Signal. 2008;20(10):1911–9.
Ho PC, Lin YW, Tsui YC, Gupta P, Wei LN. A negative regulatory pathway of GLUT4 trafficking in adipocyte: new function of RIP140 in the cytoplasm via AS160. Cell Metab. 2009;10(6):516–23.
Huq MD, Khan SA, Park SW, Wei LN. Mapping of phosphorylation sites of nuclear corepressor receptor interacting protein 140 by liquid chromatography-tandem mass spectroscopy. Proteomics. 2005;5(8):2157–66.
Huq MD, Tsai NP, Lin YP, Higgins L, Wei LN. Vitamin B6 conjugation to nuclear corepressor RIP140 and its role in gene regulation. Nat Chem Biol. 2007;3(3):161–5.
Huq MD, Ha SG, Barcelona H, Wei LN. Lysine methylation of nuclear co-repressor receptor interacting protein 140. J Proteome Res. 2009;8(3):1156–67.
Kerley JS, Olsen SL, Freemantle SJ, Spinella MJ. Transcriptional activation of the nuclear receptor corepressor RIP140 by retinoic acid: a potential negative-feedback regulatory mechanism. Biochem Biophys Res Commun. 2001;285(4):969–75.
Lee CH, Wei LN. Characterization of receptor-interacting protein 140 in retinoid receptor activities. J Biol Chem. 1999;274(44):31320–6.
Lee CH, Chinpaisal C, Wei LN. Cloning and characterization of mouse RIP140, a corepressor for nuclear orphan receptor TR2. Mol Cell Biol. 1998;18(11):6745–55.
Mostaqul HMD, Gupta P, Wei LN. Post-translational modifications of nuclear co-repressor RIP140: a therapeutic target for metabolic diseases. Curr Med Chem. 2008;15(4):386–92.
Seth A, Steel JH, Nichol D, Pocock V, Kumaran MK, Fritah A, et al. The transcriptional corepressor RIP140 regulates oxidative metabolism in skeletal muscle. Cell Metab. 2007;6(3):236–45.
Strom K, Hansson O, Lucas S, Nevsten P, Fernandez C, Klint C, et al. Attainment of brown adipocyte features in white adipocytes of hormone-sensitive lipase null mice. PLoS ONE. 2008;3(3):e1793.
Thenot S, Charpin M, Bonnet S, Cavailles V. Estrogen receptor cofactors expression in breast and endometrial human cancer cells. Mol Cell Endocrinol. 1999;156(1–2):85–93.
Tsai NP, Lin YL, Wei LN. MicroRNA mir-346 targets the 5′-untranslated region of receptor-interacting protein 140 (RIP140) mRNA and up-regulates its protein expression. Biochem J. 2009;424(3):411–8.
White R, Leonardsson G, Rosewell I, Ann Jacobs M, Milligan S, Parker M. The nuclear receptor co-repressor nrip1 (RIP140) is essential for female fertility. Nat Med. 2000;6(12):1368–74.
Zschiedrich I, Hardeland U, Krones-Herzig A, Diaz MB, Vegiopoulos A, Muggenburg J, et al. Coactivator function of RIP140 for NFkappaB/RelA-dependent cytokine gene expression. Blood. 2008;112(2):264–76.

NSP, Novel Serine Protease

► SARA

Ntk

► CSK-Homologous Kinase

NTPDase

► E-NTPDase Family

NTR

► Neurotensin Receptor (NTSR)

NTS

► Neurotensin Receptor (NTSR)

N-Type

► Voltage-Gated Calcium Channels: Structure and Function (CACNA)

Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells

► NF-κB Family

Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Zeta

► IkBz

Nuclear Factor-Kappa-B

► NF-κB Family

Nuclear Mitogen- and Stress-Activated Protein Kinase-1

▶ MSK1

Nuclear Receptor Subfamily 1, Group I, Member 1 (NR1I1)

▶ VDR, The Vitamin D Receptor

Nucleoside Triphosphate Diphosphohydrolase

▶ E-NTPDase Family

Nucleotide Receptor P2x

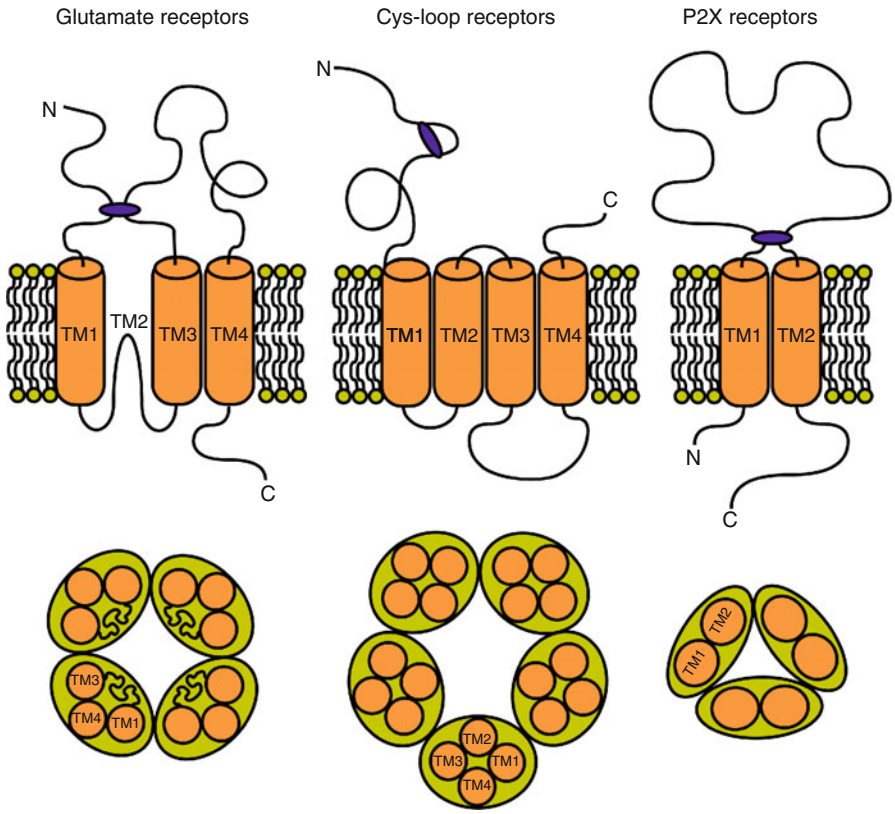
Jian-Bing Shen[1], Bruce T. Liang[1] and Florentina Soto[2]
[1]Calhoun Cardiovascular Center, University of Connecticut Health Center, Farmington, CT, USA
[2]Department of Ophthalmology & Visual Sciences, Washington University in St. Louis, St. Louis, MO, USA

Historical Background

The first evidence of purinergic signaling was described in 1929, when purines were found to underlie physiological responses in the circulatory and digestive system. After 50 years and a wealth of data supporting purine mediated effects in different systems, Burnstock presented the first direct evidence that ATP acts as a transmitter and introduced the concept of purinergic neurotransmission (Burnstock et al. 2010). Thus, ATP was recognized as both an intracellular energy source and an extracellular signaling molecule. Extracellular ATP has been implicated in intercellular communication in a wide variety of cells from different organisms and associated with a diverse array of biological effects. ATP is an ideal molecule for extracellular signaling, it is small, rapidly diffusing, highly unstable due to the presence of extracellular degrading enzymes and not abundant in the extracellular environment at resting conditions (Soto et al. 1997). ATP exerts it actions by binding to cell surface receptors called P2 receptors. P2 receptors are subsequently divided into two different families: P2Y receptors are metabotropic G-protein-coupled receptors while P2X receptors are ligand-gated ion channels (North 2002). Binding of ATP to P2X receptors opens within milliseconds an integral ion channel. While P2X receptors are functional homologues to the Cys-loop and glutamate receptor families of ligand-gated ion channels, they form a structurally distinct group of membrane receptors. Purinergic signaling via P2X receptors has a remarkably wide range of action, influencing epithelia and endocrine cell secretion, immune and inflammatory processes, cardiovascular performance, skeletal and smooth muscle contraction, and glial and neuronal function (Surprenant and North 2009). This chapter is devoted to the molecular and functional properties of P2X receptors and their involvement in physiological and pathological processes.

Molecular Properties of P2X Subunits

The first two P2X subunits (P2X1 and P2X2) were isolated from rat vas deferens smooth muscle and from PC12 cells by expression cloning. Based on sequence similarity, five additional subunits were identified in the rat and shortly after in human and mouse tissues (North 2002). The sequence identity between subunits (approx. 30–50%) and the lack of similarity to other cloned ligand-gated ion channels indicated they constitute a new family of membrane receptors. P2X subunits have been isolated and characterized from additional vertebrate classes (e.g., aves) and are present in all vertebrate species. They have also been found in fish, protozoa, trematode, fungi, and algae. In contrast, no P2X subunits have been identified in the genomes of the nematode worm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*), or in prokaryotes (Fountain and Burnstock 2009). Mammalian P2X receptor subunits are 379–595 amino acids long. Multiple splice variants of the originally cloned P2X subunits have been described, showing different amino acid lengths and properties. It was predicted using hydrophobicity plots that each subunit has two transmembrane domains linked by an



Nucleotide Receptor P2x, Fig. 1 *Ligand-gated receptor membrane topology and stoichiometry*. The membrane topology of a single subunit belonging to the three main families of ligand-gated ion channels is shown. The place of agonist interaction for the three types of receptors is shown in *purple*. In the lower part of the Figure, the arrangement of subunits around the channel pore to form a functional receptor is depicted. TM: transmembrane domain. The membrane topology and stoichoimetry is shared between the P2X receptor family and the degenerins/ENaC/ASIC family

extracellular loop comprising between 50% and 70% of the total protein length (Fig. 1). This extracellular loop contains 10 cysteine residues conserved in all cloned vertebrate P2X subunits. Both N- and C-terminus were suggested to be intracellular, with the length of the C-terminal domain being the main source of structure variation between the different subunits. The predicted membrane topology has been confirmed using multiple approaches, including mutating the extracellular domain, use of extracellular antibodies, concatemers and chimeric constructs (North 2002). The proposed membrane topology differs from that of the members of Cys-loop receptors superfamily and glutamate ionotropic receptors family but closely resembles that of the degenerins/ENaC/ASIC family (Fig. 1) (North 2006). P2X receptors are trimeric combinations of P2X subunits. Questions about the membrane topology of P2X subunits as well as the quaternary structure of P2X receptors were recently and definitely answered by X-ray crystallography (Kawate et al. 2009). The authors solved the crystal structure of zebrafish P2X4 receptor in its closed stated, as a symmetrical assembly of three P2X subunits surrounding a central ionic channel pore. This study confirms the proposed membrane structure and will further the understanding about the protein domains involved in P2X receptor function.

Genomic Organization and Splicing

The chromosomal localization (obtained from the Ensembl database, www.ensembl.org) of the seven P2X subunit genes is summarized in Table 1. Several P2X genes are localized in the same human chromosome. Thus, P2X4 and P2X7 genes are located in the long arm of chromosome 12. Similarly, P2X1 and P2X5 genes are located within 1 Mb in the short arm of chromosome 17. A co-localization of paralog genes in chromosomes could arise from tandem duplication. However, it might also indicate the formation of a gene cluster, in which the expression of functionally related genes is co-regulated (Makino and McLysaght 2008). This could be the case for P2X subunits since

Nucleotide Receptor P2x, Table 1 Chromosomal location and length of human P2X subunit genes

Gene	Chromosomal location	Gene length (Kb)
P2RX1	17p13.2	20.1
P2RX2	12q24.3	3.7
P2RX3	11q12.1	31.6
P2XR4	12q24.3	24.2
P2XR5	17p13.2	23.9
P2XR6	22q11.2	13.8
P2XR7	12q24.3	53.2

Data obtained from the Emsembl database at http://uswest.ensembl.org/index.html.

co-expression of P2X4 and P2X7 has been detected in many different tissues and cell types, including microglia, vascular endothelium, ciliated epithelium, and the immune system. Moreover, P2X1 and P2X5 subunits co-express and heteromerize to form the P2X receptor in astrocytes (Surprenant and North 2009).

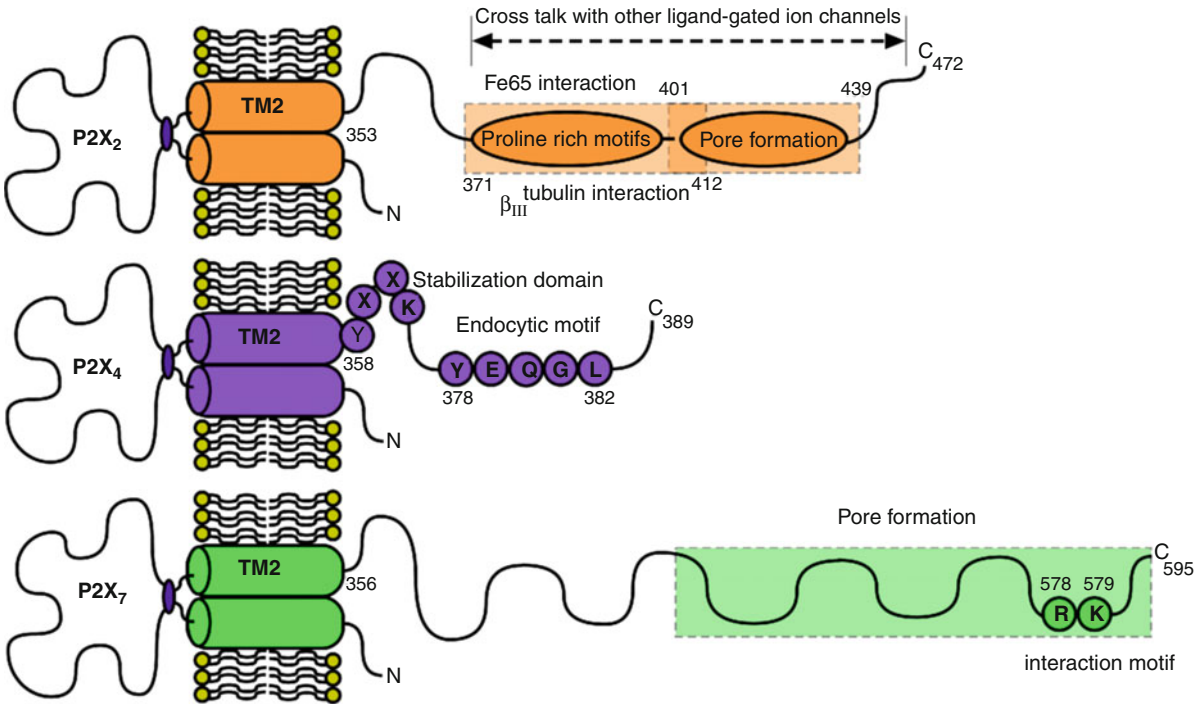
The number of exons comprising the sequence of P2X subunits varies between 11 for P2X2 and 15 for P2X7 (Cheewatrakoolpong et al. 2005, Nicke et al. 2009), while the remaining P2X genes contain 12 identified exons. Most exon–intron borders are conserved between the different genes, indicating, as expected, a common evolutionary origin. In contrast, intronic length varies largely between genes, as reflected by the differences in gene length listed in Table 1. Splice variants have been identified for all P2X subunits. Many of the isolated splice variants lack transmembrane domains or part of the extracellular domain giving rise to truncated P2X subunits that are not able to assemble in functional receptors. In addition, several subunits present a shorter version of their C-terminal domain due to the use of cryptic splice sites inside exon sequences or of inclusion of intron sequences in the corresponding DNA (Cheewatrakoolpong et al. 2005, Koshimizu and Tsujimoto 2006). The importance of this splicing in the receptor function will be described in the next section in more detail.

Regulation of P2X Receptors via Their C-terminal Domain

The C-terminal domain of P2X subunits is the least conserved part of the protein both in length and amino acid composition (North 2002), indicating that it might confer subunit specific properties. Moreover, the intracellular location of the domain makes it a candidate for interaction with intracellular adaptor and cytoskeletal proteins. Indeed, it has been shown that the P2X2 C-terminus interacts with the adaptor protein Fe65, a cytosolic protein containing several protein-binding domains (Fig. 2). P2X subunits and Fe65 colocalize in brain synapses and Fe65 has been shown to modify the functional properties of P2X2 receptors upon co-expression in heterologous systems (Masin et al. 2006). A splice variant of P2X2 with a 69 amino acid deletion in the C-terminal domain, assemble into a membrane receptor with different kinetic properties, and it does not interact with Fe65 (Masin et al. 2006).

A role for the cytosolic C-terminal domain of the P2X4 subunit in trafficking of the receptor in and out of the plasma membrane has been described. Thus, a tyrosine-based non-canonical sorting signal in the C-terminal tail of P2X4 subunits (YXXGL, Fig. 2) that directly binds to the AP2 adaptor protein has been identified. When the interaction was disrupted by mutations in the sorting signal, an increase in the surface expression of $P2X_4$ receptors in transfected neurons was observed (Murrell-Lagnado and Qureshi 2008). In addition, another tyrosine-containing domain (YXXXK) present in the proximal C-terminus of all P2X subunits was shown to be involved in membrane stabilization of P2X receptors (Murrell-Lagnado and Qureshi 2008).

Arguably, the best studied C-terminal domain is that of P2X7 receptors. In the immune system, P2X7 receptors mediate ATP-induced inflammatory responses by activating caspase-1 and the subsequent release of interleukins. Macrophages of a mouse line in which the C-terminal domain of P2X7 has been deleted by knock-in methods lack the immune response elicited by ATP. Moreover, efficient caspase-1 activation by ATP requires priming by bacterial membrane lipopolysaccharides (LPS) and an LPS binding domain has been identified in the distal C-terminal sequence (Surprenant and North 2009) (Fig. 2). P2X7 receptors have been shown to interact with many intracellular proteins, including pannexin-1, protein tyrosin-phosphatase β, heat shock, and epithelial membrane proteins (Surprenant and North 2009); however, the domains mediating the interaction are not known. In addition to playing a role in protein interaction and intracellular signaling, the C-terminal domain is modulating the channel properties of P2X7 receptors.



Nucleotide Receptor P2x, Fig. 2 *Carboxy terminus of P2X subunits.* Representation of the functional and interacting domains present in the C-termini of P2X2, P2X4, and P2X7 subunits. The surface stabilization sequence YXXK present in all P2X subunits C-terminus proximal domain is represented only for P2X4

Thus, the increases in permeability to big cations or fluorescent dyes of P2X7 receptors upon repetitive application of ATP also depend on the C-terminal domain (North 2002).

Pharmacology, Tissue Expression, and Physiological Roles of P2X Receptors

In considering the pharmacology and P2X receptors (P2XR), a number of considerations are needed. First, P2X receptors are ligand-gated ion channels, and as such serve as both cell-surface receptors and ion channels. The natural agonist is extracellular ATP or adenine nucleotides. A number of nucleotide and non-nucleotide antagonists have been developed with selectivity at each of the seven P2X receptors. Most of the agonists are relatively nonselective while the antagonists with a high degree of selectivity have been developed. Second, the endogenous or native P2XR may be homomeric or heteromeric. Each P2X channel is a subunit of the trimeric channel. Each P2X receptor is capable of complexing with itself to form a homotrimeric channel or with another P2X receptor as a heterotrimer. A total of seven heterotrimers have been demonstrated. P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6, P2X4/7. Pharmacology of heterologously expressed P2XR subtype in oocytes or HEK293 cells is often different from that of P2XR in native tissues. Third, agonist and antagonist selectivity is different at homomeric vs. heteromeric receptors. Individual P2X subunit of a heterotrimeric P2XR retains some of its selective characteristic response to agonist, antagonist, extracellular zinc, pH, or desensitization kinetics. Fourth, heteromers can exhibit unique pharmacology of agonist and/or antagonist actions or other properties that are different from those for homomeric receptors. In some heteromers, the individual characteristic properties specific to one of its subunit can become dominant and make the heteromer more like the homomeric assembly of the "dominant" subunit. Table 2 summarizes the EC50s of known agonists and IC50 of antagonists at both homomeric and heteromeric receptors (Ralevic and burnstock 1998; Gever et al. 2006; North 2002; Jarvis and Khakh 2009; Roberts et al. 2006; Burnstock and Knight 2004). Table 3

Nucleotide Receptor P2x, Table 2 Agonists and antagonists at P2X receptors

Agonists	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6	P2X7	P2X1/2	P2X1/4	P2X1/5	P2X2/3	P2X2/6	P2X4/6	P2X4/7
ATP	≤1	2–10	≤1	7–10	5–10	10	≥100	0.5–0.6	10	1–5	0.7–2	30	6	>300
2-meSATP	≤1	≤2	≤1	10	10	9	100	0.07		1	1	35	7	
α, β-meSATP	≤1	>300	≤1	>300	>300	>100	>300	0.1	10	3	5	>100	12	
BZ-ATP	0.003	0.8	0.08	7	>500		20	0.003			0.8			9
Antagonists														
Suramin	1–5	1–10	3–5	>500, 180[a]	4	>100	>300				10			
PPADS	1	1–2	1	>100, 28[a]	3	>100	3–4							
TNP-ATP	0.001–0.006	≥1	0.001	15	>10		>30		0.5	0.4	0.007			1
NF 449	0.0003, 0.0005[a]	>50	2	>100			>100, 40[a]			0.0007	0.12			
NF 023	0.2	>50	30	>100										
NF 279	0.02, 0.05[a]	0.8 –1	2	>300			10–20, 3[a]							
RO 0437626	3	>100	>100								>100			
NF 110	0.08	4	0.01	>300										
RO −3	>10	>10	0.01–0.1	>10	>10		>10				1–2	>10		
A 317491	>10	>100	0.1	>100		>100	>100				0.1			
5 -BDBD				0.5										
A 740003	>100	>100	>100	>100		>100	0.02–0.05							
A 438079	>100	>100	>100	>100		>100	0.06–0.07							
A 804598	>100	>100	>100	>100		>100	0.01							
GSK314181A	>10	>10	>10	>10			0.1							
AZ11645373	>10	>10	>10	>10	>10		0.1							
IP_5I	0.003	>300	3–7	Potentiation							3			
KN −62							0.3							

[a]Human P2XR

Value of EC50 (agonists) or EC50 (antagonists) are μM. Most of the data are taken from Antonio et al. (2009), Aschrafi et al. (2004), Ase et al. (2010), Burnstock and Knight (2004), Burnstock et al. (2010). Additional data are taken from Donnelly-Roberts et al. (2008), Evans et al. (1995), Rettinger et al. (2000), Klapperstück et al. (2000), Rettinger et al. (2005), Hechler et al. (2005), Hülsmann et al. (2003), Ford et al. (2006), Honore et al. (2006), Donnelly-Roberts et al. (2009), King (2007), Sneddon et al. (2000), Soto et al. (1999), Hausmann et al. (2006), Kassack et al. (2004), King et al. (2004), Jaime-Figueroa et al. (2005)

ATP adenosine 5′-triphosphate, *2meSATP* 2-methythioadenosine 5′-triphosphate, *α,β-meATP* 2-methythioadenosine 50-triphosphate, *BzATP* 2,3-O-(4-benzoylbenzoyl)-ATP, *PPADS* pyridoxal-5′-phosphate-6-azophenyl-20 0,40 0-disulphonic acid, *TNP-ATP* 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate, *IP5I* diinosine pentaphosphate, *KN-62* 1-[N,O-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, *A-317491* 5-({(3-phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}carbonyl)-1,2,4-benzenetricarboxylic acid, *RO-3* 5-(2-isopropyl-4,5-dimethoxybenzyl)pyrimidine-2,4-diamine, *A-740003* N-[1-(N0 0-cyano-N′-quinolin-5-ylcarbamimidamido)-2,2-dimethylpropyl]-2-(3,4-dimethoxyphenyl)acetamide, *A-438079* 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine, *A-804598* 2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine, *MRS2179* 20 0-deoxy-N^6-methyl adenosine 3′ 0,5′ 0diphosphate, *NF279* 8,80-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis(naphthalene)-1,3,5-trisulfonic acid, *NF449* 4-[({3-[({3,5-bis[(2,4-disulfophenyl)carbamoyl]phenyl}carbamoyl)amino]-5-[(2,4-disulfophenyl)carbamoyl]phenyl}carbonyl)amino]benzene-1,3-disulfonic acid, *GSK314181A* 5-{[(3R)-3-aminopyrrolidin-1-yl]methyl}-2-chloro-N-(tricyclo[3.3.1.13,7]dec-1-ylmethyl)benzamide, *AZ11645373* 3-(1-(30-nitrobiphenyl-4-yloxy)-4-(pyridine-4-yl)butan-2-yl) thiazolidine-2,4-dione, *Cibacron Blue* 1-amino-4-(4-(4-chloro-6-(2-sulfophenylamino)-1,3,5-triazin-2-ylamino)-3-sulfophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid

Nucleotide Receptor P2x, Table 3 Tissue distribution

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$	$P2X_{1/2}$	$P2X_{1/4}$	$P2X_{1/5}$	$P2X_{2/3}$	$P2X_{2/4}$	$P2X_{2/6}$	$P2X_{4/7}$
Skeletal muscle	+	–			+	+	+							
Cardical muscle	+	–	+	+	+	+								
Smooth muscle														
Urinary bladder	+	+		+	+	+	+							
Gut	+	+					+							
Blood vessels	+	+	+	+	+		+		+					
vas Deferens	+	+		+			+							
Ureter	+	+			+	+								
Overy		+												
Epithelial cell														
Nasal mucosa		+			+									
Gut		+			+		+							
Bladder			+		+	+	+							
Ureter				+	+	+	+							
Skin					+		+							
Bronchial						+								
Salivary gland				+										
Overy	+	+				+								
Thymus		+	+			+	+							
Blood vessels	+	+	+	+	+		+							
Organs														
Lung	+	–		+	+									
Trachea				+			+							
Spleen	+	–												
Liver		–		+										
Kidney	+	+	+	+	+	+	+							
Thymus	+			+		+	+							
Testis (Leydig)		+	+	+			+	+				+		
Salivary gland				+			+							
Adrenal gland		+	+	+	+	+	+							
Pancrease				+										
Uterus						+								
CNS														
Brain	+	+	+	+	+	+	+			+				
Spinal cord	+	+	+	+		+	+							

(*continued*)

Table 3 (continued)

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$	$P2X_{1/2}$	$P2X_{1/4}$	$P2X_{1/5}$	$P2X_{2/3}$	$P2X_{2/4}$	$P2X_{2/6}$	$P2X_{4/7}$
Cortical astrocytes	+				+					+				
Neuron ganglion														
Sensory	+	+	+	+		+	+				+	+		
Dorsal root	+	+	+	+		+						+		
Trigeminal	+	+	+	+	+	+								
Coeliac	+	+	+	+		+	+							
Autonomic	+	+	–	+	+	+	+	+			+			
Esophagus myenteric														
Enteric sensory neurones													+	
Otic neurones		+	+								+			
Blood cells														
Plateletes	+													
Lymphocytes	+	+		+			+							
Granulocytes							+							
Monocytes							+							
Macrophage							+							+
Neutrophils	+			+	+		+							

+ Detected
– Not present

summarizes the tissue expression pattern of both homomeric and heteromeric P2X receptors (Ralevic and burnstock 1998; Gever et al. 2006; North 2002; Burnstock and Knight 2004; Surprenant and North 2009). Each homomeric or heteromeric receptor will be discussed in the following sections.

Homomeric P2X1R

P2X1 mRNA and protein are detected in a fairly broad range of tissues, such as urinary bladder, smooth muscle of small arteries and vas deferens, brain, spinal cord and several neuron ganglions, and platelets. Low levels of P2X1 are also found in lungs, spleen, and heart. Notably, P2X1R is the significant P2X subtypes in smooth muscle of blood vessels and other hollow organs including bladder, intestine, and vas deferens. Studies demonstrated that ATP or α,β-meATP could elicite an inward current and membrane depolarization, induce the contraction in a variety of smooth muscle tissues. These effects of that ATP or α,β-meATP in smooth muscle are eliminated or reduced in P2X1R knockout (KO) mice, confirming the significant role of P2X1R in the regulation of native smooth muscle contractility (Ralevic and burnstock 1998; Gever et al. 2006).

P2X1R can also regulate platelet functions as the main ATP-gated ion channel in platelets and megakaryocytes. The endogenous platelet P2XR has a similar pharmacology as that of the heterologously expressed recombinant P2X1R. In P2X1R KO mice, functions of platelets, such as aggregation, secretion, adhesion, and thrombus formation, are impaired. Vascular disease superimposed on P2X1R KO mice showed reduced mortality in the presence of systemic thromoembolism and laser-induced vessel injury. On the other hand, hypersensitive ex vivo platelet response and increased mortality in vivo secondary to increased thromboembolism were observed in transgenic mice overexpressing human P2X1R in megakaryocytic cell line (Gever et al. 2006).

Homomeric P2X2R

P2X2R is expressed throughout the central and peripheral nervous (CNS) systems as well as other non-neuron cell types, such as in bladder, adrenal medulla, endothelial and epithelial cells, skeletal, cardiac and smooth muscles, lymphocytes, intestine, and vas deferens (see Table 3). In the heart, P2X2R mRNA was detected in smooth muscle cells of coronary artery and only in atrium myocardium. Homemeric P2X2R appears to play a significant role in ATP-mediated fast synaptic transmission at both nerve terminals and interneuronal synapses. Thus, P2X2R is likely involved in memory, learning, motor function, autonomic coordination, and sensory integration in CNS as well as afferent and efferent signal pathway in peripheral nervous system (PNS). P2X2R is also expressed in many other non-neuron tissues in which its function is still not clear, although it may have a role in autocrine/paracrine hormone release, exocytosis/endocytosis, smoother muscle contractility, and pacemaker activity.

Heteromeric P2X1/2R

Phenotypically, heteromeric P2X1/2R is identical to homomeric P2X1R except the different sensitivity to pH changes. Furthermore, Aschrafi et al. found that assembly of heteromeric P2X1//P2X2R is favored over the respective homomeric P2X1R. It suggested that ATP-stimulated currents originally attributed to homomeric P2X1R in native tissue may actually be mediated by heteromeric P2X1/2R (Aschrafi et al. 2004). Using pharmacology profile and specific P2X subunit gene knockout mice, Calvert JA et al. confirmed existence of functional heteromeric P2X1/2R in sympathetic neurons from the superior cervical ganglion and it is implied that heteromeric P2X1/2R may have a broad role in regulation of the neuron function. Although the dominant phenotype of endogenous P2XR in sympathetic neurons is P2X2-like, a subpopulation of neurons showed $P2X_1$ property such as α,β-meATP responsiveness that was reduced in P2X1R KO mice. P2X2-like means slow desensitization, sensitivity to blockade by antagonists, potentiation by acidic pH (this property is unique to P2X2 receptor) and extracellular zinc, and partial inhibition by high extracellular calcium. It is of interest that the α,β-meATP responsiveness was abrogated by high extracellular calcium and alkaline pH; the latter two properties are more characteristic of P2X2R. Thus, a presumed heteromeric P2X1/2 native receptor includes properties of both subunits (Calvert and Evans 2004).

Homomeric P2X3R

Homomeric P2X3R has restricted distribution and is only expressed in peripheral terminals of unmyelinated C-fiber and thinly myelinated afferent neurons, such as trigeminal and dorsal root ganglions. These receptors

are mainly expressed on nociceptive sensory neurons. P2X3R can be activated by both α,β-meATP and 2-methylthioATP and are sensitive to blockade by suramin, PPADS, and TNP-ATP and is selectively antagonized by NF023. The functional role of homomeric P2X3R in these neurons is to mediate the sensory neurotransmission.

Heteromeric P2X2/3R

These heteromeric receptors have a mixed property that incorporates that of both P2X2 and P2X3 receptors. Thus, they can be activated by α,β-meATP and high sensitivity by TNP-ATP (P2X3-like), and show slow desensitization and potentiation by acidic pH (P2X2-like). A feature unique to this heteromer is that diinosine pentaphosphate is a much more potent blocker at homomeric P2X3 than at heteromeric P2X2/3R. This feature is useful in characterizing whether any of the native tissue P2XR is a P2X2/3R heteromer. These heteromers are expressed in subpopulations of sensory neurons, sympathetic ganglion cells, and brain neurons. They are thought to be important in initiating sensory signaling in pathways for taste, chemocreception, visceral distension, and neuropathic pain (Ralevic and Burnstock 1998). Heteromeric P2X2/3R is the first heteromeric channel to be studied following gene knockout confirmed the role of heteromeric P2X2/3R in several sensory signaling (Roberts et al. 2006).

Homomeric P2X4R

Homomeric P2X4R is not activated by α,β-meATP but is responsive to ATP and 2-methylthioATP. Of all the P2X receptors, it is the only receptor that can be potentiated by ivermectin via allosteric enhancement. Another unique feature is its lack of sensitivity to blockade by suramin or PPADS (North 2002). It has a desensitization kinetics intermediate between that for P2X1 and P2X2 receptors. It may be the most widely distributed of all the P2X receptors with expression in brain and spinal cord, automatic and sensory ganglions, arterial smooth muscle, osteoclasts, parotid acinar cells, kidney, lung, heart, bladder, thymus, colon, pancreas, and B lymphocytes (Gever et al. 2006; Burnstock and Knight 2004). However, the functional roles in some of these tissues are not clearly defined yet. However, recent studies showed that P2X4R are expressed in microglial cells and their activation can mediate neuropathic pain. P2X4R are also expressed in the endothelium in which activation can mediate nitric oxide-mediated vasorelaxation/vasodilatation. Global P2X4R KO mice showed hypertension and smaller arteries. Recent studies have also implicated P2X4 receptor as an important subunit of the endogenous cardiac myocyte P2X receptors. Increased expression of cardiac P2X4R by cardiac myocyte-specific transgenic overexpression or by stimulation with hydrolysis-resistant P2X agonist can confer a protected phenotype in models of both ischemic and non-ischemic heart failure (Zhou et al. 2010). Expression of a P2X4-like contractile phenotype in human atrial myocardium was recently described. P2X4R may also interact with P2X7 receptors in inflammatory responses such as pain signaling (see P2X7 receptors).

Heteromeric P2X1/4R

Both the P2X1R and P2X4R are expressed in the smooth muscle in renal resistance arteries. α,β -meATP evoked a spike-like membrane depolarization followed by a sustained depolarization which could be partially blocked by nanomolar P2X1 selective antagonist, NF279. The residual current could further be blocked by millimolar NF279, consistent with the existence of heteromeric P2X1/4R. Thus, heteromeric P2X1/4R showed both agonist and antagonist pharmacology that are more P2X1-like. TNP-ATP can block this heteromer with affinity that is higher than but closer to that for the homomeric P2X1 receptor. The P2X1/4R heteromer participates in the sympathetic control and paracrine regulation of renal blood flow (Harhun et al. 2010).

Homomeric P2X5R

Homomeric P2X5R is expressed in brain, spinal cord, heart, and eye. These homomeric receptors have pharmacology and desensitization kinetics similar to those of P2X2 receptors. The current mediated by rat P2X5R has a smaller amplitude than that induced by P2X1, P2X2, P2X3, or P2X4 receptors. P2X5 channels have a uniquely high chloride conductance. Recently, high levels of homomeric P2X5R are found in differentiating tissues, such as skeletal muscle, epithelial cells of nasal mucosa, gut, bladder, utter, and skin. Activation of homomeric P2X5R inhibits proliferation while increases the differentiation of rat skeletal muscle satellite cells. Homomeric P2X5R may be also involved in the regulation of proliferation and differentiation of certain type of cancer cells in skin and prostate.

Heteromeric P2X1/5R

P2X1R and P2X5R can assemble into a heteromeric P2X1/5R. A defining property of this heteromer is its activation by α,β-meATP, which cannot activate P2X5R. Other characteristic properties of this heteromer is a greater sensitivity to activation by ATP, a biphasic response to ATP with a transient peak current followed by a sustained plateau current, and a sensitivity to TNP-ATP intermediate between the sensitive homomeric P2X1 and the insensitive homomeric P2X5 receptors. Further, this heteromer does not appear to dilate to a larger pore on prolonged ATP exposure. Although the physiological role of heteromeric P2X1/5R is not defined, it has been postulated that it may mediate excitatory junction potentials at arterial neuroeffector junctions in guinea pig (Gever et al. 2006; North 2002). Recently, Ase et al. identified the heteromeric P2X1/5R in astrocytes from mouse brain and implied that it might participate in the astroglial Ca^{2+} signaling and excitability (Ase et al. 2010).

Homomeric P2X6R

P2X6R is expressed mainly in CNS. However, P2X6R is the only P2X receptor that usually does not form the functional homomeric P2X6R without extensive glycosylation. When such functional homomeric P2X6R are formed (after glycosylation), the heteromer has significantly higher responsiveness to α,β-meATP. Since P2X4R and P2X2R are the other two P2X subunits that usually coexist with P2X6R, the physiological role of the P2X6R is mediated either by the heteromeric P2X2/6R or heteromeric P2X4/6R (Gever et al. 2006; North 2002).

Heteromeric P2X2/6R

The P2X2R and P2X6R can be co-immunoprecipitated after co-expression in HEK293 cells or oocytes. The P2X2/6R heteromer showed a hybrid sensitivity to blockade by suramin at pH 6.5. There was a bi-phasic inhibition with a high sensitivity P2X2-like component and a lower sensitivity portion that is more P2X6-like. In general, the pharmacology of P2X2/6R heteromer is similar to that of P2X2 receptors (much less like that of homomeric P2X6 receptors). A subtle difference is that the P2X2/6R heteromer has a greater sensitivity to pH and to α,β-meATP. This heteromer is expressed by respiratory neurons in the brain stem (Gever et al. 2006; North 2002).

Heteromeric P2X4/6R

P2X4 receptors can also form a heteromer with P2X6 receptors. The pharmacology of this heteromer is similar to that of the homomeric P2X4 receptor. The heteromer, like the homomeric P2X4 receptor, is relatively insensitive to blockade by PPADS or suramin or reactive blue-2. The heteromeric P2X4/6R is similar to homomeric P2X4 receptors in its potentiation by the P2X4-specific allosteric enhancer and by zinc. In fact, the P2X4/6R heteromer appears more sensitive to the potentiation effect of ivermectin. The incorporation of P2X6 receptor in this heteromer also modestly increases the sensitivity to 2-meSATP and α,β-meATP(Gever et al. 2006; North 2002). On tissue expression and function of P2X2/6R and P2X4/6R heteromers, Antonio et al. confirmed the presence of the heteromeric P2X2/6R and P2X4/6R in mouse Leydig cells using immunofluorescence and pharmacologic profiles. Both heteromers are involved in regulating testosterone secretion (Antonio et al. 2009). Heteromeric P2X2/6R and P2X4/6R are also found in rat dorsal root ganglion neuron where they contribute to the transmission of nociceptive message, especially under inflammatory condition (De Roo et al. 2003).

Homomeric P2X7R

Homomeric P2X7R is mainly localized on glia and immune cells such as mast cells, macrophages lymphocytes, erythrocytes, and erythroleumemia. With prolonged exposure to high extracellular ATP concentrations, the homomeric P2X7R becomes open to larger size molecules such as ethidium and YO-PRO-1, leading to cell death. At low ATP concentration, the receptor is a cation channel like the other P2X receptor. Activation of homomeric P2X7R has been associated with the processing and releasing of active interleukin-1β and interleukin-18 from immune cells and glia. Selective P2X7R antagonist KN-62 (known to be selective at human P2X7R) could block the release of interleukin-1 β in macrophages and microglia. This function of P2X7R is further confirmed in experiment in P2X7R KO mice, which fail to release interleukin-1 β when challenged by ATP or BzATP. P2X7R also has a role in mediating the release of cytokine, reactive oxygen species, and neurotransmitter in microglia and astrocytes. Other roles implicated include apoptosis around β-amyloid plaques in Alzheimer's disease model and neurodegeneration and cell death in models of spine

cord injury or cerebral ischemia (Gever et al. 2006; North 2002).

Heteromeric P2X4/7R

Homomeric P2X7R is structurally similar to other P2XR except its significantly longer intracellular C-terminal. Originally, P2X7R was thought not able to heteropolymorize with any other P2XR. However, Guo et al. reported a functional heteromeric P2X4/7R in macrophages, representing the latest example of heteromeric P2X receptors (Guo et al. 2007). The two P2X receptors can be co-immunoprecipitated in detergent extracts from co-transfected HEK293 cells and from murine macrophages that express endogenous P2X4 and $P2X_7$ receptors. A mutant P2X4R lacks ATP-gated channel activity but is capable of trafficking to the plasma membrane. When this mutant P2X4R (Named S341W) was co-expressed with P2X7R, ivermectin was able to potentiate the ATP-induced current in cells co-expressing these receptors. Since ivermectin could not potentiate homomeric P2X7R - mediated current, the potentiation by ivermectin of the co-expressed S341W P2X4R and P2X7R is most likely due to allosteric enhancement of the heteromer via the S341W P2X4 receptor. The overlapping expression of P2X4R and P2X7R have been identified in a number of tissues including some non-excitatory cells such as epithelial cells from salivary glands, exocrine pancreas, airway, leukocytes, microglial cells, and osteoclasts. The possible presence of heteromeric P2X4/7R in these tissues or cells raises the question that heteromeric P2X4/7R may mediate important physiological function(s) in these tissues (Dubyak 2007).

Future Directions

Further systematic examination of the effect of each agonist and antagonist will be needed at heteromeric P2X receptors. This can be performed in cells co-expressing individual P2X subunits using heterologous systems such as HEK293 cells or oocytes. Features including pharmacology, desensitization kinetics, and sensitivity to pH, extracellular calcium, or zinc will all need to be determined. Once characterized, these features should be compared to those obtained in the native tissues to ascertain whether they are similar. Similarity of features would support the particular heteromer as the native tissue receptor. Another possibility is that a heteromer may comprise three different P2X receptor subtypes. If true, this possibility will add further complexity.

Summary

P2X receptors are a family of ligand-gated ion channels. The natural agonist ligands are ATP. There are seven P2X receptors (P2X1-7) and each receptor is a subunit of the trimeric channel. Each subunit can complex with itself to form a homotrimeric channel or with other subunits to form a heterotrimeric channel. Evidence points to existence of both homo- and hetero-trimeric channels as endogenous channels in the tissue. In the heterotrimeric channel, molecular and pharmacological properties of the constituent subunit can both dominate or become masked. Evidence is accumulating to indicate potentially important biological and pathophysiolgical roles of these receptor channels. It is expected that many of these homo- or heterotrimeric channels will become novel therapeutic targets for various diseases. Much more work is needed.

Acknowledgment F.S. would like to thank Marianela Masin for help with the Figures.

N

References

Antonio LS, Costa RR, Gomes MD, Varanda WA. Mouse Leydig cells express multiple P2X receptor subunits. Purinergic Signal. 2009;5(3):277–87.

Aschrafi A, Sadtler S, Niculescu C, Rettinger J, Schmalzing G. Trimeric architecture of homomeric P2X2 and heteromeric P2X1 + 2 receptor subtypes. J Mol Biol. 2004;342(1):333–43.

Ase AR, Bernier LP, Blais D, Pankratov Y, Séguéla P. Modulation of heteromeric P2X1/5 receptors by phosphoinositides in astrocytes depends on the P2X1 subunit. J Neurochem. 2010;113(6):1676–84.

Burnstock G, Knight GE. Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol. 2004;240:31–304.

Burnstock G, Fredholm BB, North RA, Verkhratsky A. The birth and postnatal development of purinergic signalling. Acta Physiol. 2010;199(2):93–147.

Calvert JA, Evans RJ. Heterogeneity of P2X receptors in sympathetic neurons: contribution of neuronal P2X1 receptors revealed using knockout mice. Mol Pharmacol. 2004;65(1):139–48.

Cheewatrakoolpong B, Gilchrest H, Anthes JC, Greenfeder S. Identification and characterization of splice variants of the human P2X7 ATP channel. Biochem Biophys Res Commun. 2005;332(1):17–27.

De Roo M, Rodeau JL, Schlichter R. Dehydroepiandrosterone potentiates native ionotropic ATP receptors containing the P2X2 subunit in rat sensory neurones. J Physiol. 2003; 552(Pt 1):59–71.

Donnelly-Roberts D, McGaraughty S, Shieh CC, Honore P, Jarvis MF. Painful purinergic receptors. J Pharmacol Exp Ther. 2008;324(2):409–15.

Donnelly-Roberts DL, Namovic MT, Han P, Jarvis MF. Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. Br J Pharmacol. 2009;157(7):1203–14.

Dubyak GR. Go it alone no more–P2X7 joins the society of heteromeric ATP-gated receptor channels. Mol Pharmacol. 2007;72(6):1402–5.

Evans RJ, Lewis C, Buell G, Valera S, North RA, Surprenant A. Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2x purinoceptors). Mol Pharmacol. 1995;48(2):178–83.

Ford AP, Gever JR, Nunn PA, Zhong Y, Cefalu JS, Dillon MP, Cockayne DA. Purinoceptors as therapeutic targets for lower urinary tract dysfunction. Br J Pharmacol. 2006;147(Suppl 2): S132–43.

Fountain SJ, Burnstock G. An evolutionary history of P2X receptors. Purinergic Signal. 2009;5(3):269–72.

Gever JR, Cockayne DA, Dillon MP, Burnstock G, Ford AP. Pharmacology of P2X channels. Pflugers Arch. 2006;452(5):513–37.

Guo C, Masin M, Qureshi OS, Murrell-Lagnado RD. Evidence for functional P2X4/P2X7 heteromeric receptors. Mol Pharmacol. 2007;72(6):1447–56.

Harhun MI, Povstyan OV, Gordienko DV. Purinoreceptor-mediated current in myocytes from renal resistance arteries. Br J Pharmacol. 2010;160(4):987–97.

Hausmann R, Rettinger J, Gerevich Z, Meis S, Kassack MU, Illes P, Lambrecht G, Schmalzing G. The suramin analog 4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis (carbonylimino)))tetra-kis-benzenesulfonic acid (NF110) potently blocks P2X3 receptors: subtype selectivity is determined by location of sulfonic acid groups. Mol Pharmacol. 2006;69(6):2058–67.

Hechler B, Magnenat S, Zighetti ML, Kassack MU, Ullmann H, Cazenave JP, Evans R, Cattaneo M, Gachet C. Inhibition of platelet functions and thrombosis through selective or nonselective inhibition of the platelet P2 receptors with increasing doses of NF449 [4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis-(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid octasodium salt]. J Pharmacol Exp Ther. 2005;314(1):232–43.

Honore P, Donnelly-Roberts D, Namovic MT, Hsieh G, Zhu CZ, Mikusa JP, Hernandez G, Zhong C, Gauvin DM, Chandran P, Harris R, Medrano AP, Carroll W, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF. A-740003 [N-(1-{[(cyanoimino) (5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. J Pharmacol Exp Ther. 2006;319(3):1376–85.

Hülsmann M, Nickel P, Kassack M, Schmalzing G, Lambrecht G, Markwardt F. NF449, a novel picomolar potency antagonist at human P2X1 receptors. Eur J Pharmacol. 2003;470(1–2):1–7.

Jaime-Figueroa S, Greenhouse R, Padilla F, Dillon MP, Gever JR, Ford AP. Discovery and synthesis of a novel and selective drug-like P2X(1) antagonist. Bioorg Med Chem Lett. 2005;15(13):3292–5.

Jarvis MF, Khakh BS. ATP-gated P2X cation-channels. Neuropharmacology. 2009;56(1):208–15.

Kassack MU, Braun K, Ganso M, Ullmann H, Nickel P, Böing B, Müller G, Lambrecht G. Structure-activity relationships of analogues of NF449 confirm NF449 as the most potent and selective known P2X1 receptor antagonist. Eur J Med Chem. 2004;39(4):345–57.

Kawate T, Michel JC, Birdsong WT, Gouaux E. Crystal structure of the ATP-gated P2X(4) ion channel in the closed state. Nature. 2009;460(7255):592–8.

King BF. Novel P2X7 receptor antagonists ease the pain. Br J Pharmacol. 2007;151(5):565–7.

King BF, Knowles ID, Burnstock G, Ramage AG. Investigation of the effects of P2 purinoceptor ligands on the micturition reflex in female urethane-anaesthetized rats. Br J Pharmacol. 2004;142(3):519–30.

Klapperstück M, Büttner C, Nickel P, Schmalzing G, Lambrecht G, Markwardt F. Antagonism by the suramin analogue NF279 on human P2X(1) and P2X(7) receptors. Eur J Pharmacol. 2000;387(3):245–52.

Koshimizu TA, Tsujimoto G. Functional role of spliced cytoplasmic tails in P2X2-receptor-mediated cellular signaling. J Pharmacol Sci. 2006;101(4):261–6.

Makino T, McLysaght A. Interacting gene clusters and the evolution of the vertebrate immune system. Mol Biol Evol. 2008;25(9):1855–62.

Masin M, Kerschensteiner D, Dumke K, Rubio ME, Soto F. Fe65 interacts with P2X2 subunits at excitatory synapses and modulates receptor function. J Biol Chem. 2006; 160(2):281–7.

Murrell-Lagnado RD, Qureshi OS. Assembly and trafficking of P2X purinergic receptors. Mol Membr Biol. 2008;25(4): 321–31.

Nicke A, Kuan YH, Masin M, Rettinger J, Marquez-Klaka B, Bender O, Gorecki D, Murrell-Lagnado RD, Soto F. A functional P2X7 splice variant with an alternative transmembrane domain 1 escapes gene inactivation in P2X7 knock-out mice. J Biol Chem. 2009;284(38):25813–22.

North RA. Molecular physiology of P2X receptors. Physiol Rev. 2002;82(4):1013–67.

North RA. Families of ion channels with two hydrophobic segments. Curr Opin Cell Biol. 2006;8(4):474–83.

Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998;50(3):413–92.

Rettinger J, Schmalzing G, Damer S, Müller G, Nickel P, Lambrecht G. The suramin analogue NF279 is a novel and potent antagonist selective for the P2X(1) receptor. Neuropharmacology. 2000;39(11):2044–53.

Rettinger J, Braun K, Hochmann H, Kassack MU, Ullmann H, Nickel P, Schmalzing G, Lambrecht G. Profiling at recombinant homomeric and heteromeric rat P2X receptors identifies the suramin analogue NF449 as a highly potent P2X1 receptor antagonist. Neuropharmacology. 2005; 48(3):461–8.

Roberts JA, Vial C, Digby HR, Agboh KC, Wen H, Atterbury-Thomas A, Evans RJ. Molecular properties of P2X receptors. Pflugers Arch. 2006;452(5):486–500.
Sneddon P, Westfall TD, Todorov LD, Todorova SM, Westfall DP, Nickel P, Kennedy C. The effect of P2 receptor antagonists and ATPase inhibition on sympathetic purinergic neurotransmission in the guinea-pig isolated vas deferens. Br J Pharmacol. 2000;129(6):1089–94.
Soto F, Garcia-Guzman M, Stuhmer W. Cloned ligand-gated channels activated by extracellular ATP (P2X receptors). J Membr Biol. 1997;160(2):91–100.
Soto F, Lambrecht G, Nickel P, Stühmer W, Busch AE. Antagonistic properties of the suramin analogue NF023 at heterologously expressed P2X receptors. Neuropharmacology. 1999;38(1):141–9.
Surprenant A, North RA. Signaling at purinergic P2X receptors. Annu Rev Physiol. 2009;71:333–59.
Zhou S-Y, Mamdani M, Qanud K, Shen J-B, Pappano A, Kumar TS, Jacobson KA, Hintze T, Recchia FA, Liang BT. Treatment of heart failure by a methanocarba derivative of adenosine monophosphate. Implication for a role of cardiac P2X purinergic receptors. J Pharmacol Exp Ther. 2010;333(3):920–8.

Nucleotide Receptor P2Y

Didier Communi[1], Bernard Robaye[2] and Jean Marie Boeynaems[3]
[1]Institute of Interdisciplinary Research, School of Medicine, Université Libre de Bruxelles, Brussels
[2]Institute of Interdisciplinary Research, School of Medicine, Université Libre de Bruxelles, Gosselies
[3]Department of Laboratory Medicine, Erasme Academic Hospital, Université Libre de Bruxelles, Brussels, Belgium

Synonyms

Nucleotide receptors; Purinergic receptors

Historical Background

Signaling by extracellular ATP was first reported in 1929 (Drury and Szent-Györgyi 1929). Subdivision of purinergic receptors between P1 (adenosine) and P2 (ATP, ADP) was proposed in 1978 (Burnstock 1978), and further subdivision of P2 receptors between P2Y and P2X was made in 1985 (Burnstock and Kennedy 1985). The $P2Y_1$ and $P2Y_2$ receptors were the first P2Y receptors to be cloned in 1993 (Webb et al. 1993; Lustig et al. 1993).

Release of Nucleotides in the Extracellular Fluids

Although nucleotides, such as ATP and UTP, are mainly intracellular, they are released in the extracellular fluids by various mechanisms. One of them is cell damage: both necrotic and apoptotic cells release ATP and other nucleotides that thus constitute "danger signals" or DAMP (damage-associated molecular pattern) (Elliott et al. 2009). But they can also be released without cell lysis by specific mechanisms: exocytosis of secretory granules, vesicular transport, and membrane channels, such as ABC transporters, pannexins, and connexins (Abbracchio et al. 2006). Nucleotides are released by exocytosis during platelet aggregation and synaptic transmission. They are also released in response to various types of stress: mechanical stimulation (stretch, shear stress), hypoxia, or pathogen invasion. Extracellular nucleotides are rapidly degraded by a variety of ectonucleotidases such as the ENTDPases that degrade ATP into ADP and ADP into AMP, and 5'-nucleotidase that converts AMP into adenosine (Abbracchio et al. 2006). Signaling by extracellular nucleotides is mediated by two families of receptors: metabotropic G protein–coupled P2Y receptors and ionotropic P2X receptors.

Structure and Signaling Properties of P2Y Receptors

The P2Y family is composed of eight members encoded by distinct genes that can be subdivided into two groups based on their coupling to specific G proteins, as well as structural features (Abbracchio et al. 2006).

Whereas the P2X receptors are all receptors for ATP, the various P2Y receptors differ by their selectivity for distinct nucleotides (Table 1). $P2Y_{11}$ is primarily an ATP receptor, whereas $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ are ADP receptors. $P2Y_4$ and $P2Y_6$ are pyrimidinergic receptors activated by UTP and UDP respectively. $P2Y_2$ is a dual ATP and UTP receptor. $P2Y_{14}$ is a receptor for UDP-glucose and other nucleotide sugars as well as for UDP itself.

Comparisons of the structural characteristics and functionally important amino acid residues within the family have been performed using mutagenesis and

Nucleotide Receptor P2Y, Table 1 Properties of P2Y receptors

Group	Receptor	Chromosome (human)	Agonist (human)	G protein
A	$P2Y_1$	3q24-25	ADP	G_q
	$P2Y_2$	11q13.5	ATP = UTP	G_q (+ G_i)
	$P2Y_4$	Xq13	UTP	G_q (+ G_i)
	$P2Y_6$	11q13.5	UDP	G_q
	$P2Y_{11}$	19p31	ATP	G_q + G_s
B	$P2Y_{12}$	3q21-25	ADP	G_i
	$P2Y_{13}$	3q24-25	ADP	G_i
	$P2Y_{14}$	3q24-25	UDP-glucose UDP	G_i

modeling (Abbracchio et al. 2006). Conserved cationic residues that interact with the negatively charged phosphate groups have been identified in transmembrane domains 3, 6, and 7. The 8 P2Y receptors have a H-X-X-R/K motif in TM6. The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors share a Y-Q/K-X-X-R motif in TM7, whereas another motif, K-E-X-X-L is found in $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. This last motif is not specific for P2Y receptors since it is also found in GPR87, a lysophosphatidic acid receptor.

The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors couple mainly to G_q and the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors couple to G_i (Table 1). This coupling has been demonstrated directly in reconstitution experiments: ADP-activated GTP hydrolysis in vesicles containing either $P2Y_1$ and $G\alpha_q$ or $P2Y_{12}$ and $G\alpha_{i2}$ (Waldo et al. 2004). However, the dichotomy between G_q- and G_i-coupled P2Y receptors is an oversimplification. Indeed, the $P2Y_{11}$ receptor has the unique property to couple through both G_q and G_s. It is also unique by its late appearance during evolution since no $P2Y_{11}$ gene can be identified in the genome of rodents (Communi et al. 2001a). Furthermore, the $P2Y_2$ and $P2Y_4$ receptors are also coupled to G_i, as shown inter alia by a sensitivity of the responses mediated by those receptors to inhibition by pertussis toxin.

There is limited evidence for non-G-protein-mediated signaling by P2Y receptors. In particular, the $P2Y_2$ receptor has been shown to transactivate the VEGF receptor-2, by a mechanism involving the binding of ▶ Src tyrosine kinase to SH3 binding sites in the C-terminal domain of $P2Y_2$ (Seye et al. 2004).

The pharmacology of some P2Y receptors exhibits species differences: while the human $P2Y_4$ is a UTP receptor, the rat and mouse $P2Y_4$ receptors are activated equipotently by ATP and UTP.

The missing numbers in the classification represent either nonmammalian orthologs or receptors having some sequence homology to P2Y receptors, but for which there is no functional evidence of responsiveness to nucleotides.

Primary references are as follows : $P2Y_1$ (Webb et al. 1993), $P2Y_2$ (Lustig et al. 1993), $P2Y_4$ (Communi et al. 1995), $P2Y_6$ (Communi et al. 1996), $P2Y_{11}$ (Communi et al. 1997), $P2Y_{12}$ (Hollopeter et al. 2001), $P2Y_{13}$ (Communi et al. 2001b), $P2Y_{14}$ (Chambers et al. 2000).

Functions of P2Y Receptors

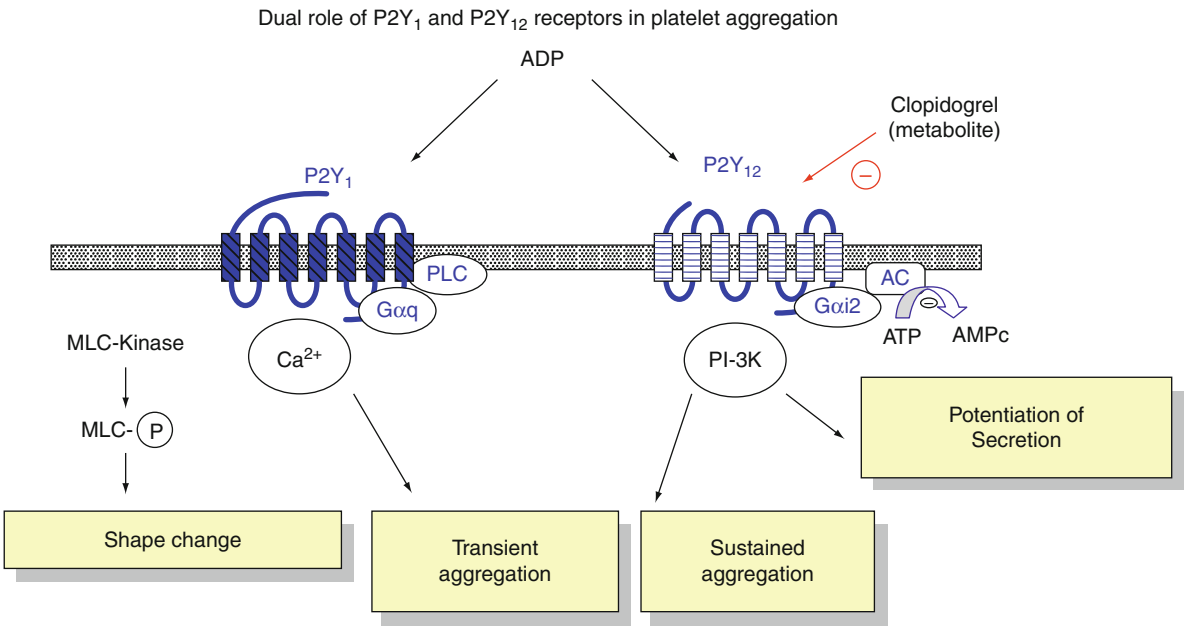
Gene silencing techniques have been instrumental in establishing the function of P2Y receptors (Table 2). ADP released from platelet dense granules amplifies platelet aggregation. This action requires the cooperation between two P2Y receptors: $P2Y_1$ and $P2Y_{12}$ (Fig. 1). $P2Y_1$ is involved in the initial platelet shape change and transient aggregation, while $P2Y_{12}$ is responsible for sustained aggregation and secretion. Both $P2Y_1^{-/-}$ and $P2Y_{12}^{-/-}$ mice show defective platelet aggregation ex vivo, increased bleeding time, and resistance to thrombosis (Leon et al. 1999; André et al. 2003). The only P2Y receptor ligands currently used as medicinal products are the thienopyridine antagonists of the $P2Y_{12}$ receptor, ticlopidine, clopidogrel, and prasugrel, which are used as antithrombotic agents.

ATP and UTP stimulate the secretion of chloride by epithelial cells through a channel distinct from CFTR (Cystic Fibrosis Transmembrane Regulator) (Fig. 2). Studies of knockout mice have demonstrated that this action is mediated by the $P2Y_2$ receptor in the airways (Cressman et al. 1999) and by $P2Y_4$ in the gut (Robaye et al. 2003). The $P2Y_2$ agonist denufosol is currently in phase III clinical development for the treatment of cystic fibrosis via mucus hydration.

Multiple P2Y receptors are expressed in the heart: $P2Y_2$ and $P2Y_6$ receptors on cardiomyocytes and $P2Y_4$ on microvascular endothelial cells. Nucleotides are released from cardiomyocytes in response to mechanical stretch or ischemia. The use of siRNA revealed that the $P2Y_6$ receptor plays a role in cardiac fibrosis resulting from pressure overload (Nishida et al. 2008).

Nucleotide Receptor P2Y, Table 2 Effects of P2Y gene silencing

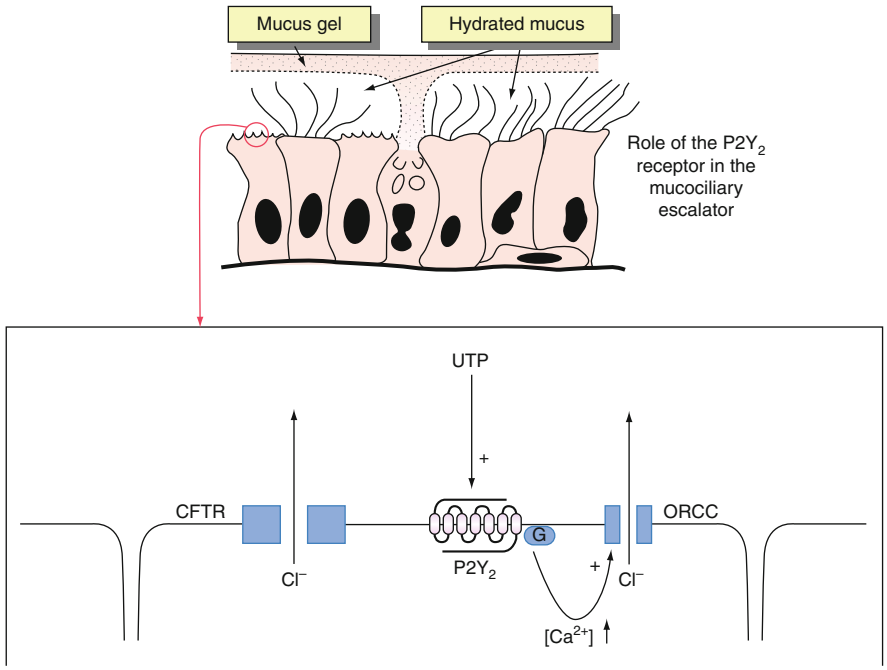
Receptor	Gene silencing method	Consequence of gene silencing
$P2Y_1$	Knockout mice	Inhibition of platelet aggregation and resistance to thromboembolism
		Smaller atherosclerotic lesions
$P2Y_2$	Knockout mice	Decreased neutrophil and monocyte/macrophage chemotaxis
		Decreased infiltration of eosinophils in asthmatic airways
		Abolition of ATP-induced Cl^- secretion in airways
$P2Y_4$	Knockout mice	Abolition of ATP-induced Cl^- secretion in the gut
$P2Y_6$	Antisense	Decreased microglial phagocytosis
	siRNA	Decreased pressure overload-induced cardiac fibrosis
$P2Y_{12}$	Knockout mice	Inhibition of platelet aggregation and resistance to thromboembolism
		Decreased microglial migration
$P2Y_{13}$	siRNA	Decreased HDL endocytosis by hepatocytes



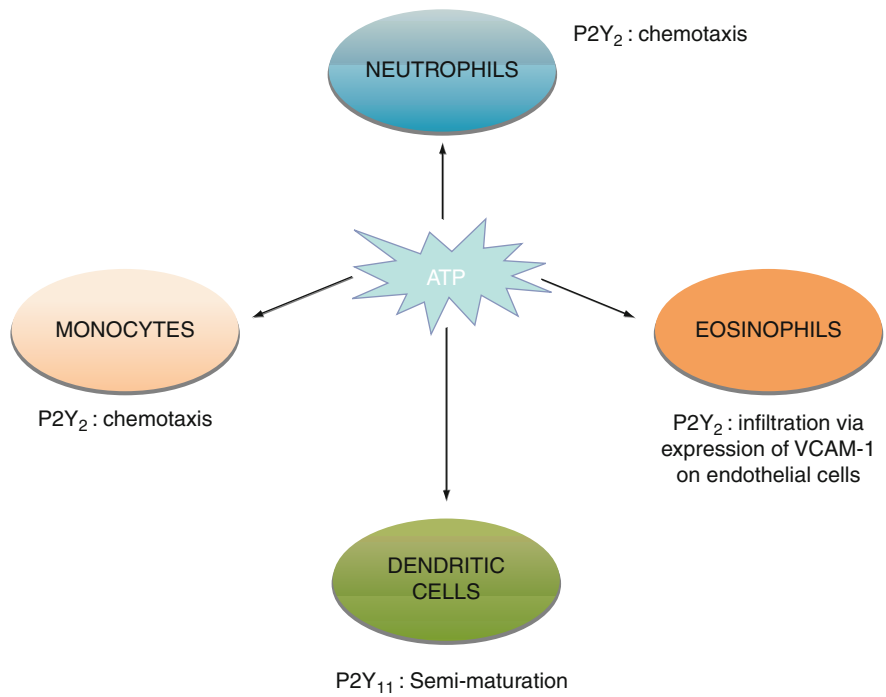
Nucleotide Receptor P2Y, Fig. 1 Cooperation between $P2Y_1$ and $P2Y_{12}$ receptors in platelet aggregationActivation of the $P2Y_1$ receptor by ADP induces a shape change of platelets and their transient aggregation, while its stimulatory effect on $P2Y_{12}$ induces a stable aggregation and potentiates the secretion of dense granules content

Multiple P2Y receptors might play a role in the development of atherosclerotic lesions, independently from their role in platelet activation. Aortic lesions were smaller in double ApoE/$P2Y_1$ knockout mice than in $ApoE^{-/-}$ mice (Hechler et al. 2008). This difference was unrelated to the role of $P2Y_1$ in platelet activation since it was unaffected by bone marrow transplantation from $P2Y_1$ wild-type mice, indicating the role of $P2Y_1$ in non-hematopoietic-derived cells, most likely endothelial cells. On the other hand, the $P2Y_{13}$ receptor plays a role in the reverse cholesterol transport, at the level of hepatocytes. It has indeed been shown that HDL Apo A-I activates an ecto-ATPase that generates ADP from ATP on the surface of hepatocytes. ADP then stimulates the endocytosis of HDL particles via the activation of $P2Y_{13}$ receptors, as demonstrated by the use of siRNA (Jacquet et al. 2005).

P2Y receptors are involved at various steps in the inflammatory process (Fig. 3). ATP released from neutrophils amplifies their attraction by chemotactic signals and its release from apoptotic cells constitutes a "find-me signal" for monocytes/macrophages



Nucleotide Receptor P2Y, Fig. 2 Role of the $P2Y_2$ receptor in the regulation of the airways mucociliary escalatorActivation of the $P2Y_2$ receptor by ATP stimulates the three components of this escalator: mucus secretion, mucus hydration, and mucus mobilization by ciliary activity. Mucus hydration results from Cl^- secretion which is mediated either by CFTR (Cystic Fibrosis Transmembrane Regulator) or ORCC (Outwardly rectifying chloride channels) that are opened by an increase in $[Ca^{2+}]_i$ in response to ATP



Nucleotide Receptor P2Y, Fig. 3 Role of P2Y receptors in various immune cellsThe $P2Y_2$ receptor plays a role in the tissue infiltration of eosinophils and the chemotaxis of neutrophils and monocytes. The $P2Y_{11}$ receptor mediates the semi-maturation of dendritic cells that favors Th2 differentiation or tolerance

(Chen et al. 2006; Elliott et al. 2009). In a murine model of asthma, the infiltration of esosinophils in the airways involves the $P2Y_2$-mediated expression of VCAM-1 (Vascular Cell Adhesion Molecule-1) on lung endothelial cells (Vanderstocken et al. 2010). P2Y receptors are also involved in adaptive immunity. In particular, ATP induces via the $P2Y_{11}$ receptor the semi-maturation of human monocyte-derived

dendritic cells, characterized by the upregulation of co-stimulatory molecules and the inhibition of IL-12 secretion, resulting in an enhanced ability to induce Th2 differentiation of T lymphocytes (Wilkin et al. 2001). Moreover, ATP confers tolerogenic and tumorigenic properties to dendritic cells (Marteau et al. 2005; Bles et al. 2010).

Microglia from $P2Y_{12}^{-/-}$ mice are unable to polarize, migrate, or extend processes toward ADP, and in vivo they showed decreased directional branch extension toward sites of laser-induced cortical damage (Haynes et al. 2006). Independently from this chemotactic action of ADP, UDP stimulates the uptake of microspheres by rat microglia, and this action was blocked by an antisense oligonucleotide targeting the $P2Y_6$ receptor (Koizumi et al. 2007). These complementary actions of ADP, a find-me signal, and UDP, an eat-me signal, involving a cooperation between $P2Y_{12}$ and $P2Y_6$, might be beneficial in neurodegenerative conditions such as Alzheimer's disease, via an increased clearance of amyloid-β deposits.

Summary

Nucleotides are released in the extracellular fluids following cell damage (necrosis or apoptosis), mechanical stimulation, or by exocytosis. They act on G protein–coupled P2Y or ionotropic P2X receptors. There are eight P2Y receptors encoded by distinct genes. They can be divided in two groups according to structural features and coupling to specific G proteins. The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors couple mainly to G_q, and the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors couple to G_i. They exhibit a selectivity for distinct nucleotides: ATP, ADP, UTP, UDP, and UDP-glucose. The study of knockout mice and other methods of gene silencing have demonstrated their involvement in multiple biological processes: platelet aggregation, epithelial surface lubrication, migration of neutrophils, monocytes and microglia, microglial phagocytosis, etc.

References

Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, et al. International Union of Pharmacology. Update and subclassification of the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 2006;58:281–341.

Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, et al. $P2Y_{12}$ regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest. 2003;112:398–406.

Bles N, Di Pietrantonio L, Boeynaems J-M, Communi D. ATP confers tumorigenic properties to dendritic cells by inducing amphiregulin secretion. Blood. 2010;116(17):3219–26 [Epub ahead of print].

Burnstock G. A basis for distinguishing two types of purinergic receptor. In: Straub RW, Bolis L, editors. Cell membrane receptors for drugs and hormones: a multidisciplinary approach. New York: Raven Press; 1978. p. 107–18.

Burnstock G, Kennedy C. Is there a basis for distinguishing two types of purinoceptor? Gen Pharmacol. 1985;16:433–40.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, et al. A G protein-coupled receptor for UDP-glucose. J Biol Chem. 2000;275:10767–71.

Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkermagel A, et al. ATP release guides neutrophil chemotaxis via $P2Y_2$ and A_3 receptors. Science. 2006;314:1792–5.

Communi D, Pirotton S, Parmentier M, Boeynaems JM. Cloning and functional expression of a human uridine nucleotide receptor. J Biol Chem. 1995;270:30849–52.

Communi D, Parmentier M, Boeynaems JM. Cloning, functional expression and tissue distribution of the human $P2Y_6$ receptor. Biochem Biophys Res Commun. 1996;222:303–8.

Communi D, Govaerts C, Parmentier M, Boeynaems JM. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. J Biol Chem. 1997;272:31969–73.

Communi D, Suarez-Huerta N, Dussossoy D, Savi P, Boeynaems JM. Cotranscription and intergenic splicing of human $P2Y_{11}$ and SSF1 genes. J Biol Chem. 2001a;276:16561–6.

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM. Identification of a novel human ADP receptor coupled to G_i. J Biol Chem. 2001b;276:41479–85.

Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, Grubb BR. Effect of loss of $P2Y_2$ receptor gene expression on nucleotide regulation of murine epithelial Cl^- transport. J Biol Chem. 1999;274:26461–8.

Drury AN, Szent-Györgyi A. The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. J Physiol. 1929;68:213–37.

Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature. 2009;461:282–6.

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D. The $P2Y_{12}$ receptor regulates microglial activation by extracellular nucleotides. Nat Neurosci. 2006;9:1512–19.

Hechler B, Freund M, Ravanat C, Magnenat S, Cazenave JP, Gachet C. Reduced atherosclerotic lesions in $P2Y_1$/apolipoprotein E double-knockout mice: the contribution of non-hematopoietic-derived $P2Y_1$ receptors. Circulation. 2008;118:754–63.

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature. 2001;409:202–7.
Jacquet S, Malaval C, Martinez LO, Sak K, Rolland C, Perez C, et al. The nucleotide receptor $P2Y_{13}$ is a key regulator of hepatic high-density lipoprotein (HDL) endocytosis. Cell Mol Life Sci. 2005;62:2508–15.
Koizumi S, Shigemoto-Mogami Y, Nasu-Tada K, Shinozaki Y, Ohsawa K, Tsuda M, et al. UDP acting at $P2Y_6$ receptors is a novel mediator of microglial phagocytosis. Nature. 2007;446:1091–5.
Léon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. J Clin Invest. 1999;104:1731–7.
Lustig KD, Shiau AK, Brake AJ, Julius D. Expression cloning of an ATP receptor from mouse neuroblasoma cells. Proc Natl Acad Sci. 1993;90:5113–17.
Marteau F, Gonzalez NS, Communi D, Goldman M, Boeynaems J-M, Communi D. Thrombospondin-1 and indoleamine 2,3-dioxygenase are major targets of extracellular ATP in human dendritic cells. Blood. 2005;106:3860–6.
Nishida M, Sato Y, Uemura A, Narita Y, Tozaki-Saitoh H, Nakaya M, Ide T, Suzuki K, Inoue K, Nagao T, Kurose H. $P2Y_6$ receptor-Galpha$_{12/13}$ signalling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis. EMBO J. 2008;27:3104–15.
Robaye B, Ghanem E, Wilkin F, Fokan D, Van Driessche W, Schurmans S, et al. Loss of nucleotide regulation of epithelial chloride transport in the jejunum of $P2Y_4$-null mice. Mol Pharmacol. 2003;63:777–83.
Seye CI, Yu N, Gonzalez FA, Erb L, Weisman GA. The $P2Y_2$ nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). J Biol Chem. 2004;279:35679–86.
Vanderstocken G, Bondue B, Horckmans M, Di Pietrantonio L, Robaye B, Boeynaems J-M, Communi D. $P2Y_2$ receptor regulates VCAM-1membrane and soluble forms and eosinophil accumulation during lung inflammation. J Immunol. 2010;185(6):3702–07.
Waldo GL, Harden TK. Agonist binding and Gq-stimulating activities of the purified human $P2Y_1$ receptor. Mol Pharmacol. 2004;65:426–36.
Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, et al. Cloning and functional expression of a brain G-protein-coupled ATP receptor. FEBS Lett. 1993;324:219–25.
Wilkin F, Duhant X, Bruyns C, Suarez-Huerta N, Boeynaems JM, Robaye B. The $P2Y_{11}$ receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. J Immunol. 2001;166:7172–7.

Nucleotide Receptors

▶ Nucleotide Receptor P2Y

N-WASP

Haein Park, Athanassios Dovas and Dianne Cox
Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, Bronx, NY, USA

Synonyms

Neural Wiskott–Aldrich Syndrome Protein; Wiskott–Aldrich Syndrome-Like (WASL)

Historical Background

Wiskott–Aldrich syndrome (WAS), a pediatric disorder, was first described in 1937 by Alfred Wiskott as a "hereditary thrombopathy" in males, presenting with thrombocytopenia, eczema, bloody diarrhea, episodes of fever, and recurrent bacterial infections. Robert A. Aldrich would later demonstrate an X-linked mode of inheritance of this disease. Other features of WAS were later recognized, including immunodeficiency involving both humoral and cellular immunity, high rate of autoimmunity and malignancies, abnormal apoptosis, and defective cell motility. The mutated gene giving rise to this disease was identified in 1994 by positional cloning and referred to as *WAS*, and mutations of the WAS protein (WASP) were demonstrated not only in patients with WAS, but also in those with X-linked thrombocytopenia (XLT), a disease showing milder clinical phenotype with a more favorable prognosis (Notarangelo et al. 2008). Northern blot analysis indicated that WASP mRNA is expressed exclusively in hematopoietic cells. Two years later, a novel protein with ~50% amino acid identity to the *WAS* gene product was reported as a binding partner for the Grb2/Ash adapter protein. In contrast to WASP, this protein was expressed ubiquitously, but strongest expression was observed in neuronal cells and was thus named Neural-WASP (▶ N-WASP) (Miki and Takenawa 2003).

▶ N-WASP and WASP are actin-nucleating promoting factors (NPF) and are the founding members of the WASP-family of NPFs that contain tandem V (Verprolin homology, also known as WH2; WASP

homology 2), C (Central or Connecting), and A (Acidic) regions, referred to as the VCA domain. Other members of this family are the WAVE/Scar, WASH, and WHAMM/JMY subfamilies. Evolutionary analyses indicate that these VCA domain-containing proteins are widely expressed among eukaryotes and evolutionarily ancient (Veltman and Insall 2010).

Structure of N-WASP

▶ N-WASP and WASP have a conserved domain organization that allows interaction with multiple distinct binding proteins (Table 1). They have a WASP homology 1 (WH1; also known as EVH1) domain that binds primarily WASP interacting protein (WIP)-family members, a basic sequence that binds phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), a GTPase-binding domain (GBD), containing a Cdc42 and Rac interactive binding (CRIB) motif that binds Cdc42-GTP, and a proline-rich region that binds SH3 proteins. The C-terminus of both proteins contains a VCA domain that binds actin via the V region and the Arp2/3 complex via the CA region but ▶ N-WASP has an additional V region (Takenawa and Suetsugu 2007) (Fig. 1). In vitro data using the purified VVCA domain of ▶ N-WASP showed that this domain alone was sufficient for actin polymerization and its activity was higher than the isolated VCA domain of WASP (Zalevsky et al. 2001).

Regulation of N-WASP

Autoinhibition and Activation by Cdc42

▶ N-WASP is regulated by diverse signals including Rho family GTPases, phospholipids, kinases, many SH3 domain-containing proteins, and both bacterial and viral pathogen proteins (Takenawa and Suetsugu 2007) (Table 1). In the resting state, ▶ N-WASP and WASP are autoinhibited through intramolecular interactions between the C-terminal VCA region and a hydrophobic pocket in the GBD but other regions, such as from the WH1 domain, may also participate in autoinhibition. In addition, it is generally considered that favorable electrostatic interactions between the basic region and the A region further stabilize the GBD–VCA interface. The Rho family GTPase Cdc42 was the first protein shown to bind ▶ N-WASP/WASP and is an important regulator of both proteins. The active, GTP bound form of Cdc42 disrupts the hydrophobic core of the GBD and releases the VCA domain, which can now bind G actin and the Arp2/3 complex. The Arp2/3 complex, which is composed of seven polypeptides, nucleates actin polymerization and contributes to branched filamentous actin (Takenawa and Suetsugu 2007). Another Rho family GTPase, Rac1, can activate ▶ N-WASP in vitro, but is probably not involved in WASP activation (Tomasevic et al. 2007). Evidence also suggests that Cdc42 may require an additional intermediate for the activation of ▶ N-WASP, such as F-BAR proteins of the CIP4 subfamily, which include Toca-1, CIP4, and FBP17. These proteins bind directly to Cdc42 and also contain a SH3 domain that binds to the polyproline region of ▶ N-WASP, and is required for ▶ N-WASP functions, such as endocytosis.

Regulation by Phosphorylation

Although it is generally accepted that the binding of Cdc42-GTP to WASP is important for allosteric release from autoinhibition, other signaling events can also modulate ▶ N-WASP function. One such event is phosphorylation of a conserved tyrosine residue (Y256 in human ▶ N-WASP and Y291 in human WASP) by non-receptor tyrosine kinases, e.g., ▶ Src, and is required for several cellular functions of ▶ N-WASP, such as neurite extension. Remarkably, this residue is located in the C-terminus of the GBD, and its phosphorylation is thought to alter the charge and therefore stability of the autoinhibited form of both proteins shifting the proteins toward the open, active conformation (Thrasher and Burns 2010). Additionally, tyrosine phosphorylation may prime the molecule for activation by proteins that contain SH2 domains contributing to an additional regulatory input. However, tyrosine phosphorylation may also target both WASP and ▶ N-WASP to various protein degradation pathways, thus resulting in signal termination (Dovas and Cox 2010).

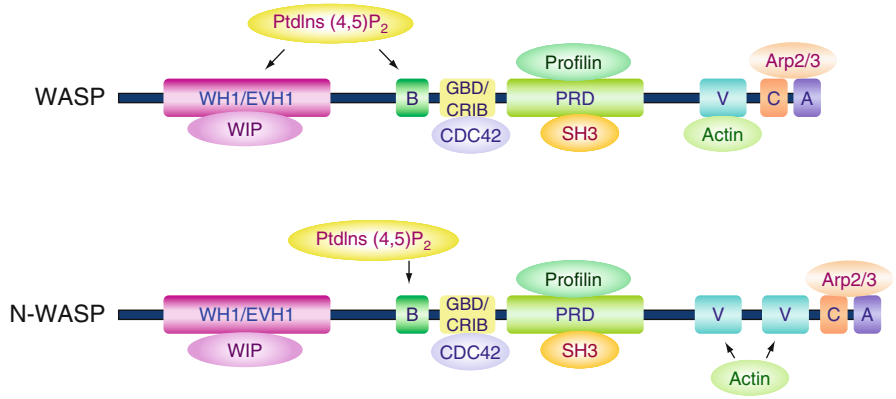
PtdIns(4,5)P_2, SH3 Domains, and EspFu

Additional levels of control of ▶ N-WASP activity following allosteric activation also exist, facilitating the integration of multiple signals in the efficient control of ▶ N-WASP activity. These are generally

N-WASP, Table 1 ▶ N-WASP/WASP binding proteins

Binding protein	Binding domain	N-WASP/WASP binding region	Effect on (N)WASP/function
WIP, CR16, WICH/WIRE	Proline-rich	WH1	Stabilization, targeting, inhibition of activity
CIB		WH1	Coupling to integrin $\alpha_{IIb}\beta_3$
Cdc42/Chp		CRIB	Activation
TC10/RhoT		CRIB	Activation
mDab1	PTB	CRIB	
PtdIns(4,5)P_2		Basic	Localization, synergistic activation with Cdc42 and Nck
Hsp90		Basic	Stabilization
FBP11	WW	Proline-rich	
Src-family kinases (Src, Hck, Lck, Lyn, Fyn, Fgr)	SH3 (SH2?)	Proline-rich	Phosphorylation, activation, protein degradation
Grb2	SH3	Proline-rich	Activation, localization
Nck	SH3	Proline-rich	Activation, localization
Crk adaptors (CrkII, CrkL)	SH3	Proline-rich	Activation, localization
Vinexin beta	SH3	Proline-rich	
Cortactin, HS1	SH3	Proline-rich	Targeting, activation, invadopodium/ podosome formation
Abi1	SH3	Proline-rich	Activation, endocytosis
Endophilin	SH3	Proline-rich	Activation, endocytosis
Amphiphysin	SH3	Proline-rich	Endocytosis
Tuba	SH3	Proline-rich	Activation via Cdc42
CIP4, Toca-1, FBP17	SH3	Proline-rich	Cdc42-mediated activation
Tec family kinases (Tec, Btk, Itk)	SH3	Proline-rich	Phosphorylation, activation
WISH/DIP	SH3	Proline-rich	Activation
PSTPIP1	SH3	Proline-rich	Activation, localization
Syndapin	SH3	Proline-rich	Activation, endocytosis
Nostrin	SH3	Proline-rich	Endocytosis
srGAP	SH3	Proline-rich	
Profilin	SH3	Proline-rich	Actin polymerization
IRSp53	SH3	Proline-rich	Activation, localization
PTP-PEST		Proline-rich	Dephosphorylation, inactivation
Sorting nexin (SNX9, SNX18)	SH3	Proline-rich	Activation, endocytosis
Arg	SH3	Proline-rich	Phosphorylation, activation
Intersectin-1, -2	SH3	Proline-rich	Localization, activation
Abp1	SH3	Proline-rich	Activation, endocytosis
VASP		Proline-rich	Localization, actin polymerization
Casein kinase	?	?	VCA phosphorylation
Arp2/3		CA	Actin polymerization
G-actin		V	Actin polymerization
F-actin		Basic	Branching
Merlin, ERM (ezrin/radixin/moesin)	FERM domain	WH1	Inhibition of actin polymerization
CD44			Stabilization, localization
IQGAP1		Basic-CRIB	Activation

? unknown



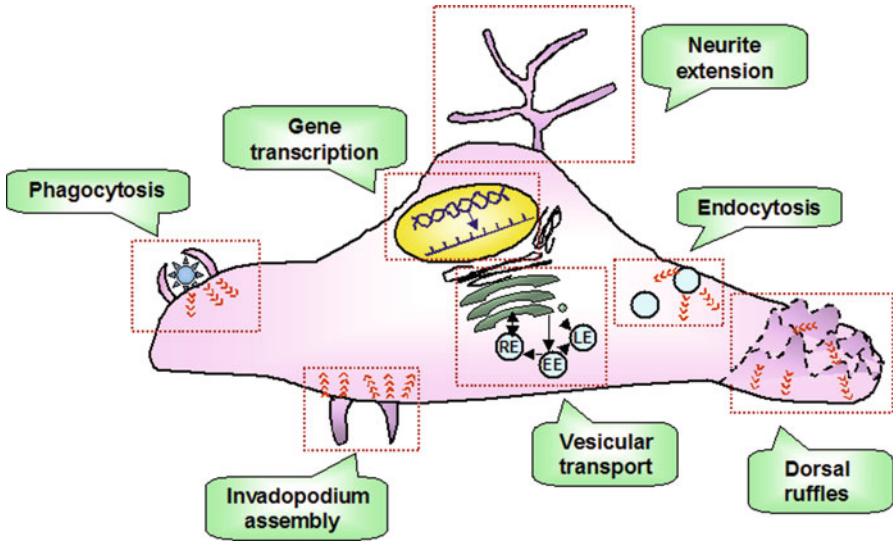
N-WASP, Fig. 1 *Domain organization and molecular interactions of* ▶ *N-WASP and WASP*. ▶ N-WASP and WASP have similar overall domain organization, with an additional V region present in ▶ N-WASP. The WH1 domain interacts with WIP and its related proteins. The basic domain mediates interactions with PtdIns(4,5)P_2 on membranes. The CRIB motif inside the GBD region binds to Cdc42-GTP and is instrumental in ▶ N-WASP activation. The polyproline stretch is a site for docking of SH3 domains, while distinct regions mediate binding to profilin. Finally, the VCA domain binds G-actin and the Arp2/3 complex and nucleates actin polymerization

thought of as influencing the activity of ▶ N-WASP by correctly localizing the molecule and/or by influencing its dimerization status (Padrick and Rosen 2010). The latter is an important means of control of ▶ N-WASP-dependent actin polymerization since VCA domain dimers bind the Arp2/3 complex with higher affinity than monomers, thus contributing to enhanced actin polymerization. PtdIns(4,5)P_2 is an important regulator of actin organization mediated by both ▶ N-WASP and WASP. PtdIns(4,5)P_2 synergizes with Cdc42 in the activation of ▶ N-WASP. Increased PtdIns(4,5)P_2 density hyperactivates ▶ N-WASP in vitro, and is required for actin comet tail formation in vivo, pointing to both membrane targeting and dimerization as means by which PtdIns(4,5)P_2 regulates ▶ N-WASP.

Adaptor proteins that contain multiple SH3 domains participate in an additional level of control of ▶ N-WASP activity via dimerization. One prominent example is Nck1, which contains five SH3 domains and is able to hyperactivate ▶ N-WASP in vitro, while dimeric BAR domain proteins that contain SH3 domains may also act by inducing ▶ N-WASP dimers. Importantly, the enhanced activity of ▶ N-WASP upon dimerization is exploited by enterohemorrhagic *Escherichia coli*, to induce its spread. The bacterial protein EspFu, which is injected into the host cell cytoplasm, contains multiple repeats of a segment that can bind the GBD of ▶ N-WASP. Therefore, EspFu activates ▶ N-WASP allosterically by competing for GBD binding with the VCA domain while at the same time clustering multiple ▶ N-WASP molecules to stimulate Arp2/3-dependent actin polymerization and pedestal formation. Therefore, these data suggest that the proline-rich region may receive activation signals as well as localization signals from proteins containing SH3 domains, and that similar mechanisms are exploited by pathogens to induce their spread.

WIP: A Major Binding Partner

WIP interacts with the WH1 domain of ▶ N-WASP and WASP and performs critical regulatory functions (Ramesh and Geha 2009). Primarily, it is required for the stability of WASP protein levels in vivo. This is highlighted by the fact that the most frequent WAS mutations are clustered at the WH1 domain and affect amino acids important for the interaction with WIP. WIP also targets WASP to sites of activity. For example, WIP targets WASP to sites of T cell receptor clustering and also recruits ▶ N-WASP to vaccinia virus particles. However, WIP also inhibits the ability of ▶ N-WASP to stimulate actin polymerization, at least in vitro. It may be possible that WIP does not dissociate from ▶ N-WASP but may undergo conformational changes that relieve inhibitory interactions and allow ▶ N-WASP activation in vivo (Ramesh and Geha 2009).



N-WASP, Fig. 2 *Cellular functions of* ▶ *N-WASP*. ▶ N-WASP plays important functions inside cells. It mediates endocytosis and vesicle motility; participates in plasma membrane extensions, such as in the outgrowth of neurites, during phagocytosis, dorsal ruffle formation and invadopodium formation, and regulates gene expression by shuttling in and out of the nucleus

The Role of N-WASP

▶ N-WASP exerts its functions primarily via the regulation of Arp2/3-mediated actin polymerization. As such, it participates in a plethora of actin-dependent functions including endocytosis, phagocytosis, invadopodium assembly, neurite extension, vesicular transport, pathogen infection, and dorsal ruffle formation (Fig. 2). ▶ N-WASP is also found in the nucleus where it participates in gene transcription through association with a nuclear complex that contains RNA polymerase II. This role of ▶ N-WASP also relies on its ability to polymerize nuclear actin (Wu et al. 2006). Similar nuclear functions have been described for WASP, where it is important in the transcription of key genes required for the differentiation of $CD4^+$ T_H1 cells (Taylor et al. 2010). ▶ N-WASP also regulates sarcomeric actin assembly in skeletal muscle, though independently of the Arp2/3 complex (Takano et al. 2010).

▶ N-WASP has not been associated with human disease and appears to be essential in mammals as ▶ N-WASP deficiency in mice results in embryonic death at day E12. Tissue-specific ablation of ▶ N-WASP, however, has revealed roles for ▶ N-WASP in T cell development (Cotta-de-Almeida et al. 2007), hair follicle cycling (Lefever et al. 2010), and myelin sheath formation by Schwann cells (Novak et al. 2011). The importance of tight regulation of ▶ N-WASP activity is suggested by the hematopoetic-restricted WASP in which activating mutations have been shown to induce a separate syndrome, X-linked neutropenia (XLN). Therefore, both inactivating and activating mutations in WASP contribute to human disease.

Common and Distinct Functions of N-WASP and WASP

Although there is a high degree of homology in the functional domains of ▶ N-WASP and WASP, they may not be able to completely substitute for one another. WASP and ▶ N-WASP may serve both redundant and nonredundant functions depending on the cellular context. For example, only ▶ N-WASP, and not WASP, can support *Shigella* motility in cells (Snapper et al. 2001). Also, while WASP is expressed exclusively in hematopoietic cells, ▶ N-WASP is also expressed in these cells, albeit at low levels (Isaac et al. 2010). Recently, unique functions for ▶ N-WASP were demonstrated in macrophages. A striking feature of WASP-deficient macrophages is the lack of podosomes. Podosomes mediate adhesion to the extracellular matrix and perform matrix degradation. WASP localizes to the F-actin-rich core along with other actin-regulatory proteins, such as cortactin/HS1 and Arp2/3. Interestingly, certain aggressive cancer

cells and ▶ Src-transformed cells possess podosome-like structures called invadopodia that appear to be directly responsible for extracellular matrix degradation. Invadopodia have similar organization and actin regulatory machinery localization compared to podosomes. However, invadopodia are regulated by ▶ N-WASP given the absence of WASP expression in these cells. When ▶ N-WASP was reduced in macrophage cells, podosomes still formed, but they were unable to perform matrix degradation (Nusblat et al. 2011). This defect was rescued by re-expression of ▶ N-WASP, but not by overexpression of WASP. Additionally, reducing ▶ N-WASP levels mistargets the matrix-degrading enzyme MT1-MMP and it no longer localizes to podosomes. Additionally, ▶ N-WASP only co-localizes with MT1-MMP positive vesicles at podosomes, suggesting that ▶ N-WASP may play a role on the targeting or fusion of MMP-containing vesicles to podosomes in macrophage cells (Nusblat et al. 2011). A unique role for ▶ N-WASP was also found for phagocytosis by macrophages and a study indicated that ▶ N-WASP may play a role in membrane delivery to the growing phagocytic cup while WASP may be required for the actin polymerization during phagocytosis (Park and Cox 2009). ▶ N-WASP and WASP have both overlapping and unique functions when expressed in the same cell.

Summary

Both ▶ N-WASP and WASP proteins play critical roles in rapid reorganization of actin filaments induced in response to diverse extracellular stimuli. Although they have high homology in their functional domains, ▶ N-WASP and WASP also have different requirements for activation and participate in distinct cellular processes. Studies in leukocytes, which express both proteins, may reveal unique functions and distinct aspects of regulation and can contribute to a more profound understanding of their cellular activities and further delineate the immunological abnormalities in WAS. Identification of different binding partners between ▶ N-WASP and WASP will provide important answers to the intriguing questions about the differential activation and unique roles of these proteins. Tissue-specific ablation of ▶ N-WASP will also help reveal its functions in vivo. Novel findings on the nuclear or Arp2/3-independent functions of ▶ N-WASP suggest that many aspects of this molecule remain unknown and promise exciting new avenues for research.

References

Cotta-de-Almeida V, Westerberg L, Maillard MH, et al. Wiskott Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development. Proc Natl Acad Sci USA. 2007;104(39):15424–9.

Dovas A, Cox D. Regulation of WASp by phosphorylation: activation or other functions? Commun Integr Biol. 2010;3(2):101–5.

Isaac BM, Ishihara D, Nusblat LM, et al. N-WASP has the ability to compensate for the loss of WASP in macrophage podosome formation and chemotaxis. Exp Cell Res. 2010;316(20):3406–16.

Lefever T, Pedersen E, Basse A, et al. N-WASP is a novel regulator of hair-follicle cycling that controls antiproliferative TGF{beta} pathways. J Cell Sci. 2010;123 (Pt 1):128–40.

Miki H, Takenawa T. Regulation of actin dynamics by WASP family proteins. J Biochem. 2003;134(3):309–13.

Notarangelo LD, Miao CH, Ochs HD. Wiskott-Aldrich syndrome. Curr Opin Hematol. 2008;15(1):30–6.

Novak N, Bar V, Sabanay H, et al. N-WASP is required for membrane wrapping and myelination by Schwann cells. J Cell Biol. 2011;192(2):243–50.

Nusblat LM, Dovas A, Cox D. The non-redundant role of N-WASP in podosome-mediated matrix degradation in macrophages. Eur J Cell Biol. 2011;90(2–3):205–12.

Padrick SB, Rosen MK. Physical mechanisms of signal integration by WASP family proteins. Annu Rev Biochem. 2010;79:707–35.

Park H, Cox D. Cdc42 Regulates Fc gamma receptor-mediated phagocytosis through the activation and phosphorylation of Wiskott-Aldrich syndrome protein (WASP) and neural-WASP. Mol Biol Cell. 2009;20(21):4500–8.

Ramesh N, Geha R. Recent advances in the biology of WASP and WIP. Immunol Res. 2009;44(1–3):99–111.

Snapper SB, Takeshima F, Anton I, et al. N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility. Nat Cell Biol. 2001;3(10):897–904.

Takano K, Watanabe-Takano H, Suetsugu S, et al. Nebulin and N-WASP cooperate to cause IGF-1-induced sarcomeric actin filament formation. Science. 2010;330(6010):1536–40.

Takenawa T, Suetsugu S. The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat Rev Mol Cell Biol. 2007;8(1):37–48.

Taylor MD, Sadhukhan S, Kottangada P, et al. Nuclear role of WASp in the pathogenesis of dysregulated TH1 immunity in human Wiskott-Aldrich syndrome. Sci Transl Med. 2010;2(37):37–44.

Thrasher AJ, Burns SO. WASP: a key immunological multitasker. Nat Rev Immunol. 2010;10(3):182–92.

Tomasevic N, Jia Z, Russell A, et al. Differential regulation of WASP and N-WASP by Cdc42, Rac1, Nck, and PI(4,5)P2. Biochemistry. 2007;46(11):3494–502.

Veltman DM, Insall RH. WASP family proteins: their evolution and its physiological implications. Mol Biol Cell. 2010;21(16):2880–93.

Wu X, Yoo Y, Okuhama NN, et al. Regulation of RNA-polymerase-II-dependent transcription by N-WASP and its nuclear-binding partners. Nat Cell Biol. 2006;8(7):756–63.

Zalevsky J, Lempert L, Kranitz H, et al. Different WASP family proteins stimulate different Arp2/3 complex-dependent actin-nucleating activities. Curr Biol. 2001;11(24):1903–13.

O

OC120 (Osteoclast Maturation-Associated Gene 120 Protein)

► CKIP-1

Odorant Receptors

► Olfactory Receptors

Olfactory Receptors

Lisa Stowers and Sandeepa Dey
Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA

Synonyms

Odorant receptors; Olfrs; ORs

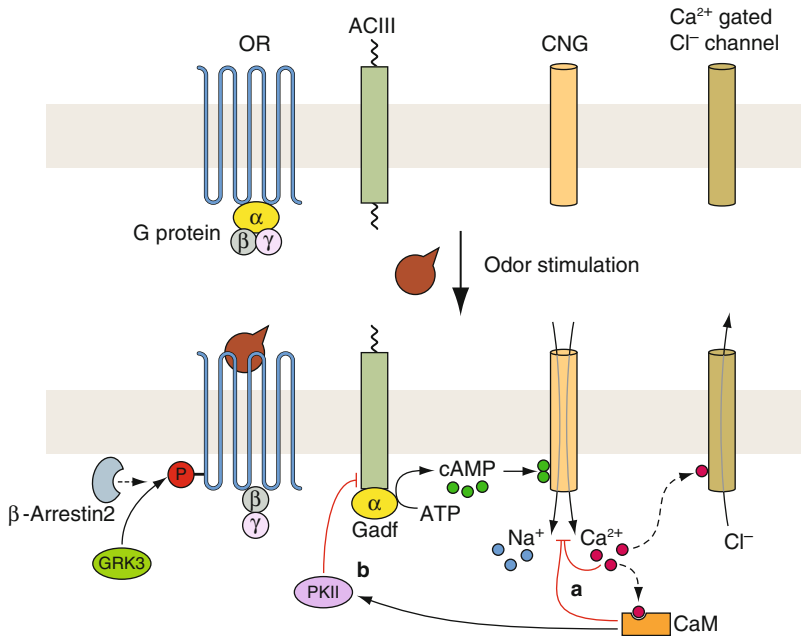
Historical Background

Among the five senses, smell is unique in that it is dedicated to discriminating an enormous diversity of stimulating ligands. Olfactory sensory neurons achieve this detection by the expression of a multigene family of seven-transmembrane G protein–coupled receptors (GPCRs) originally identified by Linda Buck and Richard Axel (Buck and Axel 1991). These receptors, termed olfactory receptors (OR, plural ORs), are expressed in the olfactory tissue of all terrestrial vertebrates examined thus far, and have been shown to respond directly to odorant binding. The identification and subsequent study of ORs has provided great insight into the molecular and neuronal organization of the olfactory system. Indeed, in 2004, Buck and Axel were jointly awarded the Nobel Prize in Physiology for their pioneering work in odorant receptor discovery. Most of the current understanding of ORs results from experiments from the mouse model which is the focus of this review.

General Physiology and Classification of ORs

Olfactory sensory neurons reside in the main olfactory epithelium (MOE) which is located in the distal recess of the nasal cavity. The MOE covers the evaginated nasal terbinates resulting in increased surface capacity to sample ligands. The dendrite of each olfactory neuron projects toward the nasal lumen providing direct sensory contact with the odor environment. The dendrite is tufted with 20–30 sensory cilia and OR proteins localized to these cilia membranes further increase surface exposure to the ligand environment. Active sniffing draws odorant ligands into the nasal cavity enabling binding and subsequent activation of ORs. Each odorant receptor is expressed from a single coding gene. Together, individual odorant receptor genes comprise the largest GPCR family in the mammalian genome (in the human genome they account for approximately 3% of predicted exons). ORs belong to class A GPCRs (Rhodopsin family) and can be broadly divided into classes I and II (Zhang and Firestein 2002). Class I is highly conserved across evolution;

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



Olfactory Receptors, Fig. 1 Endogenous G protein signaling and desensitization pathway activated by odorant receptors on odor binding. Signaling: On binding an odor molecule, the odorant receptor activates the Gαolf, which stimulates ACIII to generate cyclic AMP. cAMP regulates ion channel CNG to allow influx of sodium and calcium ions. Calcium ions in turn regulate a chloride channel to induce efflux of chloride ions. Influx and efflux of the said ions result in depolarization of the olfactory sensory neuron. Desensitization: Calcium and calcium-binding proteins like calmodulin CaM act as feedback inhibitors of OR-induced signaling by (**a**) binding and blocking CNG or (**b**) inducing PKII to phosphorylate and inhibit ACII. GRK3 may phosphorylate the odorant receptor so it can now bind β arrestin for desensitization and internalization

related to chemosensory receptors in fish. Sequence analysis shows class II to be more divergent, suggesting that this OR class may have evolved to provide specific ecological adaptations.

Evolution of Odorant Receptors

Comparative genomics has revealed that the odorant receptor family shows rapid gene birth and death events across evolution. Tandem gene duplications have resulted in chromosomal clusters of closely related ORs with high sequence homology and similar ligand-binding profiles. In the mouse, there are 43 odorant receptor clusters found on every chromosome. Old world primates, including humans, have a significantly smaller OR repertoire (390 putatively functional genes) compared to other sequenced vertebrates such as mice (1,468 putatively functional genes), rats (1,750 putatively functional genes), and dogs (922 putatively functional genes). The evolutionary loss of functional OR coding regions has been attributed to the correlated evolution of trichromatic vision and an increased utilization of visual stimuli.

OR-Mediated Signal Transduction

Upon ligand stimulation, odorant receptors initiate a signal transduction cascade that results in neuron depolarization and transmission of the signal to the brain (Fig. 1). In most olfactory sensory neurons, detection of a cognate odor ligand alters the confirmation of the OR to activate a specialized heterotrimeric ▶ guanine nucleotide binding protein (Gαolf) (Bakalyar and Reed 1991; Dhallan et al. 1990; Jones and Reed 1989) which converts guanosine tri phosphate (GTP) to guanosine di phosphate (GDP). GTP-bound Gαolf stimulates adenylate cyclase III (ACIII) to produce secondary messenger cyclic adenosine monophosphate (cAMP); and cAMP regulates

a heteromeric calcium channel composed of cyclic nucleotide gated (CNG) channels alpha 2 (CNGA2), alpha 4 (CNGA4), and beta 1b (CNGB1b) to permit entry of calcium ions. Calcium entry subsequently gates a chloride channel resulting in the efflux of chloride ions to depolarize the sensory neuron (Brunet et al. 1996; Song et al. 2008). Neural depolarization transmits the sensory signal to second-order neurons in the olfactory bulb in the brain. In addition to these canonical mechanisms, there are specialized subsets of sensory neurons in the MOE that alternatively signal through the transient receptor potential channel (TrpM5), or utilize cyclic guanidyl mono phosphate (cGMP) produced by the expression and activation of a membrane-receptor guanylyl-cyclase (GC-D). The smell of an odor is rapidly sensitizing. This is due, in part, to signaling events in the sensory neuron as activated olfactory signal transduction molecules are targeted for negative feedback regulation. Calcium and calcium-binding protein, calmodulin (CaM), bind and close the CNG channels. Calcium/Calmodulin (Ca/CaM)-dependent protein kinase II phosphorylates ACIII, reducing cAMP production. Desensitization of the odorant receptors appears to occur through phosphorylation by G protein–coupled receptor kinase 3 (GRK3) interaction with β-arrestin2 (Mashukova et al. 2006).

Olfactory Receptor Choice: The Singularity of OR Gene Expression

A remarkable property of the olfactory system is the ability to detect and distinguish among a seemingly endless variety of odor molecules. To achieve this precise discrimination, each individual olfactory sensory neuron expresses only one of the many OR genes present in the genome. Indeed this has been referred to as the "one neuron-one receptor" rule. Expression of a single OR dedicates each sensory neuron is responsive only to the cognate ligands of the expressed receptor, and sensory neurons that express different receptors display differential response profiles to odor ligands. The mechanisms that enable a neuron to activate the expression of a single receptor, and additionally silence all other OR loci distributed across the genome, are only partially understood. Recent whole genome chromatin immunoprecipitation analysis suggests that MOE neurons initially employ a distinct type of methylation of OR loci to generate heterochromatin silencing of all ORs. In a second step, one receptor reverses this silencing. The choice of which particular OR gene will be expressed in each neuron is thought to be largely a random process. However, each OR is restricted to a stereotypic expression "zone" in the MOE which may be regulated by zone-specific transcription factors. *OR* genes are polyallelic, and analysis of the expression of maternal and paternal genes have revealed that only one allele is expressed per neuron. This allelic exclusion simplifies the mechanistic challenge of coordinating receptor activation between both alleles and further increases the ability of genetic variation to functionally diversify odorant detection. Activation of the chosen receptor requires physical interaction of the OR promoter with a *cis*-acting locus control region (LCR). For one of the OR clusters, containing mouse olfactory receptor28 (MOR28), the LCR known as the "H-region," was identified as a 2.1 kb noncoding sequence that is evolutionary conserved between mouse and human. Deletion of the H-region abolishes the expression of MOR28 *OR* genes. Other LCRs that act in *cis* to regulate the expression of ORs within a cluster, or in *trans* to coordinate receptor choice across the genome, are still largely unknown. Additional experiments with transgenes have shown that if the chosen receptor cannot produce a functional protein (either through a frameshift or a deleltion) a second receptor will be activated. This suggests that the OR protein itself generates a negative feedback signaling to ultimately repress the activation of additional ORs. How functional ORs repress expression of additional ORs in olfactory sensory neurons remains unknown as mutating an ORs ability to activate G-protein mediated signaling has no effect on OR negative feedback regulation. Future experiments will be necessary to uncover the mechanisms that stabilize the expression of one receptor.

Role of ORs in Axon Pathfinding

Olfactory sensory neurons that express the same OR converge in specific neuropil, called glomeruli, in the olfactory bulb in the brain. Sensory neurons use both dorsal/ventral and anterior/posterior coordinates to

direct their axons to the correct glomeruli. While the general process of receptor choice is stochastic, each OR is expressed in a stereotypic "zone" within the olfactory epithelium. The mechanisms that regulate this zonally restricted anatomic organization have not been identified; however, the positional organization of ORs correlates with the expression of complementary gradients of guidance molecules including Robo-2, Neuropilin-2 (Nrp2), and Sema-3f in the sensory neurons. Corresponding gradients of Slit-1 and Slit-3 are present in the olfactory bulb thereby creating a topographic organization of glomeruli along the dorsal/ventral axis that mirrors the general spatial organization of ORs in the MOE (Cho et al. 2007).

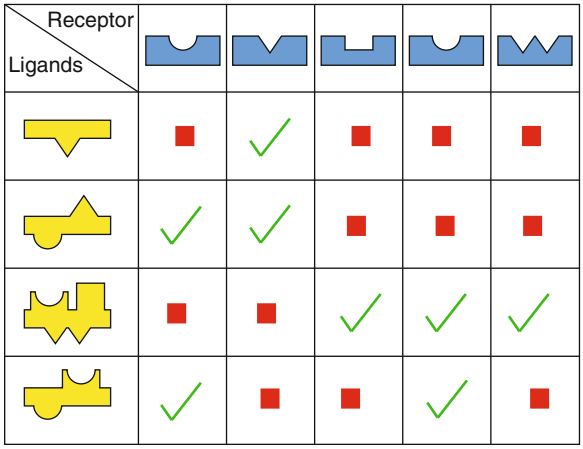
In contrast to these genetically determined mechanisms, an elegant series of experiments has shown involvement of an activity driven mechanism, signaling from the OR itself, to direct anterior/posterior axon guidance. The first indication of this arose from generation of mice in which pairs of genomic OR coding regions were swapped. These mutant mice displayed OR-driven mis-localization of their glomeruli. Moreover, OR protein was found to be localized to the axon terminus; a pivotal position to direct axon guidance. How do ORs guide axons? Key insights came from the observation that ORs mutant in Gs signaling failed to form proper glomeruli. Complementary studies of constitutively active Gs and PKA mutants indicate that high levels of OR-mediated cAMP directs axons to the posterior, while low cAMP levels directs axons to the anterior. cAMP levels directly correspond to the transcription of neuropilin-1 (Nrp1) an axon guidance molecule. Nrp1 and Sema3A expression levels form complementary anterior/posterior gradients in olfactory neurons. Axon–axon interactions are thought to sort and organize neurons as they develop toward their targets (Imai et al. 2009). While these mechanisms account for general axon targeting, it is thought that OR-mediated cAMP signaling additionally further refines glomerular patterning. Levels of neuronal activity regulate additional sets of axon guidance and adhesion molecules including Kirrel2, Kirrel3, EphA5, and ephrin-A. The repulsive and adhesive effects of these molecules are thought to promote homotypic formation of individual glomeruli (Serizawa et al. 2006). The mechanism of how individual ORs generate differential levels of cAMP activity that can distinguish the position of neighboring glomeruli remains to be understood.

The Combinatorial Code of ORs to Encode Odor Identity

How do the repertoire of ORs (390 in humans or 1,468 in mice) discriminate a seemingly endless array of odor ligands? Elegant experiments have shown that ORs can respond to a variety of structurally distinct odor molecules (Fig. 2) (Malnic et al. 1999). Each neuron (and therefore OR) is not tuned to the entire ligand per se, but instead is responsive to molecular features of the given ligand, such as the carbon chain length or functional group components of the odor molecule. Therefore, an individual OR can detect many ligands that differ in overall structure as long as they share a common molecular feature recognized by the OR. Moreover, this principle results in each ligand activating multiple receptors each able to bind different features of the molecule's overall structure. Pure ligands have been shown to activate approximately 10% of the expressed ORs. This distributed activity is referred to as the "combinatorial code" of odor processing. Assuming that the OR repertoire is able to detect a large variety of molecular features, this strategy would enable the detection of potentially unlimited number of odor molecules. The combinatorial coding strategy immediately deconstructs each odorant at the sensory receptor interface and because this information is separated into anatomically distinct glomeruli in the olfactory bulb, each odorant generates multiple lines of distinct activity processed in parallel. How this information is ultimately bound into an odor percept is still unknown.

Other Olfactory Receptors

In addition to the ORs, the main olfactory epithelium also expresses a family of trace amine receptors (TAARs) which have been shown to detect volatile amines (Liberles and Buck 2006). Additionally most vertebrates have an accessory olfactory system, the vomeronasal organ (VNO), which is physiologically and morphologically distinct from the main olfactory epithelium. The VNO has been shown to express at least three classes of chemosensory receptors that are evolutionarily distinct both from the odorant receptors and from each other: vomeronasal receptors class I (V1Rs, Vmn1Rs) (Dulac and Axel 1995), vomeronasal receptors class II (V2Rs, Vmn2Rs) (Herrada and Dulac



Olfactory Receptors, Fig. 2 Combinatorial receptor codes for odors. *Green* tick mark shows receptors (*top panel*) that recognize odorants (*left panel*). The identity of each odor molecule is encoded by a unique combination of receptors. Each receptor can act as one component of the combinatorial code for more than one odor molecule. This model allows for the recognition and discrimination of almost unlimited number and variety of odor molecules

1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997), and formyl peptide receptors (FPRs) (Liberles et al. 2009; Riviere et al. 2009). GPCRs expressed in the VNO are thought to largely detect volatile and peptide pheromonal ligands that mediate social behaviors like courtship, territorial aggression, gender and individual recognition, maternal aggression, and interspecies defense (Bean and Wysocki 1989; Chamero et al. 2007; Del Punta et al. 2002; Haga et al. 2010; Kimoto et al. 2005; Leinders-Zufall et al. 2000; Papes et al. 2010; Wysocki and Lepri 1991).

Summary

Environmental odor cues are detected by hundreds of seven-membrane-spanning odorant receptors, expressed in the olfactory system. These olfactory receptors can be broadly classified into type I and type II, type I being evolutionarily more ancient and conserved. On binding odors, ORs can signal through a canonical pathway involving cAMP second messenger that regulates the activity of the CNG channel, depolarizing the neurons. Additionally there are other noncanonical signal transductions involving TrpM5 or cGMP. Each olfactory neuron expresses a specific receptor and activation of different combinations of receptors by an odor or mixture of odors helps an organism to distinguish an endless repertoire of chemicals.

References

Bakalyar HA, Reed RR. The second messenger cascade in olfactory receptor neurons. Curr Opin Neurobiol. 1991;1(2):204–8.

Bean NJ, Wysocki CJ. Vomeronasal organ removal and female mouse aggression: the role of experience. Physiol Behav. 1989;45(5):875–82.

Brunet LJ, Gold GH, Ngai J. General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron. 1996;17(4):681–93.

Buck L, Axel R. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 1991;65(1):175–87.

Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A, et al. Identification of protein pheromones that promote aggressive behaviour. Nature. 2007;450(7171):899–902.

Cho JH, Lepine M, Andrews W, Parnavelas J, Cloutier JF. Requirement for Slit-1 and Robo-2 in zonal segregation of olfactory sensory neuron axons in the main olfactory bulb. J Neurosci. 2007;27(34):9094–104.

Del Punta K, Leinders-Zufall T, Rodriguez I, Jukam D, Wysocki CJ, Ogawa S, et al. Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. Nature. 2002;419(6902):70–4.

Dhallan RS, Yau KW, Schrader KA, Reed RR. Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature. 1990;347(6289):184–7.

Dulac C, Axel R. A novel family of genes encoding putative pheromone receptors in mammals. Cell. 1995;83(2):195–206.

Haga S, Hattori T, Sato T, Sato K, Matsuda S, Kobayakawa R, et al. The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. Nature. 2010;466(7302):118–22.

Herrada G, Dulac C. A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. Cell. 1997;90(4):763–73.

Imai T, Yamazaki T, Kobayakawa R, Kobayakawa K, Abe T, Suzuki M, et al. Pre-target axon sorting establishes the neural map topography. Science. 2009;325(5940):585–90.

Jones DT, Reed RR. Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. Science. 1989;244(4906):790–5.

Kimoto H, Haga S, Sato K, Touhara K. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature. 2005;437(7060):898–901.

Leinders-Zufall T, Lane AP, Puche AC, Ma W, Novotny MV, Shipley MT, et al. Ultrasensitive pheromone detection by mammalian vomeronasal neurons. Nature. 2000;405(6788):792–6.

Liberles SD, Buck LB. A second class of chemosensory receptors in the olfactory epithelium. Nature. 2006;442(7103):645–50.
Liberles SD, Horowitz LF, Kuang D, Contos JJ, Wilson KL, Siltberg-Liberles J, et al. Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. Proc Natl Acad Sci USA. 2009;106(24):9842–7.
Malnic B, Hirono J, Sato T, Buck LB. Combinatorial receptor codes for odors. Cell. 1999;96(5):713–23.
Mashukova A, Spehr M, Hatt H, Neuhaus EM. Beta-arrestin2-mediated internalization of mammalian odorant receptors. J Neurosci. 2006;26(39):9902–12.
Matsunami H, Buck LB. A multigene family encoding a diverse array of putative pheromone receptors in mammals. Cell. 1997;90(4):775–84.
Papes F, Logan DW, Stowers L. The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. Cell. 2010;141(4):692–703.
Riviere S, Challet L, Fluegge D, Spehr M, Rodriguez I. Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. Nature. 2009;459(7246):574–7.
Ryba NJ, Tirindelli R. A new multigene family of putative pheromone receptors. Neuron. 1997;19(2):371–9.
Serizawa S, Miyamichi K, Takeuchi H, Yamagishi Y, Suzuki M, Sakano H. A neuronal identity code for the odorant receptor-specific and activity-dependent axon sorting. Cell. 2006;127(5):1057–69.
Song Y, Cygnar KD, Sagdullaev B, Valley M, Hirsh S, Stephan A, et al. Olfactory CNG channel desensitization by Ca2+/CaM via the B1b subunit affects response termination but not sensitivity to recurring stimulation. Neuron. 2008;58(3):374–86.
Wysocki CJ, Lepri JJ. Consequences of removing the vomeronasal organ. J Steroid Biochem Mol Biol. 1991;39(4B):661–9.
Zhang X, Firestein S. The olfactory receptor gene superfamily of the mouse. Nat Neurosci. 2002;5(2):124–33.

Olfrs

► Olfactory Receptors

Oncogene c-Mel

► Rab8

Opb

► Rab23

Opb2

► Rab23

Open Brain

► Rab23

Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function

Vladana Vukojević, Yu Ming and Lars Terenius
Department of Clinical Neuroscience,
Karolinska Institute, Stockholm, Sweden

Synonyms

Nociceptin opioid receptor; δ-opioid receptor; κ-opioid receptor; μ-opioid receptor

Historical Background

The existence of opioid receptors (OR) was postulated in 1954 (Beckett and Casy 1954), at a time when evidence for specific drug-responsive substances was evasive and the concept of biochemical receptors was still in its infancy. Unequivocal confirmation of specific binding sites for opioids in the brain was first reported in 1973 (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). This breakthrough was shortly followed by evidence that opioid ligands bind specifically to different anatomical locations and show different pharmacological activity, suggesting that several types of opioid receptors exist (Martin et al. 1976; Lord et al. 1977). Based on extensive pharmacological and animal model studies that followed, opioid receptors were originally classed in four types: mu (μ), kappa (κ), sigma (σ) and delta (δ). They were named after specific ligands used for their characterization: μ after morphine, κ after ketocyclazocine, and σ after SKF-10047, whereas the δ-opioid receptor was

identified in mouse and named after the thick-walled tube in the male reproductive system that transports sperm cells from the epididymis (called *vas deferens*) in which it was characterized (Lord et al. 1977). The ultimate proof for opioid receptor existence and partitioning into different types was provided by the isolation of mouse δ-opioid receptor mRNA, leading to cDNA synthesis and receptor cloning (Kieffer et al. 1992; Evans et al. 1992). Subsequent studies have led to the identification of receptor sequences for the μ and κ opioid receptors, dismissed the σ-opioid receptor from the opioid receptor family, and revealed the existence of an unknown sequence that was similar to opioid receptors but differed in opioid ligand binding. This "opioid receptor-like" (ORL) or nociceptin/orphanin (N/OFQ) protein remained an "orphan" receptor until its corresponding endogenous peptide ligand nociceptin/orphanin FQ was identified in 1995 (Meunier et al. 1995; Reinscheid et al. 1995). It is nowadays commonly regarded that the opioid receptor family contains four types, the classical opioid receptors μ, δ, and κ, and the nonclassical nociceptin opioid receptor. The Committee on Receptor Nomenclature and Drug Classification of the International Union of Basic and Clinical Pharmacology (IUPHAR) has recommended that μ, δ, κ and nociceptin opioid receptors should be denoted as MOP, DOP, KOP, and NOP, respectively.

Opioid Receptor Relevance

Opioid receptors MOP, DOP, KOP, and NOP, and their respective peptide ligands endorphins, enkephalins, dynorphins, and nociceptin/orphanin constitute the endogenous opioid neuromodulatory system. This system plays a critical role in regulating vital physiological, sensory, cognitive and emotional functions (Bodnar 2010). For example, the opioid system modulates the nociceptive pathways and plays a decisive role in substance and behavioral addiction, mental disorders, memory, learning, and response to stress. In addition, the opioid system is implicated in the regulation of the neuroendocrine, cardiovascular, gastrointestinal, renal and hepatic functions, hormonal activity, respiration, thermoregulation, and immunological responses.

Opioid receptors are also very important pharmacological targets – natural alkaloid opiates like morphine, or synthetic opiates like methadone are potent pain-killers but also highly addictive drugs. Opioid antagonists, such as naltrexone are clinically used to reduce craving in substance addiction (heroin, alcohol, and amphetamine), food abuse in obesity, and the hedonic responses in non-substance-related, that is, behavioral addictions (gambling and gaming).

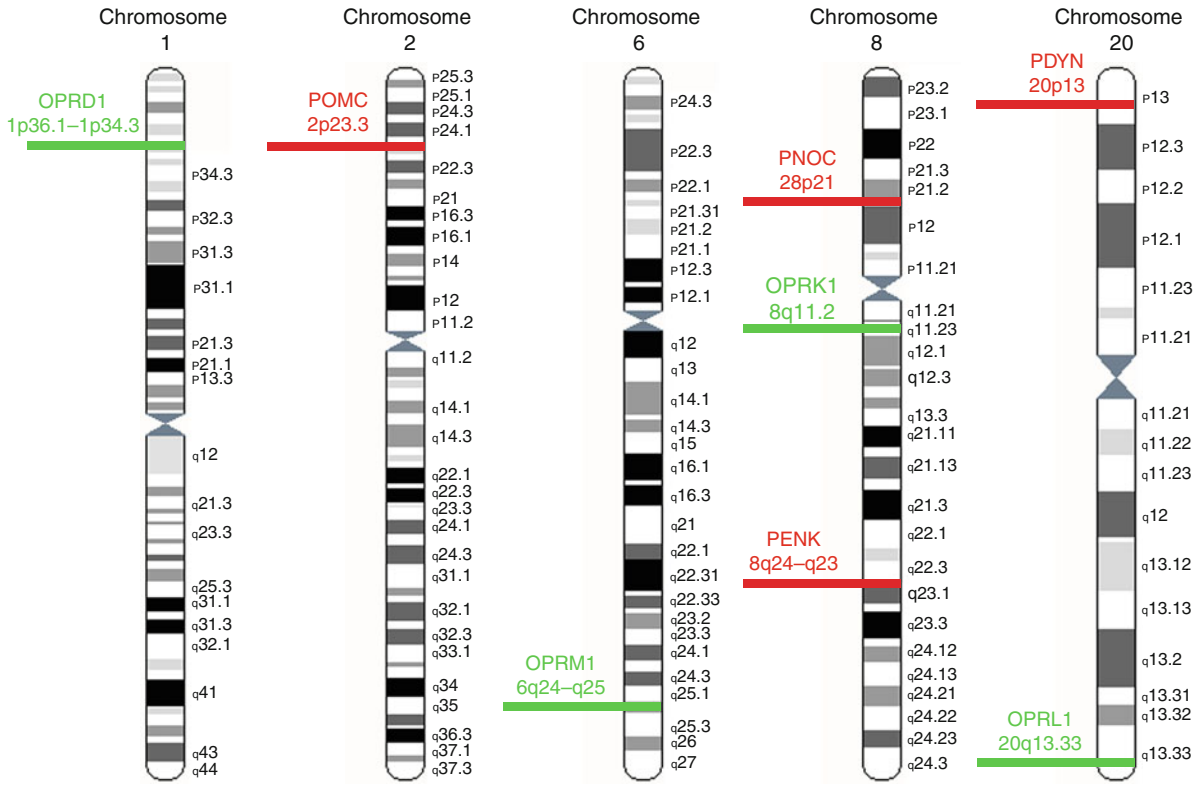
Opioid Receptor Evolution

The opioid system is an ancient neuromodulatory system. Chromosomal phylogeny studies suggest that the opioid system, with four receptor types and four opioid peptide precursors, was already present approximately 450 million years ago (Dreborg et al. 2008). These studies imply that the ancestral opioid peptide precursor and opioid receptor gene were located on the same chromosome and concomitantly duplicated in two whole genome duplication events. The first genome duplication incident yielded two genes DOP/MOP and NOP/KOP and their precursors. A second genome duplication event has led to the development of four opioid receptor types, and three opioid precursor molecules. The fourth opioid peptide precursor gene appeared to be created later on, through local gene duplication (Dreborg et al. 2008).

The opioid system, with four types of receptors and opioid peptide precursors has been identified in all vertebrate organisms, in neuronal and nonneuronal cells. In humans, opioid receptors are encoded by opioid receptor (OPR) genes: OPRM1 encoding MOP, OPRD1 encoding DOP, OPRK1 encoding KOP, and OPRL1 encoding NOP. They are located on four different chromosomes: OPRD1 on chromosome 1, OPRM1 on chromosome 6, OPRK1 on chromosome 8, and OPRL1 on chromosome 20 (Fig. 1). Opioid peptides are derived from four prepropeptide genes: the pro-opiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN), and prepronociceptin (PNOC), encoding the precursors of endorphins, enkephalins, dynorphins, and nociceptin/orphanin, respectively (Fig. 1).

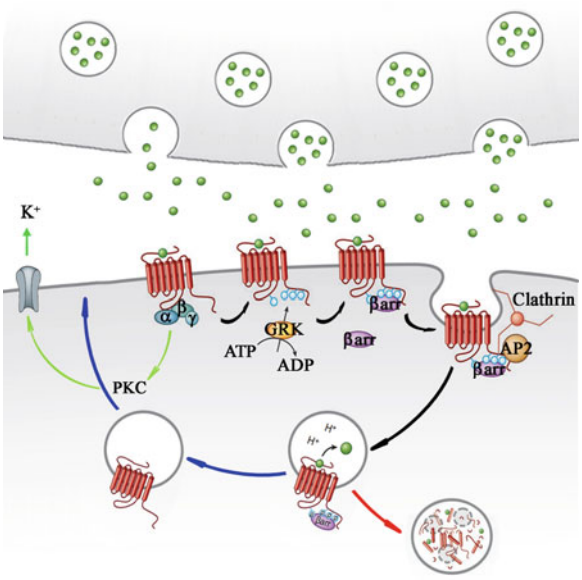
Opioid Receptor Cellular Physiology and Function

The function of opioid receptors is to transmit chemical signals across the cellular plasma membrane.



Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function, Fig. 1 Chromosomal mapping of human opioid system genes. Opioid receptors genes: OPRM1 encoding MOP, OPRD1 encoding DOP, OPRK1 encoding KOP, and OPRL1 encoding NOP sequences are located on four different chromosomes: 6, 1, 8, and 20, respectively. The opioid peptide precursors' genes: pro-opiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN), and pronociceptin (PNOC) are located on three different chromosomes: 2, 8, 8, and 20, respectively. Chromosomal phylogeny studies suggest that the ancestral opioid peptide precursor and opioid receptor genes were located on the same chromosome and suggest that the current system with four opioid receptor types and four opioid peptide precursors had evolved through two whole genome duplication events, where the ancestral opioid receptor and opioid peptide precursor genes were concomitantly duplicated. Through this process all four opioid receptor genes and three opioid peptide precursor genes were derived, whereas the fourth opioid peptide precursor gene was suggested to evolve through a local duplication event (Dreborg et al. 2008). (The image is adapted using the *Ensembl* genome databases for vertebrates and other eukaryotic species as template http://Jun2011.archive.ensembl.org/Homo_sapiens/Gene/Family/Genes?db=core;family=ENSFM00500000269674;g=ENSFM00500000269674, Flicek et al. 2011)

The complex cellular physiology of opioid receptors is an integral part of opioid receptor function. In brief, the opioid receptors are assembled by ribosomes, transferred to the endoplasmic reticulum (ER), the Golgi complex, and the trans-Golgi network (TGN) and sorted into transporting vesicles. During this process, opioid receptor molecules undergo several posttranslational modifications such as disulfide bond formation, palmitoylation, acetylation, phosphorylation, and glycosylation. At the nerve termini transporting vesicles fuse with the plasma membrane, enabling opioid receptor presentation at the neuronal surface. Opioid peptides secreted by presynaptic neurons bind to opioid receptors on the postsynaptic neurons and activate the secondary messenger system (Fig. 2). Opioid receptors have no direct link with effector proteins. Instead, the message is relayed via the so-called G proteins, heterotrimeric guanine nucleotide-binding proteins –opioid receptors are therefore classed as G protein-coupled receptors (GPCR). It has been demonstrated that activation of opioid receptors may lead to the closing of voltage sensitive calcium channels (VSCC), stimulation of potassium efflux or reduced cyclic adenosine monophosphate (cAMP) production



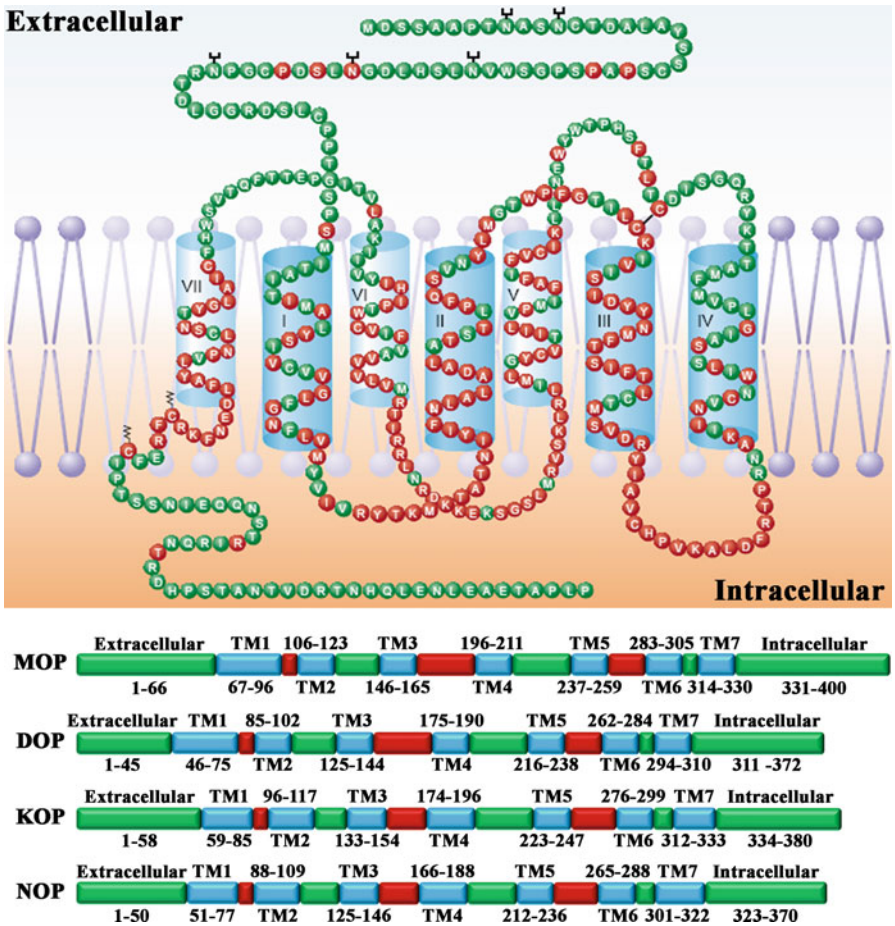
Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function, Fig. 2 Schematic presentation of a synaptic cleft with opioid receptor signaling and intracellular trafficking. Endogenous opioid ligand molecules (*green sphere*) secreted from the presynaptic neuron (on *top*) bind to an opioid receptor (*red*, far *left*) located at the postsynaptic neuron (*down*). The agonist-bound receptor initiates cellular signaling by activating the heterotrimeric G protein and causing its dissociation into α- and βγ-subunits. The subunits can activate a number of cellular responses, such as protein kinase C (PKC)-mediated enhancement of K_{ATP} channel activity (*green arrows*), whereas the agonist-bound opioid receptor devoid of the G protein rapidly undergoes phosphorylation by GPCR kinases (GRK) (*black arrows*). Selective phosphorylation of the activated receptor and subsequent binding of β-arrestin prevents further interactions of the activated receptor with G proteins and enables receptor sorting to clathrin-coated pits for receptor endocytosis, thereby effectively terminating the G protein-mediated signaling. Further sorting of endocytosed opioid receptors between divergent downstream pathways may produce additional distinct effects on cellular signaling. For example, sorting of internalized receptors to lysosomes promotes proteolytic degradation of receptors (*red arrow*), preventing receptors from signaling again and producing a prolonged attenuation of cellular signaling. In contrast, sorting of internalized receptors into a rapid recycling pathway promotes the return of intact receptors to the plasma membrane and effectively resensitizes cells to respond again to the extracellular ligand (*blue arrow*). This mechanism has been inferred from numerous studies; however, quantitative information on the cellular dynamics of opioid receptors and the kinetics of the underlying interactions is still limited (Image adapted and reprinted with permission from Hanyaloglu and von Zastrow 2008)

via inhibition of adenylyl cyclase, etc. In this way, opioid ligands cause a range of physiological and behavioral effects that are lasting several seconds to several days at the organism level (Dean et al. 2009). Selective phosphorylation of the activated receptor and subsequent binding of β-arrestin prevents further interactions of the activated receptor with G proteins and enables receptor sorting to clathrin-coated pits for receptor endocytosis, thereby effectively terminating the G protein-mediated signaling (Fig. 2).

Increasing experimental evidence supports the notion that opioid receptor cellular dynamics – the time-dependent change in receptor properties and localization in the cell, is central to opioid receptor signaling. Cellular dynamics of surface receptors is a critical factor in determining receptor availability, function, and the downstream consequences of receptor activation (Hanyaloglu and von Zastrow 2008). For example, endocytic membrane trafficking is an important regulator of opioid receptor density at the cell surface (Fig. 2). Through this dynamic process the surface density of opioid receptors and their availability for ligand activation can be controlled without modifying the overall number of receptors through receptor synthesis (up- or downregulation). Opioid receptor ligands differ in their ability to induce receptor trafficking. For example, opioid peptides such as DAMGO cause internalization of MOP, whereas the alkaloid morphine produces little MOP internalization.

Opioid Receptor Structure

The crystal structures of opioid receptors are not yet determined. Therefore, opioid receptor's structures and structural requirements for ligand binding and selectivity have to be assessed indirectly, using methods such as site-directed mutagenesis, affinity labeling, and chimeric receptor design. In addition, computational techniques enabling homology modeling are used in order to predict the three-dimensional (3D) receptor structure and the possible binding sites for different ligands (docking studies). Over the years, these methods have been successfully used to build realistic models of the opioid receptors primary, secondary, and tertiary structures in their native environment, the cellular plasma membrane and identify the key contacts in ligand recognition sites (Kane et al. 2006). These studies have shown that opioid receptors are integral, rhodopsin-like proteins



Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function, Fig. 3 Opioid receptor structure. Schematic drawing of human MOP structure. Amino acids conserved between human MOP, DOP, and KOP are indicated in red. Putative asparagine-linked glycosylation sites in the extracellular N-terminus and alleged palmitoylation sites at cysteine residues in the C-terminus are indicated. A potential intramolecular disulfide bond between cysteine residues in extracellular loops 1 and 2 is also shown. The sites for opioid ligand binding are not unequivocally determined, but the role of Asp in the third transmembrane domain (TM3:08) and His in the sixth transmembrane domain (TM6:17) have been repeatedly shown to be relevant for opioid ligand binding. The large intracellular loop between TM5 and TM6 is presumed to be the contact site with the G-protein α-subunit. A schematic representation of the secondary structure all for types of opioid receptors is presented below

that span the plasma membrane by seven helical transmembrane (TM) domains (Fig. 3). Opioid receptor types show a high sequence identity in their transmembrane (TM) domains (about 75%) and the cytoplasmic loops (about 65%), whereas the sequence identity in the N- and C-terminal domains and extracellular loops appears to be rather modest (about 37%). The sites for opioid ligands binding are not unequivocally determined, but the role of Asp in the third transmembrane domain (TM3:08) and His in the sixth transmembrane domain (TM6:17) have been repeatedly shown to be relevant for opioid ligands binding. The large intracellular loop between TM5 and TM6 is presumed to be the contact site with the G-protein α-subunit.

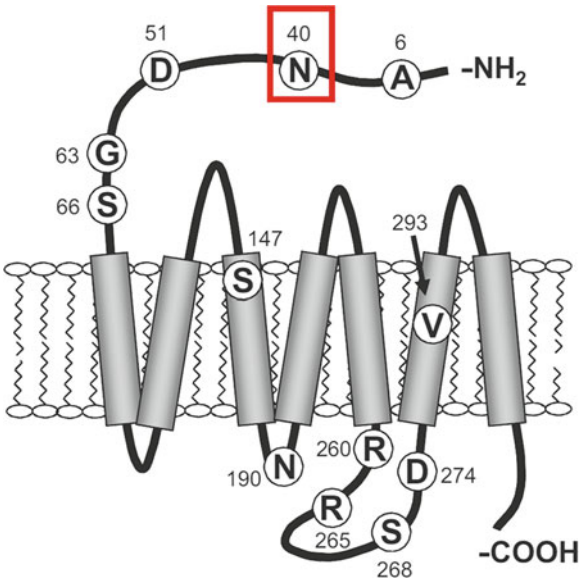
Opioid Receptor Isoforms

Naturally occurring differences in opioid receptor primary structure can alter opioid receptor binding properties and signaling, thereby predisposing for susceptibility to disease and receptiveness to therapy.

Two principle mechanisms through which opioid receptor variants are generated are missense mutations and alternative splicing.

In missense mutations, a single nucleotide is changed yielding a protein molecule with only one amino acid different. To this point, 18 missense mutations have been identified in the human MOP gene OPRM1, with 12 missense mutations occurring naturally at a frequency >1%. Consequences of the most commonly occurring missense mutations in the OPRM1 gene on MOP primary structure are schematically depicted in Fig. 4. For example, the N40D polymorphism (Fig. 4, highlighted in red) is suggested to be related with improved response to naltrexone, an opiate antagonist used clinically for treating addiction to opiate drugs and alcohol (Anton et al. 2008).

Splicing is a regulated modification of the pre-mRNA, generated by gene transcription in which introns are removed and exons are joined. Alternative splicing of pre-mRNA may result in the creation of different mRNAs, which may be translated into opioid receptor isoforms differing in several amino acids. So far, at least 6 alternative splicing variants of hMOP have been described (Pan et al. 2003).



Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function, Fig. 4 MOP variants generated by naturally occurring missense mutations. Schematic drawing of MOP structure with alterations in amino acid sequence due to the 12 most frequently encountered missense mutations. The N40D polymorphism (Fig. 4, highlighted in *red*) is suggested to be related to improved response to naltrexone, an opiate antagonist used clinically for treating addiction to opiate drugs and alcohol 2006; Fortin et al. 2010)

Opioid Receptor Oligomerization

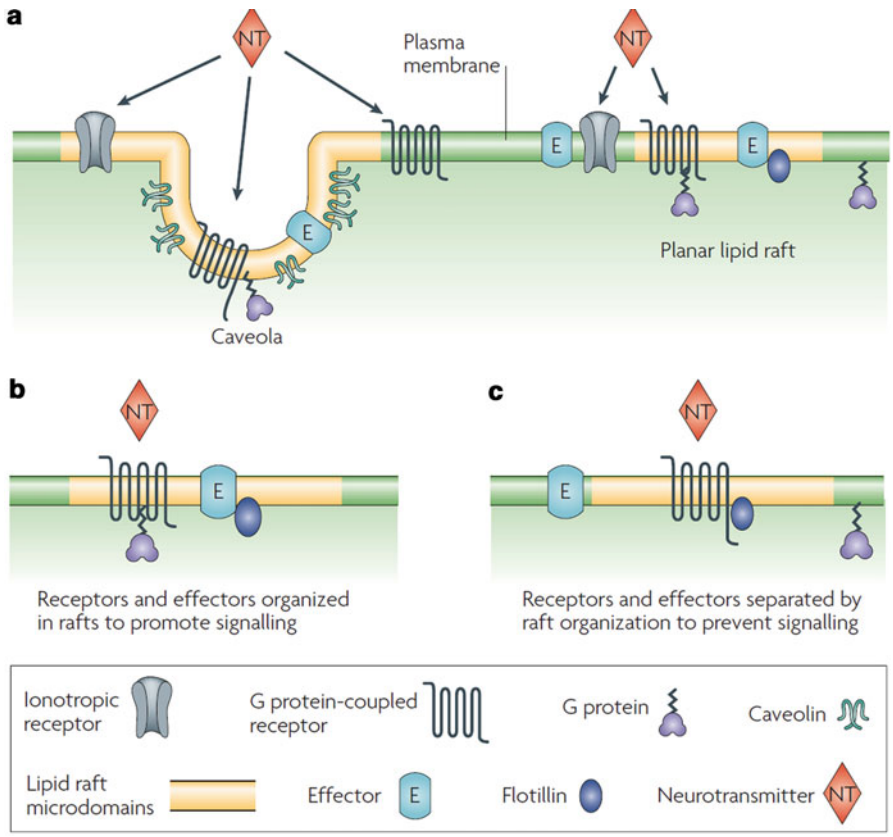
Surface receptor oligomerization is a common way to increase the functional repertoire of the receptor and represents a key regulatory step in the function of several GPCRs (Milligan 2010). In addition, receptor oligomerization may be a critical step for rapid "fine-tuning" of receptor density at the plasma membrane. The dynamic equilibrium between monomers and oligomers can be changed quickly in one direction or the other, rendering the receptor available/not available for the ligand (Vukojević et al. 2008). Opioid receptor homo- and hetero-oligomerization has been inferred from a variety of studies, however quantitative information on the cellular dynamics of opioid receptor dimers is still limited and many molecular details of the oligomerization process have yet to be resolved.

Opioid Receptor Association with Lipid Rafts

The cellular dynamics and function of opioid receptors can be significantly affected by its micro-environment in the cell membrane. The plasma membrane is highly organized into structurally and functionally distinct microdomains. Opioid receptors, G-proteins and signaling effectors, such as second-messenger-generating enzymes appear to be primarily localized to membrane microdomains called lipid rafts (Fig. 5) (Baker et al. 2007). Disruption of lipid rafts may alter the binding properties and signal transduction of opioid receptors localized in rafts. For example, it has been shown that disruption of lipid rafts in rat caudate putamen membranes decreased the E_{max} values of both DAMGO and morphine at MOP without affecting their EC_{50} (Huang et al. 2008).

Summary

The opioid neuromodulatory system is a dynamic, spatiotemporally organized structure comprising signaling circuitries at different levels of organization – the cellular, organ, and organism levels. The opioid



Opioid Receptors: Cellular and Molecular Mechanisms Underlying Opioid Receptor Function, Fig. 5 Schematic representation of lipid raft's role in opioid receptor function. (**a**) Lipid rafts are cholesterol- and sphingolipids-enriched submicroscopic assemblies about 25–100 nm in diameter, which are spontaneously generated in the plasma membrane. In mammalian cells, these dynamic assemblies can be planar or flask-shaped invaginations of the plasma membrane to which signaling and effectors molecules, such as opioid receptors, G proteins, and second-messenger generating enzymes may be located forming functional signaling units. (**b**) The lipid raft signaling hypothesis proposes that signaling molecules are spatially organized to promote kinetically favorable interactions that are necessary for signal transduction. (c) Alternatively, lipid raft microdomains might inhibit interactions by separating the signaling molecules, thereby dampening signaling responses image reprinted permission from Allen et al. 2007)

system is implicated in the regulation of physiological, sensory, cognitive, and emotional functions. The importance of the opioid system for normal physiology is well accepted and drugs addressing this innately complex system have been used for centuries recreationally and for medical purposes. In spite of this vast experience, competence in opiate pharmacotherapy, and intensive research, the opioid system function is not fully understood and it is still not known how to control the potentially deleterious effects of opioids – given the importance of the opioid system it is not surprising that drugs addressing it show numerous unwanted side effects, such as inhibition of gastrointestinal motility, respiratory depression, muscle rigidity, altered thermoregulation, sedation, dependence, and abuse.

This review summarizes current understanding of the cellular and molecular mechanisms underlying opioid system function, with a focus on the opioid receptor role in cellular signaling. In our opinion, the most pertinent information still missing are the 3D structures of opioid receptors at atomic resolution, quantitative information on the kinetics of opioid receptor interactions in living cells, opioid receptors organization in the plasma membrane, and cellular trafficking dynamics.

The necessity of knowing the 3D receptor structure is obvious from the notion that structure determines

selectivity. In addition, the 3D opioid receptor structure is expected to shed more light on an unusual feature of the MOP receptor, its potential to successfully accommodate and elicit a specific response to more than dozens of compounds differing largely in chemical composition and reactivity.

Cellular mechanisms are essential for understanding opioid receptor function because drug-induced signals are converted in long-term alterations in cellular function at this level of organization. Changes in opioid receptor organization in the plasma membrane and altered cellular trafficking may be the first steps in a cascade of events leading to remodeling of opioid cellular signaling circuits, and eventually to altered behavior, drug-seeking, and compulsive use. Such changes at the neuronal level may even underlie long persistence of addiction and risk for relapse. Quantitative studies of opioid receptor function at the molecular and cellular levels are therefore of relevance for understanding the integral opioid system function. Such studies may also deepen our understanding of the opioid system role in development of tolerance and addiction, adaptive transformations specific for MOP-mediated signaling pathways that are largely restricting the useful clinical application of opiates.

References

Allen JA, Halverson-Tamboli RA, Rasenick MM. Lipid raft microdomains and neurotransmitter signalling. Nat Rev Neurosci. 2007;8:128–40.

Anton RF, Oroszi G, O'Malley S, Couper D, Swift R, Pettinati H, Goldman D. An evaluation of mu-opioid receptor (OPRM1) as a predictor of naltrexone response in the treatment of alcohol dependence: results from the Combined Pharmacotherapies and Behavioral Interventions for Alcohol Dependence (COMBINE) study. Arch Gen Psychiatry. 2008;65:135–44.

Baker A, Saulière A, Dumas F, Millot C, Mazères S, Lopez A, Salomé L. Functional membrane diffusion of G-protein coupled receptors. Eur Biophys J. 2007;36:849–60.

Beckett AH, Casy AF. Synthetic analgesics: stereochemical considerations. J Pharm Pharmacol. 1954;6:986–1001.

Bodnar RJ. Endogenous opiates and behavior. Peptides. 2010;31:2325–59.

Dean R, Bilsky EJ, Stevens Negus S. Opiate receptors and antagonists. From Bench to Clinic. New York: Humana Press; 2009.

Dreborg S, Sundström G, Larsson TA, Larhammar D. Evolution of vertebrate opioid receptors. Proc Natl Acad Sci USA. 2008;105:15487–92.

Evans CJ, Keith Jr DE, Morrison H, Magendzo K, Edwards RH. Cloning of a delta opioid receptor by functional expression. Science. 1992;258:1952–5.

Flicek P, Amode MR, Barrell D, Beal K, Brent S, Chen Y, Clapham P, Coates G, Fairley S, Fitzgerald S, Gordon L, Hendrix M, Hourlier T, Johnson N, Kähäri A, Keefe D, Keenan S, Kinsella R, Kokocinski F, Kulesha E, Larsson P, Longden I, McLaren W, Overduin B, Pritchard B, Riat HS, Rios D, Ritchie GR, Ruffier M, Schuster M, Sobral D, Spudich G, Tang YA, Trevanion S, Vandrovcova J, Vilella AJ, White S, Wilder SP, Zadissa A, Zamora J, Aken BL, Birney E, Cunningham F, Dunham I, Durbin R, Fernández-Suarez XM, Herrero J, Hubbard TJ, Parker A, Proctor G, Vogel J, Searle SM. Ensembl 2011. Nucleic Acids Res. 2011;39:D800–6.

Fortin JP, Ci L, Schroeder J, Goldstein C, Montefusco MC, Peter I, Reis SE, Huggins GS, Beinborn M, Kopin AS. The μ-opioid receptor variant N190K is unresponsive to peptide agonists yet can be rescued by small-molecule drugs. Mol Pharmacol. 2010;78:837–45.

Hanyaloglu AC, von Zastrow M. Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu Rev Pharmacol Toxicol. 2008;48:537–68.

Huang P, Chen C, Xu W, Yoon SI, Unterwald EM, Pintar JE, Wang Y, Chong PL, Liu-Chen LY. Brain region-specific N-glycosylation and lipid rafts association of the rat mu opioid receptor. Biochem Biophys Res Commun. 2008;365:82–8.

Kane BE, Svensson B, Ferguson DM. Molecular recognition of opioid receptor ligands. AAPS J. 2006;8:E126–37.

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG. The delta-opioid receptor: Isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA. 1992;89:12048–52.

Levac BA, O'Dowd BF, George SR. Oligomerization of opioid receptors: generation of novel signaling units. Curr Opin Pharmacol. 2002;2:76–81.

Lord JAH, Waterfield AA, Hughes J, Kosterlitz HW. Endogenous opioid peptides: multiple agonists and receptors. Nature. 1977;267:495–9.

Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther. 1976;197:517–32.

Meunier J-C, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour J-L, Guillemot JC, Ferrara P, Monsarrat B. Isolation and structure of the endogenous agonist of opioid receptorlike ORL1 receptor. Nature. 1995;377:532–5.

Milligan G. The role of dimerisation in the cellular trafficking of G-protein-coupled receptors. Curr Opin Pharmacol. 2010;10:23–9.

Pan YX, Xu J, Mahurter L, Xu M, Gilbert AK, Pasternak GW. Identification and characterization of two new human mu opioid receptor splice variants, hMOR-1O and hMOR-1X. Biochem Biophys Res Commun. 2003;301:1057–61.

Pert CB, Snyder SH. Opiate receptor: demonstration in nervous tissue. Science. 1973;179:1011–4.

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ, Civelli O. Orphanin FQ: a neuropeptide that

activates an opioidlike G protein-coupled receptor. Science. 1995;270:792–4.
Simon EJ, Hiller JM, Edelman I. Stereospecific binding of the potent narcotic analgesic (3 H) Etorphine to rat-brain homogenate. Proc Natl Acad Sci USA. 1973;70:1947–9.
Terenius L. Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. Acta Pharmacol Toxicol (Copenh). 1973;32:317–20.
Vukojević V, Ming Y, D'Addario C, Hansen M, Langel U, Schulz R, Johansson B, Rigler R, Terenius L. Mu-opioid receptor activation in live cells. FASEB J. 2008;22:3537–48.

OR51E2

► PSGR

OR51E3P

► PSGR

OR52A2

► PSGR

ORs

► Olfactory Receptors

Other Myosins, "Unconventional" Myosins

► Myosins

Ovarian Cancer Antigen OA3

► CD47

OX44

► CD53

Ox-44

► CD53

P

P/CAF

► PCAF Lysine Acetyltransferase

P/Q-Type

► Voltage-Gated Calcium Channels: Structure and Function (CACNA)

P1/eIF2A Protein Kinase

► PKR

p120 GAP

► RASA1

p120 RasGAP

► RASA1

p130Cas

Dominique M. Donato[1] and Steven K. Hanks[2]
[1]Physics of Life Processes, Leiden Institute of Physics, Leiden University, CA, Leiden, The Netherlands
[2]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN, USA

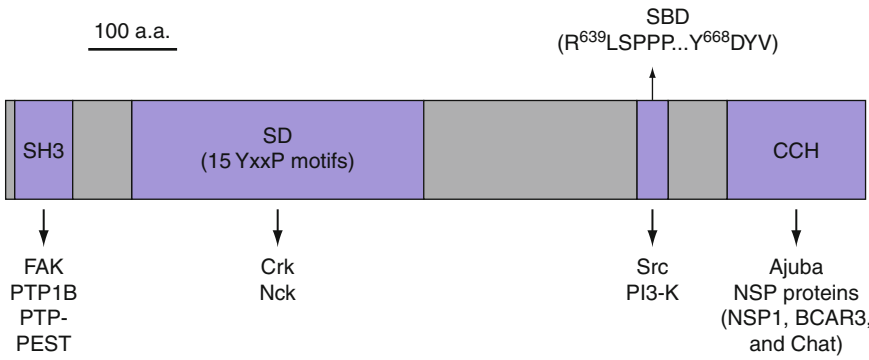
Synonyms

Breast cancer antiestrogen resistance protein 1 (BCAR1); Cas scaffolding protein family member 1 (CASS1); Crk-associated substrate (CAS)

Historical Background

p130Cas is a major substrate of the ► Src tyrosine kinase that functions in integrin signaling to promote cell motility, invasion, proliferation, and survival (reviewed by Defillipi et al. 2006; Tikhmyanova et al. 2010). p130Cas was first recognized in the late 1980s by immunoblot detection with an anti-phosphotyrosine antibody in cells transformed by retroviral oncogenes v-*crk* and v-*src*. The p130 designation indicates the apparent molecular mass of ~130 kDa during SDS polyacrylamide gel electrophoresis (PAGE), and Cas is acronymic for "Crk-associated substrate." Characterization of the direct interactions of p130Cas with the v-Crk and v-Src proteins was significant in the recognition of Src homology 2 (SH2) domains as phosphotyrosine-binding modules in signal transduction. p130Cas was independently identified as

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



p130Cas, Fig. 1 The primary structure of p130Cas and major interacting proteins. The primary structure is depicted to scale with major domains emphasized. *SH3* Src homology 3, *SD* substrate domain, *SBD* Src-binding domain, *CCH* C-terminal Cas-homology. A nonexclusive list of key binding partners are indicated below the domains with which they associate. For more information on these and other p130Cas binding partners, see also the following reviews: Chodniewicz and Klemke 2004; Defillipi et al. 2006; Tikhmyanova et al. 2010

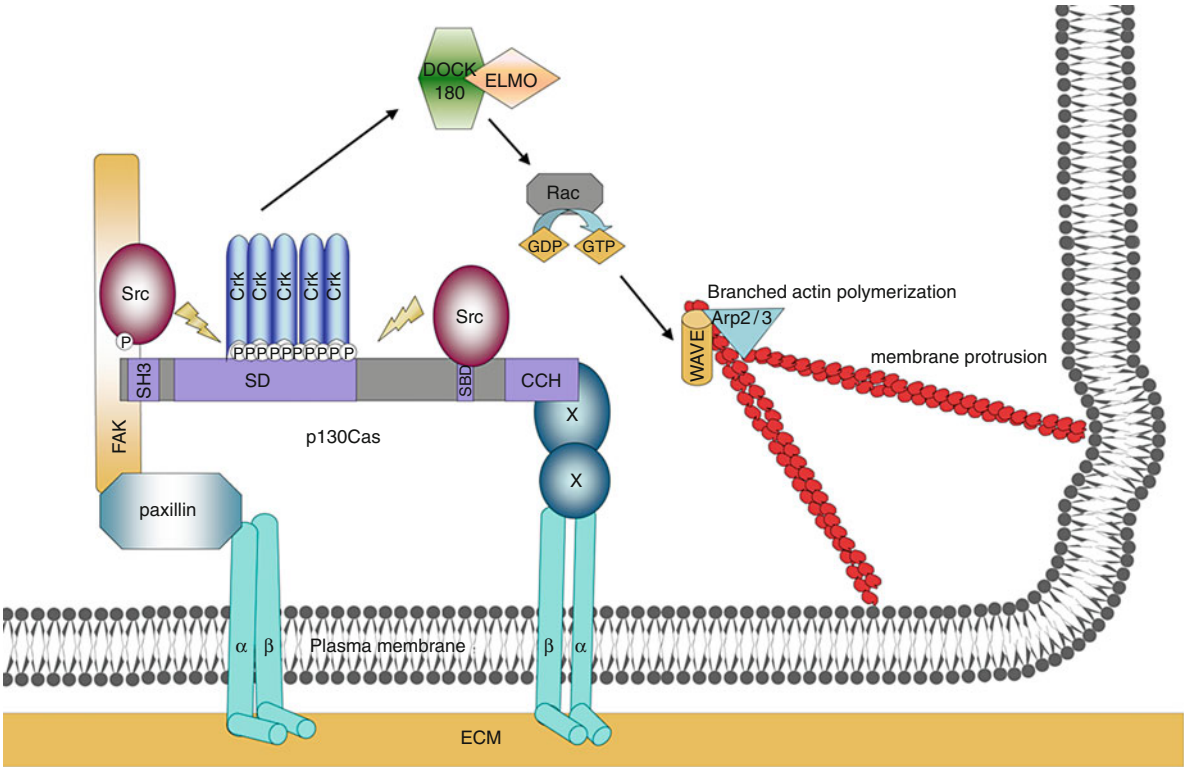
a binding partner of the integrin-associated tyrosine kinase FAK (focal adhesion kinase) (Polte and Hanks 1995). The interaction with FAK occurs by virtue of a Src-homology 3 (SH3) domain at the p130Cas N-terminus. Molecular cloning revealed that the primary structure of p130Cas includes various domains and motifs for mediating protein–protein interactions (Sakai et al. 1994; Polte and Hanks 1995), but lacks domains indicative of intrinsic enzymatic activity (Fig. 1). Hence, p130Cas is considered to be a docking or scaffolding protein in signal transduction. The molecular mass of p130Cas predicted from cDNA clones is only ~94 kDa, far less than the apparent mass by SDS-PAGE. This unusual feature of the protein suggests the presence of an extended structural component that resists full denaturation.

p130Cas is the founding member of the "Cas family" of proteins that, in mammals, also includes HEF-1/Cas-L/NEDD9, Efs/Sin, and HEPL (Tikhmyanova et al. 2010). One Cas family member is present in *Drosophila*, but there are none in yeast or *C. elegans*. All Cas family proteins have the following domain organization: an N-terminal SH3 domain, a central substrate domain (SD), and a C-terminal Cas-family homology (CCH) domain (Fig. 1). However, they appear to be functionally nonredundant due to differences in their tissue distribution and small nuances in primary structure. Of the vertebrate Cas family members, p130Cas is most widely expressed in embryonic and adult tissues. In adult tissues, highest p130Cas expression levels are in the brain, lung, intestine, kidney, and testis (Sakai et al. 1994).

Early studies implicated p130Cas as a signaling component of the focal adhesion protein complex that assembles at sites of integrin-mediated cell adhesion to the extracellular matrix (ECM). Focal adhesions are major cellular sites of tyrosine kinase signaling, in addition to their functions in establishing the transmembrane linkage between the ECM and actin cytoskeleton. Like its binding partner FAK, p130Cas localizes prominently to focal adhesions where it undergoes tyrosine phosphorylation in response to cellular adhesion and contractile force generation. Both the SH3 and CCH domains have important functions in targeting p130Cas to focal adhesions (Donato et al. 2010). FAK aids in the recruitment of p130Cas to focal adhesions via interactions made by the p130Cas SH3 domain (Donato et al. 2010). The CCH region of p130Cas is known to bind NSP family proteins (NSP1, NSP2/AND-34/BCAR3, NSP3/Chat/Shep-1) as well as a zyxin family member Ajuba, but none of these proteins are implicated in p130Cas focal adhesion targeting. They may, however, play a role in targeting p130Cas to plasma membrane ruffles and protrusions.

Regulation of p130Cas Tyrosine Phosphorylation

Despite the direct interaction with FAK, tyrosine phosphorylation of p130Cas has been attributed largely to the kinase activity of Src (Ruest et al. 2001). Nevertheless, FAK has a role in promoting p130Cas tyrosine phosphorylation by serving as a scaffold in the



p130Cas, Fig. 2 Schematic of p130Cas pro-motile signaling through the Crk-Rac pathway. Docking of p130Cas at focal adhesions allows the SD to be efficiently tyrosine phosphorylated by Src and then recruit SH2-adaptor proteins including Crk. Focal adhesion localization of p130Cas is mediated by the SH3 and CCH domains. The CCH-binding partner(s) regulating p130Cas association with focal adhesions is yet unknown. Coupling of p130Cas/Crk is associated with enhanced Rac activity via Crk association with the guanine nucleotide exchange factor complex DOCK180/ELMO. Rac is a member of the Rho family of small GTPases and is known to promote branched actin polymerization through the Arp2/3 complex

recruitment of Src to p130Cas (with the p130Cas SH3 domain bound to FAK proline-rich motifs and the Src SH2 domain bound to the FAK autophosphorylation site). The Src SH3 and/or SH2 domains can also bind directly to motifs near the p130Cas C-terminal region termed the "Src binding domain" (SBD), further contributing to tyrosine phosphorylation of p130Cas in integrin signaling (Fig. 2).

Major sites of p130Cas tyrosine phosphorylation reside in the SD, which is defined by 15 scattered Tyr-x-x-Pro (YxxP) motifs. From phospho-proteomics studies, all but one of the YxxP tyrosines have been detected as in vivo phosphorylation sites. Most notable are nine major sites within Tyr-Asp-x-Pro (YDxP) motifs, where tyrosine phosphorylation generates optimal binding sites for SH2 domains of Crk and Nck adaptor proteins (Shin et al. 2004). Crk and Nck proteins are the best-characterized downstream effectors in p130Cas-mediated signaling pathways.

Cellular stretching enhances tyrosine phosphorylation of the p130Cas SD in focal adhesions, and mechanical extension of the SD increases the accessibility of SD tyrosines for phosphorylation by Src (Sawada et al. 2006). These observations implicate p130Cas SD tyrosine phosphorylation in the process of mechanotransduction whereby cells sense a physical force and initiate a biochemical response. While the tertiary structure of the SD is undetermined, intramolecular interactions within the SD may maintain a conformational state wherein the YDxP tyrosine hydroxyl groups are hidden from Src until exposed by traction forces. The unique structural properties of the SD also appear to account for the retarded SDS-PAGE mobility of p130Cas, since deletion of this domain results in a protein that migrates according to the predicted molecular mass.

In addition to integrin-mediated cell adhesion, many studies have shown that p130Cas tyrosine

phosphorylation occurs in response to ligand stimulation of various receptor tyrosine kinases and G-coupled protein receptors (see Tikhmyanova et al. 2010 for more details). In many of these cases, p130Cas phosphorylation may be a secondary response brought about by increased cellular contractility.

Tyrosine phosphorylation of the p130Cas SD is rapid and greatly enhanced upon inhibition of cellular tyrosine phosphatases, indicating a signaling transience subject to negative regulation by phosphatases. p130Cas has been characterized as a substrate of several tyrosine phosphatases including PTP1B, PTP-PEST, RPTP-α, and SHP-2. PTP1B and PTP-PEST bind the p130Cas SH3 domain.

Critical Roles for p130Cas in Development

As a major signaling component in focal adhesions, it is not surprising that p130Cas is critical for many aspects of development. Heart and brain development are two well-documented examples. *Cas* −/− mice die at embryonic day 12.5 due to severe cardiac abnormalities (Honda et al. 1998). In the myocardium of *cas* −/− embryos, myofibrils and Z-disks show extensive disorganization. In cultured cardiac myocytes, p130Cas is prominently localized in the Z-disks where, via its interaction with FAK, it functions to regulate sarcomeric organization as well as the program of gene expression associated with hypertrophy (Kovacic-Milivojević et al. 2001).

Cas −/− mouse embryos exhibit an overall growth retardation, and have decreased brain size. p130Cas is expressed to particularly high levels in the developing cerebellum, and p130Cas tyrosine phosphorylation is enriched in the growth cones of extending neurites (Huang et al. 2006). Impairment of p130Cas expression or SD tyrosine phosphorylation in cultured cerebellar granule cells results in defects in growth cone elongation (Huang et al. 2006). The *Drosophila* p130Cas homolog was similarly shown to function in integrin-dependent neurite outgrowth and axon guidance during development (Huang et al. 2007), indicating conserved neuronal functions of Cas family proteins.

The above studies indicate a general role for p130Cas in mediating actin cytoskeletal rearrangement in various settings. *Cas* −/− mouse embryo fibroblasts (MEFs) also have defects in actin organization and cell motility (Honda et al. 1998), and these cells have been instrumental to understanding p130Cas-activated signaling events impacting the actin cytoskeleton.

p130Cas Signaling Functions Leading to Enhanced Cell Motility

The requirements for p130Cas functional domains in promoting cell motility have been evaluated through expression of wild-type versus mutational variants in *cas* −/− MEFs (Shin et al. 2004; Donato et al. 2010; Meenderink et al. 2010). These studies demonstrated that p130Cas functions to enhance cell motility, while also showing requirements for all major p130Cas functional domains (SH3, SD, SBD, and CCH). Mutation or deletion of any of the domains has a negative impact on tyrosine phosphorylation of the SD, which is a key signaling function of p130Cas in cell motility. The small adaptor proteins Crk and Nck, which bind to tyrosine phosphorylated motifs in the SD, have been implicated in p130Cas-motility responses. These adaptors consist essentially of SH2 domains (with high binding affinity for phosphorylated YDxP motifs in the SD) and SH3 domains for mediating interactions with downstream effector proteins. Most notably, p130Cas coupling to Crk adaptors (Crk-II and Crk-L) has been implicated in promoting plasma membrane protrusion by activating Rac1 GTPase via a Crk SH3 domain interaction with the guanine nucleotide exchange factor complex DOCK180/ELMO (reviewed in Chodniewicz and Klemke 2004). Activated Rac1 can promote membrane protrusion by activating the Arp2/3 complex to stimulate branched actin polymerization (Fig. 2). The p130Cas-Crk-Rac pathway has been implicated in other actin-mediated cellular processes, including the phagocytosis of the pathogenic bacteria *Yersinia* (reviewed in Tikhmyanova et al. 2010). Nck adaptors (Nck1 and Nck2) can also promote Arp2/3 complex activation via the interaction of their SH3 domains with other proteins including N-WASP and ▶ Pak1. The interaction of p130Cas with Nck adaptors has been linked to cytoskeletal reorganization and chemotaxis stimulated by platelet-derived growth factor (PDGF) (Rivera et al. 2006). It is not clear, however, if p130Cas/Nck coupling in response to PDGF stimulation occurs solely in dorsal membrane ruffles or if this coupling also has a focal adhesion signaling component.

In addition to its role in promoting plasma membrane protrusion via coupling to Crk and/or Nck, p130Cas has another role in cell motility to sustain the disassembly of mature focal adhesions (Meenderink et al. 2010). SD tyrosine phosphorylation was implicated in the focal adhesion disassembly process, while the SBD was also found to have a role in this process distinct from its ability to promote SD phosphorylation (Meenderink et al. 2010). These observations emphasize two distinct signaling functions of p130Cas: (1) SD tyrosine phosphorylation to recruit SH2-containing effectors and (2) SBD-mediated activation of Src (and possibly other signaling proteins that bind to this site) to act on substrates other than p130Cas.

p130Cas and Cancer

While p130Cas is essential to normal developmental processes, elevated p130Cas expression and/or SD tyrosine phosphorylation is seen in various forms of cancer and is likely to contribute to the malignant progression of these diseases (reviewed in Defillipi et al. 2006; Tikhmyanova et al. 2010). A role for p130Cas in invasive and metastasic cell behavior was demonstrated in studies of Src-transformed fibroblasts, where the formation of invasive structures called podosomes, activation of matrix-degrading metalloproteases, and experimental metastasis were all shown to be greatly reduced in the absence of p130Cas SD tyrosine phosphorylation (Brabek et al. 2005). The p130Cas-Crk-Rac1 pathway is implicated in both plasma membrane protrusion and cell survival during invasion (reviewed in Chodniewicz and Klemke 2004).

Much attention has been directed to the role of p130Cas in breast cancer. In breast cancer patients, high p130Cas levels are associated with a poor response to tamoxifen therapy, early disease recurrence, and lower long-term survival (for details, see Dorssers et al. 2001; Tikhmyanova et al. 2010). In mouse transgenic studies, p130Cas overexpression in mammary gland epithelia was associated with hyperplasia and, in combination with overexpression of the HER2/Neu oncogene, with shorter tumor latency (Defillipi et al. 2006). Interestingly, p130Cas was independently identified as the product of the human gene *BCAR1* that confers resistance to antiestrogen drugs (e.g., tamoxifen) in breast cancer cells (Dorssers et al. 2001). Tamoxifen is a common and effective treatment for estrogen-receptor positive (ER+) breast cancer, but patients frequently develop resistance to this treatment. The discovery that p130Cas confers tamoxifen resistance provided new mechanistic insight into antiestrogen resistance. Tamoxifen resistance conferred by p130Cas does not appear to be regulated by activation of ER target genes, but rather has been linked to Src-driven cell proliferation and survival pathways. These pathways appear to be mediated either by p130Cas-Src complexes formed with the ER where MAPK and cell cycle signaling has been implicated (reviewed in Defillipi et al. 2006; Tikhmyanova et al. 2010), or through an ER-independent pathway involving the epidermal growth factor receptor and Stat5b (Riggins et al. 2006). Other studies revealed a role for adhesion-dependent p130Cas signaling in promoting activation of the PI3K-Akt cell survival pathway in response to ER antagonism by antiestrogens (Cowell et al. 2006). Thus, p130Cas may confer antiestrogen resistance through multiple mechanisms.

p130Cas has also been implicated in the more aggressive ER-negative breast cancers. p130Cas SD tyrosine phosphorylation is commonly elevated in ER-negative breast cancer cell lines and linked to enhanced migration, invasion, and survival of ER-negative breast cancer cells (Cunningham-Edmondson and Hanks 2009).

Summary

p130Cas, first recognized as a prominent tyrosine-phosphorylated protein in cells transformed by retroviral oncogenes v-*crk* and v-*src*, is a widely expressed docking/scaffolding protein that functions as a major Src substrate in integrin-mediated signaling and mechanotransduction. p130Cas was independently identified as a FAK-interacting protein and as the product of the *BCAR1* gene that confers antiestrogen resistance in breast cancer cells. Src is recruited to phosphorylate p130Cas by directly binding to the SBD and indirectly by binding to FAK. Src phosphorylates multiple YDxP motifs in the p130Cas SD to create high-affinity binding sites for Crk and Nck adaptor proteins that act as downstream effectors in p130Cas-mediated signaling pathways. p130Cas SD

couplings to Crk and Nck are implicated in promoting leading edge actin polymerization and plasma membrane protrusion in cell motility and invasion processes. SBD-mediated binding and activation of Src is a distinct signaling function of p130Cas that may be involved in maintaining focal adhesion disassembly during cell migration. A general role for p130Cas in mediating actin and adhesion dynamics is further indicated by developmental studies showing that p130Cas deficiency causes functional defects in sarcomere organization of cardiac myocytes and neuronal outgrowth and axon guidance in the central nervous system. p130Cas expression and/or SD tyrosine phosphorylation is commonly elevated in various forms of cancer where it likely contributes to the malignant disease progression. In conferring antiestrogen resistance in breast cancer cells; p130Cas SD signaling has been linked to the activation of Src-driven cell proliferation and survival pathways.

References

Brabek J, Constancio SS, Siesser PF, Shin NY, Pozzi A, Hanks SK. Crk-associated substrate tyrosine phosphorylation sites are critical for invasion and metastasis of SRC-transformed cells. Mol Cancer Res. 2005;3:307–15.

Chodniewicz D, Klemke RL. Regulation of integrin-mediated cellular responses through assembly of a CAS/Crk scaffold. Biochim Biophys Acta. 2004;1692:63–76.

Cowell LN, Graham JD, Bouton AH, Clarke CL, O'neill GM. Tamoxifen treatment promotes phosphorylation of the adhesion molecules, p130Cas/BCAR1, FAK and Src, via an adhesion-dependent pathway. Oncogene. 2006;25:7597–607.

Cunningham-Edmondson AC, Hanks SK. P130Cas substrate domain signaling promotes growth, migration, and invasion of estrogen-receptor-negative breast cancer cells. Breast Cancer Targets Ther. 2009;1:39–52.

Defillipi P, Di Stefano P, Cabodi S. p130Cas: a versatile scaffold in signaling networks. Trends Cell Biol. 2006;16:257–63.

Donato DM, Ryzhova LM, Meenderink LM, Kaverina I, Hanks SK. Dynamics and mechanism of p130Cas localization to focal adhesions. J Biol Chem. 2010;285:20769–79.

Dorssers LC, van der Flier S, Brinkman A, van Agthoven T, Veldscholte J, Berns EM, et al. Tamoxifen resistance in breast cancer: elucidating mechanisms. Drugs. 2001;61:1721–33.

Honda H, Oda H, Nakamoto T, Honda Z, Sakai R, Suzuki T, et al. Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. Nat Genet. 1998;19:361–5.

Huang J, Sakai R, Furuichi T. The docking protein Cas links tyrosine phosphorylation signaling to elongation of cerebellar granule cell axons. Mol Biol Cell. 2006;17:3187–96.

Huang Z, Yazdani U, Thompson-Peer KL, Kolodkin AL, Terman JR. Crk-associated substrate (Cas) signaling protein functions with integrins to specify axon guidance during development. Development. 2007;134:2337–47.

Kovacic-Milivojević B, Roediger F, Almeida EA, Damsky CH, Gardner DG, Ilić D. Focal adhesion kinase and p130Cas mediate both sarcomeric organization and activation of genes associated with cardiac myocyte hypertrophy. Mol Biol Cell. 2001;12:2290–307.

Meenderink L, Ryzhova LM, Donato DM, Gochberg DF, Kaverina I, Hanks SK. P130Cas Src-binding and substrate domains have distinct roles in sustaining focal adhesion disassembly and promoting cell migration. PLoS ONE. 2010;5:e13412.

Polte TR, Hanks SK. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc Natl Acad Sci USA. 1995;92:10678–82.

Riggins RB, Thomas KS, Ta HQ, Wen J, Davis RJ, Schuh NR, et al. Physical and functional interactions between Cas and c-Src induce tamoxifen resistance of breast cancer cells through pathways involving epidermal growth factor receptor and signal transducer and activator of transcription 5b. Cancer Res. 2006;66:7007–15.

Rivera GM, Antoku S, Gelkop S, Shin NY, Hanks SK, Pawson T, et al. Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. Proc Natl Acad Sci USA. 2006;103:9536–41.

Ruest PJ, Shin NY, Polte TR, Zhang X, Hanks SK. Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. Mol Cell Biol. 2001;21:7641–52.

Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, et al. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J. 1994;13:3748–56.

Sawada Y, Tamada M, Dubin-Thaler BJ, Cherniavskaya O, Sakai R, Tanaka S, et al. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell. 2006;127:1015–26.

Shin NY, Dise RS, Schneider-Mergener J, Ritchie MD, Kilkenny DM, Hanks SK. Subsets of the major tyrosine phosphorylation sites in Crk-associated substrate (CAS) are sufficient to promote cell migration. J Biol Chem. 2004;279:38331–7.

Tikhmyanova N, Little JL, Golemis EA. CAS proteins in normal and pathological cell growth control. Cell Mol Life Sci. 2010;67:1025–48.

p160ROCK

► ROCK Kinases

p26

► Recoverin

p^{26}

▶ Recoverin

P2Y$_{14}$ Receptor

Kenneth A. Jacobson, M. P. Suresh Jayasekara, Zhan-Guo Gao and Francesca Deflorian
Laboratory of Bioorganic Chemistry & Molecular Recognition Section, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

Synonyms

GPR105; KIAA0001

Historical Background

Extracellular purine and pyrimidine nucleotides act as signaling molecules through the activation of P2X ion channels and P2Y G protein-coupled receptors (GPCRs) (Abbracchio et al. 2006). Among the eight members of the P2Y receptor family, four respond to extracellular uracil nucleotides: P2Y$_2$, P2Y$_4$, P2Y$_6$, and P2Y$_{14}$ receptors. None of the P2X ion channels are substantially activated by uracil nucleotides.

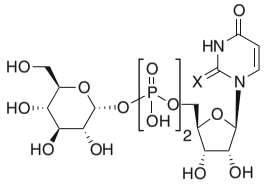
P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors belong to the P2Y$_1$-like subgroup of G$_q$-coupled receptors, and the P2Y$_{14}$ receptor belongs to the P2Y$_{12}$-like subgroup that couples to ▶ G protein αi to inhibit ▶ adenylyl cyclase. The P2Y$_{14}$ receptor is distributed in various tissues, that is, placenta, adipose, stomach, intestine, brain, spleen, thymus, lung, heart, placenta, mast cells, and discrete brain regions (Harden et al. 2010). It is activated by uridine-5′-diphosphoglucose (UDPG, **1**, Fig. 1), other endogenous UDP-sugars, and uridine-5′-diphosphate (UDP, **3**). The P2Y$_{14}$ receptor plays a role in the neuroimmune system, with expression occurring in T cells, dendritic cells, and hematopoietic stem cells. It is of interest in the following therapeutic areas: immune system disorders, inflammation, pain, asthma, glaucoma, and other diseases.

Originally, Chambers et al. (2000) cloned a human GPCR designated GPR105 or KIAA0001 that was found to respond to uracil nucleotide sugars, such as UDPG, but lacked a response to most other nucleotides. Soon thereafter this sequence was designated the P2Y$_{14}$ receptor, because it was recognized to belong structurally and functionally to the P2Y receptor family, which was previously known to be activated by various nucleotides other than UDPG.

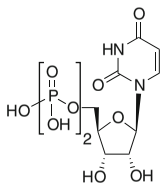
Structure and Signaling Pathways

X-ray crystallographic structural information is not yet available for any of the P2Y subtypes, but sequence analysis and modeling studies predict that the general structural and functional features of the family A of GPCRs are conserved for the P2Y receptors: seven hydrophobic transmembrane (TM) domains connected by three extracellular loops (ELs) and three intracellular loops (ILs) with an extracellular N-terminus and an intracellular C-terminus. From the sequence comparison, the P2Y$_{14}$ receptor belongs to the P2Y$_{12}$-like subfamily, with a sequence identity of about 47% to the P2Y$_{12}$ receptor (human subtypes, Communi et al. 2001). There are four cysteine residues in the extracellular region of the P2Y$_{14}$ receptor forming two possible disulfide bridges: One disulfide bridge is highly conserved in the family A of GPCRs and it connects the extracellular part of TM3 with the EL2; the other putative disulfide bridge connects the N-terminus to the EL3. A recent homology model of the P2Y$_{14}$ receptor (Deflorian and Jacobson 2011) concluded that the most applicable structural template was that of the CXCR4 receptor. Residues from the extracellular regions of TM3, TM6, TM7, and the EL2 likely coordinate the ligand(s), and specifically, basic residues of TM6 (Arg253) and TM7 (Lys277), conserved among the P2Y$_{12}$-like family, are predicted to coordinate the phosphate groups of nucleotides agonists.

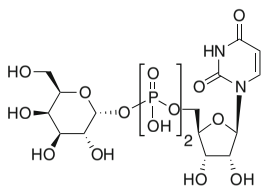
In model functional studies of the P2Y$_{14}$ receptor activities of nucleotide analogs, it has been convenient to use the recombinant human or rat homologue expressed in cell lines, such as COS, CHO, or HEK-293 cells (Fricks et al. 2008; Carter et al. 2009). Heterologous coexpression of a chimeric G protein that responds to G$_i$-coupled receptors allows the use of phosphoinositide hydrolysis as assay readout, but this also can affect the observed agonist efficacy. Cell



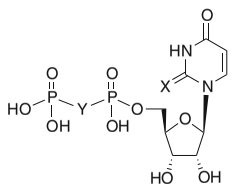
1 UDP-glucose (X=O)
EC_{50} 400 nM
2 MRS2690 (X=S)
EC_{50} 11 nM



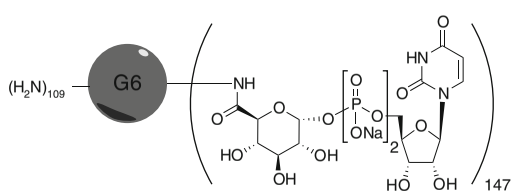
3 UDP
EC_{50} 160 nM



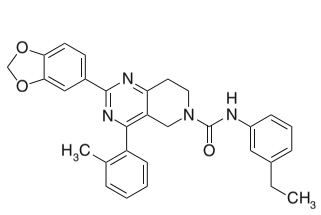
4 UDP-galactose
EC_{50} 670 nM



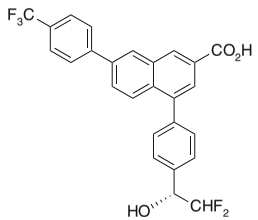
5 MRS2802 (X=O, Y=CF2)
EC_{50} 63 nM
6 MRS2905 (X=S, Y=CH2)
EC_{50} 1.92 nM



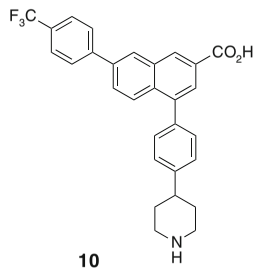
7 Representative conjugate of UDP-glucuronic acid on polyamidoamine (PAMAM) dendrimer of generation 6 (G6), average structure shown. EC_{50} 0.8 nM



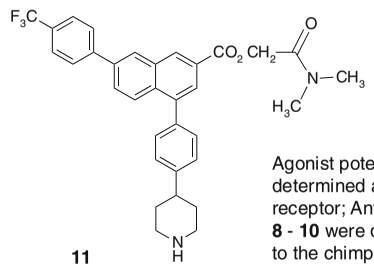
8
IC_{50} 10 nM



9
IC_{50} 8 nM



10
IC_{50} 1.9 nM



11
(prodrug of **10**)

Agonist potencies of **1** - **7** were determined at the human $P2Y_{14}$ receptor; Antagonist potencies of **8** - **10** were determined in binding to the chimpanzee $P2Y_{14}$ receptor.

$P2Y_{14}$ Receptor, Fig. 1 Structures of selective $P2Y_{14}$ againsts (1–7) and antagainsts (8–11) and their potencies

lines in which a native $P2Y_{14}$ receptor occurs are: RBL-2H3 mast cells, C6 glioma, and A549 and BEAS-2B epithelial cells (Gao et al. 2010; Muller et al. 2005). Synergistic signaling of the $P2Y_{14}$ receptor has been proposed in combination with the $P2Y_6$ receptor to promote various cell functions including activation of the MAP kinase isozymes ▶ Erk1/Erk2 (Harden et al. 2010).

Physiological Role

Identification of the physiological functions of the $P2Y_{14}$ receptor has been difficult to establish, and isolated reports of the involvement in immune function, chemotaxis, differentiation, and chemokine release have appeared. Extracellular release of **1** upon trafficking of glycoproteins to the plasma membrane has been demonstrated, suggesting its widespread role in signaling (Lazarowski et al. 2003). UDP-sugars are required for glycosylation of proteins in the endoplasmic reticulum (ER) and Golgi apparatus as they are trafficked to the surface and are present there in high concentrations and dependent on specific nucleotide-sugar transporters (Sesma et al. 2009). Thrombin enhanced UDPG release (Kreda et al. 2008), and the $P2Y_{14}$ receptor was detected in platelets, but its role is undetermined. In the rodent stomach, Bassil et al. (2009) used agonists and $P2Y_{14}$ knockout (KO) mice to demonstrate differential effects of the $P2Y_{14}$ receptor in contractility and gastric emptying.

The $P2Y_{14}$ receptor has been studied in epithelial tissues. In cultured mouse uterine cells, UDPG triggered innate immunity through the production of inflammatory cytokines (Arase et al. 2009), but not all of the pharmacological observations were consistent with the $P2Y_{14}$ receptor.

The $P2Y_{14}$ receptor is endogenously expressed in RBL-2H3 mast cells (Gao et al. 2010), where it mediates degranulation, suggesting it as a potential novel therapeutic target for allergic conditions. $P2Y_{14}$ receptor agonists also induced [^{35}S]GTPγS binding to RBL-2H3 cell membranes, and phosphorylation of ▶ MAP kinases: ▶ Erk1/Erk2, P38 and JNK. UDPG **1** and selective agonist MRS2690 **2** concentration-dependently enhanced hexosaminidase release with EC_{50} values of 1,150 and 103 nM, respectively, which was blocked by pertussis toxin and significantly diminished by $P2Y_{14}$ receptor-specific siRNA. UDP was a partial agonist in RBL-2H3 cells. The $P2Y_{14}$ receptor was recently identified to be an early pivotal regulator in mesenchymal stem cell commitment and was shown to be active in adipogenic differentiation (Zippel et al. 2011).

In the central nervous system, $P2Y_{14}$ receptors are associated with astrocytes. mRNA for the receptor is prominently expressed in immune cells including neutrophils, lymphocytes, and megakaryocytic cells. The inflammatory effects of UDPG in N9 microglia are not mediated by $P2Y_{14}$ receptor activation (Brautigam et al. 2008).

UDP also activates the $P2Y_6$ receptor, and the relative degree of involvement of multiple native agonists of the $P2Y_{14}$ receptor, various UDP-sugars that originate in a vesicular compartment and UDP, is still unclear. Nevertheless, this observation led to the synthesis of a variety of UDP analogues as selective agonists.

Structure-Activity Relationship (SAR)

Depending on the model examined, UDP has been reported to either be a full agonist, a partial agonist, or a competitive antagonist at the human $P2Y_{14}$ receptor and a potent agonist at the rat $P2Y_{14}$ receptor (Carter et al. 2009). In cellular assay systems utilizing a native G protein rather than a chimeric one, UDPG (pIC50 6.5) and UDP (pIC50 6.5) are both potent agonists of the human $P2Y_{14}$ receptor.

The SAR of synthetic analogues of both UDP and UDPG has been probed at the $P2Y_{14}$ receptor. The $P2Y_{14}$ receptor appears to be one of the least permissive among P2Y receptors (Das et al. 2010). Most modifications of the nucleobase or ribose moieties abolished activity, but the glucose moiety was amenable to substitution with other sugars (e.g., UDP-galactose **4**, pIC50 6.2). Stereochemistry of this moiety influenced potency, for example, the β-glucoside was only twofold less potent than the native α-isomer. One of the few modifications of the uracil ring possible was 2-thiol, which in the analogue of UDPG (MRS2690, **2**, pIC50 7.3) increased potency by sevenfold and prevented activation of the $P2Y_2$ receptor. Stabilizing phosphonate groups have been introduced in analogues of UDP. For example, α,β-difluoromethylene-UDP, MRS2802 **5** (pIC50 7.2) is inactive at the $P2Y_6$ receptor and fully activates the human $P2Y_{14}$ receptor. MRS2905 **6** (pIC50 8.7) is of

>2,000 selective for the $P2Y_{14}$ in comparison to the $P2Y_6$ receptor.

The carboxylate group of uridine-5′-diphosphoglucuronic acid proved to be suitable for flexible substitution by chain extension through an amide linkage. This led to the design of multivalent, polymeric conjugates having greatly increased potency as $P2Y_{14}$ receptor agonists (Das et al. 2009). For this purpose, G4 (fourth-generation) through G6 polyamidoamine (PAMAM) dendrimers containing amino groups for conjugation of ligands were amide-linked to uridine-5′-diphosphoglucuronic acid moieties. High potencies in $P2Y_{14}$ receptor activation were observed in conjugates such as **7**, in which an average of 147 out of 256 amino groups were conjugated to the nucleotide.

Two classes of nonnucleotide antagonists of the $P2Y_{14}$ receptor were recently identified in high throughput screening using a FLIPR-based calcium flux assay in HEK cells overexpressing the mouse or chimpanzee $P2Y_{14}$ receptor. The dihydropyridopyrimidine derivative **8** is a $P2Y_{14}$ antagonist with good oral bioavailability and potency (pIC50 8.0), but noncompetitive with UDP (Guay et al. 2011). The 4,7-disubstituted naphthoic acid antagonists **9** and **10** (binding K_i values at the chimpanzee $P2Y_{14}$ receptor of 4 and 1.9 nM, respectively) are competitive with UDP (Gauthier et al. 2011). **10** was also derivatized through its carboxylic acid as a prodrug **11** to greatly improve bioavailability in this hydrophobic series (Robichaud et al. 2011).

Summary

The $P2Y_{14}$ receptor is a cell-surface signaling protein (G protein coupled) that is activated by extracellular uracil nucleotides and couples to inhibition of ▶ adenylyl cyclase. It occurs in the immune system, including T cells, dendritic cells, and mast cells, and in various tissues, that is, placenta, adipose, stomach, intestine, brain, spleen, lung, heart, and brain. In mast cells, this receptor promotes the release of inflammatory mediators, suggesting that it is a possible target for allergic diseases. Chemical modification of the native agonist uridine-5′-diphosphoglucose has led to novel, selective nucleotide agonists, and nonnucleotide antagonists have been derived by optimization of screening hits. UDP, which also activates the $P2Y_6$ receptor, appears to be an endogenous agonist. Multivalent, polymeric conjugates of $P2Y_{14}$ receptor agonists, based on covalent tethering through the structurally permissive glucose moiety, have been shown to greatly increase the potency in receptor activation.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, et al. International union of pharmacology LVIII. Update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 2006;58:281–341.

Arase T, Uchida H, Kajitani T, Ono M, Tamaki K, Oda H, et al. The UDPglucose receptor $P2RY_{14}$ triggers innate mucosal immunity in the female reproductive tract by inducing IL-8. J Immunol. 2009;182:7074–84.

Bassil AK, Bourdu S, Townson KA, Wheeldon A, Jarvie EM, Zebda N, et al. UDP-glucose modulates gastric function through $P2Y_{14}$ receptor-dependent and -independent mechanisms. Am J Physiol Gastrointest Liver Physiol. 2009;296:G923–30.

Brautigam VM, Dubyak GR, Crain JM, Watters JJ. The inflammatory effects of UDP-glucose in N9 microglia are not mediated by $P2Y_{14}$ receptor activation. Purinergic Signal. 2008;4:73–8.

Carter RL, Fricks IP, Barrett MO, Burianek LE, Zhou Y, Ko H, et al. Quantification of Gi-mediated inhibition of adenylyl cyclase activity reveals that UDP is a potent agonist of the human $P2Y_{14}$ receptor. Mol Pharmacol. 2009;76:1341–8.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, et al. A G protein-coupled receptor for UDP-glucose. J Biol Chem. 2000;275:10767–71. doi:10.1074/jbc.275.15.10767.

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM. Identification of a novel human ADP receptor coupled to Gi. J Biol Chem. 2001;276:41479–85.

Das A, Zhou Y, Ivanov AA, Carter RL, Harden TK, Jacobson KA. Enhanced potency of nucleotide-dendrimer conjugates as agonists of the $P2Y_{14}$ receptor: multivalent effect in G protein-coupled receptor recognition. Bioconjug Chem. 2009;20:1650–9.

Das A, Ko H, Burianek LE, Barrett MO, Harden TK, Jacobson KA. Human $P2Y_{14}$ receptor agonists: Truncation of the hexose moiety of uridine-5′-diphosphoglucose and its replacement with alkyl and aryl groups. J Med Chem. 2010;53:471–80.

Deflorian F, Jacobson KA. Comparison of three GPCR structural templates for modeling of the $P2Y_{12}$ nucleotide receptor. J Comput Aided Mol Des. 2011;25:329–38.

Fricks I, Maddiletti S, Carter R, Lazarowski ER, Nicholas RA, Jacobson KA, Harden TK. UDP is a competitive antagonist at the human $P2Y_{14}$ receptor and a full agonist at the rat $P2Y_{14}$ receptor. J Pharmacol Exp Therap. 2008;325:588–94.

Gao ZG, Ding Y, Jacobson KA. UDP-glucose acting at $P2Y_{14}$ receptors is a mediator of mast cell degranulation. Biochem Pharmacol. 2010;79:873–9.

Gauthier JY, Belley M, Deschênes D, Fournier JF, Gagné S, Gareau Y, et al. The identification of 4,7-disubstituted

naphthoic acid derivatives as UDP-competitive antagonists of $P2Y_{14}$. Bioorg Med Chem Lett. 2011;21:2836–9. doi:10.1016/j.bmcl.2011.03.081.
Guay D, Beaulieu C, Belley M, Crane SN, DeLuca J, Gareau Y, et al. Synthesis and SAR of pyrimidine-based, non-nucleotide $P2Y_{14}$ receptor antagonists. Bioorg Med Chem Lett. 2011;21:2832–5. doi:10.1016/j.bmcl.2011.03.084.
Harden TK, Sesma JI, Fricks IP, Lazarowski ER. Signalling and pharmacological properties of the $P2Y_{14}$ receptor. Acta Physiol. 2010;199:149–60. doi:10.1111/j.1748-1716.2010.02116.x.
Kreda SM, Seminario-Vidal L, Heusden C, Lazarowski ER. Thrombin-promoted release of UDP-glucose from human astrocytoma cells. Br J Pharmacol. 2008;153:1528–37.
Lazarowski ER, Shea DA, Boucher RC, Harden TK. Release of cellular UDP-glucose as a potential extracellular signaling molecule. Mol Pharmacol. 2003;63:1190–7.
Muller T, Bayer H, Myrtek D, Ferrari D, Sorichter S, Ziegenhagen MW, et al. The $P2Y_{14}$ receptor of airway epithelial cells: coupling to intracellular Ca^{2+} and IL-8 secretion. Am J Respir Cell Mol Biol. 2005;33:601–9.
Robichaud J, Fournier JF, Gagne S, Gauthier JY, Hamel M, Han Y, Henault M, Kargman S, Levesque JF, Yael Mamane Y, Mancini J, Morin N, Mulrooney E, Wu J, Black WC. Applying the pro-drug approach to afford highly bioavailable antagonists of $P2Y_{14}$. Bioorg Med Chem Lett. 2011;21:4366–8.
Sesma JI, Esther CR, Kreda SM, Jones L, O'Neal W, Nishihara S, et al. Endoplasmic reticulum/golgi nucleotide sugar transporters contribute to the cellular release of UDP-sugar signaling molecules. J Biol Chem. 2009;284:12572–83.
Zippel N, Limbach CA, Ratajski N, Urban C, Pansky A, Luparello C, Kassack MU, Tobiasch E. Purinergic receptors influence the differentiation of human mesenchymal stem cells. Stem Cells Dev. 2011. doi:10.1089/scd.2010.0576.

p38 MAPK Family of Signal Transduction Proteins

John Papaconstantinou[1,2], Ching-Chyuan Hsieh[1] and James H. DeFord[1]
[1]Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA
[2]Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX, USA

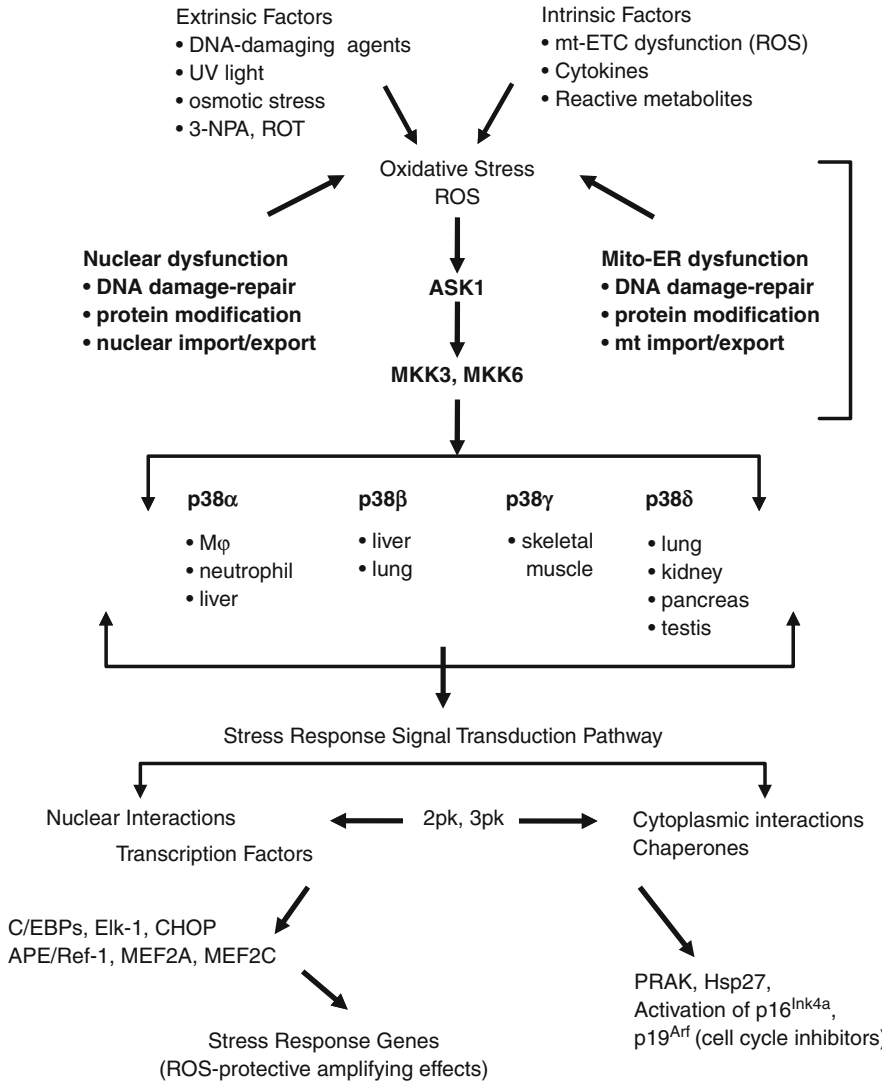
Synonyms

2-, 3pk (MAPKAPK-2, -3); CREB (cyclic AMP-responsive element-binding protein); Hsp 27 (heat shock protein 27); MAPK (mitogen activating protein kinase); MAPKAPK (MAP kinase-activated kinase); MKK (MAP kinase kinase); MKKEE (activated form of MKK6); MKKK (MAP kinase kinase kinase); MKP (MAPK phosphatase); Mnk (MAP kinase-interacting kinase); mtDNA (mitochondrial DNA); NES (nuclear export sequence); NLS (nuclear localization sequence); P-38α (phospho p38α); PRAK; ROS (reactive oxygen species); TNFα (tumor necrosis factor α)

Historical Background: The p38 MAPK Family of Stress Response Signaling

The biological transduction of physiological and environmental signals involves highly specific protein–protein interactions and posttranslational modifications that regulate both genetic and epigenetic processes in response to intrinsic and extrinsic challenges. The overall biochemical phenotype of tissues at all stages of the life cycle, i.e., growth and development, maturation, and aging are therefore consequences of those biological signaling systems that respond to intrinsic and extrinsic challenges. In mammals, the ▶ p38 mitogen activating protein kinase(p38 MAPK) family consists of α, β, γ, and δ isoforms. These isoforms are ubiquitously expressed at significantly different levels and are important factors in tissue-specific responses to growth factors, hormones, intermediates of metabolism, and environmental factors. In particular, members of the p38 MAPK family are activated by a wide variety of intracellular and extracellular signals (Fig. 1) which include such extracellular stresses as UV irradiation, oxidative stress (ROS), osmotic stress, and inflammatory cytokines. Activation of MAPK signaling leads to the regulation of gene clusters that mediate such complex biological responses as inflammation, cell proliferation, differentiation, apoptosis, senescence, and aging (Ono and Han 2000; Zer et al. 2007; Cuadrado and Nebreda 2010).

The proteins of the p38 MAPK family comprise a cascade of multiple upstream tyrosine and downstream serine/threonine kinases and phosphatases; the signals are transduced by phosphorylation events that enhance specific serine/threonine kinase activities and dephosphorylation of their catalytic sites that attenuate the signaling activities. There are numerous reviews on the structure and function of the p38 MAPK family of signaling proteins (Ono and Han 2000; Zer et al. 2007;



p38 MAPK Family of Signal Transduction Proteins, Fig. 1 A summary of the signaling pathways of the p38 MAPK isoforms. Members of the p38 family of signaling proteins are activated by a variety of intracellular (intrinsic) and extracellular (extrinsic) factors. The intrinsic and extrinsic factors are sources of oxidative stress (ROS) which activate the pathway, i.e., ASK1 – MKK3/MKK6 – p38. Activation of the p38 MAPK isoforms targets multiple physiological processes involved in the cellular response to stress. (For a more detailed presentation of proteins targeted by p38 MAPK isoforms see Cuadrado and Nebreda (2010)). The upstream tyrosine kinases that transduce signals to the ASK1 are not included in this map. (**a**) Environmental factors (UV, osmotic shock, anisomycin, rotenone, 3-nitropropionic acid, and antimycin A) activate stress response physiological processes via the p38 MAPK signaling pathway. The p38 MAPK isoforms are depicted as central components of the pathway because of their role in the translocation of signals to nuclear and cytoplasmic stress response processes. (**b**) The upstream activators of the p38 MAPK pathway are MAP kinase kinase kinases (ASK1, and MKKKs) and the MAP kinase kinases (MKKs 3 and 6). (**c**) A nuclear complex of p38 pk2/pk3-MAPK-phosphatase represents a potential mechanism for the activation of transcription factors by p38 MAPK and 2pk/3pk

Cuadrado and Nebreda 2010). In this review, we focus on the serine/threonine kinase components of the p38 MAPK stress response signaling pathway, its regulation, and its relevance to tissue responses to extrinsic and intrinsic stress factors.

The p38 MAPK Family of Stress Signaling Pathways: Tissue Specificities

The p38 MAPK family of signaling proteins are serine/threonine kinases that regulate stress response genes

(nuclear) or chaperones (cytoplasmic) via upstream cytoplasmic activator proteins (tyrosine kinases; Fig. 1). The p38 MAPK isoforms are, therefore, major nucleocytoplasmic trafficking proteins and are a major crossroads for the delivery of biological responses to environmental challenges (Fig. 1). The protein–protein interactions of the MAPK pathways are, therefore, major mechanisms that control the activation of transcription factors and their targeted genes in response to intrinsic (biological) and extrinsic (environmental) factors.

The p38α and p38β MAPK are 60% identical to p38γ and p38δ, indicating that they represent related but distinct MAPK subgroups (Ono and Han 2000; Zer et al. 2007; Cuadrado and Nebreda 2010). The four isoforms are ubiquitously expressed but at significantly different levels in each tissue. Both p38α and p38β are the predominant isoforms in the liver whereas p38γ is predominant in skeletal muscle (Lechner et al. 1996; Li et al. 1996) and δ is enriched in lung, kidney, testis, pancreas, and small intestine (Jiang et al. 1997). The isoforms have distinct biological functions due, in part, to their ability to selectively phosphorylate and activate specific transcription factors, by extracellular stimuli via the upstream tyrosine kinases. Importantly, the p38 MAPK kinases serve as a distribution center for the receipt and dissemination of biological signals within the cytoplasm as well as to their nuclear gene targets. The pathway consists, therefore, of a cascade of three serine/threonine kinase families, i.e., MAP kinase kinase kinases (MKKK), ▶ MAP kinase kinases (MKK), and ▶ MAP kinases i.e., the p38 MAPK proteins (Fig. 1). These proteins are activated in series such that the MKKKs phosphorylate the MKK activation loop serines, which then activate the p38 MAPKs by phosphorylation of threonine-180 (Thr^{180}) and tyrosine-182 (Tyr^{182}).

Tissue-Specific Distribution, Substrate Specificity, and Mechanism of Transduction of Stress Signals by p38 MAPK Isoforms

The importance of the tissue-specific expression of the p38 MAPK isoforms is indicated by the observation that sustained p38α activity is associated with ischemia-induced lethality in cultured rat neonatal cardiomyocytes (Saurin et al. 2000). Specific inhibition of p38α activity is protective against inflammatory insults. There is also evidence that activation of p38β correlates with cell viability. Thus, inhibition of the lethal effects of p38α and maintenance of the protective effects of p38β result in increased cell viability (Conrad et al. 1999). This is consistent with the differences in targets of p38α and p38β in cardiomyocyte ischemia, and is further evidence for specific biological consequences of differential isoform activation.

The specificities of p38 MAPK isoform activities, i.e., lethality, apoptosis, survival, etc., indicate the importance of the balance of p38 MAPK isoforms in a particular tissue, e.g., lung tissues and their response to external challenges. For example, the lung epithelial cells express p38β and p38δ where the pool levels of p38β $>>$ p38δ. However, the numbers of macrophages and neutrophils are elevated in inflamed, injured lungs and the pool level of p38α is threefold higher than that of p38δ in macrophages; and ninefold higher than p38δ in neutrophils. In view of the differential levels of p38 MAPK isoforms in various cell types of the diseased/inflamed lung tissues, the higher levels of p38α in the inflammatory cell lineages may overpower those of the lung tissue epithelium and favor pathological consequences such as inflammation, apoptosis, and ultimately lethality which may then enhance fibrosis.

The specificity of p38 biological functions are also supported by the observation that activation of p38α (phosphorylation of its catalytic site) by overexpression of MKK6EE, a constitutively active version of ▶ MKK6 in 3T3 fibroblasts, enhances their entry into replicative senescence (Haq et al. 2002). Thus, the over production of P-p38α in the p38β and p38δ dominant lung tissue may play a role in the development of senescence and aging characteristics in response to environmental factors. This also applies to other respiratory tissues such as vascular cells and/or inflammatory cells that may exhibit accelerated senescence and overall respiratory disease of the lung.

Substrate Specificity of p38 MAPK Isoforms

Formation of p38 MAPK to transcription factor complexes is a critical determinant of kinase specificity. For example, p38α and p38β phosphorylate and activate the muscle-specific transcription factors, MEF2A and MEF2C (Yang et al. 1999). These p38 isoforms target transcription factors by a docking

domain distinct from the phosphoacceptor motifs that confer responsiveness for p38α and p38β but not p38γ or p38δ (Engel et al. 1995; Jiang et al. 1997).

The diverse functions of the four MAPK isoforms are seen in their role in the Fas-mediated apoptosis of endothelial cells of the murine liver sinusoids (Cardier and Erickson-Miller 2002) versus their role as the MAPK required for pathogen defense against *Pseudomonas* infection in *C. elegans* (Kim et al. 2002). Furthermore, it has been shown that the *B. anthracis* lethal factor selectively induces apoptosis by cleaving the amino-terminal domain of map kinases kinases (MKKs) thereby eliminating the docking domain that is required for the activation of p38 MAPK (Park et al. 2002). This dismantling of the p38 MAPK–MKK interaction is the mechanism by which *B. anthracis* paralyzes the host inmate immunity. Similarly, in HeLa cells, p38α induces apoptosis while p38β promotes cell survival suggesting both overlapping and distinct physiological roles of the p38 isoforms, but clearly demonstrating their roles in establishing a biochemical phenotype. The p38 MAPKs also exhibit differential responses to specific drugs and inflammatory agents (Lee et al. 1999; Peifer et al. 2009). One subgroup (p38α and p38β) is inhibited by pyridinyl imidazole derivatives, drugs which inhibit the production of proinflammatory cytokines, while the others (p38γ and p38δ) are insensitive to these drugs (Lee et al. 1999). Thus, the four p38 MAPK isoforms can target genes in response to specific drugs and inflammatory agents (Lee et al. 1999; Peifer et al. 2009; Cuadrqado and Nebreda 2010).

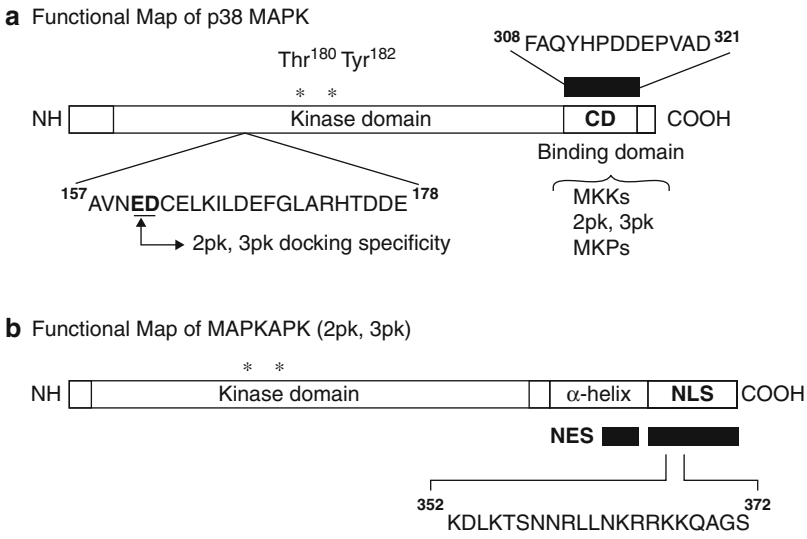
p38 MAPK signaling is mediated by interaction with upstream serine/threonine MAPK kinases, ▶ MKK3 and MKK6 (Fig. 1). While MKK6 is a common activator of p38α, β, γ, and δ, MKK3 activates only p38α, γ, and δ (Yang et al. 1998). This exclusive activation of p38β by MKK6 contributes to this pathway's signaling specificity. Targeted disruption of the *mkk3* and *mkk6* genes has shown their non-redundant functions (Enslen et al. 1998; Lee et al. 1999). For example co-expression of MKK3 with p38β enhances hypertrophy, whereas co-expression with p38α enhances apoptosis (Enslen et al. 1998; Wang et al. 1998). This signaling specificity exemplifies the importance of how specific complexing of pathway proteins target and affect specific biological processes.

Factors that contribute to the specificity of p38 MAPK activation are (Jiang et al. 1997; Enslen et al. 1998): (a) the selective formation of functional complexes between MKKs and the p38 MAPK isoforms, which requires the presence of a p38 MAPK docking site at the N-terminus of the MKKs (Fig. 2); (b) selective recognition of the activation loop (T-loop) or catalytic domain of p38 MAPK isoforms; (c) the T-loop contains the Thr^{180}-Tyr^{182} residues involved in kinase activation. Together, these provide a mechanism for the selective activation of p38 MAPKs in response to activated MKKs (Fig. 2).

The activation (phosphorylation) of all four p38 MAPK isoforms requires the interaction of the MKKs with a p38 docking domain which is located in the N-terminal region of the MKKs (Fig. 2; Enslen et al. 1998). Synthetic peptides of the MKK6 docking sites inhibit activation of p38β indicating that MKK binding is necessary for the activation. On the other hand, MKK3, which lacks this docking site, can activate p38α, p38γ, and p38δ, but not p38β; this accounts for the selective activation of p38α but not p38β by MKK3. Differences in the primary sequences of the T-loop of the p38 MAPK isoforms also contribute to signaling specificity (discussed below). Thus, the specificity of p38 MAPK activation by MKKs requires multiple intramolecular domains present in both kinases, i.e., p38 MAPK and MKKs.

Other important p38 MAPK protein–protein interactions involve the nuclear localization of the MAP kinase-activated protein kinases (▶ MAPKAPKs; 2pk, 3pk; Fig.2b). The formation of 2pk- or 3pk-p38 MAPK functional signaling complexes occurs at a conserved docking motif localized to the C-terminal region. This domain is used for binding to MKKs, nuclear localized 2pk, 3pk and ▶ MAPK phosphatases (MPKs; Fig. 2). The p38α CD domain is located outside of the active center (Fig. 2). Conceptually, therefore, recognition between p38 MAPKs and interacting proteins involves both the docking interaction domains and the transient enzyme–substrate interaction at the T-loop (Thr^{180}-Tyr^{182}), both of which regulate the efficiency and specificity of the enzymatic (kinase) reactions (Jiang et al. 1997; Muda et al. 1998; Wang et al. 1998; Yang et al. 1998; Gavin and Nebreda 1999).

The ED site, shown by a 3D molecular model of MAPK, determines docking specificity toward 2pk and 3pk, which are the nuclear enzymes that associate with p38 MAPK and transcription factors (Fig. 2a, b ; Gavin and Nebreda 1999). This structure would explain their ability to serve as a common docking groove



p38 MAPK Family of Signal Transduction Proteins, Fig. 2 Maps showing the functional domains of the p38 MAPK isoforms and the pk2/3pk docking sites. (**a**) The p38α docking sites for upstream activators (MKKs) and downstream substrates (2pk/3pk). (**b**) A map of the pk2/pk3 domains. The nuclear localization signal (NLS), nuclear export signal (NES), and p38 docking domains are at the C-terminal of pk2/pk3

(Tanoue et al. 2001). In p38 this site is located at Glu^{160} and Asp^{161}. This 3D molecular model of MAPK shows the proximity of the CD and the ED domains. Thus, every MAPK-interacting molecule may bind to this docking groove and each residue therein is differentially involved in each docking interaction (Ben-Levy et al. 1998). Since a major function of the p38 MAPKs involves the activation of transcription factors, the mechanism of this function involves the interaction of p38 MAPK isoforms with 2pk or 3pk to form a complex that activates transcription factors (Figs. 3 and 4). This complex also contains a map kinase phosphatase (MKP) which terminates the transcription activation (Fig. 3). Thus, the 2pk and 3pk proteins contain trafficking domains that mediate the import as well as the export of a p38 MAPK-2pk/3pk complex from the nucleus to the cytoplasm (Figs. 3 and 4; Ben-Levy et al. 1998).

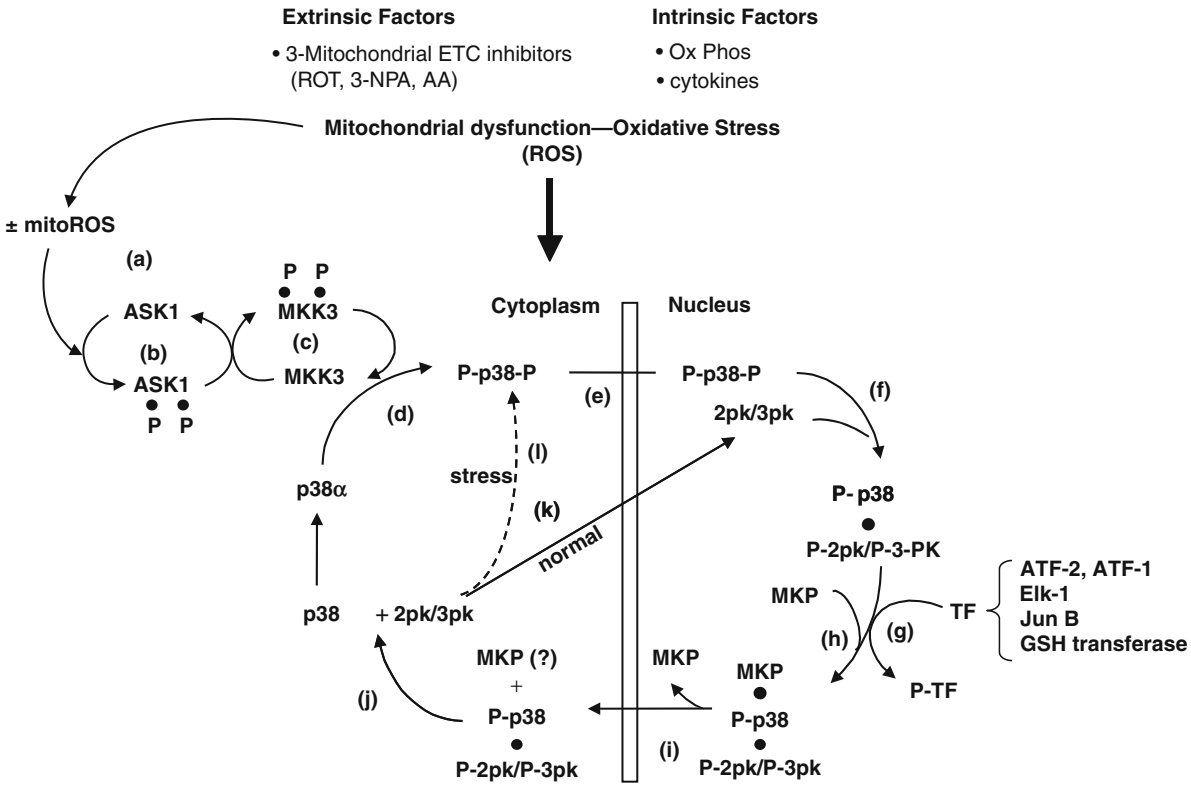
Activation of the p38 Catalytic Site

Activation of the p38 MAPKs occurs by phosphorylation of Thr^{180} and Tyr^{182} in the T-loop (Fig. 2). While p38α is phosphorylated preferentially on Tyr^{182} by MKK3 due to its lack of a MAPK docking site those with a MAPK docking site (MKK6) phosphorylate both Thr^{180} and Tyr^{182} residues of p38α MAPK. Thus, this differential phosphorylation by MKK3 versus MKK6 participates in the specificity of signaling of these upstream activators.

Subcellular Localization and Nucleocytoplasmic Transport of the p38 MAPK Signal

Subcellular localization is an integral part of the diverse functions of the p38 MAPK signaling pathway (Figs. 3 and 4). The mechanism of complex formation involves the nucleocytoplasmic trafficking of 2pk, 3pk which are p38 MAPK nuclear substrates. Both 2pk, 3pk contain nuclear localization sequences (NLS) and nuclear export sequences (NES) as an integral part of their structure. These proteins are localized in the nucleus in unstimulated cells. Upon stimulation, they dock with and are phosphorylated by p38 MAPK (Gavin and Nebreda 1999). Although this complex phosphorylates (activates) specific transcription factors, it is also exported to the cytoplasm. Furthermore, phosphorylation of 2pk and 3pk by p38 MAPK not only activates the kinase so that it can phosphorylate its transcription factor substrates, but it has also been postulated to expose the nuclear export sequence (NES) that results in cytoplasmic localization of both proteins (Fig. 4; Engel et al. 1995; Ben-Levy et al. 1998; Enslen et al. 1998).

The NES and nuclear localization sequence (NLS) of 2pk and 3pk are located at their C-terminal ends (Fig. 2; Engel et al. 1995; Gavin and Nebreda 1999). In addition, 3pk is localized in the nucleus before osmotic stress, and in the cytoplasm upon recovery. The docking of p38 with 2pk or 3pk is essential for their phosphorylation and nucleocytoplasmic export of the



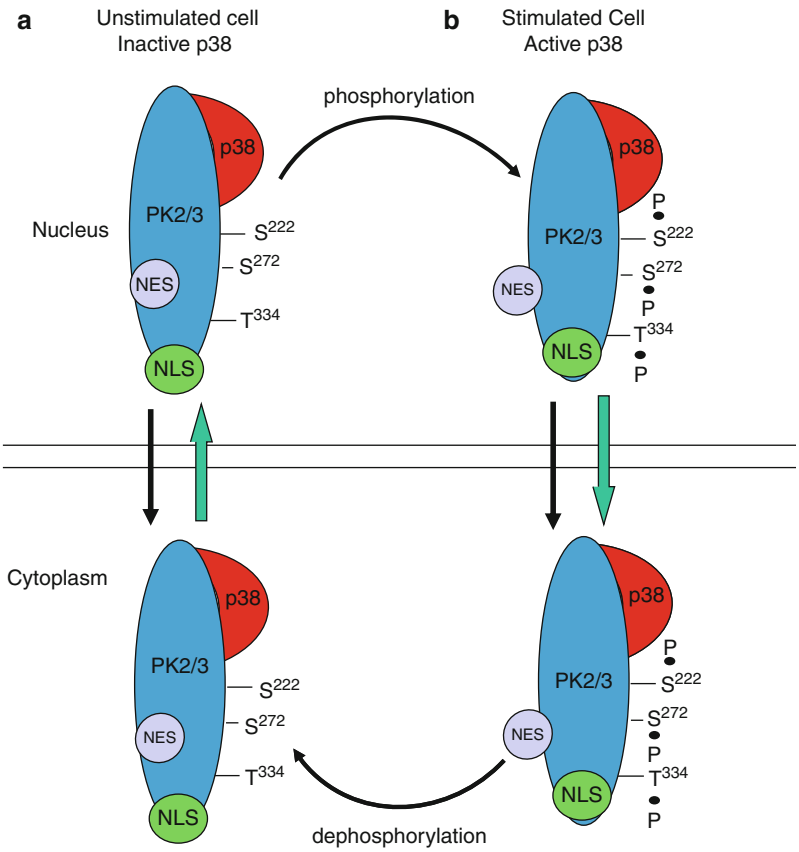
p38 MAPK Family of Signal Transduction Proteins, Fig. 3 A proposed pathway for the nucleocytoplasmic trafficking of p38 signaling proteins, their upstream activators (MKKs), and downstream substrates (pk2/pk3 and transcription factors). (**a**) Activation of p38 (P-p38-P) occurs in the cytoplasm in response to a challenge such as oxidative stress (ROS); (**b**, **c**) the activated ASK1 transduces its signal to MKK3; (**d**, **e**) MKK3 activates p38 which is translocated to the nucleus where, (**f**) it complexes with and activates (by phosphorylation) 2pk/3pk; (**g**) this complex associates and activates transcription factors (TFs); (**h**) interaction of MAPK-phosphatase (MKP) with the complex inactivates the signaling processes by dephosphorylation and, (**i**) translocation of the complex to the cytoplasm; (**j**) the p38-2pk/3pk-MKP complex dissociates; (**k**) the dephosphorylated 2pk/3pk are translocated to the nucleus where they reenter the TF activation cycle. The p38 is reactivated in the cytoplasm in response to a new stress

complex. Thus, the docking interaction between p38 and 3pk is achieved via direct interaction of the CD domain and the ED site of p38 with the C-terminal portion of 3pk (Figs. 2 and 4). The model in Fig. 4 suggests, therefore, that a p38-2pk/3pk complex may be formed in the cytoplasm of unstimulated cells and translocated to the nucleus where it may be activated.

The fact that there are cytoplasmic substrates, e.g., PRAK and Heat shock protein 27 (Hsp27), that are phosphorylated by p38 MAPK in the cytoplasm points to the diversity of its activity for downstream targets. Interestingly, the activation of p38 MAPK by DNA-damaging agents supports the idea that the p38 cascade might be initiated in the nucleus (Suh 2001). Activation of p38 MAPK in the nucleus, on the other hand, would indicate that MKK3 and MKK6 must be localized there. Ben-Levy et al. (1998) found that both MKK3 and MKK6 are localized in both the cytoplasm and nucleus. Thus, 2pk and 3pk may serve a dual function, both as effectors of p38 MAPK by phosphorylating substrates such as Hsp27 (cytoplasmic) and the nuclear cAMP responsive element binding protein (CREB), and as determinants of p38 MAPK localization (Figs. 3 and 4).

Feedback Control of MAPK-Regulated Transcription

Inactivation of the MAPKs is achieved by the dual-specificity MAP kinase phosphatases (MKPs) which target the two regulatory phosphorylation sites of the catalytic domain (Keyse 2000). There are ~9 mammalian MKPs, divided into two groups according to their



p38 MAPK Family of Signal Transduction Proteins, Fig. 4 A model of the regulation of nuclear and cytoplasmic localization of a MAPKAPK-2/3-p38 MAPK complex as a mechanism for the activation of p38 MAPK in the nucleus. (**a**) In the unstimulated cell a MAPKAPK-2/3-p38 MAPK complex is formed in the cytoplasm. The nuclear localization signal (NLS) of MAPKAPK is exposed and mediates the translocation of the complex to the nucleus. (**b**) In the stimulated cell, the active complex mediates its transcription factor activation. The nuclear export signal (NES) is exposed and the complex is translocated to the cytoplasm. In the cytoplasm of the recovered cell, MAPKAPK-2/3 is dephosphorylated and the NLS is exposed. This results in the translocation of the complex to the nucleus. In the model the p38 MAPK may be phosphorylated in either the cytoplasm or nucleus

patterns of transcriptional regulation and subcellular localization (Keyse 2000). The nuclear MKPs are rapidly and highly inducible by many of the stimuli that activate the MAPKs. It is postulated, therefore, that these MKPs play an important role in the feedback control of MAPK signaling in the nucleus. Hutter et al. (2000) showed that activation of MKP-1 involves its interaction with the C-terminal end of nuclear p38 MAPK to activate MKP-1 catalytically. This raises the question of whether a 2pk/3pk-p38-MKP complex forms in the nucleus and is exported into the cytoplasm (Fig. 3) and whether this complex is targeted in the process of inactivation of p38 MAPK.

Several cytosolic MKPs can be triggered by direct interaction with MAPKs. MKP-3 interacts specifically with ERK; binding of ERK2 to MKP-3 dramatically enhances the latter's catalytic activity (Tanoue et al. 2001). On the other hand, MKP-4 interacts with all members of the three major MAPK subfamilies to become catalytically activated (Keyse 2000). These novel mechanisms ensure the tight feedback control of MAPK signaling in the cytosol.

Role of p38 MAPK Signaling in Response to Stress Challenges and Aging

Elevated and sustained expression of p38 MAPK signaling activity is a major physiological characteristic of aging (Papaconstantinou and Hsieh 2010). This

age-associated state of chronic stress is attributed to mitochondrial ETC dysfunction and is a key factor that promotes stress-induced aging characteristics. Thus, endogenous reactive oxygen species (ROS) are important factors that regulate signaling pathways that control the development of senescence and aging phenotypes. It has been proposed that mitochondrial generated ROS may activate p38 MAPK through the ASK1-signalosome → p38 MAPK pathway (Papaconstantinou and Hsieh 2010). Activation of ASK1 initiates the activation of MKK3/6 and p38 MAPK. Thus, the mechanism of regulation of p38 MAPK in response to ROS involves activation of the ASK1-signalosome. Through this mechanism mitochondrial generated ROS activates p38 MAPK and links the activation of senescence pathways ($p16^{Ink4a}$ and $p19^{Arf}$) via p38 MAPK. Based on this hypothesis, it might be expected that (a) the increased and persistent level of oxidative stress may affect the activity of the p38 MAPK stress signaling pathways; (b) as aging progresses, the constitutive activity of stress-activated signal pathways, would increase; and (c) this new level of activity is stabilized – becoming a basic factor in the development of chronic stress in aged tissues.

Summary

Biological signaling is a highly specific process that enables cells and tissues to regulate their responses to their environment, both intrinsic and extrinsic. This review focused on the p38 MAPK family of signaling proteins that respond to mitogenic and stress-activating signaling molecules. Although the p38 MAPK stress response pathway was first discovered in response to an inflammatory challenge by bacterial endotoxin (LPS), its diverse functions have been rapidly identified thus making it a major and critical pathway that regulates numerous biological processes. This is seen in its regulation of differentiation, apoptosis, senescence, and aging. The demonstration that the mechanism of cellular destruction by anthrax lethal factor involves blocking the p38 pathway adds to the significance of the diversity of this pathway. Most certainly, there are other equally important signaling pathways that play a key role in the response to stress factors, mitogens, hormones, etc., such as the ERK and SAPK/JNK pathways, and the hormone-activated pathways such as insulin/IGF-1 and GH pathways. All of these pathways share several mechanisms of signal transduction, e.g., protein–protein interactions (docking), protein modifications (phosphorylation), and intracellular trafficking (nucleocytoplasmic, mitochondrial). Thus, in our discussion of the mechanisms of p38 MAPK pathways, our purpose was to familiarize the reader with these mechanisms which occur in most signaling processes. Furthermore, our focus on how aging affects the function of the p38 MAP kinase pathway is meant to demonstrate that in addition to environmental and intrinsic factors, signaling pathways also play a key regulating role in biological processes during the entire life cycle, i.e., spanning the embryonic young adult and aging phases of life.

References

Ben-Levy R, Hooper S, Wilson R, Paterson HF, Marshall CJ. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. Curr Biol. 1998; 8:1049–57.

Cardier JE, Erickson-Miller CL. Fas (CD95)-and tumor necrosis factor mediated apoptosis in liver endothelial cells; role of caspase-3 and the p38 MAPK. Microvasc Res. 2002;63:10–8.

Conrad PW, Rust RT, Han J, Milkorn DE, Beitner-Johnson D. Selective activation of p38α and p38β by anoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. J Biol Chem. 1999;274:23570–6.

Cuadrado A, Nebreda AR. Mechanism and functions of p38 MAPK signaling. Biochem J. 2010;429:403–17.

Engel K, Schultz H, Martin F, Kotlyarov A, Plath K, Hahn M, et al. Constitutive activation of mitogen-activated protein kinase 2 by mutation of phosphorylation sites and an A-helix motif. J Biol Chem. 1995;270:27213–21.

Enslen H, Raingeaud J, Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem. 1998; 273:1741–8.

Gavin AC, Nebreda AR. A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKP kinase-1. Curr Biol. 1999;11:281–4.

Haq R, Brenton JD, Takahashi M, Finan D, Finkelsztein A, Damaraju S, Rotapel R, Zanke B. Constitutive p38HOG mitogen-activated protein kinase activation induces permanent cell cycle arrest and senescence. Cancer Res. 2002;62:5076–82.

Hutter D, Chen P, Barnes J, Liu Y. Catalytic activation of mitogen-activated protein (MAP) kinase phosphatase-1 by binding to p38 MAP kinase: critical role of the p38 C-terminal domain in its negative regulation. Biochemistry. 2000;352:155–63.

Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Padova F, Ulevitch R, Han J. Characterization of the structure and

function of the fourth member of p38 group mitogen-activated protein kinases, p38δ. J Biol Chem. 1997;333:30122–8.
Keyse SM. Protein phosphatases and the regulation of mitogen-activated protein kinase signaling. Curr Opin Cell Biol. 2000; 12:186–92.
Kim DH, Feinbaum R, Alloing G, Emerson FE, Garsin DA, Inoue H, Tanaka-Hino M, Hisamoto N, Matsumoto K, Tan V-W, Ausubel FM. A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. Science. 2002; 297:623–6.
Lechner C, Zahalka MA, Giot F-F, Moler NPH, Ullrich A. ERK6, a mitogen-activated protein kinase involved in G2C12 myoblast differentiation. Proc Natl Acad Sci USA. 1996; 93:4355–9.
Lee JC, Kassis S, Kumar S, Badger A, Adams JL. p38-mitogen activated protein kinase inhibitors—mechanisms and therapeutic potentials. Pharmacol Ther. 1999;82:389–97.
Li Z, Jiang Y, Ulevitch RJ, Han J. The primary structure of p38gamma: a new member of p38 group of MAP kinases. Biochem Biophys Res Commun. 1996;228:334–40.
Muda M, Theodosiou A, Gillieron C, Smith A, Chabert C, Camps M, Boschert U, Rodrigues N, Davies K, Ashworth A, Arkinstall S. The mitogen-activated protein kinase phosphatase-3 N-terminal noncatalytic region is responsible for tight substrate binding and enzymatic specificity. J Biol Chem. 1998;273:9323–9.
Ono K, Han J. The p38 signal pathway activation and function. Cell Signal. 2000;12:1–13.
Papaconstantinou J, Hsieh C-C. Activation of senescence and aging characteristics by mitochondrially generated Ros: how are they linked? Cell Cycle. 2010;9:1–3.
Park JM, Greten FR, Li ZW, Karin M. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. Science. 2002;297:2048–51.
Peifer C, Abadleh M, Bischof J, Hauser D, Schattel V, Hirner H, Knippschild U, Laufer S. 3,4-diaryl-isoxazoles and –imidazoles as potent dual inhibitors of p38α mitogen activated protein kinase and casein kinase 1δ. J Med Chem. 2009;52:7618–730.
Saurin AT, Martin JL, Heads RJ, Foley C, Mockridge JW, Wright MJ, Wang Y, Marber MS. The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes. FASEB J. 2000; 14:2237–46.
Suh Y. Age-specific changes in expression, activity, and activation of the c-Jun NH(2)-terminal kinase and p38 mitogen-activated protein kinases by methyl methanesulfonate in rats. Mech Age Dev. 2001;122:1797–811.
Tanoue T, Ryota M, Adachi M, Nichida E. Identification of docking groove on ERK and p38 MAP kinases that regulate the specificity of docking interactions. EMBO J. 2001; 20:466–79.
Wang YU, Huang S, Sah VP, Ross Jr J, Brown JH, Han J, Chien KR. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. J Biol Chem. 1998;273:2161–8.
Yang SH, Whitemarsh AJ, Davis RJ, Sharrocks AD. Differential targeting of MAP kinases to the ETS-domain transcription factor E1k-1. EMBO J. 1998;17:1740–9.
Yang SH, Galanis A, Sharrocks AD. 1999 Targeting of p38 mitogen-activated protein kinase to MEF2 transcription factors. Mol Cell Biol. 1999;19:4028–38.
Zer C, Sachs G, Shin JM. Identification of genomic targets downstream of p38 mitogen-activated protein kinase pathway mediating tumor necrosis factor-α signaling. Physiol Genomics. 2007;31:343–51.

p38MAPKK

▶ Mek3

P-38α (Phospho p38α)

▶ p38 MAPK Family of Signal Transduction Proteins

p42/p44 MAPK

▶ ERK1/ERK2

p42IP4

▶ ADAP1

p45MAPKK

▶ Mek

p47 GTPases

▶ Immunity-Related GTPases (IRG)

p50

▶ CD40

p53

Krassimira Alexieva-Botcheva and Carl W. Anderson
Biology Department, Brookhaven National Laboratory, Upton, NY, USA

Synonyms

FLJ92943; LFS1; TP53; TRP53

Historical Background

As the major tumor suppressor in multicellular organisms, p53 arguably is one of the most intensively studied human proteins (over 54,000 publications including nearly 7,000 reviews) because it is critical for maintaining genomic stability and cellular homeostatic processes in response to multiple stresses. The p53 protein is a tetrameric, sequence-specific, DNA-binding transcription factor, stabilized and activated in response to genotoxic and non-genotoxic stresses; estimates are that the activation of p53 directly or indirectly induces or represses the expression of about 1,500 genes. These genes coordinate the cellular response to protect cells and/or the organism from damage by arresting the cell cycle and inducing repair, by initiating apoptosis, a program of cell death, or by triggering senescence, a permanent arrest of the cell cycle (Vousden and Prives 2009). Discovered almost simultaneously just over 30 years ago (Table 1) by several groups as an antigen associated with the large tumor antigen of the small DNA tumor virus SV40, p53 initially was considered an oncogene (Levine and Oren 2009), partly because the initial, mutant cDNA clones derived from tumors cooperated with several oncogenes, notably *HRAS*, to transform primary cell cultures. On its own, mutant p53 facilitated cell immortalization. Only after cloning of the wild-type cDNA from normal mouse cells and comparing its sequence with earlier mutant clones was p53 firmly established to be a tumor suppressor that became known as the "guardian of the genome" (Lane 1992). Several groups then demonstrated that the p53 gene (*TP53*) is mutated in approximately half of all human cancers, and now it is known that a variety of mechanisms inactivate the p53 pathway in virtually all other cancers (Olivier et al. 2010; Vousden and Prives 2009). Indeed, *TP53* knockout mice rapidly develop tumors, confirming the importance of p53 in tumor suppression (Donehower and Lozano 2009).

p53, Table 1 p53: A short timeline

Date	Event
1979	p53 discovered
1983	p53 cDNA cloned
1984	"p53" demonstrated to be oncogenic
1984	p53 shown to be induced by DNA damage
1986	First p53 phosphorylation sites identified
1988	Wild-type p53 cDNA cloned
1989	p53 determined to be a tumor suppressor and mutated in human cancers
1990–1992	p53 identified as a transcription factor
1991	p53 discovered to induce cell cycle arrest and apoptosis
1992	MDM2 proven to negatively regulate p53
1992	*TP53* knockout mice revealed as cancer prone
1994	p53 DNA-binding-domain and tetramerization-domain structures revealed
1997	MDM2 shown to ubiquitinate p53; p53 shown to be acetylated
1997	p63 and p73 described
1998	ATM demonstrated to phosphorylate p53 Ser15
2000	First mouse p53 phosphorylation site knock-in mutant made
2003	p53 shown to induce transcription-independent apoptosis via mitochondria
2004	Methylation shown to regulate p53 activity
2005	p53 established as regulating metabolism and having an antioxidant function
2007	p53 demonstrated to be required for embryo implantation
2009	Structure of full p53 tetramer described
2010	Processing of miRNAs and regulation of lincRNAs modulated by p53

But how does p53 prevent tumors? The mechanistic answer to this question began to emerge in the early 1990s with the discoveries that p53 possessed a potent transactivation domain and was a site-specific, DNA-binding protein that could induce the expression of genes controlling the progression of the cell cycle (e.g., *CDKN1A* ($p21^{Waf1}$)) and apoptosis (e.g., *BAX*) (Lane 1992; Levine and Oren 2009). Subsequently, the induction of p53 was shown to promote cellular senescence, although the underlying mechanism(s) are just emerging. Thus, p53 was established as

a transcription factor that regulates the expression of genes which control and implement the major cellular mechanisms for preventing cancer by arresting the cell cycle so to allow time to repair DNA damage or by inducing senescence or apoptosis. Subsequently, transcription-independent functions of p53 were uncovered. Among these is the ability of p53 to induce apoptosis through an interaction with the mitochondrial membrane causing the subsequent release of pro-apoptotic factors such as cytochrome C (Vaseva and Moll 2009).

The early history of p53 research is coupled intimately with that of the small DNA tumor viruses, SV40, polyomavirus, adenovirus, and papillomavirus (Levine 2009). Each virus produces a protein, respectively, the SV40- and polyoma-T-antigens, the adenovirus E1b-55kd protein, and the papillomavirus E6 protein, that during the early phase of a productive infection inactivates p53 (and suppresses apoptosis) as part of the viral strategy for maximizing virus yields. The expression of these proteins from integrated viral sequences also is required to initiate and maintain cell transformation; remarkably, except for a few papillomavirus serotypes, these viruses seemingly do not produce tumors in humans. During the past 20 years, much research on p53 focused on cellular responses to DNA damage. In normal unstressed cells, p53 has a low basal concentration of about 17,000 molecules per cell; however, after exposing cells to UV light or ionizing radiation, both of which damage DNA and are carcinogenic, p53 is "induced" and accumulates to higher levels. These seminal findings, which subsequently have been strengthened and enhanced, provide the rationale for p53 functioning as a multifaceted tumor suppressor in multicellular organisms (Zilfou and Lowe 2010; Vousden and Prives 2009).

The complex mechanisms that regulate p53 activity are far from being completely understood. Earlier, p53 was shown to be a phosphoprotein, and, as detailed below, it is now known that p53 is posttranslationally modified in multiple ways on more than 10% of its 393 (human protein) amino acid residues (Anderson and Appella 2009; Meek and Anderson 2010). An allosteric model for p53 activation as a DNA-binding protein was proposed which suggested that phosphorylation of residues in p53's carboxyl-terminal regulatory domain relieved an inhibition of DNA binding and promoted activation as a transcription factor. The oncoprotein, MDM2 (mouse double minute 2) binds to the N-terminal transactivation domain of p53 and blocks its activity as a transcription activator; subsequently, MDM2 was identified as an E3 ligase that ubiquitylates p53 at multiple sites, marking it for degradation by the 26S proteasome and thus maintaining its low cellular concentration in normal, unstressed cells. Several groups demonstrated that the ATM kinase, which is mutated in the disease ataxia telangiectasia and is activated in response to DNA damage, phosphorylates serine 15 (Ser15) in the transactivation domain of p53. Subsequently, phosphorylation of Ser15 was suggested to induce the dissociation of MDM2 from p53, thereby stabilizing it. While it is now known that the mechanisms regulating p53 are more complex, these early findings undoubtedly stimulated interest in clarifying the signaling mechanisms regulating p53 function. These studies were aided substantially by mouse models that use site-specific knock-in technology to explore p53 biology in a living animal (Broz and Attardi 2010).

Origin of the p53 Family

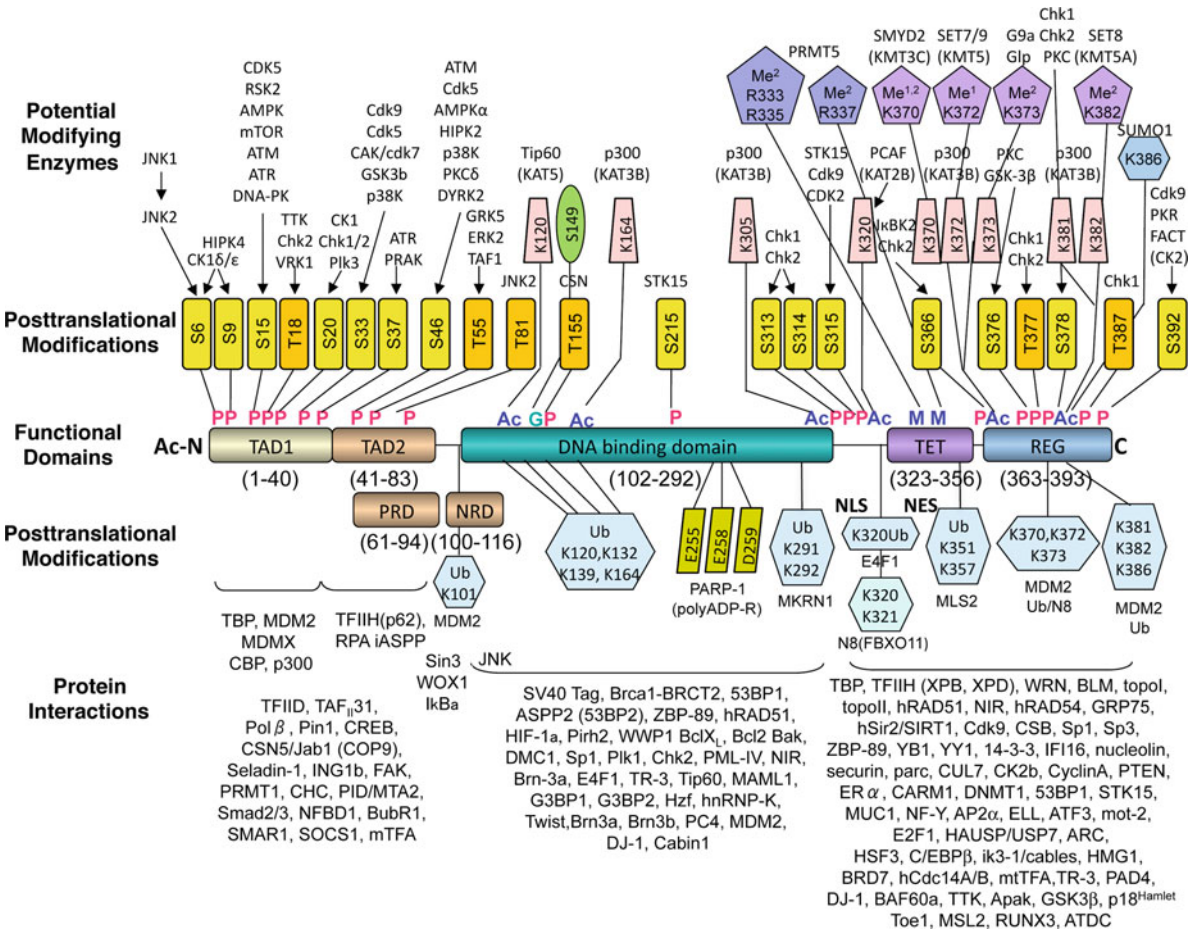
Knowledge of the primordial p53 and its signaling pathways might well offer insights to elucidate more p53 functions and potential cancer therapies (Lu et al. 2009). In addition to *TP53*, the genomes of most vertebrates, including that of humans, contain genes for two closely related homologues, namely, *TP63* and *TP73* (Dötsch et al. 2010). The domain structures of these three proteins are similar (see below), but p63 and p73 also contain an additional carboxy-terminal sterile alpha motif (SAM) that probably facilitates additional protein–protein interactions. All three proteins in this p53 family bind similar DNA sequences and activate several common target genes, but their cellular functions have diverged significantly. Although p63 and p73 have tumor-suppressor functions in mouse models, neither is mutated often in human cancers. The development of squamous epithelia requires p63, as does maintaining a pool of proliferating epithelia stem cells; p73 is indispensable for development of the central nervous system and the olfactory and immune systems. All three genes generate a plethora of isoforms due to alternative modes of initiating transcription and splicing; up to nine different isoforms have been reported for the

TP53 gene. Transactivation (TA) competent isoforms of p63 and p73 are important for maintaining genome stability in human oocytes; however, of particular importance for development are the so-called Δ isoforms of p63 and p73 that lack the N-terminal transactivation domain and function as inhibitors of the three full-length proteins. Interestingly, mice that lack a functional p53 gene develop normally but are highly cancer prone, while those without either p63 or p73 suffer from developmental defects and die soon after birth. Rare germ-line mutations in p63 are associated with developmental defects in humans. While signaling by the p53 family should be considered collectively, readers should consult recent reviews for further information on the functions of p63 and p73.

p53 and its family members do not occur in prokaryotes, fungi, or plants, but extensive genome sequencing revealed p53- and p63/73-like genes in many invertebrate species, including clams, mollusks, insects, and worms, as well as in unicellular choanoflagellates and amoeba (Lu et al. 2009). p53 from non-vertebrates is most extensively characterized in the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabdites elegans* (*CEP-1*). However, instead of a vertebrate-like p53, both species express p63/73-like proteins with carboxy-terminal SAM domains that function in germ-line cells; interestingly, neither species has an MDM2-like negative regulatory gene. The discovery of p63 and p73 in the late 1990s suggested that p53 was derived from an ancestral p63/p73-like gene through gene duplication and loss of the SAM domain. However, the more recent detection of p53- and p63/73-like genes in unicellular and simple multicellular eukaryotes challenges this view, indicating that the primordial function of p53 was not tumor suppression; apparently, this property evolved much more recently. Notably, D. Lane's group recently identified both *MDM2*- and *TP53*-like genes that are well conserved in placozoans, which are simple eukaryotes with only four cell types and with genomes only 10 times the size of that of *Escherichia coli*. Remarkably, not only is the basic structure of the placozoan p53 conserved, but so also are key amino acid residues involved in DNA binding and posttranslational modification. Studying the functions of p53 in more primitive species may uncover new roles for p53 and additional modes of signaling.

Structure and Posttranslational Modifications

The p53 polypeptide, with the classical features of a sequence-specific transcriptional factor, is functionally divided into three major domains (Fig. 1): an amino- (N) terminal transactivation segment (TA, Met1-Lys101, numbering for human p53) that interacts with regulatory proteins and components of the transcription machinery; a central, sequence-specific, DNA-binding domain (Thr102-Lys292); and, a carboxyl- (C) terminal tetramerization and regulatory domain (Gly293-Asp393) (Anderson and Appella 2009; Joerger and Fersht 2008). The unstructured N-terminal region contains two independent transcription activation domains, TAD1 (Met1-Met40) and TAD2 (Asp41-Pro83). The former is highly conserved and is required for most transactivation activity; it interacts with several coactivators and corepressor proteins (e.g., histone acetyltransferases (HATs) and histone deacetylases (HDACs)), and also with p53's major negative regulator, the E3-ubiquitin ligase, MDM2. Residues Glu11-Leu26 may function as a secondary nuclear export signal. TAD2 largely overlaps a proline-rich domain (PRD, Asp61-Leu94) that is important for assuring p53 stability, transactivation ability, and the induction of transcription-independent apoptosis. It also contains binding sites for the corepressor Sin3a and the proline isomerase Pin1. While TAD1 and the C-terminal region of TAD2 are highly conserved among mammals, the length and sequence of residues Asp41-Ala79 of human p53 are not well conserved. Except for Lys101, which can be ubiquitylated by the E3 ligase MDM2, the N-terminal TA region of p53 is covalently modified exclusively by phosphorylation at 10 serines/threonines, namely, Ser6, 9, 15, 20, 33, 37, and 46, and Thr18, 55, and 81. Phosphorylation of p53 at Ser33, Ser46, and Thr81 (and at Ser315) generates binding sites for the proline isomerase, Pin1. Pin1-catalyzed proline isomerization generates or removes binding sites for several p53 binding partners that regulate p53 acetylation and ubiquitylation, and consequently ensures the efficient loading of p53 on to target promoters. p53 TA has the most significant polymorphism in p53, a C or G in codon 72 that changes the amino acid from proline (CCC) to arginine (CGC) and affects its activity as a transcription factor (Whibley et al. 2009).



p53, Fig. 1 p53 domains, posttranslational modifications, and binding partners (Updated from Anderson and Appella (2009) and Meek and Anderson (2010))

In contrast to the unstructured N- and C-terminal regions, the central core of p53 consists of an immunoglobulin-like β-sandwich offering a scaffold for the DNA-binding surface (Fig. 1). This surface is formed by two large loops, L2 (Lys164-Leu194) and L3 (Met237-Pro250), stabilized by a zinc ion (Joerger and Fersht 2008; also see http://proteopedia.org/wiki/index.php/P53). p53 is the most frequently mutated tumor suppressor in humans, and most such tumor-derived mutations are missense mutations in the central domain that block or alter sequence-specific DNA binding or induce conformational changes in the domain structure. Six so-called hot-spot mutations (R175H, R248Q, R248W, R273H, R273C, and R282W) account for about 20% of the somatic mutations in human cancers (Olivier et al. 2010). X-ray crystallography and NMR revealed the three-dimensional structures of the DNA-binding domain, free or bound to DNA, and the structures for several mutant p53s in their DNA-free state. Recent high-resolution crystal structures of complexes between the p53 core domain tetramers and DNA targets point to mechanisms by which p53 may recognize specific REs in mammalian genomes and how such recognition may be modulated through post-translational modification or interactions with partner proteins. The DNA-binding domain can be posttranslationally modified by phosphorylation at Thr155 and Ser215, O-glycosylated at Ser149, acetylated at Lys120 and Lys164, ubiquitylated at Lys120, 132, 139, 164, 291, and 292, and ADP-rybosylated at Glu255, 258, and Asp259. In particular, the acetylation of Lys120, which is a DNA-contact residue, by the Tip60/hMOF histone acetylases in response to DNA damage might be important for p53-mediated transcriptional activation and for inducing apoptosis.

The carboxyl-terminal region contains a nuclear localization signal (Thr312-Asp324), a tetramerization domain (Leu323-Gly356), and a basic 30 amino acid segment that binds RNA and certain DNA structures, including short single strands, four-way junctions, and insertions/deletions in a sequence-independent manner, as well as numerous partner proteins (Fig. 1). The unmodified C-terminal domain initially was thought to negatively regulate sequence-specific binding by the core DNA-binding domain; subsequently it was shown to be necessary for efficiently activating transcription, possibly by facilitating the identification of response elements by promoting diffusion along the DNA. The C-terminal domain can be posttranslationally modified at multiple sites in many ways. Ser313, 314, 315, 366, 376, 378, and 392 may be phosphorylated along with Thr377 and 387; Lys305, 320, 370, 372, 373, 381, and 382 can be acetylated; K370, 372, 373, and 382 also can be mono- or dimethylated; Arg333, 335, and 337 can be dimethylated; K351 and K357 can be ubiquitylated; K320, 321, 370, 372, 373, 381, 382, and 386 can be ubiquitylated or neddylated; and, K386 can be sumoylated. Arg333, 335, and 337, as well as Lys351 are in the core tetramerization domain, and, though untested, their modification might directly affect p53 oligomerization. Regulation of the formation of p53 tetramers is an underappreciated aspect of p53 signaling. The dissociation constant for its formation apparently is tuned so that p53 is largely in a monomeric (or dimeric) state in unstressed cells; however, the modest approximately threefold increase in p53 concentration after stress is sufficient to push p53 toward the formation of tetramers, and only tetrameric p53 is active as a transcription factor. Thus, the effect of p53 stabilization on transcription is much greater than would be expected from the relatively small increase in its concentration. Tetramer formation also hides the p53 nuclear export signal in the tetramerization domain (Fig. 1). p53 acetylation and ubiquitylation reportedly inhibit p53 tetramer formation, while the binding of several proteins, including c-Abl, ARC, and 14-3-3, are reported to negatively or positively affect tetramer formation, respectively. Finally, some p53 posttranslational modifications only occur on tetrameric p53.

Structural studies of full-length human p53 have been impeded because ~40% of the molecule is intrinsically unstructured and because p53 has a low thermal stability. Nevertheless, A. Fersht's group determined the low-resolution structure of a stabilized variant of full-length human p53 with and without DNA using small-angle X-ray scattering (SAXS), NMR, and electron microscopy (Joerger and Fersht 2008); this structure has been confirmed and further enhanced by applying biophysical and computational techniques. The free protein in solution forms an elongated cross-shaped tetramer with loosely coupled core domain dimers and extended N- and C-termini. Upon DNA binding, p53 wraps around the DNA, and the structure becomes more rigid. All four N-termini point away from one face of the core domain/DNA complex, consistent with the fact that they become heavily posttranslationally modified and serve as an interaction scaffold for numerous interacting proteins, including MDM2, the histone acetyltransferases p300/CBP, the single-stranded binding protein RPA, and several components of the transcriptional apparatus (Fig. 1). Interestingly, a cryo-electron microscopy structure of full-length murine p53 is inconsistent with this structure. While elucidating the quaternary structure of p53 with DNA is an important achievement, the fact is that cell DNA is mostly packaged in nucleosomes, and, while some promoter regions may be nucleosome-free, this is an unlikely scenario for many p53 binding sites. To resolve this issue, A. Nagaich and colleagues have begun to examine how p53 interacts with nucleosomal response elements. Ostensibly, the bending of DNA enhances p53 binding when the response element is positioned near the nucleosomal dyad, but the response element is inaccessible if the orientation of the core nucleosome changes by ~180°. Sequestering by the nucleosome of the many potential p53 recognition sites in mammalian cells may partly explain how the relatively few p53 molecules find their important target sequences, and why different cells, with different chromatin organizations, may respond differently to the activation of p53.

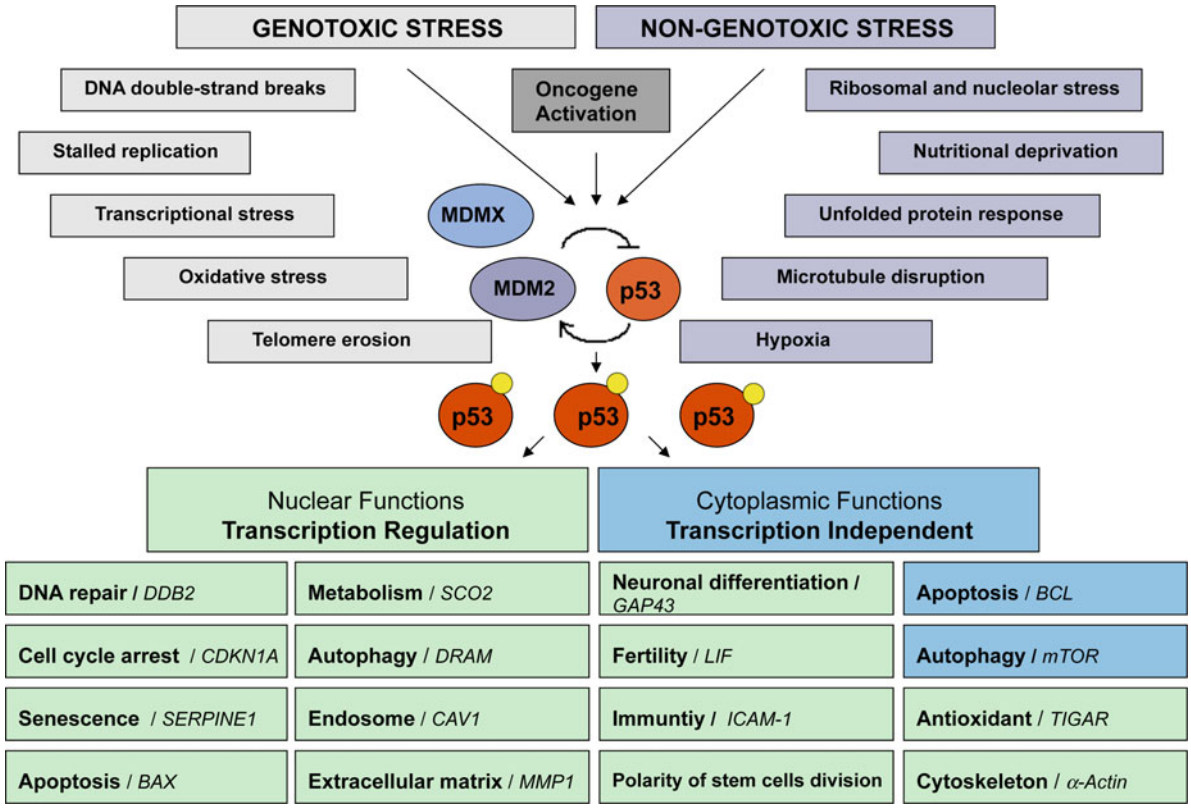
Signaling to p53

p53 activity is regulated not only through control of its cellular location and concentration, but also through posttranslational modifications that affect its structure or alter its interaction with protein-binding partners. In unstressed cells, the half-life of p53 is short (~20 min), and low levels of p53 are maintained primarily by the E3 ubiquitin ligase MDM2 (Perry 2010) that transfers ubiquitin to multiple p53 lysines (Fig. 1); however,

several additional E3 ubiquitin ligases, including Pirh2, COP1, CHIP, ARF-BP1, E6-AP, TOPORS, and TRIM24, may contribute to p53 ubiquitylation in specific cells or under specific circumstances. Polyubiquitylation of p53 targets it for degradation through the 26S proteasome, while monoubiquitylation (or oligo-, less than ~4) marks p53 for nuclear export to the cytoplasm and non-transcriptional functions (see below) or affects its binding specificity. The complex switch between inhibition of ubiquitylation, mono- or polyubiquitylation, probably is cell-type dependent and is regulated by posttranslational modifications to both p53 and MDM2, and by interactions with several binding factors, including the histone acetyltransferases p300/CBP and the proline isomerase, Pin1 (above). The switch is modulated in response to various genotoxic and non-genotoxic stresses through several signaling networks. Furthermore, MDM2 interacts with its homolog MDMX (or MDM4), which does not have ubiquitin ligase activity, but affects MDM2 activity and autoubiquitylation, and therefore, p53 levels. Deleting or inactivating either MDM2 or MDMX in mice results in a lethal embryonic phenotype, but the simultaneous deletion of p53 recovers viability in both cases. Both MDM2 and MDMX also bind the N-terminal transactivation domain of p53, preventing it from interacting with the transcriptional apparatus and activating transcription. p53 also induces the expression of MDM2 (but not MDMX) to create a negative feedback loop. Indeed, the p53 circuit communicates with several other transcriptional targets, including Wnt-β-catenin, IGF-1-AKT, Rb-E2F, p38MAP kinase, cyclin-cdk, p14/19 ARF pathways, and cyclin G-PP2A, as well as the ubiquitin ligases Cop-1 and Pirh-2 creating several negative- and positive-feedback loops that modify its activity or concentration (Harris and Levine 2005).

Although early studies suggested that p53 binding to DNA was regulated allosterically through posttranslational modifications to its C-terminus, experiments with the small-molecule inhibitor, nutlin-3a, suggested that increases in p53 concentration alone can activate many p53 target genes, although the transcriptional response may be somewhat different than those induced by DNA-damaging agents. Nutlin binds MDM2 and inhibits MDM2 binding to p53, thereby blocking p53 degradation without obviously activating stress signaling pathways that posttranslationally modify p53. Likewise, the induction of integrated, recombinant p53 under the control of exogenous regulatory elements similarly can activate p53 transcriptional responses. While unmodified p53 may be transcriptionally competent, it is widely accepted that in normal cells posttranslational modifications to p53 and its partner binding proteins in response to the many cellular stresses to which it responds (Fig. 2) not only stabilizes p53 but "activates" it, fine tuning the transcriptional responses in a stress- and cell-type-specific manner. Figure 1 shows the known sites at which human p53 can be posttranslationally modified and some of the enzymes that accomplish these modifications, at least in vitro. Also listed are many of the proteins that reportedly interact with p53 with the p53 interaction domain indicated. Not all enzymes that have been reported to modify p53 in vitro will do so in vivo; likewise, the list of interacting proteins, though certainly incomplete, may contain false positives. Nevertheless, the potential complexity is daunting ($>10^{12}$ possible posttranslational combinations), even though only a minute fraction of the possible combinations can exist at one time in any one cell.

The best characterized signaling pathways that modify and modulate p53 are those that are activated in response to DNA damage – primarily DNA double-stranded breaks (DSBs) and stalled or collapsed replication forks (Anderson and Appella 2009; Meek and Anderson 2010). The induction of DSBs triggers a complex signaling network characterized by the rapid activation of the ataxia telangiectasia–mutated (ATM) protein kinase. ATM is a member of the family of phosphatidylinositol-3 kinase-related kinases (PIKKs) that includes ATR (ATM-related), DNA-PK (double-stranded DNA-activated protein kinase), ▶ mTOR (mammalian target for the immunosuppressant drug, rapamycin), and SMG1 (homology to *C. elegans* SMG-1). Activated ATM directly phosphorylates p53 on Ser15, while also activating several effector kinases, including ▶ CHK2, that can phosphorylate p53 on several different sites in the N-terminal transactivation domain (e.g., Thr18, Ser20) as well as multiple sites in the C-terminal domain, including Ser313, 314, 366, 378, and Thr377. Under some circumstances, ATM may phosphorylate additional sites, such as Ser46, whose phosphorylation is important for p53-induced apoptosis in response to severe DNA damage. Stalled or collapsed replication



p53, Fig. 2 p53 signaling in response to genotoxic and non-genotoxic stress

forks (caused, e.g., by exposure to UV light) preferentially activate the ATR kinase, which, like ATM, phosphorylates p53 at Ser15 and also actuates effector kinases including Chk1; similarly to Chk2, Chk1 phosphorylates multiple p53 residues. Ultimately, most of the known 21 p53 phosphorylation sites may be phosphorylated in response to DNA damage, with different DNA damage-inducing agents engendering different phosphorylation profiles, thus partly accounting for different outcomes. For example, exposing fibroblasts to moderate levels of ionizing radiation primarily arrests the cell cycle, while exposure to UV light strongly induces apoptosis. Within the N-terminal transactivation domain, a complex interdependency between phosphorylation sites exists. For example, phosphorylation of Thr18, Ser20, and Ser46 may depend upon prior phosphorylation of Ser15. The molecular basis for this interdependency is unknown, but it may represent one way in which p53 integrates signals from different stress pathways, and/or insures that activation requires sustained signaling from several sources.

Phosphorylation of N-terminal sites, especially phosphorylation of Thr18, weakens binding by MDM2 and, in part, accounts for p53 stabilization in response to DNA damage. Equally important is the modification of MDM2 in response to DNA damage. Like p53, MDM2 and MDMX are highly modified proteins (Meek and Hupp 2010). Briefly, ATM directly phosphorylates MDM2 Ser395, which impairs nuclear export and p53 degradation; it also activates the cAbl kinase, which phosphorylates MDM2 Tyr394 and inhibits MDM2-mediated p53 ubiquitylation. Exposure to ionizing radiation also results in hypophosphorylation of several residues in the central, acidic domain of MDM2, thereby weakening the interaction with p53 and contributing to p53 stabilization. Similarly, DNA damage changes the phosphorylation of MDMX (Meek and Hupp 2010).

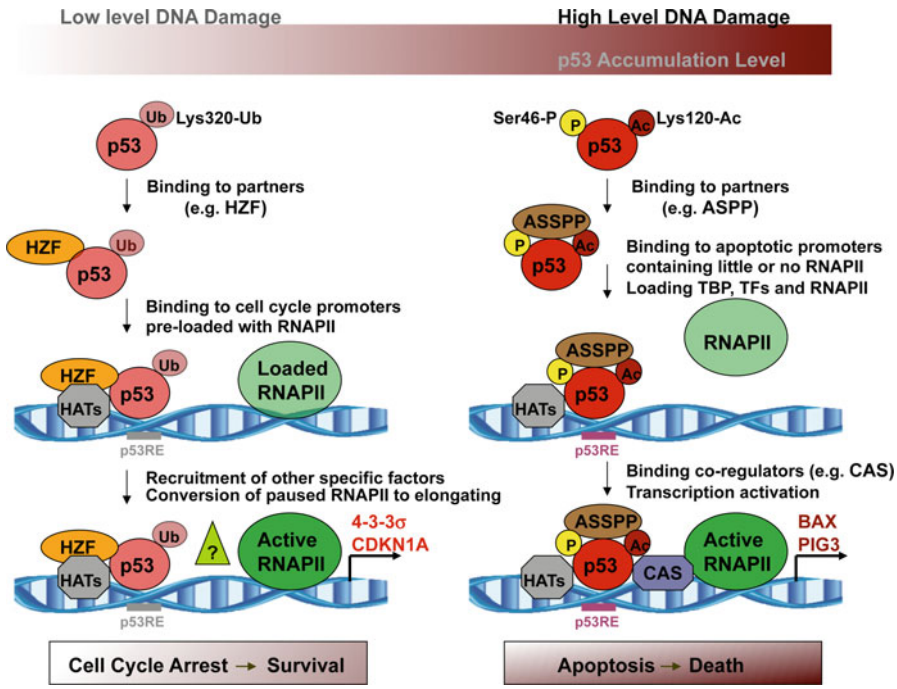
In addition to inhibiting binding to MDM2, the phosphorylation of multiple sites in the N-terminal transactivation domain of p53 also enhances p53's interaction with the HATs p300/CBP, so generating a posttranslational modification cascade that causes the

acetylation of multiple lysines in the DNA-binding and C-terminal domains of p53, including Lys164, 305, 370, 372, 381, and 382. Lysine 320 can be acetylated by the HAT ▶ pCAF. Acetylation of p53 may augment its stability by preventing ubiquitylation; acetylation has both positive and negative effects on tetramer formation and DNA binding. While no single acetylation site appears to be critical for p53 activity, when its eight C-terminal lysines were changed to arginine, it became transcriptionally "dead," suggesting that acetylation is an important p53 modification. Upon severe DNA damage, the MYST family of acetyl transferases, hMOF and TIP60, target Lys120 in the central DNA-binding domain, and the Lys120 acetylated p53 preferentially binds and activates promoters of pro-apoptotic genes (Meek and Anderson 2010). Three lysines, Lys370, 372, and 382, at the C-terminus of p53 are methylated in response to DNA damage. Mono-methylation at Lys372 and dimethylation at Lys370 activate p53, while mono-methylation at Lys370 and at 382 inhibit its activity. p53 Lys372 is targeted by the Set7/9 methyltransferase, a necessary modification for recruiting the TIP60 HAT complex. Lys372 methylation prevents mono-methylation at Lys370, which is a "repressive mark." The enzymes that acetylate and methylate p53 in response to DNA damage also may modify histones near the p53 binding site, supporting crosstalk between the p53 signaling pathway and epigenetic regulation of chromatin.

Enzymes that reverse posttranslational modifications and restore homeostasis upon recovery from stress also are important components of the cellular signaling networks regulating p53 activity. At least four phosphatases, PP1, PPM1D (Wip1), PP2A, and Cdc14A, reportedly dephosphorylate specific p53 residues (Anderson and Appella 2009; Meek and Anderson 2010). PPM1D, acting as an oncogene, is overexpressed in approximately 15% of primary breast cancers; in model rodent systems, its inhibition or elimination delays or prevents tumor formation. Several deacetylases, including HDAC1 and SIR2, the demethylase KDM1A (LSD1), and the de-ubiquitylation enzyme HAUSP, directly target p53 to remove modification marks. The lifetimes of such modifications on free and chromatin-bound p53 largely are unknown (Anderson and Appella 2009).

In addition to DNA damage, a variety of non-genotoxic physiological processes and stresses, including nutrient deprivation, microtubule distribution, hypoxia, and hyperoxia, may activate p53 through interconnected, and, as yet incompletely characterized signaling pathways that involve kinases such as AMPK and mTOR, both of which are involved in energy metabolism and can induce phosphorylation of p53 at Ser15, the ▶ Aurora kinase A (STK15) which phosphorylates Ser215 and Ser315, and the p38MAP kinase which phosphorylates Ser33 and Ser46 (Anderson and Appella 2009). An unfolded protein response (ER stress) may result from glucose starvation, aberrant protein glycosylation, expression of mutant proteins, extreme environmental conditions, and release of Ca_2+ from the lumen, thereby compromising ER homeostasis. If homeostasis cannot be restored, cells are induced to undergo apoptosis through p53-independent pathways. ER stress is the only one yet identified that leads to p53 protein destabilization, thereby preventing p53-mediated apoptosis. Hypoxia, a common form of non-genotoxic stress, triggers a p53 response resulting from abnormal development of vasculature during the growth of solid tumors or in ischemic disorders. Ser15 of p53 becomes phosphorylated and p53 accumulates under severe hypoxia (<0.2% oxygen). Unlike other types of stress, this hypoxia-triggered response predominantly leads to transcriptional repression, not activation. Nutritional deprivation activates the AMP kinase (AMPK), which senses the intracellular AMP:ATP ratio. Glucose limitation induces cell cycle arrest and causes an accumulation of p53 phosphorylated at Ser15, while glucose starvation causes p53 phosphorylation at Ser46 (a site phosphorylated after severe DNA damage) and triggers p53-mediated apoptosis. The disruption of nucleolar and ribosomal functions by normal physiological processes or various stresses release excess ribosomal proteins L5, L11, and L23 that bind and inhibit MDM2, thereby stabilizing p53 and promoting cell cycle arrest without inducing p53 phosphorylation. Deregulation of cell adhesion, and disruption of the microtubular architecture and dynamics each can engender p53 stabilization and activation. While less well defined, the non-genotoxic stress pathways activating p53 are distinct from those induced by genotoxic stress.

The activation or overexpression of oncogenes such as Ras, c▶ Myc, or E1A, as occurs during the initiation of cancer, stabilizes and activates p53 by inducing ARF, the product of the alternative reading frame of the cell cycle regulatory gene, INK4a (*CDKN2A*).



p53, Fig. 3 Models for p53-dependent transcriptional activation

ARF, in turn, binds MDM2 and inhibits p53 ubiquitylation. The activation of p53 by oncogenes undoubtedly is a driving force underlying the mutation of p53 in cancers.

Transcription-Dependent p53 Functions

The transcriptional activity of p53 crucially supports its function as a tumor suppressor. Once stabilized, modified, and accumulated in the nucleus, p53 recognizes and binds DNA as a tetramer, via its core domain, to a consensus sequence known as a response element (RE). The canonical p53 RE comprises two decamer palindromic half-sites (5′-RRRCWWGYYY-3′ wherein R is A or G, W is A or T, and Y is C or T), separated by a spacer 0–13 bp long. The core domains of two p53 monomers bind to a half-site to form a symmetrical dimer, and two such dimers assemble on a full site to form a p53 tetramer. Both the sequence and the spacer's length have important roles in the outcome of p53 binding to a RE. The C and G of the core CWWG motif are strongly conserved, while the WW motif and the flanking RRR and YYY segments exhibit considerable variation, an ambiguity that generates thousands of putative binding sites across the human genome. Once bound, p53 either drives transcriptional activation or represses large networks of target genes. Different computing algorithms predict hundreds of thousands of putative p53REs in the human genome, but based on the amount of p53 protein accumulated after stress activation in the normal cell nucleus ($\sim 2 \times 10^4$ monomers/nucleus), a given cell probably can bind no more than 1,500–2,000 p53 targets at one time. By binding and transcriptionally regulating different sets of target genes, after mild DNA damage p53 typically arrests the cell cycle, allowing time for DNA repair and cell survival; however, after severe DNA damage p53 triggers either senescence or apoptosis in a cell-type dependent manner (Fig. 3). The induction of p53-dependent cell cycle arrest in the G_1 phase is mediated through the transcriptional activation of targets, such as the cyclin-dependent kinase inhibitor gene *CDKN1A* (p21), one of the best-studied p53 targets, while the induction of 14-3-3σ contributes to inhibiting the G_2/M transition. Triggering p53-dependent apoptosis involves the transcription activation of genes in the mitochondrial apoptotic pathway, such as *PUMA*, *APAF1*, and *BAX*, and in the death receptor pathway, such as *FAS* and *TNFRSF10B*. However, as noted below, p53 also can induce apoptosis independent of transcription.

Since 1992, when El-Deiry and colleagues first identified the p53 RE (Table 1), considerable effort

has been directed toward understanding its role in p53 stress-specific responses. p53 displays well-documented different binding affinities for different REs and, depending on the level of p53 accumulation after stress, may bind different sets of REs, with the highest affinity sites being occupied first. This pattern fits well with the observation that overall cell cycle promoters have stronger binding sites than do pro-apoptotic genes. At low stress levels, p53 binds and regulates cell cycle genes, while severe stress entails higher levels of stabilized p53 (or more highly modified p53) that enable binding to weaker REs and the activation of pro-apoptotic genes. The difference between "cell cycle" and "apoptotic" p53 REs is supported by their divergent evolution and the relatively poor conservation of apoptotic REs compared to cell cycle regulating REs as determined from comparative genomic studies of mice and humans. Recently, p53 was found to utilize partial REs (half- and three-quarter-sites), in conjunction with other transcription factors such as the estrogen receptor, a feature which greatly expands the number of genes that p53 potentially regulates (Menendez et al. 2009). p53 also may bind some simple sequences (e.g., $(5'\text{-TGYCC-}3')_{n>10}$) and non-B-form DNA structures, either to regulate transcription, or, perhaps, DNA repair and chromatin structure.

According to the classical "selective binding" model (see below for "selective context" model), p53-specific posttranslational modifications and specific binding partners assure that p53 discriminates among the variety of REs available for binding, based on their sequence characteristics. Once bound to an RE, p53 loads transcriptional coactivators, such as HATs and chromatin-remodeling factors, resulting in the acetylation of histones and the opening of chromatin for binding the components of the basal transcriptional machinery, such as TBP, TFs, and RNAP II. A few lines of evidence support this model. First, p53 REs are diverse, with different affinities for p53 that specifically impact the mode of regulation; second, certain p53 partners can "direct" p53 binding to cell cycle or to pro-apoptotic promoters; and, third, at these two groups of promoters, specific p53 posttranslational modifications are enriched. Lastly, p53 loads coactivators, such as p300/CBP and PCAF, at many of its target promoters, with the consequent opening of chromatin and subsequent activation of transcription.

Specific p53 posttranslational modifications directly influence the selection of p53REs. For example, as C. Sardet's group showed, p53 ubiquitylated at Lys320 specifically activates cell cycle arrest genes without affecting pro-apoptotic targets, while, in response to severe DNA damage, Ser46 phosphorylation specifically promotes p53 binding to pro-apoptotic genes (Anderson and Appella 2009; Meek and Anderson 2010). Furthermore, as shown by Y. Xu's group, in a mouse knock-in mutant, wherein Lys319 (the equivalent of human Lys320) is changed to the non-modifiable residue Ala, the p53-dependent apoptotic response is impaired, but cell cycle arrest is unaffected. Interestingly, some of the p53 tumor-derived mutations also selectively compromise the ability to regulate transcription; for example, the R175P mutant p53 arrests the cell cycle but cannot induce apoptosis. p53 binding partners (Fig. 1) also influence target selectivity, and their interaction with p53 may be affected by posttranslational modifications. A classical example is that of the ASPP protein family, consisting of the three members ASPP1, ASPP2, and iASPP. While binding of ASPP1 and ASPP2 to p53 directs it to pro-apoptotic genes and stimulates apoptosis, iASPP inhibits these interactions. 53BP1 is a binding partner involved in both regulating cell cycle arrest and repairing DNA damage; therefore, it could contribute to coordinating these processes. Another binding partner, HZF, was described by Lee's group to selectively load p53 on cell cycle regulating promoters without affecting the regulation of pro-apoptotic genes (Anderson and Appella 2009). Although these data support the selective binding model, since clearly certain posttranslational modifications and particular binding partners provide a signal for p53's selective occupation of a target, the model recently was challenged because of findings pointing to the regulation of transcription at the p53 post-binding stage.

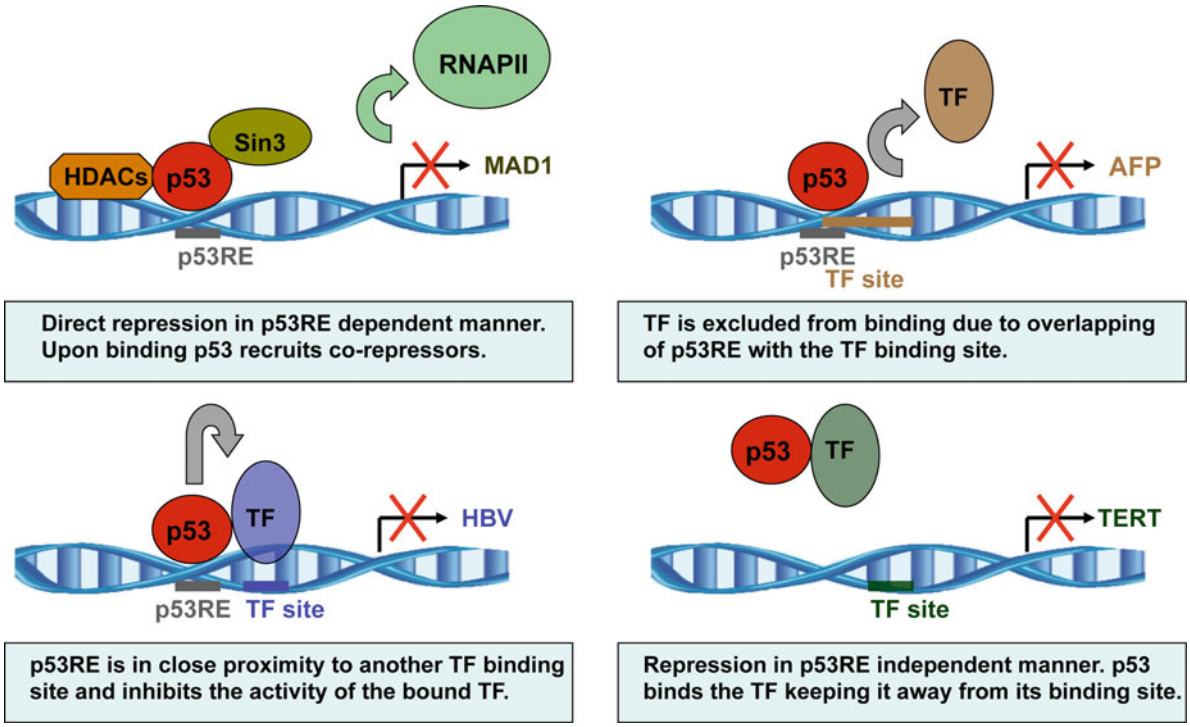
According to the "selective context" hypothesis recently proposed by Gomes and Espinosa (2010), the outcome of p53 binding to its targets might depend on additional factors that are required due to the promoter's state before binding. For example, promoters for cell cycle genes contain bound but inactive RNAP II prior to their activation by stress; in contrast, pro-apoptotic genes contain little or no pre-bound RNAP II. Accordingly, additional factors would be needed by cell cycle–regulating promoters to convert

the paused RNAP II into an elongating one, while, for pro-apoptotic promoters, basal transcription factors and RNAP II first must be loaded (Fig. 3). The "selective binding" and "selective context" models are not mutually exclusive, and it is noteworthy that different p53-regulated core promoters (the minimal DNA sequence containing promoter elements that direct initiation of transcription by RNAP II) vary in their promoter element compositions and, therefore, may require different co-regulators. Although TATA element-containing "focused" core promoters are more ancient, in vertebrates, most core promoters are of the "dispersed" type that do not contain the TATA element and commonly are found in CpG islands. Therefore, the classical model in which p53 helps to load TBP and the rest of the basic transcriptional machinery is applicable to only a fraction of regulated, focused promoters, and the need for specific co-regulator loading may depend on the particular elements of the core promoter at a given target. The epigenetic state of the targets before binding of p53 also may impose specific requirements before transcription is activated. In support of the selective context model, co-regulators recently were described as co-occupying specific sets of p53-regulated target promoters. For example, hCAS was reported by C. Prives' group to associate with a subset of pro-apoptotic p53 targets and to enhance their transcription by reversing H3 Lys27 methylation (a modification blocking transcription). In contrast, Bach1, a transcription factor that binds at a subset of oxidative stress-inducible p53 target genes and promotes histone deacetylation, as shown by K. Igarashi's group, inhibits transcription from these genes, thus suppressing p53-mediated cellular senescence. Likewise, the calcineurin-binding protein, Cabin1, physically interacts with p53 (Fig. 1) on certain promoters and, by regulating histone modifications, represses p53-dependent transcription in the absence of genotoxic stress. Finally, Gomes and Espinosa recently reported that expression of *PUMA* (*BBC3*), a pro-apoptotic p53 target gene, is regulated through a newly discovered "noncanonical" mechanism involving the insulator protein CTCF (the CCCTC-binding factor) and the cohesion complex that occupy intragenic chromatin boundaries and inhibit transcriptional elongation in the absence of stress. These findings demonstrate the role of the chromatin landscape in regulating p53-mediated transcriptional responses.

Although much is known about p53's activation of transcription, it is well-documented that p53 also can repress it. Following particular types of stress, for example, hypoxia, p53 predominantly represses transcription at many of its targets. Different mechanisms of p53-dependent repression have been reported (Fig. 4). Direct repression in a p53RE-dependent manner involves recruiting co-repressors, such as Sin3 and chromatin-modifying HDACs, which promote the formation of repressive chromatin structures and prevent the binding of transcriptional activators at target promoters. Several studies have examined the role of the p53RE structure in directing transcription repression. An early study of the survivin (*BIRC5*) promoter suggested that p53REs with a spacer length of 3 bp directed repression, while a zero-length spacer between half-sites caused activation. Subsequently, it was found that a spacer length of 3 bp which, compared to the same RE with no spacer (as is found in most activating p53REs), significantly weakened p53 binding and was insufficient to direct repression (Riley et al. 2008). More recently, E. C. Ren's group showed that when the WW in the core p53RE sequence CWWG was AT, AA, or TT, the RE activated transcription, while other dinucleotides, including CG, GG, and TG, directed transcriptional repression. Large-scale studies in vivo are needed to address the extent to which the REs at repressed p53 targets conform to that rule. Another way p53 can repress transcription is by interfering with the binding of transcription activators at neighboring binding sites, or by directly competing for binding when a p53RE overlaps with other activator binding sites. p53 may indirectly cause repression in an RE-independent manner by binding to, and sequestering other transcriptional activators (Fig. 4). A recent report from J. Rinn's lab demonstrated that p53 directs the expression of a set of large, intergenic, noncoding (linc) RNAs that are responsible for repression of some p53 responsive genes by an unknown mechanism.

Transcription-Independent p53 Functions

Several studies showed that p53 can induce apoptosis in the presence of transcriptional or translational inhibitors, strongly suggesting a transcription-independent role for p53 in the apoptotic process (Vaseva and Moll 2009). Subsequently, mutants deficient for transcriptional activation were shown to retain the ability to activate apoptosis. While initially controversial,



p53, Fig. 4 Models for p53-dependent transcriptional repression

transcription-independent p53 functions now are well recognized and characterized, particularly in regulating apoptosis and autophagy (Fig. 2). DNA damage, hypoxia, or oncogene activation promote p53 translocation to the cytoplasm where it inhibits the anti-apoptotic proteins Bcl-2 and Bcl-xL, causing permeabilization of the outer mitochondrial membrane, the release of cytochrome C, and the activation of caspases, followed by chromatin condensation, targeted proteolysis, and cell death. Since the p53 protein lacks a classical mitochondrial translocation motif, the signaling mechanisms that promote cytoplasmic and mitochondrial localization were investigated. Key studies from U. Moll's lab led to the current model for mitochondrial p53 targeting, in which MDM2 mono-ubiquitylates p53, thereby promoting its translocation to the cytoplasm and mitochondria, whereupon it rapidly is de-ubiquitylated by mitochondrial HAUSP, generating an apoptotically active p53. Autophagy is subjected to dual p53 regulation, activation, or inhibition, both transcription dependently and transcription independently (Vousden and Prives 2009). Upon genotoxic stress or oncogene activation, induced p53 activates autophagy as does p53 loss in the absence of stress, implying an inhibitory effect of basal p53 activity on autophagy in nonstressed cells. Transcription-independent p53 activation of autophagy depends on activation of the nutrient sensor AMPK, followed by mTOR repression. Besides its essential role in cellular housekeeping by regulating protein turnover, autophagy serves as another tumor-suppressor mechanism, since loss of autophagy increased DNA damage and genetic instability.

Summary

In the 30 years since its discovery (Table 1), p53 has become perhaps the best characterized mammalian transcription factor. While its roles as the central hub in cellular stress responses and as a tumor suppressor have been detailed, many questions remain and new ones are emerging. Thus, surprisingly little is understood about the biochemical mechanisms by which the many p53 posttranslational modifications regulate its function. G. Lahav's lab demonstrated oscillations in p53 abundance in stressed and unstressed cells, but transcription is activated only in the former,

presumably due to specific posttranslational modifications. This raises a plethora of questions: Where and when are these modifications accomplished, in the cytoplasm, nucleoplasm, or on chromatin? How do they differ among different cell types? What fraction of p53 is modified and which modifications coexist on the same p53 molecule and on the same p53 tetramer? What is the functional interplay among p53 family members? Furthermore, while p63 and p73 carry out different functions, they bind the same or similar REs. How do the more than a hundred p53 binding partners influence p53 activity, and how are these interactions regulated? Like p63 and p73, p53 is expressed as several different isoforms, some lacking the transactivation domain or having different carboxyl termini. How do these influence p53 "activity"? How does p53 discriminate between the thousands of potential REs available for binding in mammalian genomes? Does p53 activity and function vary among individuals? Are polymorphisms in p53REs important in human health? How does the chromatin landscape, including epigenetic modifications such as DNA methylation, modulate p53 binding and regulate transcription. Is it all about transcription? Or are there other functions for p53 sequence-specific DNA binding? Besides its major role as a cellular tumor suppressor, recent studies illuminated many stress-independent p53 functions, including the regulation of cellular metabolism and autophagy, the composition of the extracellular matrix and cell cytoskeleton, immunity and fertility in mammals, stem cell renewal, and neuronal differentiation. Unsurprisingly, p53 has been implicated in the development of a broad spectrum of pathologies other than cancer, including ischemia after stroke, myocardial infarction, and Parkinson's and Alzheimer's disease. Also, other work suggests that too much p53 activity promotes aging. The mechanisms responsible for balancing p53 activity for suppressing tumors and extending longevity remain to be defined. There is little doubt that research on p53 as a signaling molecule has a long, bright future.

Acknowledgments D. Meek, D. Menendez, M. Resnick, and K. Sakaguchi are thanked for critical comments and suggestions. CWA and KAB were supported in part by the DOE Office of Biological and Environmental Research Low Dose Radiation Research Program and by Laboratory Directed Research and Development funds at the Brookhaven National Laboratory under contract with the US Department of Energy.

References

Anderson CW, Appella E. Signaling to the p53 tumor suppressor through pathways activated by genotoxic and non-genotoxic stresses. In: Bradshaw RA, Dennis EA, editors. Handbook of cell signaling, vol. 3. 2nd ed. Oxford: Academic; 2009. p. 2185–204. Chapter 264.

Broz DK, Attardi LD. In vivo analysis of p53 tumor suppressor function using genetically engineered mouse models. Carcinogenesis. 2010;31:1311–8.

Donehower LA, Lozano G. 20 years studying p53 functions in genetically engineered mice. Nat Rev Cancer. 2009;9:831–41.

Dötsch V, Bernassola F, Coutandin D, Candi E, Melino G. p63 and p73, the ancestors of p53. In: Levine AJ, Lane D, editors. Cold spring harbor perspectives in biology, volume on the p53 family. New York: Cold Spring Harbor Laboratory Press; 2010. p. 49–62.

Gomes NP, Espinosa JM. Differential regulation of p53 target genes: it's (core promoter) elementary. Genes Dev. 2010;24:111–4.

Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. Oncogene. 2005;24:2899–908.

Joerger AC, Fersht AR. Structural biology of the tumor suppressor p53. Annu Rev Biochem. 2008;77:557–82.

Lane DP. Cancer. p53, guardian of the genome. Nature. 1992;358:15–6.

Levine AJ. The common mechanisms of transformation by the small DNA tumor viruses: the inactivation of tumor suppressor gene products: p53. Virology. 2009;384:285–93.

Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. Nat Rev Cancer. 2009;9:749–58.

Lu WJ, Amatruda JF, Abrams JM. p53 ancestry: gazing through an evolutionary lens. Nat Rev Cancer. 2009;9:758–62.

Meek DW, Anderson CW. Posttranslational modification of p53: cooperative integrators of function. In: Levine AJ, Lane D, editors. Cold spring harbor perspectives in biology, volume on the p53 family. New York: Cold Spring Harbor Laboratory Press; 2010. p. 81–96.

Meek DW, Hupp TR. The regulation of MDM2 by multisite phosphorylation–opportunities for molecular-based intervention to target tumors? Semin Cancer Biol. 2010;20:19–28.

Menendez D, Inga A, Resnick MA. The expanding universe of p53 targets. Nat Rev Cancer. 2009;9:724–37.

Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. In: Levine AJ, Lane D, editors. Cold spring harbor perspectives in biology, volume on the p53 family. New York: Cold Spring Harbor Laboratory Press; 2010. p. 123–40.

Perry ME. The regulation of the p53-mediated stress response by MDM2 and MDM4. In: Levine AJ, Lane D, editors. Cold spring harbor perspectives in biology, volume on the p53 family. New York: Cold Spring Harbor Laboratory Press; 2010. p. 97–108.

Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol. 2008;9:402–12.

Vaseva AV, Moll UM. The mitochondrial p53 pathway. Biochim Biophys Acta. 2009;1787:414–20.

Vousden KH, Prives C. Blinded by the light: the growing complexity of p53. Cell. 2009;137:413–31.

Whibley C, Pharoah D, Hollstein M. p53 polymorphisms: cancer implications. Nat Rev Cancer. 2009;9:95–107.
Zilfou JT, Lowe SW. Tumor suppressive functions of p53. In: Levine AJ, Lane D, editors. Cold spring harbor perspectives in biology, volume on the p53 family. New York: Cold Spring Harbor Laboratory Press; 2010. p. 175–86.

p58

▶ CDK11

p58CDC2L1

▶ CDK11

p58CLK-1

▶ CDK11

p58GTA

▶ CDK11

p60-Src

▶ c-Src Family of Tyrosine Kinases

p68 Kinase, dsRNA-Activated Inhibitor (DAI)

▶ PKR

p90RSK

▶ RSK (p90 Ribosomal S6 Kinase)

P97

▶ Gab2

Pak2

Sheng-Wei Yang and Yuan-Hao Hsu
Department of Chemistry, Tunghai University, Taichung, Taiwan

Synonyms

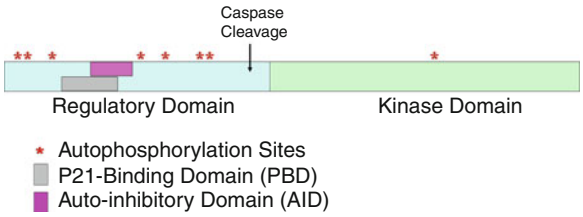
γ-Pak; Gamma-Pak; Pak65; PAKI

Historical Background

Pak2 (p21-activated protein kinase 2) is the serine/threonine protein kinase PAK I, which was first detected as a protease activated form in Dr. Traugh's laboratory in early 1980s (Tahara and Traugh 1981, 1982). Pak2 was further cloned and sequenced in the same lab in 1996 (Jakobi et al. 1996). Pak2 belongs to the PAK (p21-activated protein kinase) family, which can be activated by Rac and Cdc42 (Manser et al. 1994). The PAK family consists of group I protein kinases, including the highly homologous Pak1 (α-PAK), Pak2 (γ-PAK), and Pak3 (β-PAK), and the recently identified group II, including Pak4, Pak5, and Pak6 (Jaffer and Chernoff 2002). The 60-kDa Pak1 is expressed in brain and is also detected in muscle and spleen. The 60-kDa Pak3 is expressed mainly in brain. Pak2 is 58 kDa and expressed ubiquitously in mammalian cells (Roig and Traugh 2001) (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PAK2).

Pak2 Structural Information

Pak2 is a serine/threonine protein kinase. This 58-kDa protein contains 524 residues. The residues 1–228 is defined as the regulatory domain and the residues 229–524 is the catalytic domain (Fig. 1) (http://www.uniprot.org/uniprot/Q13177). The catalytic domain has a typical bilobol kinase structure, which is composed of a β-sheet-formed small lob and a α-helix-formed large



Pak2, Fig. 1 Pak2 schematic presentation. The p21-binding domain (PBD), autoinhibitory domain (AID), autophosphorylation sites (*), and caspase 3 cleavage site are marked. The regulatory domain is *blue*; the protein kinase domain is *green*

lob (Lei et al. 2000). The ATP binding sites are located in the active site cleft between the two lobs. The conserved catalytic loop, Mg binding loop, and activation loop also reside in the active site cleft. The regulatory domain contains seven autophosphorylation serines, 19, 20, 55, 141, 165, 192, and 197, and the catalytic domain contains one autophosphorylation threonine 402 (Walter et al. 1998).

Pak2 Inhibition and Activation

Pak2 formed a transinhibited dimer before autophosphorylation and activation. The autoinhibitory domain is located in the region 92–133 in the regulatory domain. In the inactive state, the AID interacts with the catalytic domain to inhibit its kinase activity. PAK activation is through disruption of autoinhibition, followed by autophosphorylation. The two main activation mechanisms of Pak2 are caspase-mediated activation and small G protein-mediated activation.

The small GTPases Rac and Cdc42 can activate Pak2 (Fig. 2). GTP-bound Cdc42 can disrupt autoinhibition, which, in turn, leads to autophosphorylation and activation of PAK (Tu and Wigler 1999). The basal autophosphorylation activity of Pak2 is observed, and Pak2 is autophosphorylated at five sites, serines. Additional three phosphorylation sites (serines 141 and 165 and threonine 402) are autophosphorylated in the presence of Cdc42(GTP) and ATP (Gatti et al. 1999). Autophosphorylation of Thr402 in the activation loop is required for the kinase activity of Pak2.

Pak2 can be activated in response to a lot of stresses. Moderate stresses, like hyperosmolarity, ionizing radiation, DNA-damaging agents, and serum-deprivation, induce Pak2 activation in cells and lead to cell cycle arrest at G2/M (Roig and Traugh 2001). Activated Pak2 inhibits translation by phosphorylation of various substrates. Pak2 has specific protein substrates, e.g., histone 4, myosin light chain, prolactin, c-Abl, eukaryote translation initiation factor 3 (eIF3), eIF4B, eIF4G, and Mnk1. Pak2 recognizes the consensus sequence (K/RRXS).

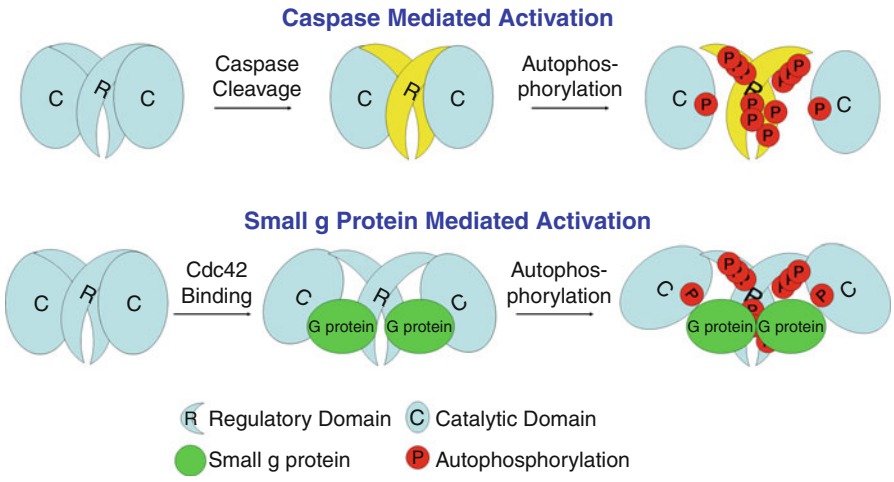
Pak2 is the only member of the PAK family that is directly activated by caspase 3. When Pak2 is cleaved and activated by caspase 3, Pak2 promotes the morphological and biochemical changes of apoptosis. The proapoptosis protease, caspase 3, cleaves Pak2 after Asp 212 and thus produces a p27 fragment containing primarily the regulatory domain and a p34 fragment containing a small piece of the regulatory domain and the entire catalytic domain (Fig. 2). This event loosens the autoinhibitory dimer structure and leads to a complete autophosphorylation, which then results in a constitutively active p34 kinase domain (Hsu et al. 2008). The nuclear import signal (245–251) is required for nuclear localization (Jakobi et al. 2003). Disruption of the region (197–246), containing nuclear export signal, results in the nuclear localization of the Pak2 p34 fragment.

Pak2 and Apoptosis

Pak2 is activated in response to various biological stress, such as hyperosmolarity, ionizing radiation, DNA-damaging agents, and serum deprivation (Roig and Traugh 2001). Among them, the heat shock, H2O2, and UV radiation have been shown to stimulate apoptosis.

Pak2 is proteolytically cleaved during apoptosis. This cleavage event generates two Pak2 fragments and leads to the autophosphorylation and activation of Pak2. The p34 fragment containing the kinase domain is the active fragment. It can localize to nuclear and plays an important role in regulation of the apoptotic cell. The caspase-cleaved Pak2 (p34) has also been reported posttranslationally myristoylated (Vilas et al. 2006). Myristoylation of p34 can induce the relocation from cytosol to membranes and induce cell death without mitochondria damage. Nef protein of HIV has been shown to bind to and activate Pak2 and activates Pak2 and induces apoptosis in Jurkat T cell (Krautkramer et al. 2004).

Proteolytic kinase activation and protein kinase phosphorylation have become an important method



Pak2, Fig. 2 Pak2 activation mechanisms

of regulation in apoptosis (Bokoch 1998). These protein kinases include Pak2, MEKK1, FAK, DNA-PK, PITSLRE, PAKCaKII, Akt and Raf-1.

Pak2 and Cancer

Pak2 can be activated by Ras-related small GTPases, which can regulate the structure, mobility, and migration of the cytoskeleton in cancer cells, indicating the role of Pak2 in tumorigenesis and metastasis of the cancer cells (Kumar et al. 2006; Dummler et al. 2009). Pak2 can react with some key substrates related to cancer development, such as PIX and MLCK in cytoskeleton remodeling, ▶ c-Myc, MNK1, prolactin, and c-Raf1 in cell growth, and BAD in cell survival.

Pak2 is a negative regulator of Myc and suggested Pak2 may be the product of a tumor suppressor gene (Huang et al. 2004). Pak2 mediates tumor invasion in breast carcinoma cells (Coniglio et al. 2008). Inhibition of RhoA in Pak2-depleted cells decreases MLC phosphorylation and restores cell invasion. Also, the NF2 tumor suppressor Merlin is a substrate of Pak2 (Kissil et al. 2002). Wilkes MC (2009) showed that Erbin regulates the function of Merlin through Pak2 binding to Merlin.

Summary

Pak2 is a highly regulated enzyme. It can stimulate both cell growth through small GTPase binding and cell death through caspase cleavage. This characteristic makes this enzyme important for the future researches in apoptosis and cancer.

References

Bokoch GM. Caspase-mediated activation of PAK2 during apoptosis: proteolytic kinase activation as a general mechanism of apoptotic signal transduction? Cell Death Differ. 1998;5(8):637–45.

Coniglio SJ, Zavarella S, Symons MH. Pak1 and Pak2 mediate tumor cell invasion through distinct signaling mechanisms. Mol Cell Biol. 2008;28(12):4162–72.

Dummler B, Ohshiro K, Kumar R, Field J. Pak protein kinases and their role in cancer. Cancer Metastasis Rev. 2009;28(1–2):51–63.

Gatti A, Huang Z, Tuazon PT, Traugh JA. Multisite autophosphorylation of p21-activated protein kinase gamma-PAK as a function of activation. J Biol Chem. 1999;274(12):8022–8.

Hsu YH, Johnson DA, Traugh JA. Analysis of conformational changes during activation of protein kinase Pak2 by amide hydrogen/deuterium exchange. J Biol Chem. 2008;283(52):36397–405.

Huang Z, Traugh JA, Bishop JM. Negative control of the Myc protein by the stress-responsive kinase Pak2. Mol Cell Biol. 2004;24(4):1582–94.

Jaffer ZM, Chernoff J. p21-activated kinases: three more join the Pak. Int J Biochem Cell Biol. 2002;34(7):713–7.

Jakobi R, Chen CJ, Tuazon PT, Traugh JA. Molecular cloning and sequencing of the cytostatic G protein-activated protein kinase PAK I. J Biol Chem. 1996;271(11):6206–11.

Jakobi R, McCarthy CC, Koeppel MA, Stringer DK. Caspase-activated PAK-2 is regulated by subcellular targeting and proteasomal degradation. J Biol Chem. 2003;278(40):38675–85.

Kissil JL, Johnson KC, Eckman MS, Jacks T. Merlin phosphorylation by p21-activated kinase 2 and effects of

phosphorylation on merlin localization. J Biol Chem. 2002;277(12):10394–9.
Krautkramer E, Giese SI, Gasteier JE, Muranyi W, Fackler OT. Human immunodeficiency virus type 1 Nef activates p21-activated kinase via recruitment into lipid rafts. J Virol. 2004;78(8):4085–97.
Kumar R, Gururaj AE, Barnes CJ. p21-activated kinases in cancer. Nat Rev Cancer. 2006;6(6):459–71.
Lei M, Lu W, Meng W, Parrini MC, Eck MJ, Mayer BJ, et al. Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. Cell. 2000;102(3):387–97.
Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature. 1994;367(6458):40–6.
Roig J, Traugh JA. Cytostatic p21 G protein-activated protein kinase gamma-PAK. Vitam Horm. 2001;62:167–98.
Tahara SM, Traugh JA. Cyclic Nucleotide-independent protein kinases from rabbit reticulocytes. Identification and characterization of a protein kinase activated by proteolysis. J Biol Chem. 1981;256(22):11558–64.
Tahara SM, Traugh JA. Differential activation of two protease-activated protein kinases from reticulocytes by a Ca^{2+}-stimulated protease and identification of phosphorylated translational components. Eur J Biochem. 1982;126(2):395–9.
Tu H, Wigler M. Genetic evidence for Pak1 autoinhibition and its release by Cdc42. Mol Cell Biol. 1999;19(1):602–11.
Vilas GL, Corvi MM, Plummer GJ, Seime AM, Lambkin GR, Berthiaume LG. Posttranslational myristoylation of caspase-activated p21-activated protein kinase 2 (PAK2) potentiates late apoptotic events. Proc Natl Acad Sci USA. 2006;103(17):6542–7.
Walter BN, Huang Z, Jakobi R, Tuazon PT, Alnemri ES, Litwack G, et al. Cleavage and activation of p21-activated protein kinase gamma-PAK by CPP32 (caspase 3). Effects of autophosphorylation on activity. J Biol Chem. 1998;273(44):28733–9.

Pak65

► Pak2

PAKI

► Pak2

Palmitoyl Acyl Transferase (PAT)

► DHHC Proteins

Palmitoylating Enzyme

► DHHC Proteins

Paracaspase

► MALT1(Mucosa-Associated Lymphoid Tissue Translocation Gene 1)

PARK6

► PTEN-Induced Kinase 1 (PINK1)

PBAF RSC

► SWI/SNF Chromatin Remodeling Complex

PC-1 (Human)

► TPD52 (Tumor Protein D52)

PC2

► Polycystin-2

pCAF

► PCAF Lysine Acetyltransferase

PCAF (p300/CBP-Associated Factor)

► PCAF Lysine Acetyltransferase

PCAF Lysine Acetyltransferase

Linya You[1,3] and Xiang-Jiao Yang[1,2,3]
[1]The Rosalind & Morris Goodman Cancer Research Center, McGill University, Montréal, Québec, Canada
[2]Departments of Biochemistry and Anatomy & Medicine, McGill University Health Center, Montréal, Québec, Canada
[3]Departments of Medicine, McGill University Health Center, Montréal, Québec, Canada

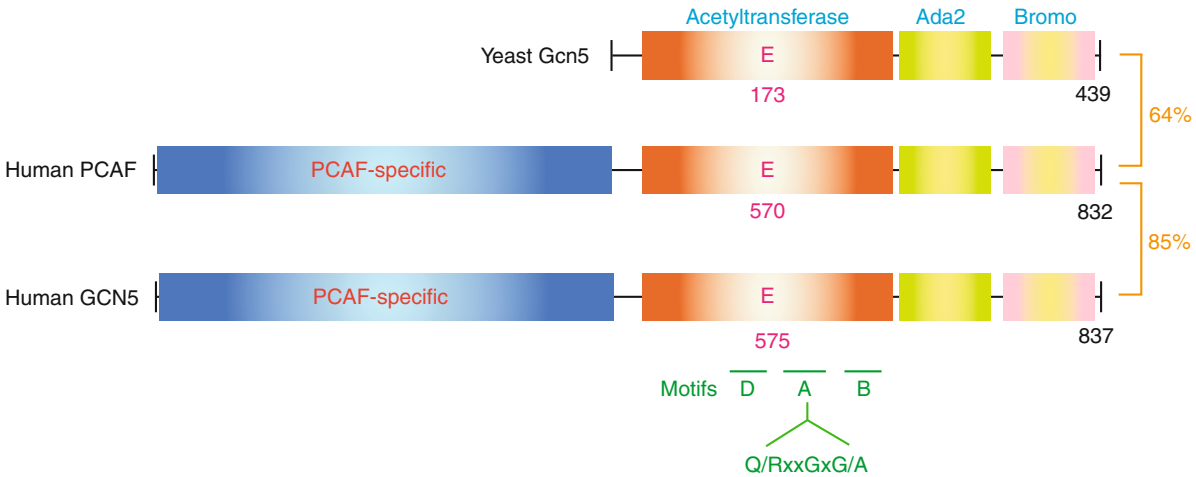
Synonyms

K-acetyltransferase 2B (KAT2B); Lysine acetyltransferase 2B; P/CAF; pCAF; PCAF (p300/CBP-associated factor)

Historical Background: Identification of PCAF as an Acetyltransferase

Human PCAF was identified in the mid-1990s based on an analogy between yeast and mammalian transcriptional control systems (Yang et al. 1996). In the budding yeast *Saccharomyces cerevisiae*, amino acid starvation results in derepression of expression of enzymes for different biosynthetic pathways. This phenomenon suggests co-regulation of different pathways and is thus referred to as "general control of amino acid biosynthesis" (Hinnebusch and Fink 1983; Thireos et al. 1984). Genetic screens in the late 1970s and early 1980s uncovered various mutant strains with defects in the gene control response (Hinnebusch and Fink 1983). Responsible genes of two such mutant strains were named *Gcn4* (*g*eneral *c*ontrol *n*on-derepressible 4, also referred to as *a*mino acid *a*nalog-*s*ensitive 3, *Aas3* or *Aas101*) and *Gcn5* (also called *Aas104*) (Hinnebusch 1984; Thireos et al. 1984). The former was cloned and found to encode a DNA-binding transcription factor (Thireos et al. 1984), whereas analysis of the latter mutant strain suggested that *Gcn5* encodes a transcriptional coactivator (Georgakopoulos and Thireos 1992). Consistent with this conclusion, subsequent cloning revealed that Gcn5 possesses a structural domain with sequence similarity to several other transcriptional regulators without obvious DNA-binding activity (Georgakopoulos and Thireos 1992). This structural domain was initially discovered in *Drosophila* Brahma and was thus named "bromodomain" (Haynes et al. 1992; Tamkun et al. 1992). Along with *Ada2* (*a*lteration/*d*eficiency in *a*ctivation 2) and *Ada3*, *Gcn5* was also uncovered in yeast genetic screens, which were carried out in the early 1990s and aimed at identifying transcriptional adaptors (Marcus et al. 1994). Biochemical data indicated that the three proteins form a transcriptional coactivator complex (Marcus et al. 1994).

In mammalian cells, studies of cAMP-dependent transcriptional responses led to the identification of CBP (*C*REB-*b*inding *p*rotein) as a coactivator able to bind CREB (*c*AMP-*r*esponsive *e*lement *b*inding protein) that was specifically phosphorylated by the cAMP-dependent protein kinase PKA (*p*rotein *k*inase *A*) (Arany et al. 1994). CBP was found to be homologous to p300, a 300 kDa cellular protein associated with adenoviral oncoportein E1A (Arany et al. 1994). Both were shown to coactivate c-Jun, a leucine-zipper transcription factor with sequence and functional similarity to yeast Gcn4. Moreover, both p300 and CBP contain bromodomains homologous to yeast Gcn5 and display sequence similarity to a small domain of yeast Ada2 (Arany et al. 1994). Together with the conclusion that Gcn5 and Ada2 are components of a coactivator complex, these similarities suggested a potential role of mammalian homologs of yeast Gcn5 and Ada2 as transcriptional coactivators. With these considerations, PCAF was cloned in the mid-1990s as an 832-residue human protein with its C-terminal half homologous to yeast Gcn5 (Fig. 1) (Yang et al. 1996). A highly relevant development was that p55, a 55 kDa *Tetrahymena* protein with significant sequence homology to yeast Gcn5, was identified as a histone acetyltransferase (Brownell et al. 1996), thereby leading to the demonstration of intrinsic histone acetyltransferase activity in PCAF (Yang et al. 1996). This activity was also shown with human GCN5 (KAT2A) (Yang et al. 1996; Wang et al. 1997), which is paralogous to PCAF (Fig. 1). These studies established that from yeast to humans, Gcn5 and its homologs form an evolutionarily conserved family of histone acetyltransferases with important roles in transcriptional regulation. This notion has been subsequently validated in numerous studies with various transcription factors.



PCAF Lysine Acetyltransferase, Fig. 1 Domain organization of yeast Gcn5 and human PCAF and GCN5. Yeast Gcn5 possesses three domains: an acetyltransferase core, an Ada2-intercation module (*Ada2*), and a bromodomain (*bromo*). Human PCAF and GCN5 are paralogous to each other. Their C-terminal parts are highly homologous to yeast Gcn5, but they possess long N-terminal extensions. The amino acid sequence similarity between yeast Gcn5 and the corresponding region of PCAF is 64% (shown at *right*), whereas the overall similarity between human PCAF and GCN5 is 85%. The *red* and *dark* numbers below each protein denote positions of the catalytic glutamate (E) residue and the C-terminal end. Rough positions of motifs A, B, and D conserved with other members of the Gcn5-related N-acetyltransferase (GNAT) family are indicated at the bottom of the acetyltransferase domains, along with the motif A signature sequence Q/RxxGxG, which is also found in the MYST family of acetyltransferases

Domain Organization and 3D Structure of PCAF

Compared to yeast Gcn5, PCAF has a long N-terminal extension (Fig. 1). Similar to yeast Gcn5, the C-terminal half of PCAF contains three domains: an acetyltransferase core, an Ada2-interacting module, and a bromodomain (Fig. 1). PCAF is paralogous to human GCN5 because they share high sequence similarity in the entire sequence, i.e., both the N-terminal extension and the Gcn5-like C-terminal half. The N-terminal extension is conserved in *Drosophila* Gcn5 and mediates the association with p300 and CBP. One report indicated that the N-terminal extension of PCAF also contains an E3 ubiquitin ligase activity. Additional studies are needed for the N-terminal extension, but lots of knowledge has been gained about the C-terminal half.

The acetyltransferase core possesses three conserved motifs, A, B, and D (Fig. 1), which are shared by members of the GNAT (*G*cn5-related *N*-*a*cetyl*t*ransferease) family (Roth et al. 2001). The peptide QVKGYG within motif A of PCAF matches nicely to the Q/RxxGxG/A signature sequence present in members of the GNAT as well as MYST families of histone acetyltransferases. Crystal structure of the PCAF acetyltransferase core has been determined and is similar to that of yeast Gcn5, with motifs A and B forming the acetyl coenzyme A-binding pocket. A key catalytic residue is E-570, which is located at the N-terminal part of motif A and serves a base to extract a proton from the amino group of an acetylatable lysine residue during catalysis. Structural comparison with other Gcn5 family members suggests how this family of acetyltransferases can accommodate various substrates with divergent sequence. Two correlations have been reported: (1) the acetylatable lysine residue itself and its C-terminal region, especially residues at positions +2 and +4, play a particularly important role in substrate selectivity, and the backbone of the substrate in this region in positions +1 and +3 also contributes to substrate binding; and (2) the substrate region N-terminal to the lysine residue modulates substrate affinity. This flexibility of substrate recognition explains that PCAF has been shown to acetylate numerous proteins substrates.

At the C-terminal end of PCAF is a bromodomain (Fig. 1). This domain was initially discovered as a structural motif that *Drosophila* Brahma and yeast Snf2 share with other proteins. NMR structural determination and peptide interaction assays revealed an acetyl-lysine binding pocket within the bromodomain of PCAF (Dhalluin et al. 1999). This property has been demonstrated in other bromodomains and plays a role in acetylation-dependent chromatin remodeling (de la Cruz et al. 2005). The PCAF bromodomain prefers acetyl-K binding site a hydrophobic residue at position +2 and an aromatic or positively charged residue at position +3.

Between the acetyltransferase core and bromodomain of PCAF is a small ADA2-interaction module (Fig. 1). As with yeast Gcn5, Ada2 interaction is required for activation of PCAF. Further studies are needed to map the details of the interaction, e.g., which residues are crucial. As detailed in the next section, there are two paralogous ADA2 proteins in humans and dependent on which one PCAF associates with, different multisubunit complexes are formed (see the next section).

Multisubunit PCAF Complexes

Yeast Gcn5 is part of high-molecular-weight multisubunit ADA and SAGA (*S*pt-*A*da-*G*cn5-*a*cetytransferase) complexes (Grant et al. 1997). As yeast genetic screens suggested, both complexes contain Ada2 and Ada3. Compared to Gcn5 alone, these complexes are more active in acetylating nucleosomes (Baker and Grant 2007). A SAGA-like PCAF complex consists of more than 20 distinct polypeptides (Nagy and Tora 2007). Interestingly, the N-terminal extension is not required for the complex formation (Ogryzko et al. 1998). Among the subunits are several TBP-associated factors (TAFs) that form a histone-octamer subcomplex. Another prominent subunit is TRRAP (TRansactivation/tRansformation domain Associated Protein), a 400 kDa protein originally isolated from a Myc-associated transcription coactivator (McMahon et al. 1998). Electron microscopy revealed that the complex forms an slightly twisted L-shape structure consisting of five modular domains, with PCAF or GCN5 occupying domain III in the middle region (Nagy and Tora 2007). As in *Drosophila*, there are two Ada2-like proteins in mammals, Ada2a and Ada2b (Barlev et al. 2003; Pankotai et al. 2005). The SAGA-like complex contains Ada2b, whereas Ada2a is present in a different complex, known as ATAC (Ada two A containing), that also contains another acetyltransferase ATAC2 and additional subunits that are absent in the SAGA complexes (Suganuma et al. 2008). Some of these additional subunits are components of the JNK pathway and confer the ability of the ATAC complex to regulate JNK signaling (Suganuma et al. 2010). Therefore, different from the SAGA complex, the ATAC complex possesses two acetyltransferases. Both GCN5 and PCAF are present in the two types of complexes.

PCAF Function in Various Cellular Processes

Consistent with its acetyltransferase activity toward numerous substrates, PCAF and its orthologs regulate different cellular processes in diverse organisms from the fly to humans. Many transcription activators and cell-cycle regulatory proteins such as nuclear receptor, c-Myc, KLF, and E2F family contact directly and recruit PCAF-containing complexes to specific promoters. The PCAF coactivators can be recruited to a target gene promoter by a transcription activator. At the gene promoter, the coactivators catalyze lysine acetylation on either histone tails, the transcription activator, and/or the basal transcription machinery components. These reactions help unmask the promoter DNA from the nucleosomes, stabilize the transcription factor, enhance its interaction with DNA or protein partners, and/or promote transcription initiation and elongation. As a result, the transcription of the target gene is activated.

PCAF Function in Development

Human PCAF is ubiquitously expressed in adult tissues, but more abundant in heart and skeletal muscle. The expression of mouse *Pcaf* is low during embryonic development but increases in adult heart and skeletal muscle. By contrast, mouse *Gcn5* expression is high during early development and decreases after birth, suggesting distinct roles of *Pcaf* and *Gcn5* during mouse development (Lin and Dent 2006). Indeed, gene disruption of mouse *Pcaf* did not yield any obvious phenotypes except some defects in short-term

memory and stress responses (Maurice et al. 2008), whereas inactivation of *Gcn5* is embryonic lethal at E8.5. Moreover, knock-in mouse embryos expressing an inactive acetyltransferase mutant (E568A/D609; mouse E568 is a key catalytic residue and is equivalent to E575 of human GCN5, Fig. 1) of Gcn5 exhibited severe cranial neural tube closure defects and exencephaly, indicating that the acetyltransferase activity is required for cranial neural tube closure. Thus, even though they are highly homologous at the amino acid sequence level and display similarity at the biochemical level, mouse Pcaf and Gcn5 have different roles in different tissues and developmental stages due to their different patterns of expression. On the other hand, although *pcaf* expression is low during early development, deletion of both *Pcaf* and *Gcn5* led to a more severe phenotype, indicating that they also have overlapping functions during early development.

Summary

PCAF was initially identified in 1996 and contains an N-terminal extension and a C-terminal Gcn5-like half that comprises an acetyltransferase core, an Ada2-interaction module, and a bromodomain. The three-dimensional structures of the acetyltransferase core and bromodomain have been solved and provide structural and mechanistic details as to how these two domains carry out lysine acetylation and recognize an acetyl-lysine peptide, respectively. Although it is absent in yeast Gcn5, the N-terminal extension of PCAF is conserved from orthologs from flies to humans. In mammals, GCN5 shares high sequence similarity with PCAF in the entire sequence. Like GCN5, PCAF exists at least two different multisubunit complexes, SAGA and ATAC. While the SAGA complex is conserved in yeast, the ATAC complex is metazoan-specific. At the functional level, PCAF is well known to serve as a transcriptional coactivator. Through acetylating histones and numerous non-histone substrates, PCAF also regulates diverse cellular programs. While little difference has been reported for them at the biochemical level, roles of PCAF and GCN5 in mouse – and perhaps also human – development are quite different owing to rather distinct spatiotemporal expression patterns.

Acknowledgments This research was supported by operating grants from CIHR and Canadian Cancer Society (to X.J.Y.).

References

Arany Z, Sellers WR, Livingston DM, Eckner R. E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators. Cell. 1994;77:799–800.

Baker SP, Grant PA. The SAGA continues: expanding the cellular role of a transcriptional co-activator complex. Oncogene. 2007;26:5329–40.

Barlev NA, Emelyanov AV, Castagnino P, Zegerman P, Bannister AJ, Sepulveda MA, Robert F, Tora L, Kouzarides T, Birshtein BK, et al. A novel human Ada2 homologue functions with Gcn5 or Brg1 to coactivate transcription. Mol Cell Biol. 2003;23:6944–57.

Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY, Allis CD. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell. 1996;84:843–51.

de la Cruz X, Lois S, Sanchez-Molina S, Martinez-Balbas MA. Do protein motifs read the histone code? Bioessays. 2005;27:164–75.

Dhalluin C, Carlson JE, Zeng L, He C, Aggarwal AK, Zhou MM. Structure and ligand of a histone acetyltransferase bromodomain. Nature. 1999;399:491–6.

Georgakopoulos T, Thireos G. Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. EMBO J. 1992;11:4145–52.

Grant PA, Duggan L, Cote J, Roberts SM, Brownell JE, Candau R, Ohba R, Owen-Hughes T, Allis CD, Winston F, et al. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev. 1997;11:1640–50.

Haynes SR, Dollard C, Winston F, Beck S, Trowsdale J, Dawid IB. The bromodomain: a conserved sequence found in human, Drosophila and yeast proteins. Nucleic Acids Res. 1992;20:2603.

Hinnebusch AG. Evidence for translational regulation of the activator of general amino acid control in yeast. Proc Natl Acad Sci USA. 1984;81:6442–6.

Hinnebusch AG, Fink GR. Positive regulation in the general amino acid control of Saccharomyces cerevisiae. Proc Natl Acad Sci USA. 1983;80:5374–8.

Lin W, Dent SY. Functions of histone-modifying enzymes in development. Curr Opin Genet Dev. 2006;16:137–42.

Marcus GA, Silverman N, Berger SL, Horiuchi J, Guarente L. Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. EMBO J. 1994;13:4807–15.

Maurice T, Duclot F, Meunier J, Naert G, Givalois L, Meffre J, Celerier A, Jacquet C, Copois V, Mechti N, et al. Altered memory capacities and response to stress in p300/CBP-associated factor (PCAF) histone acetylase knockout mice. Neuropsychopharmacology. 2008;33:1584–602.

McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell. 1998;94:363–74.
Nagy Z, Tora L. Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. Oncogene. 2007;26:5341–57.
Ogryzko VV, Kotani T, Zhang X, Schiltz RL, Howard T, Yang XJ, Howard BH, Qin J, Nakatani Y. Histone-like TAFs within the PCAF histone acetylase complex. Cell. 1998;94:35–44.
Pankotai T, Komonyi O, Bodai L, Ujfaludi Z, Muratoglu S, Ciurciu A, Tora L, Szabad J, Boros I. The homologous Drosophila transcriptional adaptors ADA2a and ADA2b are both required for normal development but have different functions. Mol Cell Biol. 2005;25:8215–27.
Roth SY, Denu JM, Allis CD. Histone acetyltransferases. Annu Rev Biochem. 2001;70:81–120.
Suganuma T, Gutierrez JL, Li B, Florens L, Swanson SK, Washburn MP, Abmayr SM, Workman JL. ATAC is a double histone acetyltransferase complex that stimulates nucleosome sliding. Nat Struct Mol Biol. 2008; 15:364–72.
Suganuma T, Mushegian A, Swanson SK, Abmayr SM, Florens L, Washburn MP, Workman JL. The ATAC acetyltransferase complex coordinates MAP kinases to regulate JNK target genes. Cell. 2010;142:726–36.
Tamkun JW, Deuring R, Scott MP, Kissinger M, Pattatucci AM, Kaufman TC, Kennison JA. Brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell. 1992;68:561–72.
Thireos G, Penn MD, Greer H. 5′ untranslated sequences are required for the translational control of a yeast regulatory gene. Proc Natl Acad Sci USA. 1984;81:5096–100.
Wang L, Mizzen C, Ying C, Candau R, Barlev N, Brownell J, Allis CD, Berger SL. Histone acetyltransferase activity is conserved between yeast and human GCN5 and is required for complementation of growth and transcriptional activation. Mol Cell Biol. 1997;17:519–27.
Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature. 1996;382:319–24.

PCCMT

► Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

PCD8 (Programmed Cell Death 8)

► AIF

pCIP

► Steroid Receptor Coactivator Family

PCMT

► Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

PDE 1

► Phosphodiesterase 1

PDE10

► Phosphodiesterase 10A

PDE10A

► Phosphodiesterase 10A

PDE4

Miles D. Houslay
Molecular Pharmacology Group, Institute of Neuroscience and Psychology, CMVLS, University of Glasgow, Glasgow, Scotland, UK

Synonyms

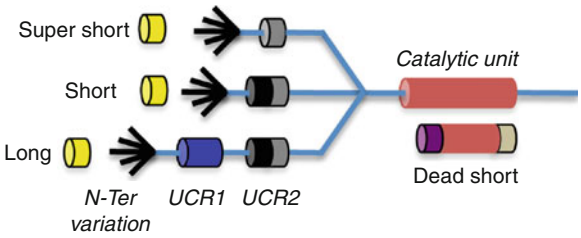
cAMP phosphodiesterase-4; Type IV cAMP phosphodiesterase

Historical Background

Cyclic 3’5’ adenosine monophosphate (cAMP) is a key second messenger that is responsible for

regulating many pivotal signaling processes in all mammalian cell types (Tasken and Aandahl 2004). It influences cell growth, differentiation, shape, and movement as well as processes such a cardiac contraction, metabolism, water retention, learning, and memory. Most intriguingly, however, cAMP can selectively regulate a variety of very different processes in any one particular cell type. Indeed, uncovering the molecular mechanisms that allow cAMP to selectively regulate disparate processes within a single cell has provided a major challenge. Only very recently has the means whereby cAMP signaling is compartmentalized in cells begun to be understood (Tasken and Aandahl 2004; Baillie et al. 2005; Willoughby and Cooper 2007). One key requirement is for spatially discrete signaling complexes to be assembled in cells that have the machinery for detecting cAMP and translating this into an appropriate action. Critically, however, is a need for machinery able to dynamically regulate the access of cAMP to the detections system(s) and this is achieved by controlling the rate of cAMP degradation in the immediate vicinity of the signaling complex by targeted phosphodiesterases (Houslay 2010). Spatially constrained cAMP degradation has the functional consequence of adjusting the threshold for activation of cAMP sensors, in particular signaling complexes, in the face of a prevailing level of cAMP formation by ▶ adenylyl cyclase activity. Carrying out this process are forms of cAMP degrading phosphodiesterases (PDEs) that have specific 'zip codes' allowing them to be targeted to particular signaling complexes (Houslay 2010). These PDEs convert cAMP to 5'-adenosine monophosphate (AMP) and so play a pivotal role in not only degrading the cAMP signal but, critically, in forming and shaping cAMP gradients in cells so as to regulate the activity of distinct signaling complexes. Indeed, recent technological advances have allowed cAMP gradients formed by PDEs to be dynamically visualized in living cells (Zaccolo 2009). These are FRET-based genetically encoded sensors that are based upon the cAMP effectors, either protein kinase A (PKA), which phosphorylates specific target proteins, or Exchange Protein Activated by cAMP (EPAC), which activates the mini G-proteins, Rap1/2.

Our understanding of how targeted cAMP degradation underpins cAMP signaling has been revolutionized by studies on members of the multigene PDE4 subfamily.



PDE4, Fig. 1 Schematic of PDE4 isoform classification. These four classes are defined by their complement of UCR1 and UCR2 domains with individual isoforms defined by distinct N-terminal domains

What Is PDE4?

Eleven PDE families describe enzymes that hydrolyse either or both cAMP and cGMP (Conti and Beavo 2007; Lugnier 2006). Four genes (*PDE4A*/*B*/*C*/*D*) encode the PDE4 family of enzymes (Houslay 2010; Conti and Beavo 2007). Through the use of distinct promoters and alternative nRNA splicing these genes encode a series of splice variants that are classified as long, short, super-short, and dead-short isoforms. Each isoform is characterized by a unique N-terminal region with the sole known exceptions being PDE4B5 and PDE4D6, which have identical N-terminal regions, albeit encoded by distinct exons (Fig. 1).

Long PDE4 isoforms possess dual regulatory domains that are unique to PDE4, namely, Upstream Conserved Region 1 (UCR1) and Upstream Conserved Region 2 (UCR2). These are located between the isoform-specific N-terminal region and the highly conserved catalytic unit (Fig. 1). UCR1 is tethered to UCR2 by the heterogeneous linker region LR1, with UCR2 tethered to the catalytic unit by the heterogeneous linker region, LR2. The PDE4 C-terminal regions from different subfamilies show no similarity but are identical for all active isoforms within any particular subfamily.

The presence or absence of UCR1/2 underpins classification; long forms have both, short forms lack UCR1, super-short forms lack UCR1 and have a truncated UCR2 while catalytically inactive dead-short forms lack both UCR1 and UCR2 and, through a unique 3' splicing event, have a truncated catalytic unit and novel C-terminal region.

Regulation by Phosphorylation

The rate of cAMP degradation by PDE4 can be dynamically regulated by phosphorylation. Pivotal to this are the UCR1 and UCR2 regulatory domains, which direct the functional outcome on the catalytic unit in response to phosphorylation (Houslay and Adams 2003).

UCR1 contains a classical site for PKA phosphorylation, which confers activation on long isoforms. Thus long isoforms play a prime role in the desensitization of cAMP signaling by facilitating cAMP degradation when cAMP levels rise sufficiently to activate PKA.

The catalytic unit of all PDE4 isoforms, save those from the PDE4A subfamily, contains a classical site for ERK phosphorylation. However, the UCR modules direct the functional outcome of such ERK phosphorylation, with long forms being inhibited by ERK phosphorylation, while short forms are activated and super-short isoforms show no activity change.

The inhibitory effect of ERK phosphorylation of PDE4 long isoforms can be overcome by PKA phosphorylation. This provides a potential feedback regulatory unit where ERK-mediated inhibition of a long PDE4 can cause local cAMP levels to rise, activating local PKA, which then phosphorylates the long PDE4 to disinhibit it, causing cAMP levels to fall. Thus, through this route, ligands activating ERK can lead to a programmed transient rise in cAMP through effects on PDE4 long forms.

Additionally, an unidentified kinase activated by reactive oxygen species can phosphorylate PDE4. Although this does not alter PDE4 activity, it reprograms the effect of inhibitory ERK phosphorylation of long isoforms to one of activation.

UCR1 is also the site of phosphorylation by the stress-activated kinase, MK2 (MAPKAPK2). This does not alter PDE4 activity, but attenuates the ability of PKA to activate long forms.

Certain PDE4 forms are also modified by ubiquitination and SUMOylation. While neither alters PDE4 activity, SUMOylation of PDE4D5 enhances its activation by PKA while reducing its inhibition by ERK phosphorylation. Very recently it has been demonstrated that GSK3 can phosphorylate PDE4D isoforms, causing their ubiquitination and targeting for degradation.

Multisite phosphorylation confers dynamic regulation upon PDE4 activity and certain targeting events. In this, the UCR1 and UCR2 domains perform critical roles in defining the effect of phosphorylation on PDE4 catalytic activity. Unfortunately there are no determined structures for full-length PDE4 isoforms due to the propensity of PDE4 enzymes to multimerize upon purification. However, biochemical studies have shown that UCR1 and UCR2 interact with each other, as does UCR2 with the catalytic unit. Indeed, recent structural studies demonstrate that UCR2 docks to the catalytic unit alongside the catalytic pocket. In fact, in certain conformational states a portion of UCR2 may even bind across the catalytic pocket, obscuring access to cAMP and engendering inhibition. This process may be regulated by phosphorylation, dimerization, and interaction with binding partner proteins.

The core PDE4 catalytic unit is a compact structure of 17 α-helices folded into three sub-domains. At the junction of the three sub-domains is a deep cAMP substrate-binding pocket that contains two essential Me^{2+} binding sites, considered natively to be Zn^{2+} at the deeper site and Mg^{2+} at the more surface exposed site. The Zn^{2+} is tightly bound by four direct ligand interactions, with the fifth position being provided by water that bridges to the loosely held Mg^{2+} and plays the key role of nucleophilic attack in hydrolysis of the cyclophosphodiester bond. Structural changes arising from post-translational modification, UCR1/2 domain interaction, and partner protein sequestration may be relayed into the catalytic center through helices 10 and 11.

Targeting of PDE4

Each PDE4 isoform has a signature N-terminal (N-Ter) domain whose major function is to confer intracellular targeting to specific signaling complexes and intracellular locations (Houslay 2010; Keravis and Lugnier 2010). The paradigm for this concept is the super-short PDE4A1 isoform that has a unique 25 amino acid N-Ter region. PDE4A1 is entirely membrane associated, being found in Golgi and vesicles that traffic from it. However, the engineered removal of its unique N-Ter region generates a soluble, cytosolic species that is clearly correctly folded as it remains fully catalytically active. This observation led to the notion that the PDE4 N-Ter region determined intracellular targeting, thus providing a functional reason for the diversity of PDE4 isoforms.

Such targeting is invariably driven by protein–protein interactions although, as in the case of PDE4A1, it may also involve protein–lipid interactions.

All of the information required for membrane association and targeting is encompassed within the unique N-Ter region of PDE4A1 as when it is fused to various soluble proteins the distribution of such chimeric species parallels that of full-length PDE4A1. NMR analysis of the PDE4A1 N-Ter region revealed two α-helical domains separated by a flexible hinge. The binding of Ca^{2+} to Asp21 in helix-2 elicits a conformational change that allows insertion of a core Trp19:Trp20 unit into the bilayer. However, PDE4A1 membrane targeting also requires helix-1, which increases the efficiency of helix-2 membrane association as well as conferring targeting to the trans-Golgi stack, probably by interacting with a Golgi-localized protein subsequent to membrane insertion of helix-1. Additionally, helix-1 allows PDE4A1 to undergo a dynamic intracellular redistribution to phosphatidic acid-rich regions upon Ca^{2+}-binding to Asp6. Thus PDE4A1 provides a paradigm for the targeting of PDE4 isoforms to specific complexes and for such interactions to be functionally, and dynamically, regulated.

Subsequently, specific PDE4 isoforms have been found to associate with signaling scaffold proteins (RACK1, myomegalin, β-arrestin, AKAPs, Ndel1, spectrin, Shank2, Lis1), receptors (p75 neurotropin receptor, ryanodine receptor, β_1-adrenoceptor), enzymes (ERK, DNA-PKc), certain SH3 domain-containing proteins (e.g., ▶ Src family tyrosyl kinases), and transcription factors (AIP/XAP2). The mapping of interaction surfaces between many of these partnerships has been greatly facilitated by peptide array approaches. These have driven mutagenesis strategies to define interaction surfaces and in developing cell-permeable peptides for use in disrupting specific PDE4 partnerships in cells and so allowing functional assessment.

Dominant negative, siRNA, and gene-targeted knockout approaches have unequivocally shown that individual PDE4 isoforms can have specific functional roles and that there is no redundancy in the system. It is now appreciated that the diversity of PDE4 isoforms allows for targeting of particular species to specific, spatially defined signaling complexes in cells in order to allow for their exquisite control by gradients of cAMP sculpted by tethered PDE4 isoforms. A paradigm for this is the sequestration of the PDE4D5 isoform with the signaling scaffold β-arrestin. This complex is recruited to the β_2-adrenergic receptor when cells are challenged with β-adrenergic receptor agonists, thus delivering an active cAMP degrading system to the site of cAMP synthesis. This complex has a dual desensitization role. Thus β-arrestin uncouples the β_2-adrenoceptor from the guanine nucleotide binding protein, Gs, and so negates activation of adenylyl cyclase, while PDE4D5 lowers local cAMP levels and switches off the activity of plasma membrane and β_2-adrenergic receptor-tethered PKA. The importance of β-arrestin-delivered PDE4D5 in the control of these PKA subpopulations was gathered by a dominant negative approach. This involved making a single point mutation deep within the cAMP-binding pocket of PDE4D5 so as to destroy catalytic activity while leaving the ability of PDE4D5 to bind β-arrestin unaltered. Overexpressing this inactive species in cells displaces active wild-type PDE4D5 from β-arrestin and allows plasma membrane PKA activity to rise unhindered in the face of challenge with β-adrenergic receptor agonists. However, no such action was seen if different catalytically inactive PDE4 isoforms were used or if a catalytically inactive PDE4D5 construct was engineered so as to disrupt its β-arrestin binding site.

Various of these PDE4 partnerships can be dynamically altered by external cues so as to reprogram cAMP gradients within cells. This can take the form of either changes in the expression of scaffolds, allowing new or competing partnerships to occur, or posttranslational modification that can alter specific partnerships. Thus the Mdm2-mediated ubiquitination of PDE4D5 enhances its interaction with β-arrestin while the PKA-mediated phosphorylation of PDE4D3 serves to enhance its interaction with the mAKAP scaffold while decreasing its interaction with the Ndel1 scaffold.

PDE4 and Disease

The first indication that PDE4 might provide an effective therapeutic target came from observations that rolipram, the first reported PDE4 selective inhibitor, exerted antidepressant properties. However, such therapeutic potential was negated by side effects, primarily severe emesis and nausea. Indeed, these issues have characterized, to a greater or lesser extent, all

active-site-directed PDE4 inhibitors to date and provided a major challenge to the pharmaceutical industry in developing selective inhibitors with effective therapeutic windows (Houslay et al. 2005). PDE4 selective inhibitors also exert potent anti-inflammatory actions and have been developed for treating inflammatory lung diseases such as asthma and COPD. Promisingly, in this regard, Roflumilast® has been approved for use in treating COPD in Europe and the USA.

The central problem with therapeutic exploitation of active-site-directed inhibitors is that they inhibit all PDE4 isoforms rather than just those that govern the target therapeutic process. As PDE4 catalytic units are identical in all isoforms from a particular subfamily and near identical between subfamilies, the chance of obtaining isoform-specific inhibitors through this route is nigh on impossible. Indeed generating subfamily selective inhibitors poses a severe challenge that has, to date, only shown scant possibilities for PDE4D and PDE4B. Further obfuscation arises from the fact that interacting proteins and posttranslational modification can alter the conformation of PDE4 isoforms. This, as shown in various instances, can translate into alterations in their susceptibility to active-site-directed inhibitors. Indeed, recent structural studies have given insight into how UCR2 interaction with the catalytic unit may drive some of these (Houslay and Adams 2010).

One possibility for the future is to determine whether the inhibition of a specific PDE4 isoform that was anchored could provide a route to treating defined diseases. If so, a novel therapeutic approach might be employed based upon developing either small molecules, peptides, or peptidomimetics that disrupt such a key partnership. This would have the effect of displacing a specific PDE4 isoform from the site where it is needed to function in the cell, thereby raising only local cAMP levels, thus eliciting a highly targeted and specific action for therapeutic advantage while minimizing/negating side effects.

PDE4 diversity has been highly conserved in evolution. Interestingly, mutations in the PDE4 gene in *Drosophila melanogaster* have been described that lead to learning defects. This correlates with studies on mammals showing that cAMP is intimately linked with learning processes and a panoply of studies showing that PDE4 inhibitors can act as cognitive enhancers, which may offer future promise for the utility of PDE4 inhibitors in Alzheimer disease. More recently, the *PDE4B* gene has been linked to schizophrenia in certain families. Indeed, in an example of converging genetic and biochemical approaches, the scaffold protein DISC1, mutations in whose gene can provide strong association with schizophrenia, can interact with PDE4 isoforms, including PDE4B.

Mutations in the *PDE4D* gene have also been linked to stroke susceptibility. However, the linkage appears likely to depend on both the type of stroke and ethnic background. The basis of the linkage is obscure to date but mutations appear to locate in noncoding regions upstream of the PDE4D7 isoform, which may indicate effects on the specific promoter for this isoform.

PDE4 inhibitors have also been suggested to have therapeutic possibilities for treating certain cancers. They, seemingly, can promote B-cell apoptosis in chronic lymphocytic leukemia and can inhibit the growth and migration of colon cancer cells, for example.

Summary

PDE4 isoforms provide a major route for underpinning compartmentalized cAMP signaling in all mammalian cells. The plethora of isoforms provides a library of species that can be targeted to specific intracellular locales/signaling complexes. This enables them to sculpt local cAMP gradients, thereby gating the activation of tethered cAMP effectors to cAMP. The selective expression of PDE4 isoforms with different regulatory regions (UCR1/2) allows them to perform distinct roles in both the cellular processes for desensitizing cAMP signaling and for cross-talk with other critical signaling systems so as to integrate cellular responses in cell-type specific fashions.

Members of the diverse PDE4 family are invariably located at nodes that link multiple signaling pathways and, as such, are set to play key roles in regulating pivotal cellular responses. Our understanding of the range of PDE4 partner proteins and multiple sites for phosphorylation, ubiquitination, and SUMOylation is only just beginning to be appreciated. Furthermore there is also the exciting possibility that the function of the PDE4 enzymes is not just to carry out targeted cAMP degradation but also to provide a signaling role for themselves (Murdoch et al. 2011).

Current studies have served to set the scene for a more detailed appreciation of the importance and pervasiveness of PDE4 isoforms, their key roles in biology, health and disease, and future therapeutic exploitation.

References

Baillie GS, Scott JD, Houslay MD. Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. FEBS Lett. 2005;579:3264–70.
Conti M, Beavo J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem. 2007;76:481–511.
Houslay MD. Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. Trends Biochem Sci. 2010;35:91–100.
Houslay MD, Adams DR. PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. Biochem J. 2003;370:1–18.
Houslay MD, Adams DR. Putting the lid on phosphodiesterase 4. Nat Biotechnol. 2010;28:38–40.
Houslay MD, Schafer P, Zhang KY. Keynote review: phosphodiesterase-4 as a therapeutic target. Drug Discov Today. 2005;10:1503–19.
Keravis T, Lugnier C. Cyclic nucleotide phosphodiesterases (PDE) and peptide motifs. Curr Pharm Des. 2010;16:1114–25.
Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacol Ther. 2006;109:366–98.
Murdoch H, Vadrevu S, Prinz A, Dunlop A, Klussmann E, Bolger GB, Norman JC, Houslay MD. LIS1/cAMP phosphodiesterase-4 (PDE4) interaction and their role in cytoplasmic dynein function. J Cell Sci. 2011;124:2253–66.
Tasken K, Aandahl ME. Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev. 2004;84:137–67.
Willoughby D, Cooper DM. Organization and Ca^{2+} regulation of adenylyl cyclases in cAMP microdomains. Physiol Rev. 2007;87:965–1010.
Zaccolo M. cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. Br J Pharmacol. 2009;158:50–60.

Pea15

Joanna E. Gawecka[1] and Joe W. Ramos[2]
[1]University of Hawai'i Cancer Center, University of Hawai'i at Manoa, Honolulu, HI, USA
[2]The Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI, USA

Synonyms

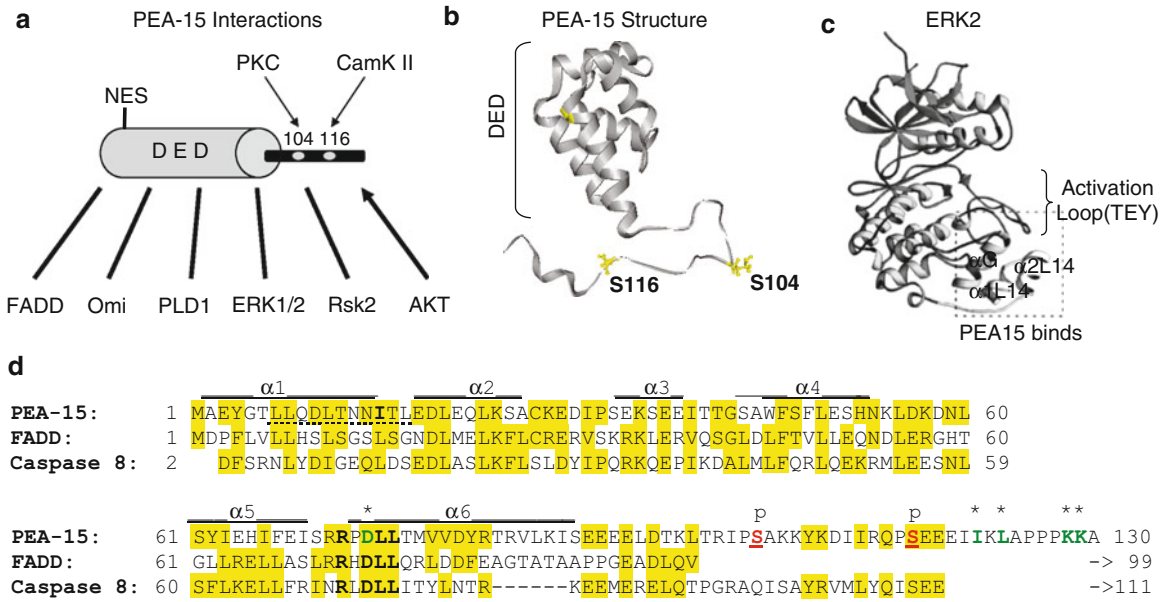
HMAT1; HUMMAT1H; MAT1; MAT1H; Pea15a; PEA-15; PED; PED/PEA-15; PED-15; Phosphoprotein enriched in astrocytes 15kDa; Phosphoprotein enriched in diabetes; Pkcs15

Historical Background

Phosphoprotein Enriched in Astrocytes 15 kDa (PEA-15), also known as PED-15, is a 15 kDa protein that is highly expressed in the nervous system with particularly high levels in astrocytes and neurons of the hippocampus. Chneiweiss and colleagues first characterized PEA-15 as a major substrate for Protein Kinase C (PKC) in astrocytes and later cloned the cDNA encoding the protein (Araujo et al. 1993; Estelles et al. 1996). Subsequently, a portion of the 3′ UTR of the PEA-15 mRNA, called MAT1, was identified as a mammary transforming gene associated with mouse mammary carcinogenesis (Bera et al. 1994). It remains unclear how this relates to the function of the full-length PEA-15 protein. The initial functional significance of PEA-15 was identified when it was shown to be a phosphoprotein overexpressed in skeletal muscle in patients with type 2 diabetes (Condorelli et al. 1998). In this context, PEA-15 was shown to inhibit insulin-stimulated glucose transport indicating it may be involved in development of type 2 diabetes. PEA-15 was further found by expression cloning to inhibit H-Ras signaling to integrins (Ramos et al. 1998). This work provided an additional potential connection to cancer and the first suggestion that PEA-15 may modulate cell adhesion. This was quickly followed by the first two reports showing that PEA-15 can act as an anti-apoptotic protein that blocks cell death by preventing Fas-Associated via Death Dominion protein (FADD) recruitment and activation of Caspase 8 in response to Fas or Tumor Necrosis Factor Receptor (TNFR) activation (Condorelli et al. 1999; Kitsberg et al. 1999). Thus from the initial handful of papers it was clear that PEA-15 is a multifunctional protein affecting many distinct cell signaling pathways.

PEA-15 Structure and Binding Partners

PEA-15 is encoded by two highly conserved mRNAs that are alternatively spliced in the large 3′ UTR. The mRNA is expressed in most tissues while the protein expression is more limited. The message encodes a nonenzymatic 15 kDa cytoplasmic protein consisting of 130 amino acids (Estelles et al. 1996). The N-terminal 80 amino acids encode a canonical death effector domain (DED), while the remaining 50 amino acid C-terminal tail lacks structure, but contains two



Pea15, Fig. 1 PEA-15 Structure and Binding Partners. (**a**) PEA-15 is 130 amino acids in length and consists of a death effector domain (DED) that constitutes amino acids 1–80. PEA-15 is phosphorylated at serine 104 by protein kinase C (PKC) and at serine 116 by either Calcium calmodulin kinase II (CamK II) or AKT. *Arrows* depict these phosphorylations and PEA-15 binding partners are indicated by *connecting lines*. (**b**) The NMR-derived structure of PEA-15. The C-terminal tail lacks structure and has an extended and flexible conformation in the unphosphorylated state. (**c**) The structure of ERK2 is shown. PEA-15 binds the MAP kinase insert that is located just below the activation loop that contains the phosphorylated TEY sequence. (**d**) The full amino acid sequence of PEA-15 is indicated aligned with the DEDs of two other DED family members, FADD and Caspase 8. Conserved amino acids are highlighted in *yellow*. The six predicted alpha helices are indicated above the sequence by the over-lined α regions. The phosphorylated serines are *underlined* and in *red* and point mutants that abrogate ERK binding are indicated by a *star (*)* and are in *green*. The consensus nuclear export sequence (NES) is underlined by a *dashed line* (aa 7–17)

serines that are phosphorylated by PKC (Ser104) or AKT and (Calcium Calmodulin Kinase II) CamKII (Ser116) (Ramos 2005). Proteins containing DEDs are known to regulate apoptosis, the process of programmed cell death and have more recently been found to also affect proliferation (Valmiki and Ramos 2009). PEA-15 is a member of this DED family of proteins (Fig. 1). PEA-15 also has a functional nuclear export sequence (NES) which, when mutated, leads to the accumulation of PEA-15 in the cell nucleus (Formstecher et al. 2001). In addition PEA-15 protein stability is regulated by ubiquitination, which is blocked by phosphorylation of Ser116 by CamKII downstream of PKCζ or phorbol esters (Fiory et al. 2009).

PEA-15 binds several proteins known to regulate apoptosis, transcription, and proliferation and thereby affects these processes (Ramos 2005). Among identified interacting partners some of the most extensively characterized are extracellular signal-regulated kinase 1 and 2 (ERK1/2) MAP Kinase, FADD, (Phospholipase D) PLD, ribosomal protein S6 kinase 2 (RSK2), and AKT. PEA-15 binding to ERK and FADD is regulated by phosphorylation at the two serines. This indicates that phosphorylation is one mechanism by which PEA-15 binding to its partners is determined. PEA-15 can also simultaneously bind ERK and the ERK substrate RSK2 and thereby act as a scaffold to target ERK to RSK2. Thus far this remains the only identified instance of PEA-15 acting as a scaffold. PEA-15 binding to FADD acts predominantly to block FADD binding to and activation of Caspase 8 while binding to PLD appears to serve to activate that lipase (Fig. 1) (Fiory et al. 2009; Ramos 2005). In this way, the function of PEA-15 is determined by its binding partners.

PEA-15 in Glucose Transport

PEA-15 was identified by differential display to be overexpressed in the adipose and skeletal muscle tissues, as well as in skin fibroblasts of type 2 diabetic

patients and their first-degree relatives. Moreover PEA-15 expression in skeletal muscle impairs glucose transporter type 4 (GLUT4) translocation and thereby inhibits insulin-stimulated glucose transport. This established early on that PEA-15 likely plays a significant role in type 2 diabetes (Condorelli et al. 1998). Subsequently, transgenic mice overexpressing PEA-15 were found to have some of the hallmarks of type 2 diabetes. These transgenic mice had impaired glucose tolerance, resistance to insulin action on glucose disposal, reduced insulin-stimulated glucose transport in fat and skeletal muscles, and impaired insulin effects on GLUT4 membrane translocation (Fiory et al. 2009). These effects may be caused in part by the ability of PEA-15 to directly bind and stabilize ▶ phospholipase D (PLD) thereby inducing PKC activity (Fiory et al. 2009). This hypothesis is further supported by the observation that inhibition of PEA-15 binding to PLD restores insulin-stimulated glucose uptake. Thus targeting the PEA-15/PLD1 interaction may provide a new approach to improving sensitivity to insulin action in diabetics (Fiory et al. 2009).

PEA-15 in Apoptosis

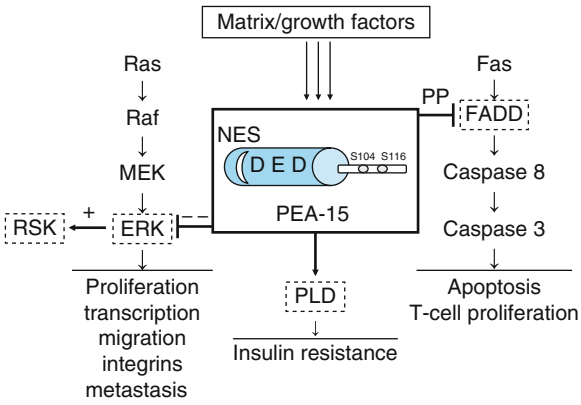
PEA-15 regulates apoptosis by inhibiting the death receptor–activated extrinsic cascade. PEA-15 is expressed at particularly high levels in astrocytes and its N-terminal DED supports its interaction with other DED-containing proteins including FADD and possibly Caspase 8. This was an early indicator that PEA-15 may play an anti-apoptotic role in these cells. Indeed, sensitivity to TNF-dependent apoptosis is significantly increased in astrocytes from PEA-15-null mice (Kitsberg et al. 1999). Moreover, this unusual sensitivity to TNF can be repaired by exogenous expression of PEA-15 in the knockout astrocytes. In separate experiments, PEA-15 was also reported to block the extrinsic apoptotic pathway in human mammary carcinoma cells (MCF-7). The likely mechanism is that by binding to both FADD and Caspase 8, PEA-15 inhibits their interaction and thereby prevents the formation of the death-inducing signaling complex (DISC). Thus Caspase 8 and downstream caspases are not activated. This is the same mechanism suggested for the anti-apoptotic effects of another DED family protein called cFLIP. The anti-apoptotic effect of PEA-15 appears to extend to other cancer cell types as well. Human malignant glioma cells are often highly resistant to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Because of this, these gliomas cannot be treated with chemotherapies based on TRAIL activation. However, knockdown of PEA-15 in these cells restores their sensitivity to TRAIL (Fiory et al. 2009; Valmiki and Ramos 2009). These results suggest that reducing PEA-15 levels in some cancer cells may improve their response to death receptor–based therapies.

Phosphorylation at Ser116 stabilizes PEA-15 levels and promotes its interaction with FADD, while phosphorylation at Ser-104 abrogates PEA-15 binding to ERK. Moreover, only the dually phosphorylated PEA-15 is found in the death-inducing signaling complex (DISC) in TRAIL-resistant glioma cells, suggesting that dual phosphorylation is necessary for its protective role (Fiory et al. 2009). Indeed, treatment with inhibitors of PKC (known to phosphorylate PEA-15 at Ser104) abrogated the anti-apoptotic function. Similarly, inhibitors of AKT/PKB block phosphorylation of PEA-15 at Ser116 and increase PEA-15 degradation leading to elevated TRAIL-mediated apoptosis. Thus dual phosphorylation of PEA-15 is essential for it to block extrinsic apoptosis initiated by Fas, TNFR, or TRAIL (Fiory et al. 2009). PEA-15 levels are also regulated in apoptosis by the serine protease Omi/HtrA2. This pro-apoptotic mitochondrial protease is released in response to different cellular stresses and exerts pro-apoptotic function by promoting Caspase 3 activation through its protease activity. Omi/HtrA2 also promotes apoptosis by degrading PEA-15 and thus preventing PEA-15 anti-apoptotic activity (Fiory et al. 2009). Hence PEA-15 anti-apoptotic function is regulated by phosphorylation, ubiquitination, and proteolytic degradation of the protein.

In addition to regulating extrinsic apoptotic cascades, PEA-15 also inhibits stress-induced apoptotic responses to oxidative agents, serum deprivation, and anisomycin treatment. In human kidney embryonic cells (HEK293), overexpression of PEA-15 decreased anisomycin- and H_2O_2-induced apoptosis and inhibited the phosphorylation of JNK1/2 and p38. Impaired activity of these stress kinases by PEA-15 correlated with inhibition of stress-induced Cdc-42, MKK4, and ▶ MKK6 activation (Fiory et al. 2009). PEA-15 is therefore well established as an important anti-apoptotic protein that functions by multiple mechanisms to promote cell survival.

PEA-15 in the ERK Mitogen-Activated Protein Kinase (MAPK) Pathway

Expression of the oncogenic small GTPase H-Ras can decrease binding of integrins to their extracellular ligands such as fibronectin. This causes reduced cell adhesion and prevents assembly of the extracellular matrix. The H-Ras signal is mediated by the Raf/ERK MAP kinase signaling cascade. PEA-15 can inhibit this H-Ras suppression of integrins when it is co-expressed. Furthermore the effect of PEA-15 is at the level of ERK but PEA-15 does not alter ERK activity (Fiory et al. 2009; Ramos et al. 1998). In subsequent studies it was found that PEA-15 binds directly to ERK and prevents translocation of active ERK into the nucleus. The cytoplasmic sequestration of ERK blocks ERK-dependent transcription and proliferation (Ramos 2008; Valmiki and Ramos 2009). PEA-15 not only restricts ERK to the cytoplasm, it can target cytoplasmic ERK to its substrate kinase RSK2. In this way, PEA-15 acts as a scaffold for ERK activation of RSK2. Moreover like most scaffold proteins, PEA-15 effects on RSK2 activation depend on the expression level of PEA-15. For example at optimum molar ratios of PEA-15, ERK, and RSK2, PEA-15 enhances RSK2 activation. Whereas expression at high molar excess of PEA-15 in comparison to ERK or RSK2 can inhibit RSK2 activation (Ramos 2008; Vaidyanathan et al. 2007; Valmiki and Ramos 2009). RSK2 is itself a serine/threonine kinase that has a number of downstream targets and can regulate transcription, proliferation, survival, and migration. Thus it is likely that some of the effects of PEA-15 on these functions are due to its effects on RSK2 activity. The mechanisms of PEA-15 regulation of cell signaling are depicted in Fig. 2.



Pea15, Fig. 2 PEA-15 regulates both apoptotic and ERK-pathways. The phosphorylation status of PEA-15 determines if it binds to ERK MAP kinase or FADD. If serine 104 is not phosphorylated, PEA-15 can bind to ERK and regulate its function by altering its location and interaction with RSK2. If PEA-15 is phosphorylated at serines 104 and 116, it binds to FADD and can regulate FADD-mediated signaling by blocking FADD binding to and activation of caspases. Phosphorylation of PEA-15 is regulated by growth factors. Finally, PEA-15 can bind and activate PLD

PEA-15 in Cancer Development and Progression

PEA-15 is reported to play diverse roles in many types of cancer including mammary, ovarian, skin, and glioma (Fiory et al. 2009; Ramos 2005). However, the effects of PEA-15 expression are not the same in each cancer and indeed PEA-15 is reported to act in the manner of both a tumor promoter and a tumor suppressor. The functions of PEA-15 that make it particularly interesting in cancer research are its effects on apoptosis, chemoresistance, senescence, adhesion, and migration.

Cancer cells frequently become resistant to apoptosis-mediated cell death. PEA-15 can effectively block apoptosis induced by death receptors in many cellular contexts. In glioma, B cell carcinoma, as well as breast and small cell lung cancer, increased PEA-15 expression levels correlate with resistance to death receptor–induced apoptosis (Fiory et al. 2009). Similarly, PEA-15 inhibits apoptosis in a skin carcinoma mouse model by abrogating TPA-dependent Caspase 3 activation (Fiory et al. 2009). The ability of PEA-15 to prevent apoptosis suggests it could be a valuable target for development of drugs to be used in combinatorial therapy to make cancer cells more prone to elimination (Ramos 2005). Thus, the ability of PEA-15 to block apoptosis may partly explain cancer resistance to radiation- and chemotherapy-induced cell death.

Cancers also typically develop the ability to undergo increased, uncontrolled cell proliferation in the absence of apoptosis or senescence. PEA-15 binds to ERK, prevents ERK translocation into the nucleus, and thereby impairs ERK-dependent transcription and proliferation. This function of PEA-15 appears to be key in the ability of PEA-15 to limit

proliferation of ovarian and breast cancers (Bartholomeusz et al. 2010; Bartholomeusz et al. 2006). PEA-15 may also prevent proliferation of some cancers by inducing cellular senescence as described in fibroblasts. In both instances, PEA-15 levels may be controlled by the adenovirus E1A. In ovarian cancer E1A upregulates PEA-15, which prevents ERK nuclear signaling and thereby decreases cell proliferation (Bartholomeusz et al. 2006). Conversely, in mouse embryo fibroblasts, E1A downregulates PEA-15, promoting ERK nuclear localization and thereby preventing Ras-induced senescence. Thus PEA-15 restriction of ERK to the cytoplasm is necessary in these cells for Ras to induce senescence. This provides an alternative method by which PEA-15 can prevent tumor cell proliferation even in the presence of an oncogene by promoting senescence (Fiory et al. 2009; Ramos 2005).

The initial discovery of PEA-15 as an inhibitor of Ras/MAPK signaling to integrins was the first indication of its potential role in cell adhesion, migration, and invasion. Since then PEA-15 has been reported to inhibit mammary tumor invasion (Glading et al. 2007) and astrocytoma migration and invasion (Renault-Mihara et al. 2006) by differing mechanisms. Immunohistochemical microarray analysis shows that PEA-15 expression is correlated with decreased invasive behavior of breast carcinoma and this effect seems to relate to the nuclear localization of activated ERK1/2. In these tumors, PEA-15 inhibits invasion by preventing ERK translocation to the nucleus. Indeed PEA-15 mutants that cannot bind or sequester ERK are unable to inhibit invasion. Additionally, membrane-localized ERK1 that is unable to translocate to the nucleus also decreases invasion. These results reveal the significance of nuclear entry of ERK1/2 in tumor behavior and indicate that the PEA-15 inhibitory effect on cell invasion depends on its ability to bind ERK (Glading et al. 2007). Additionally, the use of organotypic culture analysis of highly invasive primary astrocytomas revealed that only tumor cells expressing low levels of PEA-15 migrated away from the originating explants. Similarly, PEA-15-null astrocytes demonstrated significantly elevated motility compared to wild-type control cells and this was reversed by transfection of PEA-15. Moreover, mouse embryo fibroblasts transfected with PEA-15 displayed reduced migration (Renault-Mihara et al. 2006). Pharmacological treatments in these experiments excluded participation of ERK1/2, PI3K/AKT, CamK II in this effect of PEA-15. Rather inhibition of astrocyte migration is dependent on PEA-15 downregulation of PKCδ (Fiory et al. 2009; Renault-Mihara et al. 2006). Independently of the mechanism, the loss of cell invasiveness strongly suggests a role for PEA-15 as a potential metastasis suppressor.

High PEA-15 expression correlates to better prognosis for several cancer types. Women with high PEA-15-expressing ovarian tumors survive longer than those with low PEA-15-expressing tumors. PEA-15 was thus proposed to be a potentially important prognostic marker in ovarian cancer (Fiory et al. 2009). Similarly, PEA-15 expression correlates with World Health Organization (WHO) grading criteria for astrocytic tumors. High PEA-15 levels correlate with lower grade tumors. Hence here too PEA-15 may prove to be a useful prognostic marker (Watanabe et al. 2010). Finally, PEA-15 expression levels inversely correlate with cell motility and invasiveness in astrocytomas and mammary carcinomas (Glading et al. 2007; Renault-Mihara et al. 2006). Thus, PEA-15 expressions tend to reduce proliferation and invasion in some cancers and can thereby act as a tumor suppressor in these tumors. Whether PEA-15 acts to increase or decrease tumorigenesis therefore likely depends upon its phosphorylation status and the affected signaling pathways and microenvironment of the tumor.

PEA-15 in the Immune Response and Spatial Learning

Normal PEA-15 function has been investigated using PEA-15 null mice (Kitsberg et al. 1999). The PEA-15 null mice are born at Mendelian ratios, appear grossly normal, and did not evidence any abnormal brain size, neuron number, or structural defects. PEA-15 is highly expressed throughout the brain and regulates ERK, RSK2, and CREB function. As these proteins are important in cognitive function, the PEA-15 null mice were examined for nervous system function and cognitive defects. In these studies the effects of loss of PEA-15 was investigated in a series of experiments designed to measure stress activity, as well as learning and sensory functions. PEA-15 knockout mice exhibit impaired spatial learning, while their fear conditioning, passive avoidance, egocentric navigation, and

odor discrimination are normal. Additionally, PEA-15 knockout mice exhibit impaired forepaw strength. The knockout mice also were of normal weight, pain sensitivity, and coordination and have normal visual, auditory, and olfactory abilities (Ramos et al. 2009). It is attractive to suggest that these cognitive defects may be the result of abnormal ERK- and RSK2-dependent ▶ CREB transcription.

PEA-15 null mice were noted by Dr. Chneiweiss to suffer enlarged spleens and lymph nodes when kept in uncontrolled antigen-rich environments. This largely went away when the mice were kept in controlled Hepa-filtered cages. Thus a potential role for PEA-15 in the immune response was investigated. Pastorino and colleagues found that PEA-15 null mice have no significant defects in thymic or splenic lymphocyte cellularity or differentiation. However, activation of PEA-15 null T cells results in hyperproliferation in comparison to wild-type littermates. This increased proliferation results at least in part from increased ERK translocation into the nucleus and the resultant elevated activation of IL-2 transcription and secretion (Pastorino et al. 2010). In vitro studies conducted in Jurkat T cells confirm that PEA-15 negatively regulates T-cell receptor signaling restricting cell proliferation, ERK nuclear translocation, and IL-2 transcription (Pastorino et al. 2010). Interestingly, there is no indication that apoptosis is altered in the PEA-15 null immune cells. Hence, PEA-15 is a novel player in T-cell homeostasis and thereby contributes to the regulation of the immune responses. Significantly, the effects of PEA-15 deletion on both cognitive function and immune response can be explained by changes in ERK MAP kinase signaling.

Summary

PEA-15 modulates signaling in extrinsic apoptosis, ERK MAP kinase, and PLD pathways. This leads to changes in cell death, proliferation, and motility among others. As a result of these effects, PEA-15 plays an important role in normal glucose metabolism, the immune response and learning while its abnormal expression is associated with type 2 diabetes and cancer. Therefore, it will be important to determine if manipulation of PEA-15 is a valid therapeutic approach in diabetes and cancer. However, the fact that PEA-15 expression can both enhance and impair cancer progression provides a note of caution that the context of any PEA-15 directed therapy must be well understood before progressing. To this end, much work is still needed.

References

Araujo H, Danziger N, Cordier J, Glowinski J, Chneiweiss H. Characterization of PEA-15, a major substrate for protein kinase C in astrocytes. J Biol Chem. 1993;268(8):5911–20.

Bartholomeusz C, Itamochi H, Nitta M, Saya H, Ginsberg MH, Ueno NT. Antitumor effect of E1A in ovarian cancer by cytoplasmic sequestration of activated ERK by PEA15. Oncogene. 2006;25(1):79–90.

Bartholomeusz C, Gonzalez-Angulo AM, Kazansky A, Krishnamurthy S, Liu P, Yuan LX, et al. PEA-15 inhibits tumorigenesis in an MDA-MB-468 triple-negative breast cancer xenograft model through increased cytoplasmic localization of activated extracellular signal-regulated kinase. Clin Cancer Res. 2010;16(6):1802–11.

Bera TK, Guzman RC, Miyamoto S, Panda DK, Sasaki M, Hanyu K, et al. Identification of a mammary transforming gene (MAT1) associated with mouse mammary carcinogenesis. ProcNatlAcadSciUSA. 1994;91(21):9789–93.

Condorelli G, Vigliotta G, Iavarone C, Caruso M, Tocchetti CG, Andreozzi F, et al. PED/PEA-15 gene controls glucose transport and is overexpressed in type 2 diabetes mellitus. EMBO J. 1998;17(14):3858–66.

Condorelli G, Vigliotta G, Cafieri A, Trencia A, Andalo P, Oriente F, et al. PED/PEA-15: an anti-apoptotic molecule that regulates FAS/TNFR1-induced apoptosis. Oncogene. 1999;18(31):4409–15.

Estelles A, Yokoyama M, Nothias F, Vincent JD, Glowinski J, Vernier P, et al. The major astrocytic phosphoprotein PEA-15 is encoded by two mRNAs conserved on their full length in mouse and human. J Biol Chem. 1996;271(25):14800–6.

Fiory F, Formisano P, Perruolo G, Beguinot F. Frontiers: PED/PEA-15, a multifunctional protein controlling cell survival and glucose metabolism. Am J Physiol Endocrinol Metab. 2009;297(3):E592–601.

Formstecher E, Ramos JW, Fauquet M, Calderwood DA, Hsieh JC, Canton B, et al. PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. Dev Cell. 2001;1(2):239–50.

Glading A, Koziol JA, Krueger J, Ginsberg MH. PEA-15 inhibits tumor cell invasion by binding to extracellular signal-regulated kinase 1/2. Cancer Res. 2007;67(4):1536–44.

Kitsberg D, Formstecher E, Fauquet M, Kubes M, Cordier J, Canton B, et al. Knock-out of the neural death effector domain protein PEA-15 demonstrates that its expression protects astrocytes from TNFalpha-induced apoptosis. J Neurosci. 1999;19(19):8244–51.

Pastorino S, Renganathan H, Caliva MJ, Filbert EL, Opoku-Ansah J, Sulzmaier FJ, et al. The death effector domain protein PEA-15 negatively regulates T-cell receptor signaling. FASEB J. 2010;24(8):2818–28.

Ramos JW. Cancer research center hotline: PEA-15 phosphoprotein: a potential cancer drug target. Hawaii MedJ. 2005; 64(3):77–80.
Ramos JW. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. Int J Biochem Cell Biol. 2008;40(12):2707–19.
Ramos JW, Kojima TK, Hughes PE, Fenczik CA, Ginsberg MH. The death effector domain of PEA-15 is involved in its regulation of integrin activation. J Biol Chem. 1998; 273(51):33897–900.
Ramos JW, Townsend DA, Piarulli D, Kolata S, Light K, Hale G, et al. Deletion of PEA-15 in mice is associated with specific impairments of spatial learning abilities. BMC Neurosci. 2009;10:134.
Renault-Mihara F, Beuvon F, Iturrioz X, Canton B, De BS, Leonard N, et al. Phosphoprotein enriched in astrocytes-15 kDa expression inhibits astrocyte migration by a protein kinase C delta-dependent mechanism. Mol Biol Cell. 2006;17(12):5141–52.
Vaidyanathan H, Opoku-Ansah J, Pastorino S, Renganathan H, Matter ML, Ramos JW. ERK MAP kinase is targeted to RSK2 by the phosphoprotein PEA-15. Proc Natl Acad Sci USA. 2007;104(50):19837–42.
Valmiki MG, Ramos JW. Death effector domain-containing proteins. Cell Mol Life Sci. 2009;66(5):814–30.
Watanabe Y, Yamasaki F, Kajiwara Y, Saito T, Nishimoto T, Bartholomeusz C, et al. Expression of phosphoprotein enriched in astrocytes 15 kDa (PEA-15) in astrocytic tumors: a novel approach of correlating malignancy grade and prognosis. J Neurooncol. 2010;100(3):449–457.

PEA-15

► Pea15

Pea15a

► Pea15

Pebble (PBL) in *Drosophila melanogaster*

► Ect2 (Epithelial Cell Transforming 2 Oncogene)

PED

► Pea15

PED/PEA-15

► Pea15

PED-15

► Pea15

Peptide N-Myristoyltransferase

► NMT (N-Myristoyltransferase)

Peptidyl-Prolyl cis-trans Isomerase NIMA-Interacting 1

► Pin1

Peptidyl-Prolyl cis-trans Isomerase Pin1

► Pin1

Peripheral Type CCK Receptor

► Cholecystokinin-1 Receptor

PFT

► Protein Farnesyltransferase

PHLDA1 (Pleckstrin Homology-like Domain, Family A, Member): Alias: PHRIP; TDAG51; DT1P1B11; MGC131738

Maria Aparecida Nagai
Disciplina de Oncologia, Departamento de Radiologia da Faculdade de Medicina da, Centro de Investigação Translacional em Oncologia, Laboratório de Genética Molecular, Instituto do Cancer de São Paulo, Universidade de São Paulo, São Paulo, Brazil

Synonyms

DT1P1B11; MGC131738; PHRIP; TDAG51

Historical Background

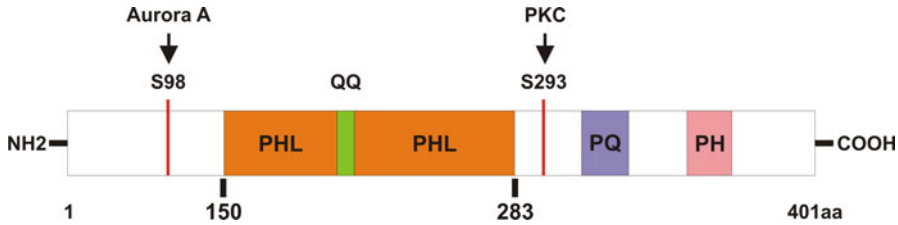
PHLDA1 (pleckstrin homology-like domain, family A, member) gene encodes for a member of an evolutionarily conserved pleckstrin homology-related domain protein family and was first identified as a potential transcription factor required for Fas expression and activation-induced apoptosis in mouse T cell hybridomes (Park et al. 1996). Further, PHLDA1 homologues were identified and isolated in rat (Gomes et al. 1999) and human (Kuske and Johnson 2000), and sequencing comparison showed that mouse, human, and rat PHLDA1 cDNAs exhibit high level of identity. The genes encoding for the members of the pleckstrin homology-related domain protein family are located in different chromosomes, PHLDA1 (also named PHRIP/TDAG51/DT1P1B11/MGC131738; 12q21.2), PHLDA2 (also named IPL/BRWR1C/HLDA2/TSSC3; 11p15.4), and PHLDA3 (also named TIH1; 1q32.1), but have a similar simple gene structure with two exons (one coding exon) and a small intron (Frank et al. 1999).

Protein Structure and Biochemical Function

The human PHLDA1 gene encodes a 401 amino acid protein containing plackstrin homologue (PHL) domain spanning amino acids from 150 to 283 interrupted by a small proline/glutamine rich sequence (QQ) and protein–protein interaction domains in its carboxy-terminal region, as proline-glutamine (PQ) and proline-histidine-rich (PH) tracts (Fig. 1). The PHL domains are evolutionary conserved and found in a wide range of eukariotic proteins (Lemmon et al. 2002). The PHL domains are composed of about 100–120 aa residues with properties to bind phosphatidylinositol lipids (Lemmon et al. 2002). Many proteins containing PHL domains (such as IRS proteins) are found to Interact with membrane components and participate in cell signaling transduction, vesicular trafficking, and cytoskeletal rearrangement (Lemmon and Furgson 2000). Poly-glutamine (QQ), proline-histidine (PH), and proline-glutamine (PO) tracts are well-conserved motifs in eukariotic proteins and were reported to be involved in transcriptional regulation and protein-protein interaction in the development of neurodegenerative diseases (Williamson 1994; Butland et al. 2007). In addition, number variations of QQ repeats are associated with the development of neurodegenerative diseases (Butland et al. 2007). Although the function of the members of the plackstrin homologue (PHL) domain protein family (PHLDA1, PHLDA2, and PHLDA3) in different types of human cells remain to be elucidate, PHLDA1 has been shown to be regulated by different stimuli and associated with cell proliferation, cell survival, and tumorigenesis.

Expression Regulation

PHLDA1 is expressed in several mammalian tissues displaying subcellular localization predominantly in the cytoplasm and nucleoli (Gomes et al. 1999; Hinz et al. 2001; Neef et al. 2002; Oberg et al. 2004; Xi et al. 2007; Nagai et al. 2007). PHLDA1 mRNA and protein expression is induced by different stimuli such as growth factors, differentiation agents, and ER-stress agents (Fig. 2). In T cell hybridome, PHLDA1 is rapidly induced upon TCR activation (Park et al. 1996). In rat neuronal cells, PHLDA1 is induced by fibroblast growth factor (FGF) and differentiation agent (Gomes et al. 1999). Phorbol ester (TPA)/ionomycin induce PHLDA1 expression in different transformed T cell lines (Hinz et al. 2001). PHLDA1 was found to be induced by both PDGFβ and AKT activation in MERAKT cells (Kuhn et al. 2001). IGF-1 induces PHLDA1 mRNA and protein in NIH-3 T3 (NWTb3) cells through IGF-1R via p38MAPK pathway and in normal skin fibroblasts via PI3K pathway (Toyoshima



PHLDA1 (Pleckstrin Homology-like Domain, Family A, Member): Alias: PHRIP; TDAG51; DT1P1B11; MGC131738, Fig. 1 Schematic representation of the modular structure of PHLDA1 protein and phosphorylation sites. PHL (plackstrin homologue domain spanning amino acids from 150 to 283; *QQ* proline/glutamine rich sequence; *PQ* proline-glutamine tracts; and *PH* proline-histidine-rich tracts

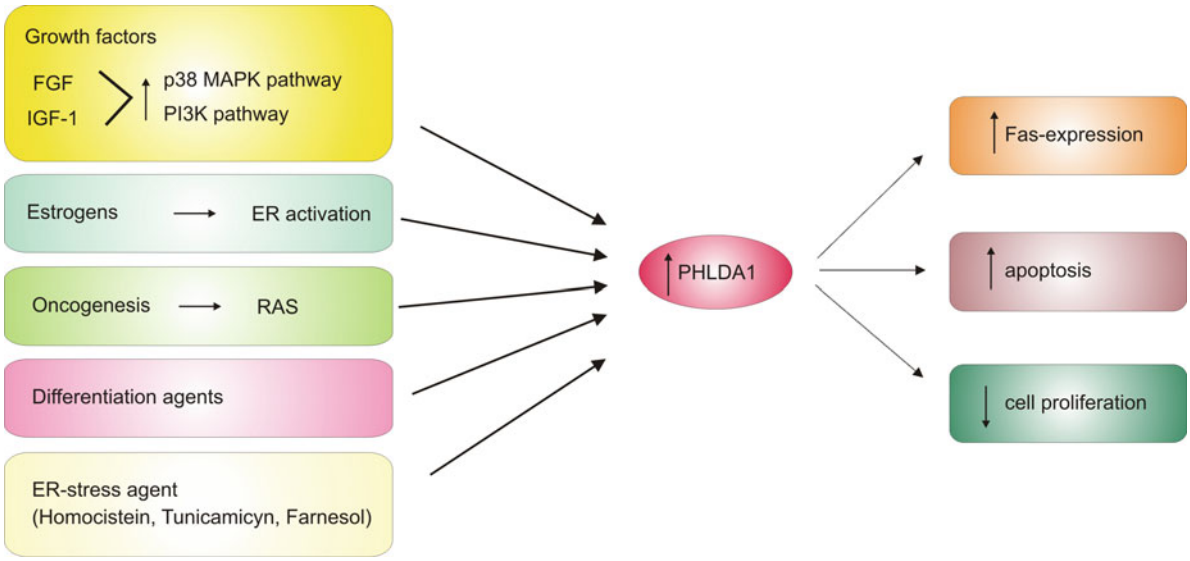
et al. 2004; Wu et al. 2010). In MCF-7 breast cancer cells PHLDA1 is upregulated by estrogen via estrogen receptor (ER) (Marchiori et al. 2008). PHLDA1 expression is also induced by ER stress-inducing agents, such as homocysteine, tunicamycin, and farnesol in different cell types (Hossain et al. 2003; Joo et al. 2007).

Role in Cell Death

Several experimental evidences consistently indicate that PHLDA1 is involved in cell death induction. The pro-apoptotic activity of PHLDA1 was first demonstrated in mouse T cell hybridome where PHLDA1 is required for Fas(CD95)-expression, which plays an important role in TCR-induced apoptosis (Park et al. 1996). PHLDA1 expression was found to be dependent upon the activity of PKC, and PKC activation only induces Fas-expression in cells expressing wild type PHLDA1 (Wang et al. 1998). However, Rho et al. (2001) demonstrated that PHLDA1−/− mice express normal levels of Fas and had normal T-cell apoptosis, indicating that PHLDA1 is not essential for Fas-expression. Microinjection of differentiating H19-7 cells with a neutralizing antibody anti-PHLDA1 increases cell survival, and transient PHLDA1 expression decreases cell survival in both differentiating and non-differentiating H19-7 cells, indicating that PHLDA1 acts as a mediator of differentiation-associated apoptosis in H19-7 hippocampal cells (Gomes et al. 1999). In 293 T cells, PHLDA1 was found to interact with two mRNA-binding proteins, eIF3-p66 and PABP, and PHLDA1 co-transfection with the luciferase gene leads to protein biosynthesis inhibition, a process important in the execution of apoptosis (Hinz et al. 2001). Overexpression of PHLDA1 in endothelial cells leads to changes in cell morphology, decreases cell adhesion, and promotes detachment-mediated cell death (Hossain et al. 2003). Knockdown of PHLDA1 abolish the ability of IGF-1 to prevent NIH-3 T3 (NWTb3) cells from undergoing apoptosis in response to serum starvation (Toyoshima et al. 2004). Constitutive PHLDA1 expression in different melanoma-derived cell lines leads to reduced cell growth, reduced colony formation, and increased basal apoptosis (Neef et al. 2002). Stress-induced heat shock proteins (Hsp70, Hsp110, and Hsp40) directly bind to the pleckstrin domain of PHLDA1 and suppress its pro-apoptotic function in MEF cells (Hayashida et al. 2006). The promoter region of the PHLDA1 has HSE-binding sites where the heat shock transcription factor 1 (HSF1) binds and modulates PHLDA1 expression; in addition, HSF1 is able to directly bind to and activate PHLDA1 in response to heat shock in male germ cells (Hayashida et al. 2006). Moreover, during the time-course induction of cryptorchidism rat testis, Hsf1 and Phlda1 are strongly expressed, suggesting that the Hsf1/Phlda1 pathways play an important role in the apoptosis of primary spermatocytes in criptorchid testis (Liu et al. 2011).

Role in Cancer

Evasion from apoptosis or programmed cell death, which is an active, energy-dependent process involving biochemical and molecular events regulated by a series of distinct genes, is a hallmark of cancer (Hanahan and Weinberg 2000, 2011). Several experimental studies provided evidences that PHLDA1 plays a role in apoptosis induction and cell proliferation, cell adhesion, and migration. Alterations in the PHLDA1 mRNA and/or protein expression were observed in different types of tumors, such as melanomas, breast



PHLDA1 (Pleckstrin Homology-like Domain, Family A, Member): Alias: PHRIP; TDAG51; DT1P1B11; MGC131738, Fig. 2 Schematic illustration for PHLDA1 in cell signaling and function. PHLDA1 can be up-regulate by several stimuli, such as growth factor cell signaling pathways, estrogen receptor activation, ras activation, and ER-stress agents. In addition, PHLDA1 up-regulation leads to an increase in Fas-expression, enhanced apoptosis and decreased cell proliferation

tumors, and intestinal tumors. Decreased PHLDA1 protein levels were demonstrated in melanomas (Neef et al. 2002) and breast carcinomas (Nagai et al. 2007; Johnson et al. 2011). On the other hand, PHLDA1 was found to be strongly expressed in human intestinal adenomas and the majority of intestinal carcinomas and PHLDA1 knockdown in colon cancer cells inhibits anchorage-independent cell growth and reduces cell migration (Sakthianandeswaren et al. 2011).

PHLDA1 reduced expression was found in metastatic as compared with primary melanoma cells, suggesting that PHLDA1 downregulation may contribute to the progression of malignant melanomas (Neef et al. 2002). Constitutive PHLDA1 in different melanoma-derived cell lines leads to reduced cell growth, colony formation, increased basal apoptosis, and increased sensitivity to apoptosis induced by chemotherapeutic agents, doxorubicin and camptothecin (Neef et al. 2002). Expression profiling analysis identified the PHLDA1 transcripts to be overexpressed in hair follicular stem cells (Ohyama et al. 2006). A recent study identified consistent positive immunoreactivity for PHLDA1 in trichoepitheliomas and negative immunoreactivity in basal cell carcinomas, indicating that PHLDA protein expression may allow differential diagnosis between trichoepitheliomas and basal cell carcinomas (Sellheyer and Nelson 2011). PHLDA1 protein downregulation was a strong predictor of poor prognosis for breast cancer patients and patients with tumors showing reduced PHLDA1 expression and paucity for ER has the worse outcome (Nagai et al. 2007). Reduction of PHLDA1 expression in HME16C mammary epithelial cells during ras-mediated cellular transformation induces proliferation under anchorage-independent conditions and results in increase in ERK activation, suggesting that PHLDA1 opposes ERK-mediated proliferation in breast cancer cells (Oberst et al. 2008). Recently, a regulatory loop between Aurora-A and PHLDA1 was observed in breast cancer cells (Johnson et al. 2011). In MDA-MB-231 breast cancer cells, Aurora-A predominantly phosphorylates PHLDA1 at serine residue 98 (Ser98) leading to its degradation and PHLDA1 overexpression results in decreased levels of Aurora-A, suggesting that PHLDA1 acts as a negative regulator of Aurora-A-mediated breast oncogenesis (Johnson et al. 2011).

Summary

In summary, several reports provide evidence that PHLDA1 plays an important role in apoptosis

induction and cell proliferation inhibition leading to reduced cell survival; however, little is known about the mechanisms or cell signaling pathways involved in the pro-apoptotic functions of PHLDA1 in different cell types. PHLDA1 is induced by different stimuli, including ER-stress agents, cell differentiation agents, and mitogenic agents, such as growth factors and estrogens, suggesting that PHLDA1 may play diverse roles in addition to apoptosis. Downregulation of PHLDA1 has been shown in skin cancer and breast cancer biopsies, indicating a tumor-suppressive function for PHLDA1; however, new clinical studies are required to evaluate if this suppressive effect is restricted to certain types of tumors or is common to the majority of human tumors. In melanoma cells, PHLDA1 expression increases chemosensitivity to doxorubicin and camptothecin; it will be interesting to see if and how PHLDA1 improves efficacy to chemotherapeutic drugs in different cell types. Further experimental studies in vitro and in informative and testable animal models are required to *improve the understanding* of PHLDA1 mechanisms of action both in normal and cancer cells and its role as a prognostic and predictive factor in cancer.

References

Butland SL, Devon RS, Huang Y, Mead CL, Meynert AM, Neal SJ, Lee SS, Wilkinson A, Yang GS, Yuen MM, Hayden MR, Holt RA, Leavitt BR, Ouellette BF. CAG-encoded polyglutamine length polymorphism in the human genome. BMC Genomics. 2007;8:126.

Frank D, Mendelsohn CL, Ciccone E, Svensson K, Ohlsson R, Tycko B. A novel pleckstrin homology-related gene family defined by Ipl/Tssc3, TDAG51, and Tih1: tissue-specific expression, chromosomal location, and parental imprinting. Mamm Genome. 1999;10(12):1150–9.

Gomes I, Xiong W, Miki T, Rosner MR. A proline- and glutamine-rich protein promotes apoptosis in neuronal cells. J Neurochem. 1999;73(2):612–22.

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57–70.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.

Hayashida N, Inouye S, Fujimoto M, Tanaka Y, Izu H, Takaki E, Ichikawa H, Rho J, Nakai A. A novel HSF1-mediated death pathway that is suppressed by heat shock proteins. EMBO J. 2006;25(20):4773–83.

Hinz T, Flindt S, Marx A, Janssen O, Kabelitz D. Inhibition of protein synthesis by the T cell receptor-inducible human TDAG51 gene product. Cell Signal. 2001;13(5):345–52.

Hossain GS, van Thienen JV, Werstuck GH, Zhou J, Sood SK, Dickhout JG, de Koning AB, Tang D, Wu D, Falk E, Poddar R, Jacobsen DW, Zhang K, Kaufman RJ, Austin RC. TDAG51 is induced by homocysteine, promotes detachment-mediated programmed cell death, and contributes to the cevelopment of atherosclerosis in hyperhomocysteinemia. J Biol Chem. 2003;278(32):30317–27.

Johnson EO, Chang KH, de Pablo Y, Ghosh S, Mehta R, Badve S, Shah K. PHLDA1 is a crucial negative regulator and effector of Aurora A kinase in breast cancer. J Cell Sci. 2011;124(Pt 16):2711–22.

Joo JH, Liao G, Collins JB, Grissom SF, Jetten AM. Farnesol-induced apoptosis in human lung carcinoma cells is coupled to the endoplasmic reticulum stress response. Cancer Res. 2007;67(16):7929–36.

Kuhn I, Bartholdi MF, Salamon H, Feldman RI, Roth RA, Johnson PH. Identification of AKT-regulated genes in inducible MERAkt cells. Physiol Genomics. 2001;7(2):105–14.

Kuske MD, Johnson JP. Assignment of the human PHLDA1 gene to chromosome 12q15 by radiation hybrid mapping. Cytogenet Cell Genet. 2000;89(1–2):1.

Lemmon MA, Ferguson KM. Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem J. 2000;350(Pt 1):1–18.

Lemmon MA, Ferguson KM, Abrams CS. Pleckstrin homology domains and the cytoskeleton. FEBS Lett. 2002;513(1):71–6.

Liu F, Xu ZL, Qian XJ, Qiu WY, Huang H. Expression of Hsf1, Hsf2, and Phlda1 in cells undergoing cryptorchid-induced apoptosis in rat testes. Mol Reprod Dev. 2011;78(4):283–91. doi:10.1002/mrd.21304.

Marchiori AC, Casolari DA, Nagai MA. Transcriptional up-regulation of PHLDA1 by 17beta-estradiol in MCF-7 breast cancer cells. Braz J Med Biol Res. 2008;41(7):579–82.

Nagai MA, Fregnani JH, Netto MM, Brentani MM, Soares FA. Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. Breast Cancer Res Treat. 2007;106(1):49–56.

Neef R, Kuske MA, Pröls E, Johnson JP. Identification of the human PHLDA1/TDAG51 gene: down-regulation in metastatic melanoma contributes to apoptosis resistance and growth deregulation. Cancer Res. 2002;62(20):5920–9.

Oberg HH, Sipos B, Kalthoff H, Janssen O, Kabelitz D. Regulation of T-cell death-associated gene 51 (TDAG51) expression in human T-cells. Cell Death Differ. 2004;11(6):674–84.

Oberst MD, Beberman SJ, Zhao L, Yin JJ, Ward Y, Kelly K. TDAG51 is an ERK signaling target that opposes ERK-mediated HME16C mammary epithelial cell transformation. BMC Cancer. 2008;8:189.

Ohyama M, Terunuma A, Tock CL, Radonovich MF, Pise-Masison CA, Hopping SB, Brady JN, Udey MC, Vogel JC. Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest. 2006;116(1):249–60.

Park CG, Lee SY, Kandala G, Lee SY, Choi Y. A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. Immunity. 1996;4(6):583–91.

Rho J, Gong S, Kim N, Choi Y. TDAG51 is not essential for Fas/CD95 regulation and apoptosis in vivo. Mol Cell Biol. 2001; 21(24): 8365–8370; Erratum in: Mol Cell Biol. 2002; 22(4):1276.

Sakthianandeswaren A, Christie M, D'Andreti C, Tsui C, Jorissen RN, Li S, Fleming NI, Gibbs P, Lipton L, Malaterre J, Ramsay RG, Phesse TJ, Ernst M, Jeffery RE, Poulsom R, Leedham SJ, Segditsas S, Tomlinson IP, Bernhard OK, Simpson RJ, Walker F, Faux MC, Church N, Catimel B, Flanagan DJ, Vincan E, Sieber OM. PHLDA1 expression marks the putative epithelial stem cells and contributes to intestinal tumorigenesis. Cancer Res. 2011;71(10):3709–19.

Sellheyer K, Nelson P. Follicular stem cell marker PHLDA1 (TDAG51) is superior to cytokeratin-20 in differentiating between trichoepithelioma and basal cell carcinoma in small biopsy specimens. J Cutan Pathol. 2011;38(7):542–50. doi:10.1111/j.1600-0560.2011.01693.x.

Toyoshima Y, Karas M, Yakar S, Dupont J, Lee Helman, LeRoith D. TDAG51 mediates the effects of insulin-like growth factor I (IGF-I) on cell survival. J Biol Chem. 2004;279(24):25898–904.

Wang R, Zhang L, Yin D, Mufson RA, Shi Y. Protein kinase C regulates Fas (CD95/APO-1) expression. J Immunol. 1998;161(5):2201–7.

Williamson MP. The structure and function of proline-rich regions in proteins. Biochem J. 1994;297(Pt 2):249–60.

Wu S, Walenkamp MJ, Lankester A, Bidlingmaier M, Wit JM, De Luca F. Growth hormone and insulin-like growth factor I insensitivity of fibroblasts isolated from a patient with an I{kappa}B{alpha} mutation. J Clin Endocrinol Metab. 2010;95(3):1220–8.

Xi ZQ, Wang LY, Sun JJ, Liu XZ, Zhu X, Xiao F, Guan LF, Li JM, Wang L, Wang XF. TDAG51 in the anterior temporal neocortex of patients with intractable epilepsy. Neurosci Lett. 2007;425(1):53–8.

Phosphatidylinositol (3,4,5)-Trisphosphate-Dependent Rac Exchanger

► P-Rex

Phosphatidylinositol 3,4,5-Trisphosphate-Dependent RAC Exchanger 2

► P-Rex

Phosphatidylinositol 3-Kinase

► Phosphoinositide 3-Kinase

Phosphatidylinositol 3-OH Kinase

► Phosphoinositide 3-Kinase

Phosphatidylinositol 5-phosphate 4-kinase

Jonathan H. Clarke and Robin F. Irvine
Department of Pharmacology, University of Cambridge, Cambridge, UK

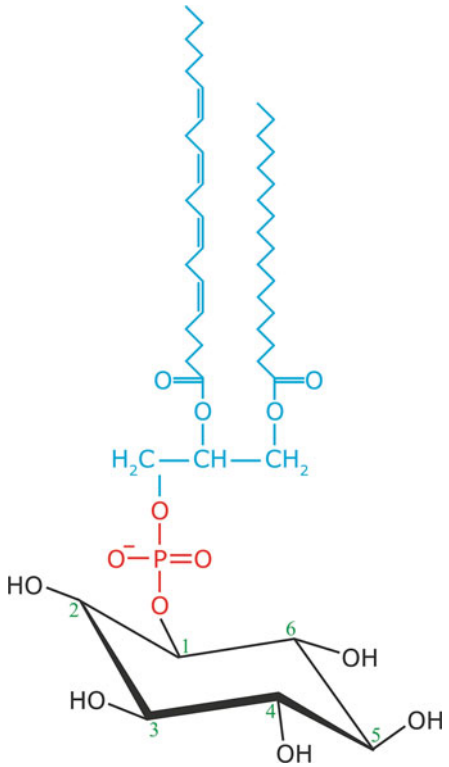
Synonyms

PIP kinase; PI5P4K; PIP4K; PIP5K2; PIPKII; PIPkin; PtdIns(5)*P* 4-kinase; PtdIns*P* kinase Type II; Type II PIP kinase; Type II PIPK

Historical Background

Phosphatidylinositol 5-phosphate 4-kinase (PIP4K) is an enzyme activity capable of converting a mono-phosphorylated lipid substrate into a bis-phosphorylated product, a reaction that is fundamental in the maintenance of the cellular phosphoinositide (PI) cycle. PIP4K catalyzes the addition of a phosphate group to position D-4 of the inositol head-group of PtdIns5*P* (Figs. 1 and 2). Downstream effects of signaling molecules generated by the PI cycle are diverse and include vesicle trafficking, ion channel activity, cytoskeletal dynamics, cell differentiation, proliferation, and apoptosis (Toker 2002; Irvine 2003; Di Paolo and De Camilli 2006; Gonzales and Anderson 2006).

Pioneering work in the early 1950s led to the elucidation of the roles of multiple kinases and phosphatases required to generate the diverse array of inositol lipid species in the PI cycle and in the numerous regulatory, signaling, and metabolic pathways that they are involved in (Hinchliffe et al. 1998; Anderson et al. 1999). The first activities, isolated from human erythrocytes, to convert PtdIns4*P* to PtdIns(4,5)P_2 were classified as Type I and Type II enzymes (Bazenet et al. 1990). Subsequent studies revealed that these two enzymes had distinct substrate



Phosphatidylinositol 5-phosphate 4-kinase, Fig. 1 Molecular structure of PtdIns (1[stearoyl], 2[arachidonoyl]-diacyl-*sn*-glycero-3-phospho-[1-D-*myo*-inositol]). Stearoyl (C18:0) and arachidonyl (C20:6) fatty acid chains on the diacylglycerol moiety are the commonest substitutions found in cellular phosphoinositides. The hydrophilic diacylglycerol "tail" sits within the membrane while positions D-3, D-4, and D-5 of the inositol head-group are available for phosphorylation

specificities, and that the Type II enzyme was in fact solely utilizing PtdIns5*P*, a contaminant of the commercial PtdIns4*P* substrate in the assay (Rameh et al. 1997). Although the preferred substrate of PIP4K is PtdIns5*P* it has also been shown to phosphorylate PtdIns3*P* in vitro (Fig. 2), producing PtdIns(3,4)P_2 (Hinchliffe et al. 1998; Clarke et al. 2007). Both of these substrates are minor components of the typical cellular phosphoinositide content, PtdIns5*P* and PtdIns3*P* constituting 2% and 5% of total PtdIns*P*, respectively, the majority being PtdIns4*P* (Toker 2002). As a consequence, the contribution of PIP4K activity to cellular PtdIns(4,5)P_2 synthesis is thought to be restricted to specific isolated compartments, or the role of the enzyme is to attenuate the putative signaling function of PtdIns5*P* itself. Three mammalian PIP4K isoforms have so far been discovered (α, β, and γ), with no known splice variants to date, and each has been shown to have a specific distribution and subcellular localization (Clarke et al. 2010).

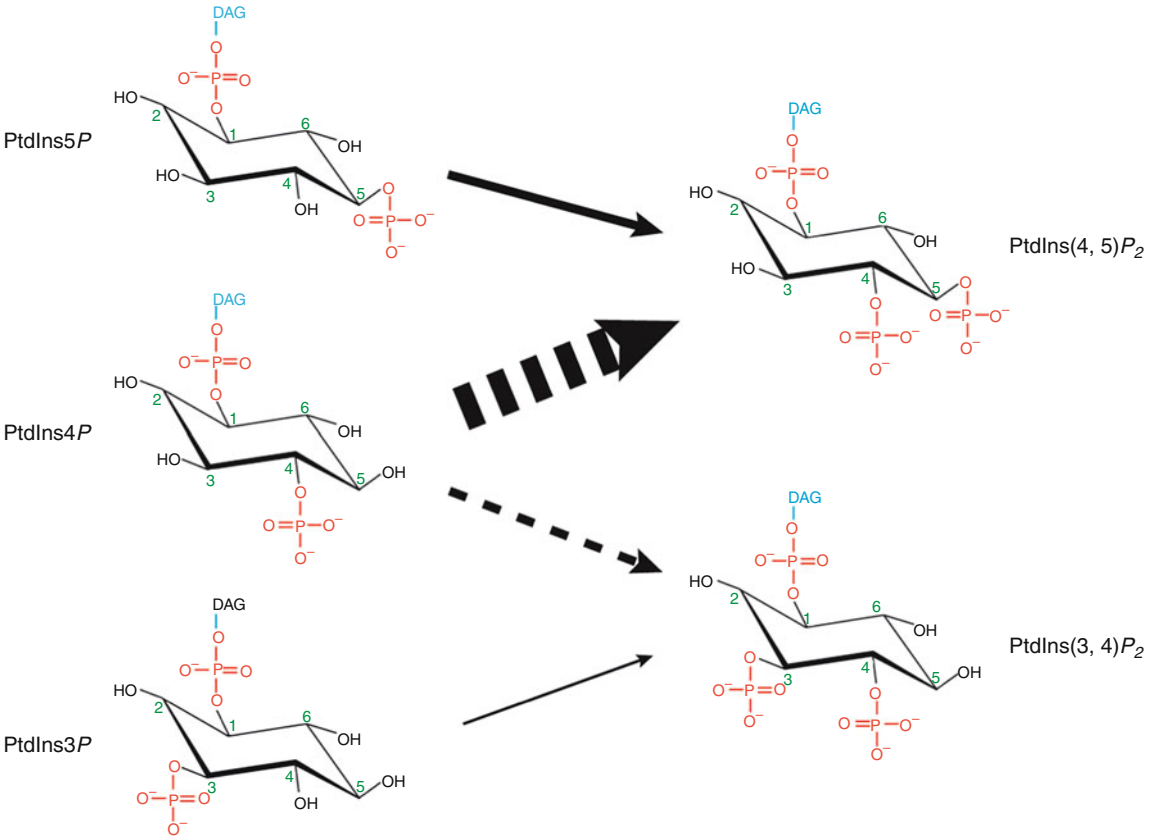
PIP4K Molecular Evolution

Homologs to genes encoding PIP4K proteins have been discovered in over 50 species with completed genomic sequences. Early divergence of the PIP4K and phosphatidylinositol 4-phosphate 5-kinase (PIP5K) enzymes (Fig. 3), which share significant identity over the conserved kinase-core domain (discussed below), presumably occurred by gene duplication, as did the later emergence of PIP5KL1, an inactive scaffolding protein for PIPKs (Chang et al. 2004). Lower eukaryotes, such as the yeast *Saccharomyces cerevisiae*, do not have an identified PIP4K activity, and within the plant kingdom, 11 of the *Arabidopsis thaliana* atPIPK proteins have sequence similarity to both PIP4K and PIP5Ks although the examples studied so far use PtdIns4*P* as substrate (Mueller-Roeber and Pical 2002). A single PIP4K activity is present in nematodes such as *Caenorhabditis elegans* (PPK-2), arthropods such as *Drosophila melanogaster* (dPIP4K), and urochordates such as *Ciona intestinalis*. The later divergences produce first PIP4Kγ, then PIP4Kα and PIP4Kβ (Fig. 3). PIP4Kα is present in all vertebrates, with a few organisms missing either PIP4Kβ or PIP4Kγ; the exception of the latter isoform from any of the bird genomes completed to date is particularly notable. Within the human genome each isoform is present as a single-copy gene, with *PIP4K2A* (coding for PIP4Kα) located on chromosome 10 (p12.2), *PIP4K2B* (coding for PIP4Kβ) on chromosome 17 (q21.2), and *PIP4K2C* (coding for PIP4Kγ) on chromosome 12 (q13.2).

PIP4K Structure

Although the PIP4K isoforms have a significant level of identity at the amino acid level, they share only 30% identity with the phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks) and constitute a novel family with no significant identity to any other lipid or protein kinases (Anderson et al. 1999).

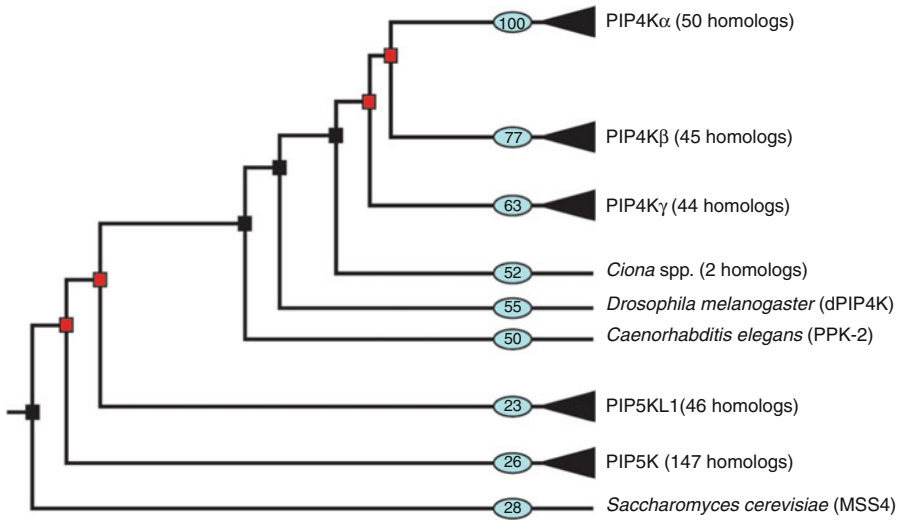
Homology between the PIPKs is mostly limited to the large kinase core domain (Fig. 4) as defined in



Phosphatidylinositol 5-phosphate 4-kinase, Fig. 2 Production of PtdInsP_2 from PtdIns$P$. PIP4K is able to use two of the three possible PtdIns$P$ species as substrates (in vitro), producing PtdIns(4,5)P_2 from PtdIns5P and PtdIns(3,4)P_2 from PtdIns3P (*filled arrows*, relative substrate preference indicated by width). Alternative routes of production (from PtdIns4P) by PI3K (PtdIns(3,4)P_2) and PIP5K (PtdIns(4,5)P_2) are also shown (*dashed arrows*, relative contribution indicated by width), although other phosphoinositides are omitted

deletion studies by Ishihara et al. and structural similarity alignment by Rao et al. (reviewed in Hinchliffe et al. 1998; Anderson et al. 1999). Regions of low similarity outside of the kinase core define the functional differences between the PIP4K and PIP5Ks. Three-dimensional structure by X-ray crystallography has been elucidated for both the PIP4Kβ (3.0 Å resolution, PDB i.d. 1BO1, Rao et al. 1998) and the PIP4Kγ (2.8 Å resolution, PDB i.d. 2GK9, Thorsell, Protein Data Bank online submission) isoforms, which share 79% similarity by structural comparison. These data show the PIP4K structure to be two domains arranged across a central cleft, which has catalytic and ATP-binding residues conserved with a protein kinase, PKA. Four conserved catalytic residues are identified, and the mutation of at least one (aspartate 273 in PIP4Kα, or aspartate 278 in PIP4Kβ) results in biochemical inactivity. This active site is open on one side, such that the proposed binding of ATP and a phospholipid head-group would accommodate PtdIns5P or PtdIns3P (Rao et al. 1998).

The N-terminal domain has seven β-sheets and four α-helices, and the crystal structure shows that the protein homodimerizes along the interface of two adjacent β1 strands (Fig. 4) to form a broad, flat face that is able to contact cell membranes by means of electrostatic interaction with lipid head-groups (Rao et al. 1998). Recent data has also shown the potential of the PIP4K isoforms to heterodimerize, suggesting a possible means of inter-isoform regulation based on protein expression levels in different tissues (Bultsma et al. 2010; Wang et al. 2010). This domain also contains a putative ATP-stabilizing G-loop motif (Rao et al. 1998).



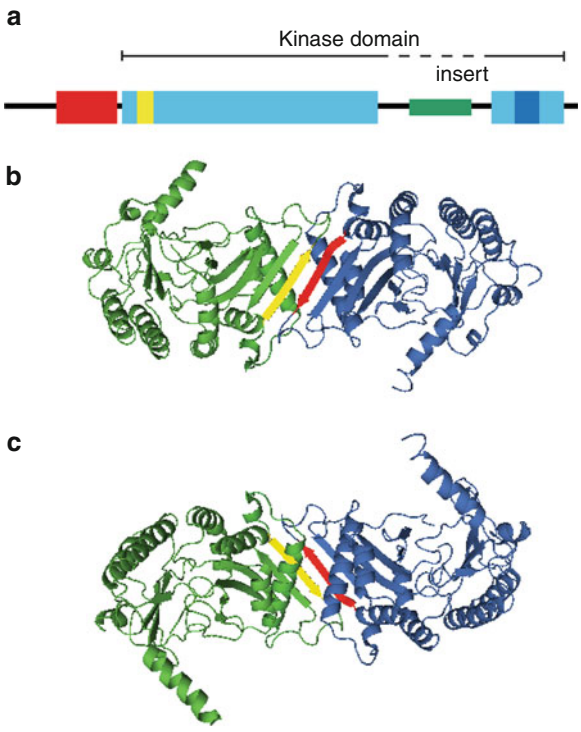
Phosphatidylinositol 5-phosphate 4-kinase, Fig. 3 Phylogenetic plot of PIP4K gene evolution with all currently annotated genes in the Ensembl database (EMBL-EBI). *Branch points* indicate divergence, *black nodes* represent speciation, and *red nodes* represent a potential gene duplication event. Species with single genes with similarity to human PIP4K are listed by name (gene name in brackets). Separate PIPK gene branches indicate number of total homologs (orthologs of PIP4Ks and PIP5KL1, orthologs plus paralogs of PIP5K) in vertebrates (48 species with PIP4Kα, 44 species with PIP4Kβ, and 38 species with PIP4Kγ). *Boxed numbers* represent percentage similarity of human homologs (or individual *D. melanogaster*, *C. elegans*, *S. cerevisiae*, and *Ciona* genes) to human PIP4Kα protein sequence (average similarity for *Ciona* species and PIP5K isoforms)

The C-terminal domain consists of five β-sheets and four α-helices and contains the "activation loop" and "variable insert" (Fig. 4). The activation loop region (amino acids 373–391) confers substrate specificity as demonstrated by domain swapping between PIP4K and PIP5K enzymes (Kunz et al. 2000). The variable insert region interrupts the catalytic kinase core and represents the region of least similarity between the PIP4K isoforms. In PIP4Kβ the α7 helix in this insert serves as a specific nuclear localization motif (Ciruela et al. 2000), and other inter-isoform differences could be accounted for in this region.

Mammalian PIP4K Isoforms

PIP4Kα

The PIP4Kα isoform was the first to be cloned independently by two groups in 1995, from human circulating-leukocyte and bone marrow cDNA libraries (Boronenkov and Anderson 1995; Divecha et al. 1995). It is the most catalytically active of the three isoforms (Table 1). Northern blotting suggests that the enzyme has a ubiquitous but low expression in tissues compared to the other isoforms, with slightly elevated levels in brain and hematocytes (reviewed in Clarke et al. 2010), which is confirmed in subsequent quantitative PCR experiments (Fig. 5). Overexpression studies in DT40 and HeLa cells suggest that PIP4Kα is predominantly cytosolic (Clarke et al. 2007; Richardson et al. 2007) and has been observed to translocate to the cytoskeleton upon integrin-mediated signaling, potentially via interaction with a PIP5K activity (Hinchliffe et al. 2002). Translocation of a PIP4K enzyme was also seen in agonist-induced platelet α-granule secretion, and this activity was regulated by PKC activation and attenuated by calpain cleavage (Rozenvayn and Flaumenhaft 2003; O'Connell et al. 2005). Recent studies, using genomically tagged PIP4Kα and high-resolution mass spectrometry, were able to assess endogenous protein in DT40 cells and show that significant amounts of this enzyme are also found in the nucleus. This distribution is thought to occur via association with the PIP4Kβ isoform (Bultsma et al. 2010; Wang et al. 2010) as discussed above. The PIP4Kα isoform activity is regulated by phosphorylation on different serine and threonine residues. Cell stimulation results in both removal of inhibitory phosphorylation, such as at threonine 376 by protein kinase D, and further activating



Phosphatidylinositol 5-phosphate 4-kinase, Fig. 4 PIP4K structure. (**a**) The PIP4K domain structure shows that the kinase catalytic core domain (*light blue*) is interrupted by the "variable insert," a region with the least sequence similarity between the three isoforms (*green*). The C-terminal kinase domain contains the "activation loop" (*dark blue*) and the N-terminal domain contains the "G-loop" motif (*yellow*). The dimerization region is shown in *red*. (**b**, **c**) Ribbon diagrams of the crystallographic structure of PIP4Kβ as a homodimer (Rao et al. 1998) with the dimerization region highlighted (*green* monomer with *yellow* region, *blue* monomer with *red* region). Views are from the membrane contact surface (**b**), showing the flattened face, and from the cytosolic side (**c**), showing the stabilization of the dimerization interface by α-helices

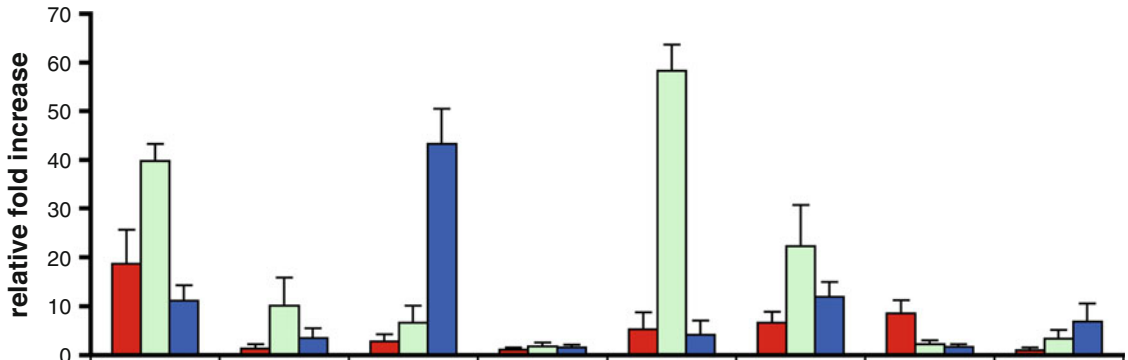
Phosphatidylinositol 5-phosphate 4-kinase, Table 1 Comparison of activities of the PIP4K isoforms. Experiments were carried out as in (Wang et al. 2010). Specific activity is defined as nmoles of ATP incorporated into lipid per minute per mg of protein under the same assay conditions. Turnover number (k_{cat}) is defined as the number of ATP (hence PtdIns5*P* to PtdIns(4,5)P_2) reactions per second

PIP4K isoform	Specific activity	K_M for ATP (μM)	k_{cat} (s^{-1})
PIP4Kα	3.7×10^{-2} (± 0.12)	03.9	2105.1
PIP4Kβ	1.6×10^{-5} (± 0.08)	67.1	0019.1
PIP4Kγ	3.7×10^{-7} (± 0.12)	94.2	0001.1

phosphorylation (Hinchliffe and Irvine 2006). Protein kinase CK2 also specifically phosphorylates serine 304, although this has not been shown to affect enzyme activity (Hinchliffe et al. 1999). Upregulation of PIP4Kα activity has also been observed on tyrosine phosphorylation in bovine photoreceptor rod outer segments, although this is thought to be an indirect response (Huang et al. 2001).

PIP4Kβ

Yeast two-hybrid screening of a murine cerebellar cDNA library, using a cytoplasmic domain of the human p55 TNF-α receptor as bait, resulted in the cloning of the PIP4Kβ isoform (Castellino et al. 1997). Although this isoform has 77% amino acid similarity to PIP4Kα, it has 100-fold less in vitro activity by catalytic turnover (Table 1). PIP4Kβ mRNA was detected in all tissues tested, and was found to be specifically enriched in skeletal muscle and brain (Fig. 5 and Castellino et al. 1997). At a subcellular level, although PIP4Kβ has been shown to associate with the TNF-α, EGF, and ErbB2 (HER2) receptors (Castellino et al. 1997; Castellino and Chao 1999), and undoubtedly has a cytoplasmic role, it is also the only isoform to specifically localize to the cell nucleus. The targeting of PIP4Kβ to the nucleus is affected by a nuclear localization sequence unique to this isoform (Ciruela et al. 2000), and genomic tagging indicates that the majority of endogenous PIP4Kβ is transported across the nuclear membrane in DT40 cells (Richardson et al. 2007). Activation of PIP4Kβ has been shown to be mediated by TNF-α in specific cytokine-responsive cells (Castellino et al. 1997). Within the nucleus, PIP4Kβ activity is inhibited by p38 mitogen activated protein kinase (MAPK) phosphorylation of serine 326 after ultraviolet irradiation, leading to increases in nuclear PtdIns5*P*, which is sensed by the cell stress–responsive nuclear adapter protein ING2 (Jones et al. 2006); note that this residue is also present in PIP4Kα, so it is possible that nuclear PIP4Kα (see above) is similarly regulated. PIP4Kβ that is co-localized to nuclear speckles (pre-mRNA processing sites) with the Cul3-SPOP ubiquitin ligase complex is itself targeted for degradation by ubiquitylation (Bunce et al. 2008). Intriguingly, PtdIns5*P* activation of p38 MAPK stimulates the activity of the Cul3-SPOP complex, suggesting that a feed-forward mechanism of regulation exists, which could in part be explained by the interaction between PIP4Kβ and PIP4Kα in the nucleus (Bultsma et al. 2010). There is also evidence that



	BRAIN	LIVER	KIDNEY	LUNG	MUSCLE	HEART	SPLEEN	TESTIS
PIP5K2A	18.67	1.36	2.83	1.11	5.34	6.64	8.62	1.00
PIP5K2B	39.78	10.11	6.71	1.80	58.24	22.26	2.31	3.38
PIP5K2C	11.16	3.45	43.19	1.54	4.14	11.93	1.72	6.87

Phosphatidylinositol 5-phosphate 4-kinase, Fig. 5 Expression of PIP4K mRNA in mouse tissues. Expression of *PIP4K2A*, *PIP4K2B*, and *PIP4K2C* was determined by quantitative PCR using the comparative threshold cycle (CT) method (As described in Clarke et al. 2008)

PIP4Kβ is able to regulate insulin-induced signaling in cells by an indirect mechanism. Carricaburu et al. showed that PIP4Kβ regulated PtdIns5*P* levels were responsible for activation of a PtdIns(3,4,5)P_3-specific 5-phosphatase, which removed this PI 3-kinase (PI3K) signaling product, resulting in reduced Akt/PKB protein kinase phosphorylation (Carricaburu et al. 2003).

PIP4Kγ

The PIP4Kγ isoform is the most recent to be discovered, and was isolated from a rat brain cDNA library by Itoh et al. in 1998. PIP4Kγ shares approximately 63% similarity with PIP4Kα and PIP4Kβ at the protein level, but has a 2,000-fold lower in vitro catalytic turnover of PtdIns5*P* than the PIP4Kα isoform (Table 1), and no detectable activity against any other phosphoinositide substrates. Highest levels of PIP4Kγ expression are observed in the kidney and brain (Fig. 5), with significant levels also in the heart, ovary, and testis (Itoh et al. 1998; Clarke et al. 2008, 2009). Within the kidney, the expression is limited to the distal nephron and restricted to the epithelial cells of the thick ascending limb of the loop of Henle and to intercalated cells of the collecting duct (Clarke et al. 2008). Expression of PIP4Kγ is also seen at early developmental stages in the zebra fish embryo, localized to the pronephric duct and brain. In the mouse brain PIP4Kγ has a restricted expression, found only in specific neuronal subpopulations, such as cerebellar Purkinje cells, hippocampal pyramidal cells, and mitral cells in the olfactory bulb, and is excluded from granule cells (Clarke et al. 2009). At a subcellular level, endogenous PIP4Kγ is partially associated with the *cis*-Golgi matrix and predominantly with an unidentified vesicular compartment, which in kidney epithelial cells is concentrated at the apical secretory membrane (Clarke et al. 2008). PIP4Kγ undergoes protein phosphorylation on serine residues in vivo, and at least two phosphorylated forms are differentially expressed in mouse brain regions (Itoh et al. 1998; Clarke et al. 2009). In a β-pancreatic cell line, the mTORC1 complex is thought to be responsible for phosphorylation of PIP4Kγ at two specific sites (L. Rameh, personal communication 2011), although it is not known whether these modifications lead to in vivo activation of the enzyme. Phosphorylation may be regulated by mitogenic cell stimulation with EGF or PDGF, and to a lesser extent with lysophosphatidic acid or bradykinin (Itoh et al. 1998). Enzyme expression may also be regulated in thyrocytes by thyroid-stimulating hormone (Park et al. 2001).

PIP4Ks and Disease

Aberrant regulation of cellular phosphoinositides has been implicated in a number of diseases including cancer and diabetes. Interference with PtdIns(3,4,5)P_3 regulation of Akt signaling via enzymes such as PI3K and the phosphatases PTEN and SHIP1, are linked to many forms of cancer (McCrea and De Camilli 2009). PtdIns(4,5)P_2 can also regulate ion channels, the dysfunction of which can lead to epilepsy and rare forms of congenital channelopathies (Halstead et al. 2005). In these cases any involvement of the PIP4Ks would be indirect, via the downstream production of lipid substrates and second messengers themselves. However, some direct effects of the PIP4Ks in disease have been reported.

PIP4Kβ, as discussed above, has been implicated in the regulation of Akt/PKB activation during insulin signaling by reducing PtdIns5*P* levels that inhibit the enzymatic removal of PtdIns(3,4,5)P_3 (Carricaburu et al. 2003). In agreement, PIP4Kβ-/- knockout mice are viable but hypersensitive to insulin, suggesting that the presence of the PIP4Kβ substrate, PtdIns5*P*, is directly required for maintenance of PtdIns(3,4,5)P_3 levels for enhanced Akt/PKB phosphorylation. Removal of PtdIns5*P* by PIP4Kβ reduces the effectiveness of insulin signaling, which may have consequences in type 2 diabetes (Lamia et al. 2004).

Early reports suggest that PIPK activity was upregulated in malignant tumors (Singhal et al. 1994). More recent studies have shown that as well as PIP4Kβ interacting with the HER2b receptor (see above), the *PIP4K2B* gene is located close to the *HER2* locus on chromosome 17. Gene amplification of *HER2* occurs in 25% of breast cancer cases, and increased levels of PIP4Kβ have also been detected in these tumors (Luoh et al. 2004). *PIP4K2B* has also been linked to neuroblastoma development, as this gene is one of three to be disrupted by a translocation breakpoint characteristic of this tumor type (Schleiermacher et al. 2005).

Treatment of bipolar disorder with lithium is thought to be involved with phosphoinositide metabolism. Subsequent linkage studies have implicated PIP4Kα as a candidate gene for susceptibility to schizophrenia, either through a common single nucleotide polymorphism or transcriptional repression (Stopkova et al. 2003).

Summary

PIP4K enzymes phosphorylate their preferred phospholipid substrate, PtdIns5*P*, to produce PtdIns(4,5)P_2. The relative scarcity of PtdIns5*P* in cells suggests that the PIP4Ks function to remove a lipid signal provided by this phosphoinositide, or to produce a small pool of PtdIns(4,5)P_2 that is required in an isolated cellular compartment. PIP4K exists as three distinct isoforms (α, β, and γ) with PIP4Kα being significantly more active than PIP4Kβ or PIP4Kγ. Each isoform has a different tissue distribution and subcellular localization, and this is mediated in part by differences in highly variable regions within the more conserved common catalytic core domain. The isoforms form dimers by interaction between β1-sheets, and the possibility of forming heterodimers suggests a mechanism by which the more active isoform is targeted to different cellular locations by other isoforms in a tissue and possibly cell-type-dependent manner. PIP4Ks may have direct links to diabetes and psychological disorders, and PIP4Kβ has recently been shown to be upregulated in human cancers, suggesting that this family of enzymes may be potential targets for disease treatments.

References

Anderson RA, Boronenkov IV, Doughman SD, Kunz J, Loijens JC. Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. J Biol Chem. 1999;274(15):9907–10.

Bazenet CE, Ruano AR, Brockman JL, Anderson RA. The human erythrocyte contains two forms of phosphatidylinositol-4- phosphate 5-kinase which are differentially active toward membranes. J Biol Chem. 1990;265(29):18012–22.

Boronenkov IV, Anderson RA. The sequence of phosphatidylinositol-4-phosphate 5-kinase defines a novel family of lipid kinases. J Biol Chem. 1995;270(7):2881–4.

Bultsma Y, Keune WJ, Divecha N. PIP4Kbeta interacts with and modulates nuclear localization of the high-activity PtdIns5P-4-kinase isoform PIP4Kalpha. Biochem J. 2010;430(2):223–35.

Bunce MW, Boronenkov IV, Anderson RA. Coordinated activation of the nuclear ubiquitin ligase Cul3-SPOP by the generation of phosphatidylinositol 5-phosphate. J Biol Chem. 2008;283(13):8678–86.

Carricaburu V, Lamia KA, Lo E, Favereaux L, Payrastre B, Cantley LC, et al. The phosphatidylinositol (PI)-5-phosphate 4-kinase type II enzyme controls insulin signaling by regulating PI-3,4,5-trisphosphate degradation. Proc Natl Acad Sci U S A. 2003;100(17):9867–72.

Castellino AM, Chao MV. Differential association of phosphatidylinositol-5-phosphate 4-kinase with the EGF/ErbB family of receptors. Cell Signal. 1999;11(3):171–7.

Castellino AM, Parker GJ, Boronenkov IV, Anderson RA, Chao MV. A novel interaction between the juxtamembrane region of the p55 tumor necrosis factor receptor and phosphatidylinositol-4-phosphate 5-kinase. J Biol Chem. 1997;272(9): 5861–70.

Chang JD, Field SJ, Rameh LE, Carpenter CL, Cantley LC. Identification and characterization of a phosphoinositide phosphate kinase homolog. J Biol Chem. 2004;279(12): 11672–9.

Ciruela A, Hinchliffe KA, Divecha N, Irvine RF. Nuclear targeting of the beta isoform of type II phosphatidylinositol phosphate kinase (phosphatidylinositol 5-phosphate 4-kinase) by its alpha-helix 7. Biochem J. 2000;346(Pt 3): 587–91.

Clarke JH, Richardson JP, Hinchliffe KA, Irvine RF. Type II PtdInsP kinases: location, regulation and function. Biochem Soc Symp. 2007;74:149–59.

Clarke JH, Emson PC, Irvine RF. Localization of phosphatidylinositol phosphate kinase IIgamma in kidney to a membrane trafficking compartment within specialized cells of the nephron. Am J Physiol Renal Physiol. 2008;295(5):F1422–30.

Clarke JH, Emson PC, Irvine RF. Distribution and neuronal expression of phosphatidylinositol phosphate kinase II gamma in the mouse brain. J Comp Neurol. 2009;517(3): 296–312.

Clarke JH, Wang M, Irvine RF. Localization, regulation and function of type II phosphatidylinositol 5-phosphate 4-kinases. Adv Enzyme Regul. 2010;50(1):12–8.

Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. Nature. 2006;443(7112):651–7.

Divecha N, Truong O, Hsuan JJ, Hinchliffe KA, Irvine RF. The cloning and sequence of the C isoform of PtdIns4P 5-kinase. Biochem J. 1995;309(Pt 3):715–9.

Gonzales ML, Anderson RA. Nuclear phosphoinositide kinases and inositol phospholipids. J Cell Biochem. 2006;97(2):252–60.

Halstead JR, Jalink K, Divecha N. An emerging role for PtdIns (4,5)P2-mediated signalling in human disease. Trends Pharmacol Sci. 2005;26(12):654–60.

Hinchliffe KA, Irvine RF. Regulation of type II PIP kinase by PKD phosphorylation. Cell Signal. 2006;18(11):1906–13.

Hinchliffe KA, Ciruela A, Irvine RF. PIPkins1, their substrates and their products: new functions for old enzymes. Biochim Biophys Acta. 1998;1436(1–2):87–104.

Hinchliffe KA, Ciruela A, Letcher AJ, Divecha N, Irvine RF. Regulation of type IIalpha phosphatidylinositol phosphate kinase localisation by the protein kinase CK2. Curr Biol. 1999;9(17):983–6.

Hinchliffe KA, Giudici ML, Letcher AJ, Irvine RF. Type IIalpha phosphatidylinositol phosphate kinase associates with the plasma membrane via interaction with type I isoforms. Biochem J. 2002;363(Pt 3):563–70.

Huang Z, Guo XX, Chen SX, Alvarez KM, Bell MW, Anderson RE. Regulation of type II phosphatidylinositol phosphate kinase by tyrosine phosphorylation in bovine rod outer segments. Biochemistry. 2001;40(15):4550–9.

Irvine RF. Nuclear lipid signalling. Nat Rev Mol Cell Biol. 2003;4(5):349–60.

Itoh T, Ijuin T, Takenawa T. A novel phosphatidylinositol-5-phosphate 4-kinase (phosphatidylinositol- phosphate kinase IIgamma) is phosphorylated in the endoplasmic reticulum in response to mitogenic signals. J Biol Chem. 1998;273(32): 20292–9.

Jones DR, Bultsma Y, Keune WJ, Halstead JR, Elouarrat D, Mohammed S, et al. Nuclear PtdIns5P as a transducer of stress signaling: an in vivo role for PIP4Kbeta. Mol Cell. 2006;23(5):685–95.

Kunz J, Wilson MP, Kisseleva M, Hurley JH, Majerus PW, Anderson RA. The activation loop of phosphatidylinositol phosphate kinases determines signaling specificity. Mol Cell. 2000;5(1):1–11.

Lamia KA, Peroni OD, Kim YB, Rameh LE, Kahn BB, Cantley LC. Increased insulin sensitivity and reduced adiposity in phosphatidylinositol 5-phosphate 4-kinase beta-/- mice. Mol Cell Biol. 2004;24(11):5080–7.

Luoh SW, Venkatesan N, Tripathi R. Overexpression of the amplified Pip4k2beta gene from 17q11-12 in breast cancer cells confers proliferation advantage. Oncogene. 2004;23(7):1354–63.

McCrea HJ, De Camilli P. Mutations in phosphoinositide metabolizing enzymes and human disease. Physiology (Bethesda). 2009;24:8–16.

Mueller-Roeber B, Pical C. Inositol phospholipid metabolism in *Arabidopsis*. Characterized and putative isoforms of inositol phospholipid kinase and phosphoinositide-specific phospholipase C. Plant Physiol. 2002;130(1):22–46.

O'Connell DJ, Rozenvayn N, Flaumenhaft R. Phosphatidylinositol 4,5-bisphosphate regulates activation-induced platelet microparticle formation. Biochemistry. 2005;44(16):6361–70.

Park S, Lee W, You KH, Kim H, Suh JM, Chung HK, et al. Regulation of phosphatidylinositol-phosphate kinase IIgamma gene transcription by thyroid-stimulating hormone in thyroid cells. J Mol Endocrinol. 2001;26(2): 127–33.

Rameh LE, Tolias KF, Duckworth BC, Cantley LC. A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature. 1997;390(6656):192–6.

Rao VD, Misra S, Boronenkov IV, Anderson RA, Hurley JH. Structure of type IIbeta phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. Cell. 1998;94(6):829–39.

Richardson JP, Wang M, Clarke JH, Patel KJ, Irvine RF. Genomic tagging of endogenous type IIbeta phosphatidylinositol 5-phosphate 4-kinase in DT40 cells reveals a nuclear localisation. Cell Signal. 2007;19(6):1309–14.

Rozenvayn N, Flaumenhaft R. Protein kinase C mediates translocation of type II phosphatidylinositol 5-phosphate 4-kinase required for platelet alpha-granule secretion. J Biol Chem. 2003;278(10):8126–34.

Schleiermacher G, Bourdeaut F, Combaret V, Picrron G, Raynal V, Aurias A, et al. Stepwise occurrence of a complex unbalanced translocation in neuroblastoma leading to insertion of a telomere sequence and late chromosome 17q gain. Oncogene. 2005;24(20):3377–84.

Singhal RL, Prajda N, Yeh YA, Weber G. 1-Phosphatidylinositol 4-phosphate 5-kinase (EC 2.7.1.68): a proliferation- and malignancy-linked signal transduction enzyme. Cancer Res. 1994;54(21):5574–8.

Stopkova P, Saito T, Fann CS, Papolos DF, Vevera J, Paclt I, et al. Polymorphism screening of PIP5K2A: a candidate gene for chromosome 10p-linked psychiatric disorders. Am J Med Genet B Neuropsychiatr Genet. 2003;123B(1):50–8.
Toker A. Phosphoinositides and signal transduction. Cell Mol Life Sci. 2002;59(5):761–79.
Wang M, Bond NJ, Letcher AJ, Richardson JP, Lilley KS, Irvine RF, et al. Genomic tagging reveals a random association of endogenous PtdIns5P 4-kinases IIalpha and IIbeta and a partial nuclear localization of the IIalpha isoform. Biochem J. 2010;430(2):215–21.

Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

Su Jun Lim[1] and Willis X. Li[2]
[1]University of Rochester Medical Center, Rochester, NY, USA
[2]Department of Medicine, University of California San Diego, La Jolla, CA, USA

Synonyms

Marelle; Signal transducers and activators of transcription; *STAT1*; *STAT2*; *STAT3*; *STAT4*; *STAT5A*; *STAT5B*; *STAT6*; STAT92E

Historical Background

The STAT (Signal Transducers and Activators of Transcription) proteins were first identified as signaling proteins that function as second messengers and transcription factors in response to cytokines and growth factors (Santos and Costa-Pereira 2011; Li 2008). Mammals have seven STAT genes, namely, *STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B*, and *STAT6* (Santos and Costa-Pereira 2011). All the STAT proteins are highly conserved and contain six domains including the SH2, linker, coiled-coil, and DNA binding domain (Bromberg and Darnell 2000). Traditionally, unphosphorylated STAT is believed to reside in the cytoplasm in an inactive form; upon phosphorylation by JAK or another tyrosine kinase, the phosphorylated (active) STAT translocates into the nucleus to induce transcription of target genes (Bromberg and Darnell 2000). However, recent studies have prompted revisions to this paradigm. In addition to the canonical signaling pathway, there is increasing evidence that unphosphorylated STAT shuttles back and forth between the nucleus and cytoplasm (Santos and Costa-Pereira 2011). In this noncanonical mode of signaling, unphospho-STAT seems to carry additional functions, such as chromatin remodeling and heterochromatin dynamics (Li 2008).

STAT's Protein Structure and Isoforms

STAT proteins are 750–850 amino acids long and possess six functionally conserved domains (Fig. 1). The crystal structures of both phospho- and unphospho-STAT dimers have been solved for STAT1; both of them adopt a nutcracker-like structure (Lim and Cao 2006). The six conserved domains have been extensively studied and can have overlapping functions. Although most of the domains are functionally conserved across species, the differences within the conserved domains among the different STAT proteins allow specificity in their responses in signaling. There are also multiple isoforms and posttranslational modifications that can determine the specificity of STAT regulation.

The N-terminal domain (NTD) is required for dimerization of unphosphorylated STATs and tetramerization between STAT1, STAT3, and STAT5. In addition, the NTD can also recruit transcriptional co-activators, such as CREB binding protein (CBP)/p300 by promoting protein-protein interaction, and is also involved in tyrosine phosphorylation and dephosphorylation of STATs. The coiled-coil domain (CCD) is important for the interaction of STATs with other proteins to mediate transcription. For example, STAT3 interacts with c-Jun to induce transcription of target genes in response to IL-6 induction and binds to epidermal growth factor (EGF) receptors via its CCD domain. Besides, a leucine-rich segment is located within the CCD domain, which also regulates nuclear translocation of STAT proteins. Even though the DNA-binding domain (DBD) is highly conserved across the protein family, the small differences in the amino acid sequences of DBD across different STATs are responsible for their different DNA-binding specificities. Upon cytokine stimulation, the heterodimers formed by phosphorylated STAT1 and STAT3 require the DBD for nuclear translocation as well. Next to the DBD is the α-helical linker domain

Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT), Fig. 1 The structural organization of STAT proteins. All the STATs have six main domains, namely, the NTD at the N-terminal end of the peptide, coiled-coil domain (CCD), DNA binding domain (DBD), linker domain (LD), the Src-homology 2 domain (SH2D), and transactivation domain (TAD) at the very carboxyl-terminal end. Phosphorylated sites are as indicated

(LD). It links two crucial domains in STAT, namely, DBD and the ▶ Src-homology 2 (SH2) domain (Santos and Costa-Pereira 2011). Additionally, LD is also crucial for the transcriptional activation of STAT1 following ▶ IFN-γ stimulation and has been implicated in the constitutive nucleocytoplasmic shuttling of unphophorylated STATs in resting cells (Yang et al. 1999; Marg et al. 2004). The 100 amino acid long SH2 domain, located at the C-terminal end of the peptide, is probably the most well-conserved domain in STAT; it plays a crucial role in protein-protein interactions between STATs and other proteins (Santos and Costa-Pereira 2011). SH2 domain recognizes phosphorylated tyrosine residue and binds to phosphorylated Janus kinase (JAK) or STAT (Huang et al. 2008; Watanabe and Arai 1996). Moreover, a number of residues within this domain are important for STAT to mediate cellular functions. In particular, the phosphorylation of STAT1 on tyrosine 701 and its subsequent homodimerization are dependent on the arginine 602 residue within the SH2 domain (Li 2008); when proline 630 is mutated, tyrosine phosphorylation of STAT2 is impaired and it no longer dimerizes with STAT1 (Santos and Costa-Pereira 2011). The last domain on the C-terminal end is the transactivation domain (TAD). In addition to its function in activating transcription, TAD is used to interact with other proteins as well. For instance, TAD is required for the interaction of STAT1 with breast cancer 1 (BRCA1) and CBP/p300 (Santos and Costa-Pereira 2011).

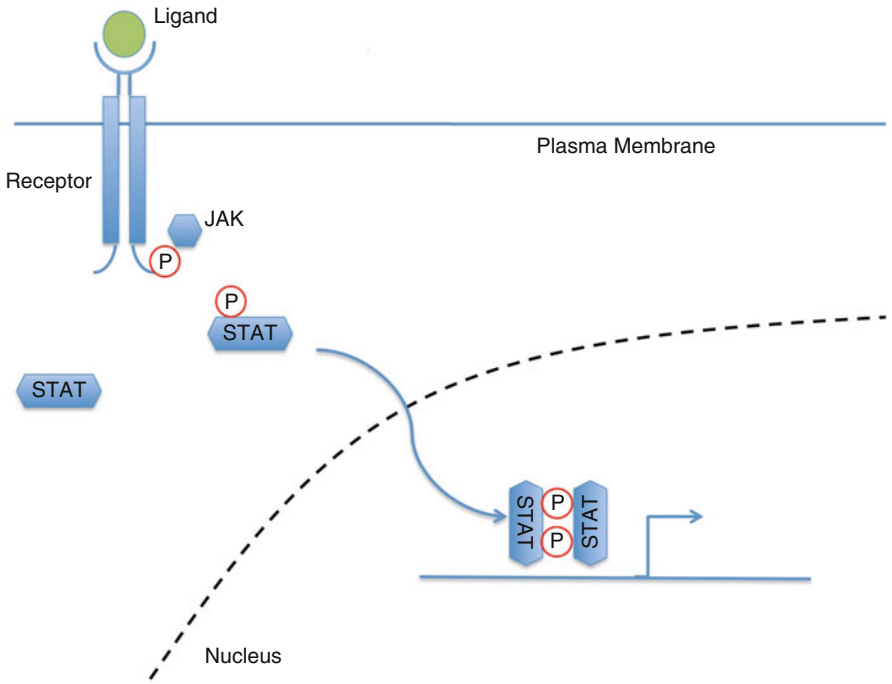
STAT in the Canonical JAK-STAT Pathway

In the canonical pathway, when a ligand (e.g. a cytokine or growth factor) binds to its transmembrane receptor, conformational changes lead to receptor dimerization and cross-activation of the receptor-associated tyrosine kinase, Janus kinase (JAK). As a result, multiple phosphorylation events occur and the phospho-tyrosines on the cytoplasmic tail of the receptor serve as docking sites for the binding of latent STATs residing in the cytoplasm (Fig. 2). Once bound, JAK can then phorphorylate associated STATs and result in dimerization of the STAT proteins via phosphorylated tyrosine residue located in the SH2 domain. These activated STAT dimers then translocate into the nucleus to regulate transcription of STAT target genes in response to cytokine or growth factor stimulation (Li 2008).

STAT is involved in regulating many cellular processes such as cell proliferation, apoptosis, growth, hematopoiesis, antiviral responses, and immune systems (O'Shea et al. 2004). Typically, STAT binds to well-defined DNA consensus sequences and initiates transcription of interferon (IFN)-stimulated genes (ISGs). It is also known to regulate the transcription of many genes including the B-cell lymphoma 2 (Bcl-2) family proteins, cyclin D1, ▶ Myc, and many genes implicated in angiogenesis or metastasis (Li 2008). In addition to the consensus sequence, STAT tetramer complexes can also recognize and bind weakly to non-consensus regions via the NTD domain for added specificity in its regulation . Besides inducing transcription, STAT can also act as a negative regulator of transcription in some instances (Boucheron et al. 1998). As an example, STAT3β, a naturally occurring splice variant of STAT3, inhibits transcription of STAT3's target genes (Caldenhoven et al. 1996).

Unphosphorylated STATs in Gene Regulation

The canonical JAK/STAT pathway had to be revised when much evidence has suggested that at least a fraction of the unphosphorylated STATs may have biological functions and shuttle continuously into the nucleus, instead of maintaining its latency in the cytoplasm (Li 2008). Work done in *Drosophila* has shown that loss of *STAT* strongly suppresses position effect variegation (PEV), a heterochromatin-dependent gene



Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT), Fig. 2 The canonical JAK/STAT signaling pathway. Canonically, unphosphorylated STATs maintain their latency and reside in the cytoplasm. When ligand binds to the transmembrane receptors, they homo-dimerize and are phosphorylated by JAK, a tyrosine kinase. Following that, a series of phosphorylation events result in conformational changes on the receptor that open up binding sites for recruiting STATs. Consequently, JAK can then phosphorylate STATs. Phospho-STATs then dimerize and translocate into the nucleus to mediate transcription of target genes

silencing process (Li 2008). Further investigation has shown that unphospho-STAT associates with heterochromatin protein 1 (HP1) in the nucleus to maintain heterochromatin stability (Li 2008; Shi et al. 2008a). However, upon phosphorylation, STAT disperses from heterochromatin, causing HP1 displacement and heterochromatin destabilization (Shi et al. 2008b). Consistent with the observation in *Drosophila*, mammalian unphospho-STATs, STAT3 and STAT5A in particular, have also been shown to localize at the nucleus as well (Liu et al. 2005). Even though it is still unclear if mammalian STATs also associate with HP1 and localize at heterochromatin, unphospho-STATs in mammals clearly regulate gene transcription by a mechanism distinct from the canonical JAK-STAT signaling pathway (Yang and Stark 2008). Due to their effects on chromatin structure, it is not surprising that STATs are crucial for gene regulation in stem cells and during organisms' development (Boyer et al. 2006; Hochedlinger and Jaenisch 2006). Therefore, as opposed to the traditional view of STAT, unphospho-STATs are not completely dormant and play important roles in the regulation of gene transcription.

The Role of STATs in Cancer

STAT1, STAT3, and STAT5 are often misregulated in many cancer cells, especially STAT3, which is a known oncogene (Silva 2004). STAT3 is believed to contribute to cancer cell transformation via its inflammatory response (Yu and Jove 2004). However, no STAT mutations have been identified in association with cancer incidents. In most cases, the abnormal activities of the upstream kinases are the cause of STAT signaling misregulation in cancer cells. In fact, a well-established tumor model in *Drosophila* involves a hyperactive JAK kinase called $hop^{Tumorous\text{-}L}$ ($hop^{Tum\text{-}L}$) that causes STAT over-activation and over-proliferation of blood cells, analogous to human leukemia (Li 2008). The role of STAT activation in these pathological conditions has so far been attributed mostly to over-activation of the canonical

JAK-STAT pathway that upregulates STAT target genes. In addition to the canonical pathway, disruption of STAT's function in stabilizing heterochromatin may also contribute to tumorigenesis since heterochromatin has been implicated in tumor suppression. Therefore, respective contributions of canonical and noncanonical JAK-STAT signaling in cancer development are currently under investigation.

Summary

In conclusion, STAT is a crucial signaling molecule in animal cells that translates extracellular signals into changes in gene transcription. In the canonical JAK-STAT pathway, only phosphorylated STATs translocate into the nucleus and act as transcription factors, whereas unphosphorylated STATs are assumed to remain latent in the cytoplasm. In the noncanonical mode of JAK-STAT signaling, unphosphorylated STATs are also found, at least in fruit flies, to localize in the nucleus and play important roles in regulating gene expression by modulating heterochromatin formation. It has recently been shown that mammalian JAK2 plays a role in regulating heterochromatin formation (Dawson et al. 2009). Whether STAT's function in heterochromatin is conserved in mammals is yet to be determined. Proper function of STAT is essential for different stages of development and maintaining the health of individual organisms and misregulation of STAT has been implicated in multiple human diseases. STAT thus poses as an attractive potential therapeutic target, because it mediates a rather straightforward signaling pathway and the phospho-tyrosine residue can conceivably be targeted. However, since STAT has proven to be versatile and involved in multiple functions via distinct mechanisms, targeting STAT may cause unexpected cellular responses. Therefore, the biological functions and cellular effects of STAT proteins must be thoroughly investigated even though STAT remains a potential therapeutic target.

References

Boucheron C, Dumon S, Santos SC, Moriggl R, Hennighausen L, Gisselbrecht S, et al. A single amino acid in the DNA binding regions of STAT5A and STAT5B confers distinct DNA binding specificities. J Biol Chem. 1998;273(51):33936–41.

Boyer LA, Mathur D, Jaenisch R. Molecular control of pluripotency. Curr Opin Genet Dev. 2006;16(5):455–62.

Bromberg J, Darnell Jr JE. The role of STATs in transcriptional control and their impact on cellular function. Oncogene. 2000;19(21):2468–73.

Caldenhoven E, van Dijk TB, Solari R, Armstrong J, Raaijmakers JA, Lammers JW, et al. STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. J Biol Chem. 1996;271(22):13221–7.

Dawson MA, Bannister AJ, Gottgens B, Foster SD, Bartke T, Green AR, et al. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature. 2009;461(7265):819–22.

Hochedlinger K, Jaenisch R. Nuclear reprogramming and pluripotency. Nature. 2006;441(7097):1061–7.

Huang H, Li L, Wu C, Schibli D, Colwill K, Ma S, et al. Defining the specificity space of the human SRC homology 2 domain. Mol Cell Proteomics. 2008;7(4):768–84.

Li WX. Canonical and non-canonical JAK-STAT signaling. Trends Cell Biol. 2008;18(11):545–51.

Lim CP, Cao X. Structure, function, and regulation of STAT proteins. Mol Biosyst. 2006;2(11):536–50.

Liu L, McBride KM, Reich NC. STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3. Proc Natl Acad Sci U S A. 2005;102(23):8150–5.

Marg A, Shan Y, Meyer T, Meissner T, Brandenburg M, Vinkemeier U. Nucleocytoplasmic shuttling by nucleoporins Nup153 and Nup214 and CRM1-dependent nuclear export control the subcellular distribution of latent Stat1. J Cell Biol. 2004;165(6):823–33.

O'Shea JJ, Pesu M, Borie DC, Changelian PS. A new modality for immunosuppression: targeting the JAK/STAT pathway. Nat Rev Drug Discov. 2004;3(7):555–64.

Santos CI, Costa-Pereira AP. Signal transducers and activators of transcription-from cytokine signalling to cancer biology. Biochim Biophys Acta. 2011;1816(1):38–49.

Shi S, Larson K, Guo D, Lim SJ, Dutta P, Yan SJ, et al. Drosophila STAT is required for directly maintaining HP1 localization and heterochromatin stability. Nat Cell Biol. 2008a;10(4):489.

Shi S, Larson K, Guo D, Lim SJ, Dutta P, Yan SJ, et al. Drosophila STAT is required for directly maintaining HP1 localization and heterochromatin stability. Nat Cell Biol. 2008b;10(4):489–96.

Silva CM. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. Oncogene. 2004;23(48):8017–23.

Watanabe S, Arai K. Roles of the JAK-STAT system in signal transduction via cytokine receptors. Curr Opin Genet Dev. 1996;6(5):587–96.

Yang J, Stark GR. Roles of unphosphorylated STATs in signaling. Cell Res. 2008;18(4):443–51.

Yang E, Wen Z, Haspel RL, Zhang JJ, Darnell Jr JE. The linker domain of Stat1 is required for gamma interferon-driven transcription. Mol Cell Biol. 1999;19(7):5106–12.

Yu H, Jove R. The STATs of cancer–new molecular targets come of age. Nat Rev Cancer. 2004;4(2):97–105.

Phosphodiesterase 1

Sujeet Kumar, Ponniah Selvakumar and Rajendra K. Sharma
Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada
Cancer Research Unit, Saskatchewan Cancer Agency, Saskatoon, SK, Canada

Synonyms

Calmodulin-dependent cyclic nucleotide phosphodiesterase; PDE 1

Historical Background

Cyclic nucleotide phosphodiesterase (PDE) was demonstrated after the discovery of cAMP (Sutherland and Rall 1958). In most tissues, PDE exists in multiple forms which differ in subcellular localization, relative substrate specificity toward cAMP and cGMP, regulatory and immunological properties (Beavo 1995; Kakkar et al. 1999; Goraya and Cooper 2005). Most tissues examined have been shown to contain Ca^{2+} and calmodulin (CaM)-dependent cyclic nucleotide phosphodiesterase 1 (PDE1) and this enzyme has been intensively studied (Beavo 1995; Kakkar et al. 1999). Earlier, it was suggested that PDE1 consists of a single species (Wells and Hardman 1977), but later found to exist as a tissue-specific and immunological distinct enzyme (Sharma et al. 1984). With the rapidly expanding list of PDE1 isozymes, a new nomenclature was developed based on the primary structure of different PDEs (Beavo et al. 1994). It is now known that at least three different genes encode the PDE1 family; they are named PDE1A, PDE1B, and PDE1C. The complementary DNAs (cDNAs) encoding the brain 63 kDa PDE1 isozyme (PDE1B1), brain 60 kDa PDE1 isozyme (PDE1A2), heart PDE1 (PDE1A1), and 70 kDa PDE1 (PDE1C) were suggested to represent subfamily members. In addition, new splice variants of mouse and human PDEs (PDE1C1, PDE1C2, PDE1C3, PDE1C4, PDE1C5, PDE1A3, and PDE1B1) have been reported. They have been characterized in terms of their regulation by Ca^{2+}, sensitivity to inhibitors, and tissue/cell-specific expression. The genes for different PDEs undergo tissue-specific alternative splicing that generates structurally and functionally diverse gene products.

Differential Stimulation of PDE1 by CaM and Ca^{2+}

PDE1 can be distinguished from other forms of PDE by the stimulation with CaM in the presence of Ca^{2+}. Although brain PDE1A2 and heart PDE1A1 isozymes are almost identical immunologically and kinetically in their properties (Sharma et al. 1984; Sharma and Kalra 1994), they are differentially activated by CaM (Sharma and Kalra 1994). It may be possible that differential affinity for CaM may reflect subtle differences in Ca^{2+} activation of these PDE1 isozymes. The differences in CaM affinity exhibited by these isozymes may be related to the relative concentration of CaM in these tissues. It has been suggested that the differential of CaM affinity is an important mechanism by which the regulatory action of CaM may be fine-tuned (Klee 1988). However, the physiological significance of differential CaM affinity requires further research in this area. It is noteworthy that CaM concentration in mammalian brain is approximately 10 times higher than in mammalian heart (Klee and Vanaman 1982). The PDE1A1 isozyme have a higher affinity for CaM than brain PDE 1A2 (Hansen and Beavo 1986; Sharma 1991). Similarly, the pig brain PDE1 has been shown to have a lower affinity for CaM than the isozymes from pig artery (Keravis et al. 1986). In addition, Ca^{2+} and CaM interact synergistically in activation of PDE1 isozymes (Huang et al. 1981). When the CaM concentration is increased, the Ca^{2+} concentration required for half-maximal activation is decreased. Such synergistic interactions have been repeatedly shown for various CaM-dependent enzymes. Although the physiological significance of the observed differential Ca^{2+} sensitivity of the PDE1 isozymes is not known, these studies suggest that the differential Ca^{2+} affinity of the tissue-specific isozymes may be a mechanism by which CaM regulatory reactions are adapted in the respective tissues. The lung PDE1 isozyme has the highest apparent affinity for CaM, since it contains CaM as a subunit (Sharma and Wang 1986a). A change in CaM concentration had no effect on the Ca^{2+} concentration of the lung enzyme, suggesting that this isozyme does not undergo a Ca^{2+}-dependent reversible association with CaM. At present, the significance of CaM as a subunit is not known.

Kinetic Properties of PDE1 and Its Inhibitors

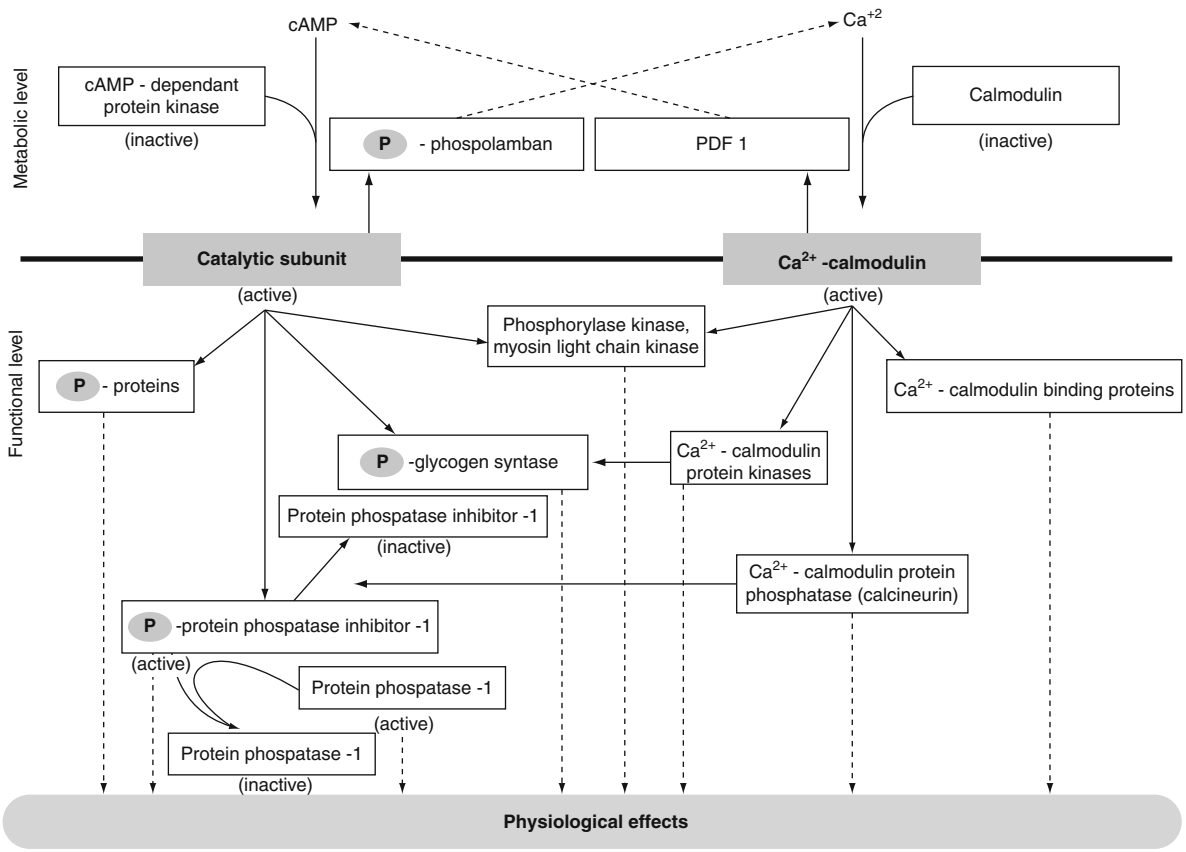
Originally, it was reported that PDE1 displays a much higher affinity for cGMP than for cAMP (Beavo 1995; Kakkar et al. 1999). The earlier studies from various laboratories reported marked differences in kinetic parameters; the discrepancy may be due to the purity of enzyme, as well as varying assay conditions. To understand the physiological role of PDE1 isozymes, purified PDE1 isozymes were used to determine kinetic parameters. PDE1 isozymes have a higher affinity toward cGMP than cAMP (Kakkar et al. 1999). However, PDE1B1 isozyme exhibits two- to threefold higher affinity for both the substrates, cAMP and cGMP, compared to PDE1A2 and PDE1A1 and a higher V_{max} for cGMP and cAMP (Kakkar et al. 1999). It is interesting to note that PDE1A2 and PDE1A1 isozymes have very similar kinetic properties, whereas PDE1B1 isozyme is kinetically distinct from other PDE1 isozymes (Kakkar et al. 1999). However, PDE1C1 has approximately similar K_m and V_{max} for cAMP and cGMP (Vandeput et al. 2007).

Early studies on inhibitors were carried out by using purified or partially purified PDE1. Therefore, it was not clear from previous studies which of the purified or partially purified PDE1 isozymes were used. Ginsenosides were found to be potent inhibitors of heart PDE1A1 and brain PDE1A2 but not of brain PDE1B1 (Kakkar et al. 1999). However, deprenyl (selegiline hydrochloride), an antiparkinsonian agent also inhibits brain PDE1A2 but is a poor inhibitor of brain PDE1B1 (Kakkar et al. 1999). In addition, amantadine only inhibits brain PDE1A2 isozyme but not brain PDE1B1, heart PDE1A1, and lung PDE1 isozymes (Kakkar et al. 1999). Since the inhibition of these isozymes is overcome by increasing the concentration of CaM, this suggests that these compounds act specifically and reversibly against the action of CaM. Therefore, these compounds may be valuable tools to investigate the diverse physiological roles of distinct PDE1 isozymes. Unlike other CaM-dependent enzymes, PDE1 has been suggested to be inhibited by dihydropyridine calcium antagonists, which act as direct vasodilator drugs rather than indirectly through their actions on CaM. In previous studies, it was not clear which of the specific PDE1 isozymes were used (Kakkar et al. 1999). The effect of dihydropyridine Ca^{2+} channel blockers felodipine and nicardipine on purified brain PDE1 isozymes has been examined. The results indicate that both brain isozymes are inhibited by felodipine and nicardipine by partial competitive inhibition, and these two Ca^{2+} antagonists appear to counteract each other (Sharma et al. 1997). The K_i values for felodipine (1.8 and 2.8 μM) and nicarpidine (2.3 and 5.8 μM) for PDE1A2 and PDE1B1, respectively, suggest that the two brain PDE1 isozymes have similar affinities for the Ca^{2+} antagonists, and both the isozymes bind felodipine slightly tighter than they bind nicarpidine. This study further demonstrated the existence of a specific site, distinct from the active site on PDE1 isozyme, which exhibits high-affinity binding of these drugs.

Interaction of the Ca^{2+} and cAMP Second Messenger Systems

All cells have the ability to identify and respond to changes in their environment. They recognize extracellular signals through specific cell membrane receptors. The stereospecific binding of the signal molecules to the receptors results in a series of rapid events which translate the external signal into specific cellular responses. Most of these intracellular reactions depend on second messenger systems, which are composed of second messenger molecules such as cAMP and Ca^{2+} and a host of enzymes, protein factors, and cell organelles which regulate the metabolism of and mediate the regulatory actions of the messenger molecules. In most cases, different second messenger systems undergo complex yet precise interactions, on one hand to achieve integrated regulation of cellular activities and on the other hand to terminate cell activities in an orderly manner. The interaction between the Ca^{2+} and cAMP second messenger system is among the most extensively studied. The two systems appear to interact at multiple levels and in either a synergistic or an antagonistic manner depending on the cellular process and/or the cell type.

The Ca^{2+} regulatory cascade (calcium signaling) is mediated by a Ca^{2+} binding protein, CaM, and involves a Ca^{2+}-dependent, reversible association of CaM with its target protein(s) (Klee 1988). This results in changes in targeted protein activity. In the case of the cAMP second messenger system, cAMP-dependent protein kinase(s) is considered to be the essential molecule which regulates wide-ranging physiological effects. Upon binding of cAMP to a regulatory subunit, the subunits dissociate and two catalytically active subunits are released which



Phosphodiesterase 1, Fig. 1 Schematic interactions between the cAMP and Ca^{2+} second messenger systems. The second messenger molecules cAMP and Ca^{2+} interact in several important fashions to integrate a physiological response. Some of these interactions are involved in the regulation of PDE1 (Adapted from Kakkar et al. 1999)

catalyze phosphorylation of specific proteins. In addition, numerous cyclic nucleotide-binding proteins have been characterized. Firstly, the high-affinity allosteric binding sites on cGMP-stimulated phosphodiesterase constitute a family of homologous cAMP-binding sites on several proteins. Secondly, the cGMP and cAMP-binding sites on cyclic nucleotide-gated channels include the potassium channel on cardiac pacemaker cells (Fesenko et al. 1985). Initially, such channels were thought to be present only in the retina and cardiac pacemaker cells. However, these channels have since been identified in many other cell types as well. Recently, Epac (Eoac1, Epac2, exchange protein directly activated by cAMP) has been recognized as a genomic sequence with homology to cAMP-binding sites and GEFs (guanine nucleotide exchange factors) for Ras-like proteins. Originally, Epac1 and Epac2 were also identified as a cAMP-GEF1 and cAMP-GEF2, respectively (Kowasski et al. 1998).

In many biological systems, the two second messengers, Ca^{2+} and cAMP, act in a concerted fashion to control regulatory processes. Several regulatory pathways for the interactions between Ca^{2+} and cAMP second messenger systems have been observed (Fig. 1). At the metabolic level, one second messenger regulates the metabolism of the other second messenger. For example, PDE1 controls cAMP concentration, and the phospholamban/Ca^{2+}-ATPase system regulates cytoplasmic Ca^{2+} concentration.

On a functional level, there are at least three mechanisms by which the two messenger systems exert their mutual effects (Fig. 1). Firstly, CaM-stimulated enzymes are substrates of cAMP-dependent protein kinase, such as phosphorylase kinase and smooth muscle ▶ myosin light chain kinase. A second mechanism

of Ca^{2+}-CaM and cAMP interaction involves phosphorylation of common protein substrates by Ca^{2+}-CaM and cAMP-dependent protein kinases. For example, glycogen synthase can be phosphorylated by cAMP-dependent protein kinase, by the CaM-dependent glycogen synthase kinase and phosphorylase kinase, as well as by at least four other kinases. These phosphorylations inactivate both muscle and liver glycogen synthases with the expectation that phosphorylation of the liver enzyme by CaM-dependent glycogen synthase kinase has no effect on enzyme activity. A third mode of interaction between the Ca^{2+} and cAMP signal systems involves the dephosphorylation of protein phosphatase inhibitor-1. Upon phosphorylation, protein phosphatase inhibitor-1 is active and inhibits the activity of protein phosphatase-1. When it is dephosphorylated by CaM-dependent protein phosphatase (calcineurin, CaN), protein phosphatase inhibitor-1 is inactivated, allowing protein phosphatase-1 to be active.

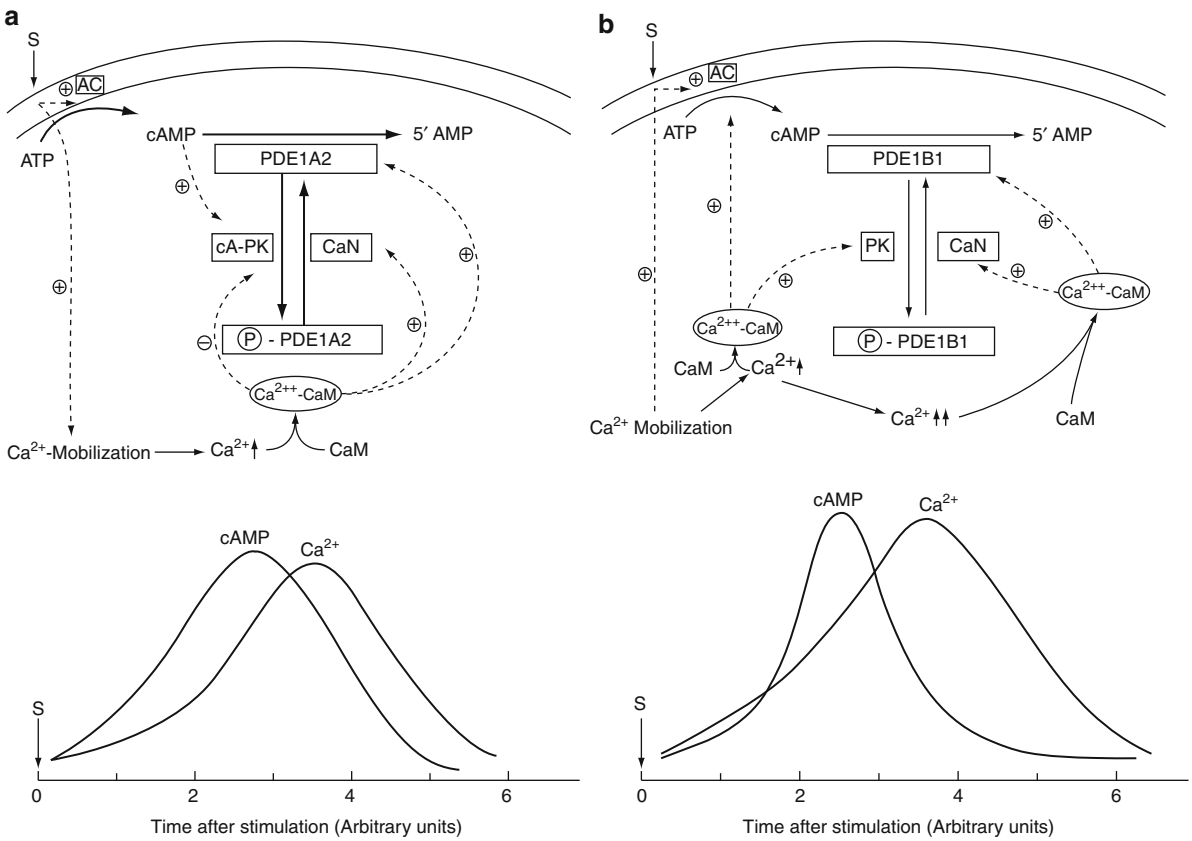
Regulation of PDE1 Isozymes by Phosphorylation

The main difference is that the brain PDE1A2 and heart PDE1A1 are substrates of cAMP-dependent protein kinase (Sharma and Wang 1985; Sharma 1991; Florio et al. 1994), and phosphorylation is inhibited by Ca^{2+} and CaM whereas brain PDE1B1 is phosphorylated by CaM-dependent protein kinase II in a Ca^{2+}/CaM-dependent manner (Sharma and Wang 1986b). The phosphorylation of PDE1A2 and PDE1A1 is accompanied by a decrease in the isozymes affinity toward CaM and an accompanying increase in the Ca^{2+} concentrations required for the isozyme activation by CaM. PDE1A2 and PDE1B1 are phosphorylated by different protein kinases; however, both can be dephosphorylated by CaM-dependent protein phospatase. This dephosphorylation is accompanied by an increase in the affinity of the isozymes for CaM as well as decrease in the Ca^{2+} concentration for the activation of isozyme by CaM. It is interesting to note that PDE1C is phosphorylated by cAMP-dependent protein kinase and upon phosphorylation of PDE1C activity is inhibited with increased EC_{50} for CaM (Ang and Antoni 2002).

Role of PDE1A2 and PDE1B1 in the Regulation of cAMP Concentration

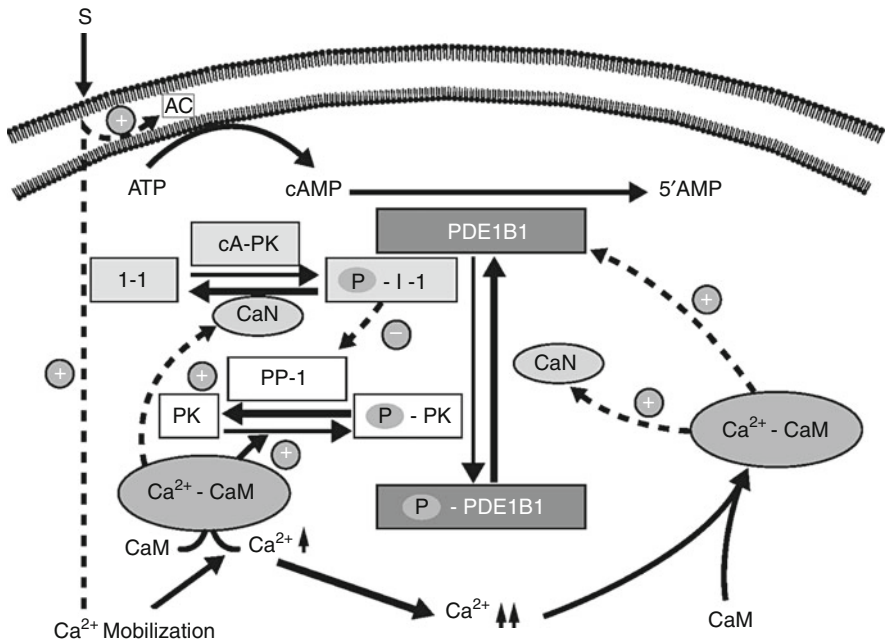
A working hypothesis describing the role of PDE1A2 in the coupling between the two messenger fluxes is presented in Fig. 2a. In most cases, cell activation involves transitory increase in both cAMP and cell Ca^{2+}. The operation of the different regulatory mechanisms on PDE1 may be temporally separated during the signal fluxes. The temporal separation of the regulatory reactions is a result of the Ca^{2+} and cAMP signal fluxes on the one hand and contributes to determining the intensity and duration of the fluxes on the other hand. Thus, and initial increase in cAMP concentration during cell activation may bring about phosphorylation of PDE1A2 and thereby prevent the enzyme from being activated by the low concentrations of Ca^{2+} existing at the early stages of cell activation. The hydrolysis of cAMP, therefore, would be inhibited coordinately with the stimulation of ▶ adenylate cyclase by external signals. This would ensure a rapid and sharp rise in intracellular cAMP. At later stages of cell activation, when intracellular fee Ca^{2+} concentration is increased, the phosphatase reaction may be activated to reverse phosphorylation of the PDE1A2. The PDE1A2 then becomes fully activated by Ca^{2+} and CaM. Since Ca^{2+}-CaM can block PDE1A2 phosphorylation, the dephosphorylated state of the enzyme will be maintained even though the cAMP concentration may still be high in the cell. The concerted actions of these regulatory mechanisms on PDE1A2, namely, the phosphatase reaction, Ca^{2+}-CaM stimulation of PDE1A2, and Ca^{2+}-CaM inhibition of phosphorylation, may bring about a rapid decline in cAMP concentration. A similar hypothesis has been proposed for heart PDE1A1.

The multiple regulatory actions of the PDE1B1 isozyme are dependent on Ca^{2+} and CaM (Fig. 2b), whereas phosphorylation of PDE1A2 by cAMP-dependent protein kinase is inhibited by Ca^{2+} and CaM. Regulation of PDE1B1 by Ca^{2+} and CaM can occur by at least three mechanisms: (1) the PDE1B1 isozyme depends on Ca^{2+} and CaM for full activity, (2) it requires higher concentrations of Ca^{2+} for activation upon phosphorylation by Ca^{2+} and CaM-dependent protein kinase(s), and (3) the phosphorylation of PDE1B1 is reversed by the CaM-dependent protein phosphatase. Therefore, these three distinct CaM-dependent reactions can regulate PDE1B1 and can produce opposite effects on PDE1B1. Based on the findings from studies (Sharma and Wang 1985, 1986b), a working hypothesis has been proposed to indicate how these regulatory actions, separated temporally, could bring about meaningful interactions



Phosphodiesterase 1, Fig. 2 Hypotheses of the temporally separated regulation of PDE1A2 (**a**) and PDE1B1 (**b**) by Ca^{2+} and cAMP. Symbols: *AC* adenylate cyclase, *CaN* Ca^{2+}-CaM dependent protein phosphatase (calcineurin), *cA-PK* cAMP-dependent protein kinase, *PK* CaM-dependent protein kinase, *P*- phosphorylated, ⊕ activation, Θ inhibition. *Upper panel*, organization of regulatory reactions; *lower panel*, simulated Ca^{2+} and cAMP fluxes; *S* stimulus (Adapted from Kakkar et al. 1999)

between the Ca^{2+} and cAMP signal systems during cell activation (Fig. 2b). It is postulated that adenylate cyclase and the CaM-dependent protein kinase(s) can be turned on at lower concentration of Ca^{2+} during cell activation, whereas the activation of the PDE1B1 and CaM-dependent protein phosphatase require higher concentration of Ca^{2+}. The first two reactions which act in concert to increase cAMP concentration predominate at the early stage of surge in cytosolic Ca^{2+}, whereas the other two reactions will reduce cAMP concentrations at the higher concentrations of the Ca^{2+} flux. For this proposed hypothesis, it is necessary that adenylate cyclase and the CaM-dependent protein kinase(s) be activated by lower concentrations of Ca^{2+} than the CaM-dependent protein phosphatase and PDE1B1 (Fig. 2b). It has been reported that brain adenylate cyclase is indeed activated by much lower concentration of Ca^{2+} than the PDE1. The validity of the working hypothesis depicted in Fig. 1b has been tested by purification and characterization of CaM-dependent protein kinase from bovine brain. Bovine brain contains two CaM-dependent protein kinases which were separated on a Sephacryl S-300 column (Zhang et al. 1993a). The high molecular weight 500 kDa protein kinase has been purified close to homogeneity. On the basis of its molecular mass, subunit size, and protein substrate specificity, the purified bovine brain CaM-dependent protein kinase is considered to belong to the CaM-dependent protein kinase II family. The phosphorylation of PDE1B1 by the CaM-dependent protein kinase II is dependent on the presence of Ca^{2+} and CaM, and after phosphorylation a further increase in Ca^{2+} concentrations is required for enzyme activation.



Phosphodiesterase 1, Fig. 3 Schematic representation of the regulation of PDE1B1 by Ca^{2+} and cAMP as mediated by the autophosphorylation mechanism of CaM-dependent protein kinase II. The scheme depicts the complex interactions among *cA-PK* cAMP-dependent protein kinase, *PP-1* protein phosphatase-1, *I-1* protein inhibitor-1, *P-* phosphorylated, *CaN* Ca^{2+}-CaM dependent protein phosphatase (calcineurin), ⊕ activation, Θ inhibition, *light arrow* early events, *dark arrow* late events (Adapted from Kakkar et al. 1999)

Earlier, it was postulated (Fig. 2b) that the CaM-dependent protein kinase is activated by CaM at much lower concentrations of Ca^{2+} than the CaM-dependent protein phosphatase and PDE1B1. However, this suggestion was not supported when the dose-dependent activation of PDE1B1 by Ca^{2+} was compared with the purified CaM-dependent protein kinase II at identical concentrations of CaM. The results suggest that CaM-dependent protein kinase II and the PDE1B1 have similar Ca^{2+} concentration dependence at identical concentrations of CaM (Zhang et al. 1993a). However, the observation that the CaM-dependent protein kinase II becomes Ca^{2+}-independent and fully activated upon autophosphorylation suggests an alternative mechanism (Zhang et al. 1993b). Therefore, the CaM-dependent protein kinase may use the autophosphorylation reaction to override its requirements for higher concentrations of Ca^{2+}.

Bovine brain CaM-dependent protein kinase II is autophosphorylated rapidly in the presence of Ca^{2+} and CaM; however, it is converted into a Ca^{2+}-independent protein kinase, that is, the phosphorylation of PDE1B1 isozyme by autophosphorylated CaM-dependent protein kinase II becomes Ca^{2+}-independent. It is postulated that the autophosphorylation reaction can be used to achieve the required temporal separation of the activation of protein kinase from that of phosphatase and/or PDE1B1. Therefore, upon very brief exposure to high concentrations of Ca^{2+}, the CaM-dependent protein kinase II becomes active and insensitive to subsequent increases in Ca^{2+} concentrations, whereas the activation of PDE1B1 requires the continued presence of high concentrations of Ca^{2+}.

There are a number of possible ways by which such a brief exposure of CaM-dependent protein kinase II to high concentrations of Ca^{2+} can occur at onset of Ca^{2+} flux. Studies of agonist-induced Ca^{2+} flux in single cells have suggested that overall Ca^{2+} surge may be composed of a series of rapid Ca^{2+} transients. Such Ca^{2+} transients may, therefore, be used to trigger autophosphorylation of protein kinases at onset of Ca^{2+} surge. Alternatively, it is possible that CaM-dependent protein kinase II may be localized proximal to the sites of Ca^{2+} entry and, therefore, may be autophosphorylated rapidly at onset of Ca^{2+} flux. Immunocytochemical studies have shown that CaM-dependent protein kinase II is localized at inner surface of plasma membranes, as well as at outer surface of mitochondria and at synaptic vesicles and microtubules. Therefore, autophosphorylation of CaM-dependent protein kinase II may provide an additional mechanism that can be incorporated into a revised hypothesis for regulation of PDE1B1 isozyme, which is presented schematically in Fig. 3. In addition to temporal separation, a hypothesis is required to include a number of other regulation possibilities. For

example, autophosphorylation of CaM-dependent protein kinase II can be reversed by protein phosphatase-I and this protein phosphatase-I is regulated by protein inhibitor-I. When cAMP levels rise in the cell, cAMP-dependent protein kinase phosphorylates protein inhibitor-I to activate it. Phosphorylated protein inhibitor-I can then inhibit protein phosphatase-I. When protein inhibitor-I is dephosphorylated and inactivated by CaM-stimulated protein phosphatase, protein phosphatase-I is reactivated. As a result, cAMP may exert an inhibitory effect on PDE1B1 isozyme through a regulatory cascade involving protein phosphatase inhibitor-I, protein phosphatase-I, and CaM-dependent protein kinase II. This complex regulatory interaction is in agreement with the previously suggested role for PDE1B1 isozyme in the dynamic coupling of cAMP and Ca^{2+} fluxes in the cell.

In summary, during the early stage of cell activation, initial increase in cAMP and Ca^{2+} causes a temporary suppression of PDE1B1 isozyme activity to maintain the rise in cAMP concentration. As Ca^{2+} concentration in the cell is subsequently elevated, CaM-dependent protein phosphatase is activated to reverse the phosphorylation of PDE1B1 isozyme and reactivate PDE1B1. Since CaM-dependent protein phosphatase also dephosphorylates protein phosphatase inhibitor-I to cause reactivation of protein phosphatase-I, autophosphorylation of CaM-dependent protein kinase II is also reversed. Therefore, rephosphorylation of PDE1B1 isozyme will no longer occur as Ca^{2+} concentration subsides in the cell.

The main feature of the working hypothesis for the regulation of PDE1 isozymes is that the multiple regulatory actions exerted by second messengers on a single PDE1 isozyme are temporally separated. This is possible because the concentrations of both cAMP and Ca^{2+} undergo continuous change during cell activation. When the cell Ca^{2+} flux changes, cAMP flux will change accordingly. The transitory elevations of two messengers during cell activation are, therefore, coupled to each other with PDE1 isozymes playing key roles in this signal coupling phenomenon.

Summary

One of the most intensively studied cyclic nucleotide phosphodiesterase enzymes is the calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1) which is stimulated by the binding of Ca^{2+} and calmodulin. The earlier notion that PDE1 consists of a single species has been surpassed and it is clear that PDE1 exists as different isozymes, namely, PDE1A, PDE1B, PDE1C, and their various splice variants. These isozymes are regulated by multiple second messenger–dependent regulatory systems. One of the main features of the findings is that during cell activation, the various regulatory activities are temporally separated. Therefore, a temporal separation of second messenger–dependent reactions can be considered as natural consequences of dynamic fluxes of the messengers. These studies indicate that the activity of PDE1 is precisely regulated by cross talk between the Ca^{2+} and cAMP-signaling pathways.

Despite substantial progress, it is important to acknowledge that the understanding of the functions of PDE1 in disease states is far from complete. In the future, it is important to identify adaptive responses where PDE1 isozyme's activity and expression are changed and the change in levels of proteins which regulate and target PDE1 isozymes.

References

Ang KL, Antoni FA. Reciprocal regulation of calcium dependent and calcium independent cyclic AMP hydrolysis by protein phosphorylation. J Neurochem. 2002;81:422–33.

Beavo JA. Phosphodiesterases: functional implications of multiple isoforms. Physiol Rev. 1995;75:725–43.

Beavo JA, Conti M, Healip RJ. Multiple cyclic nucleotide phosphodiesterases. Mol Pharmacol. 1994;75:399–405.

Fesenko EE, Kolesnikov SS, Lyubarsky AL. Induction of cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. Nature. 1985;313:310–3.

Florio VA, Sonnenburg WK, Johnson R, Kwak KS, Jensen GS, Walsh KA, Beavo JA. Phosphorylation of the 61 kDa calmodulin-stimulated cyclic nucleotide phosphodiesterase at serine 120 reduces its affinity for calmodulin. Biochemistry. 1994;33:8948–54.

Goraya TA, Cooper DM. Ca^{2+}-calmodulin-dependent phosphodiesterase (PDE1): current perspectives. Cell Signal. 2005;17:789–97.

Hansen RS, Beavo JA. Differential recognition of calmodulin-enzyme complexes by a conformation-specific anti-calmodulin monoclonal antibody. J Biol Chem. 1986;261:14636–45.

Huang CY, Chau V, Chock PB, Wang JH, Sharma RK. Mechanism of activation of cyclic nucleotide phosphodiesterase: requirement foer the binding of four Ca^{2+} to calmodulin for activation. Proc Natl Acad Sci USA. 1981;78:871–4.

Kakkar R, Raju RVS, Sharma RK. Calmodulin-dependent cyclic nucleotide phosphodiesterase. Cell Mol Life Sci. 1999;55:1164–85.

Keravis TM, Duemler BH, Wells JN. Calmodulin-sensitive phosphodiesterase of porcine cerebral cortex: kinetic behavior, calmodulin activation and stability. J Cyclic Nucleotide Protein Phosphor Res. 1986;11:365–72.
Klee CB. Interaction of calmodulin with Ca^{2+} and target proteins. In: Cohen P, Klee CB, editors. Molecular aspects of cell regulation, vol. 5. New York: Elsevier Science; 1988. p. 35–56.
Klee CB, Vanaman TC. Calmodulin. Adv Protein Chem. 1982;35:213–321.
Kowasski H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybrel AM. A family of cAMP-binding proteins that directly activate Rep1. Science. 1998;282:2275–79.
Sharma RK. Phosphorylation and characterization of bovine heart calmodulin-dependent phosphodiesterase. Biochemistry. 1991;30:5963–8.
Sharma RK, Kalra J. Characterization of calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes. Biochem J. 1994;299:97–100.
Sharma RK, Wang JH. Differential regulation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes by cyclic AMP-dependent protein kinase and calmodulin-dependent phosphatase. Proc Natl Acad Sci USA. 1985;82:2603–7.
Sharma RK, Wang JH. Purification and characterization of bovine lung calmodulin-dependent cyclic nucleotide phosphodiesterase. An enzyme containing calmodulin as a subunit. J Biol Chem. 1986a;261:14160–6.
Sharma RK, Wang JH. Calmodulin and Ca^{2+}-dependent phosphorylation and dephosphorylation of 63 kDa subunit-containing bovine brain calmodulin-stimulated cyclic nucleotide phosphodiesterase isozyme. J Biol Chem. 1986b;261:1322–8.
Sharma RK, Adachi AM, Adachi K, Wang JH. Demonstration of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes by monoclonal antibodies. J Biol Chem. 1984;259:9248–54.
Sharma RK, Wang JH, Wu Z. Mechanisms of inhibition of calmodulin-stimulated cyclic nucleotide phosphodiesterase by dihydropyridine calcium antagonists. J Neurochem. 1997;69:845–50.
Sutherland EW, Rall TW. Factionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J Biol Chem. 1958;232:1077–91.
Vandeput F, Wolda SL, Krall J, Hambleton R, Uher L, McCaw KN, Radwanski PB, Florio V, Movsesian MA. Cyclic nucleotide phosphodiesterase PDE1C1 in human cardia myocytes. J Biol Chem. 2007;82:32749–57.
Wells JN, Hardman JG. Cyclic nucleotide phosphodiesterases. Adv Cyclic Nucleotide Res. 1977;8:119–43.
Zhang GY, Wang JH, Sharma RK. Purification and characterization of bovine brain calmodulin-dependent protein kinase II. The significance of autophosphorylation in the regulation of 63 kDa calmodulin-dependent cyclic nucleotide phosphodiesterase isozyme. Mol Cell Biochem. 1993a;122:159–69.
Zhang GY, Wang JH, Sharma RK. Bovine brain calmodulin-dependent protein kinase II: molecular mechanisms of autophosphorylation. Biochem Biophys Res Commun. 1993b;191:669–74.

Phosphodiesterase 10A

Takashi Sasaki, Jun Kotera and Kenji Omori
Advanced Medical Research Laboratories, Mitsubishi Tanabe Pharma Corporation, Yokohama, Kanagawa, Japan

Synonyms

PDE10; PDE10A

Historical Background

Identification of phosphodiesterase 10A (PDE10A) was first reported in mice and humans at the same period from three laboratories (Soderling et al. 1999; Fujishige et al. 1999a; Loughney et al. 1999). Primary sequence of PDE10A possesses a catalytic domain (HD domain) conserved within the 3, 5′-cyclic nucleotide phosphodiesterase family. PDE10A shows substrate specificity for both cyclic AMP (cAMP) and cyclic GMP (cGMP), and hydrolyzes these molecules to 5′-AMP and 5′-GMP, respectively. PDE10A mRNA and protein are highly expressed in the brain, particularly, in the striatal medium spiny neurons (Fujishige et al. 1999b; Seeger et al. 2003). Genetic deletion of PDE10A gene in mice as well as PDE10A inhibition by papaverine, a first reported PDE10 inhibitor, showed altered behavioral responses to several schizophrenia models (Siuciak et al. 2006; Siuciak et al. 2008); therefore, therapeutic implications of PDE10A inhibitor for psychiatric diseases have been received with considerable attention. To date, several potent and selective inhibitors for PDE10A have been reported to be efficacious in rodent models for positive, cognitive, and negative symptoms of schizophrenia (Schmidt et al. 2008; Grauer et al. 2009), while it remains to be demonstrated that whether these inhibitors provide a therapeutic benefit for schizophrenia patients or not.

Enzymatic Property

Kinetic analyses of human, mouse, and rat PDE10A indicated that PDE10A has higher affinity for cAMP

(K_m value of 0.05–0.158 μM) than cGMP (K_m value of 0.26–9.3 μM) (Soderling et al. 1999; Fujishige et al. 1999a; Loughney et al. 1999). On the other hand, V_{max} values for cAMP is twofold lower than that for cGMP. As expected from this observation, the catalytic activity for cAMP is significantly inhibited by the presence of cGMP (Fujishige et al. 1999a). Two major N-terminal splice variants have been reported in humans: PDE10A1 and PDE10A2 (Kotera et al. 1999). PDE10A2 is the most predominant splice form in the brain. Fujishige et al. reported various N-terminal splice variants in rats (PDE10A2-PDE10A6), but enzymatic properties and biological functions of minor splice variants have not determined yet (Fujishige et al. 1999b). Another feature in the primary structure of PDE10A is the two GAF domains (stands for cGMP binding and stimulated phosphodiesterases, Anabaena ▶ adenylyl cyclases, and *Escherichia coli* FhlA) that are situated in the N-terminus of the catalytic domain. Initial study using chimeric constructs with PDE10A GAF domain and bacterial adenylyl cyclase indicated that the cyclase activity was activated by cAMP binding to the GAF domain (Gross-Langenhoff et al. 2006). Subsequent study conducted by Matthiesen et al. has demonstrated that binding of cAMP does not stimulate hydrolytic activity of full-length human PDE10A2 (Matthiesen and Nielsen 2009). Crystal structure of PDE10A GAF-B domain with cAMP has been reported (Handa et al. 2008). This study has demonstrated that a cNMP-binding pocket tightly binds cAMP, and the β1 and β2 strand in the pocket contribute to the recognition of adenine base. At present, implication of the nucleotide binding to GAF domain for the biological pathway in vivo remains to be determined.

Tissue Expression and Subcellular Localization

PDE10A transcripts are highly expressed in basal ganglia, particularly in striatum and caudate nucleus in the central nervous system (Fujishige et al. 1999a, b). In the peripheral tissues in humans, moderate expression was detected in the thyroid, testis, heart, and kidney (Fujishige et al. 1999a; Loughney et al. 1999). Immunohistochemical analysis has revealed that PDE10A protein is expressed exclusively in the cell body of the striatal GABArgic medium spiny neurons and their projecting axons in substantia nigra (Seeger et al. 2003). Striatal expression of PDE10A transcripts and proteins is conserved among humans and rodents, but there are slight species differences in other region in the brain. For example, in rats, marked expression was observed in hippocampus and cerebellum, whereas only minimum expression was detected in these tissues in mice (Coskran et al. 2006). PDE10A proteins are clearly detectable in cerebral cortex in humans. On the other hand, the expression in this area is relatively low in mice and rats. Growing evidence has suggested the idea that subcellular localization of PDEs contributes to the compartmentation of cyclic nucleotide signals. Human and rat PDE10A2 proteins were demonstrated to be localized in membrane fraction in PC12 cells, while human PDE10A1, and rat PDE10A3 were mainly expressed in cytosolic fraction (Kotera et al. 2004). Rat PDE10A2, a major splice variant in the striatum, has been mainly detected in the membrane fraction prepared from striatum by using specific antibody for PDE10A2 (Kotera et al. 2004). Xie et al. reported detailed subcellular localization of PDE10A in striatum by immunoelectron microscopy study (Xie et al. 2006). The report has indicated that the PDE10A protein was confined in vesicle-filled presynaptic terminals of striatal neurons in rats. Two modes of posttranscriptional regulation are known to alter subcellular localization of PDE10A. Kotera et al. has reported that phosphorylation at Thr-16 of PDE10A2 by PKA-mediated pathway causes alteration of subcellular localization from membrane to cytosol in PC12 cells (Kotera et al. 2004). Charych et al. has demonstrated that palmitoylation of Cys-11 of PDE10A2 is required for membrane association in vivo and distal dendritic trafficking of the protein in cultured striatal neurons (Charych et al. 2010). Phosphorylation of PDE10A2 at Thr-16 interfered with palmitoylation at Cys-11, resulting in the retention of PDE10A2 in cytosolic fraction. Zinc finger domain-containing DHHC domain-containing protein (ZDHHC)-7 and -19 were identified as candidates for the enzyme-mediating palmitoylation of PDE10A2.

Implication for Cellular Signaling

Striatal medium spiny neurons can be categorized into two distinct neurons: dopamine D1 receptor-containing (direct pathway) neurons and dopamine

D2 receptor-containing (indirect pathway) neurons, which are involved in the regulation of cortico-striatal-thalamic loop. Involvement of PDE10A on corticostriatal signaling was investigated by Threlfell et al. using electrophysiological technique in vivo (Threlfell et al. 2009). Single-cell recording from direct or indirect pathway neurons, which were identified based on the response to antidromic stimulation, has revealed that administration of PDE10A inhibitors, papaverine and TP-10, increase the responsiveness of striatal neurons to cortical stimulation in vivo. Moreover, robust increase of the activity in response to cortical input was observed in striatopallidal neurons but not striatonigral neurons. As well as PDE10A, ▶ PDE4 is expressed in stiratum. Nishi et al. have reported distinct role of PDE4 and PDE10A in striatum (Nishi et al. 2008). Expression of PDE10A was detected in all ▶ DARPP-32 positive neurons, while PDE4B was mainly expressed in striatopallidal neurons rather than striatonigral neurons. PDE10A inhibition by papaverine, but not PDE4 inhibition by rolipram, caused DARPP-32 and GluR1 phosphorylation in stiatum. On the other hand, phosphorylation of tyrosine hydroxylase was increased by rolipram at presynaptic dopaminergic terminals. In striatonigral neurons, PDE10A inhibition activated cAMP-pathway (measured by DARPP-32 phosphorylation) resulting in the potentiation of dopamine D1 receptor pathway. In striatopallidal neurons, papaverine activated cAMP-pathway by potentiating adenosine A2a receptor pathway and inhibiting dopamine D2 receptor pathway simultaneously.

Knockout (KO) Mouse Phenotype

Behavioral characteristics of PDE10A KO mice (C57BL/6 background) have been reported by Siuciak et al. (2008). PDE10A KO mice showed slight decrease in spontaneous locomotor activity and delayed acquisition in conditioned avoidance response (CAR). MK-801-induced increase of locomotor activity was blunted in KO mice compared with wild-type mice, while there was no difference in PCP-induced locomotor response. Unexpectedly, basal cyclic nucleotide concentration and ▶ CREB phosphorylation were unchanged in striatum from KO mice, whereas dopamine turnover was increased in KO mouse striatum. KO mice demonstrated increased response to amphetamine and methamphetamine administration. These observations suggested the hypothesis that PDE10A may influence cortical glutamatergic neuronal activity via both D1 receptor-mediated direct pathway and D2 receptor-mediated indirect pathway. Sano et al. has reported increased social interaction behavior in KO mice of PDE10A2, a major splice form in the brain (Sano et al. 2008). Although elevation of cAMP and CREB phosphorylation in striatum was observed, PDE10A2 KO mice did not exhibit abnormalities in spontaneous locomotor activity. Behavioral test battery has revealed that responses in elevated plus maze, rotor rod, conditioned fear, and forced-swim were unchanged between PDE10A2 KO and wild-type mice.

Inhibitors

Papaverine, an opium alkaloid used for the treatment of visceral spasm and smooth muscle relaxation, was a first-identified inhibitor for PDE10A (IC_{50} value = 36 nM) (Siuciak et al. 2006). Papaverine has shown to increase cGMP and CREB phosphorylation in mouse striatum; in addition, these observations were completely abolished in PDE10A KO mice. In 2008, a research group from Pfizer has reported the discovery of a novel class of inhibitor, TP-10 (Schmidt et al. 2008). TP-10 potently inhibited rat PDE10A (K_i value = 0.3 nM) and showed great selectivity (> 3,000-fold) against other PDEs. TP-10 caused dose-dependent increase of cAMP, cGMP, and phosphorylated CREB levels in mouse striatum in vivo. Administration of TP-10 inhibited CAR and reversed amphetamine-induced auditory gating deficits in rodents. Clinically used D2-antagonists and atypical antipsychotics have been known to induce catalepsy. Although TP-10 induced weak catalepsy, the response was not increased by the dose elevation in contrast to the D2-antagonists and atypical antipsychotics. In 2009, detailed evaluation of MP-10, a novel compound that is closely related analogue of TP-10, in several animal models for schizophrenia was reported (Grauer et al. 2009). MP-10 demonstrated IC_{50} value of 1.3 nM for human PDE10A and at least 1,300-fold selectivity for other PDEs. As expected from the results in TP-10 treatment, MP-10 caused significant increase of cAMP and cGMP in vivo. In addition, phosphorylation of CREB, DARPP-32, and GluR1 was increased by MP-10 treatment. MP-10 administration demonstrated

weak catalepsy and disruption of CAR in rodents. Increased phosphorylation of GluR1 at Ser-845 and upregulation of enkephalin and substance-P mRNA accompanying with MP-10 treatment further supports the idea that PDE10A modulate dopaminergic and glutamatergic neurotransmission. The fact that MP-10 improved social odor memory, and increased time spent in social side in social approach/social avoidance model in mice suggest that MP-10 has a potential for the improvement of cognitive function and negative symptoms in schizophrenia patients. To date, many structurally different classes of PDE10A inhibitors have been reported from various laboratories. Comprehensive review of these inhibitors was published by Kehler and Kilburn (2009). Currently, phase 1 clinical trial using MP-10 has been conducted.

Summary

PDE10A is a dual-specific and cGMP-inhibited phosphodiesterase that is highly expressed in the striatal medium spiny neurons. This unique expression property has suggested association of this enzyme for basal ganglia function. Recent advances in the research of PDE10A using genetic deletion and selective inhibitors have suggested that PDE10A is involved in the control of dopaminergic circuit as well as the modulation of gultamatergic neuronal transmission. D2 antagonist and atypical antipsychotic drugs are widely used for the treatment of schizophrenia, while these medications exhibit several side effects such as extrapyramidal disorder and obesity, and ineffectiveness for negative symptom in schizophrenia. Distinct roles of PDE10A in the regulation of neuronal circuit may provide novel hypothesis in the regulation of brain function by PDE10A and therapeutic opportunity for schizophrenia by PDE10A inhibitor.

References

Charych EI, Jiang LX, Lo F, Sullivan K, Brandon NJ. Interplay of palmitoylation and phosphorylation in the trafficking and localization of phosphodiesterase 10A: implications for the treatment of schizophrenia. J Neurosci. 2010;30:9027–37.

Coskran TM, Morton D, Menniti FS, Adamowicz WO, Kleiman RJ, Ryan AM, Strick CA, Schmidt CJ, Stephenson DT. Immunohistochemical localization of phosphodiesterase 10A in multiple mammalian species. J Histochem Cytochem. 2006;54:1205–13.

Fujishige K, Kotera J, Michibata H, Yuasa K, Takebayashi S, Okumura K, Omori K. Cloning and characterization of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). J Biol Chem. 1999a;274:18438–45.

Fujishige K, Kotera J, Omori K. Striatum- and testis-specific phosphodiesterase PDE10A isolation and characterization of a rat PDE10A. Eur J Biochem. 1999b;266:1118–27.

Grauer SM, Pulito VL, Navarra RL, Kelly MP, Kelley C, Graf R, Langen B, Logue S, Brennan J, Jiang L, Charych E, Egerland U, Liu F, Marquis KL, Malamas M, Hage T, Comery TA, Brandon NJ. Phosphodiesterase 10A inhibitor activity in preclinical models of the positive, cognitive, and negative symptoms of schizophrenia. J Pharmacol Exp Ther. 2009;331:574–90.

Gross-Langenhoff M, Hofbauer K, Weber J, Schultz A, Schultz JE. cAMP is a ligand for the tandem GAF domain of human phosphodiesterase 10 and cGMP for the tandem GAF domain of phosphodiesterase 11. J Biol Chem. 2006;281:2841–6.

Handa N, Mizohata E, Kishishita S, Toyama M, Morita S, Uchikubo-Kamo T, Akasaka R, Omori K, Kotera J, Terada T, Shirouzu M, Yokoyama S. Crystal structure of the GAF-B domain from human phosphodiesterase 10A complexed with its ligand, cAMP. J Biol Chem. 2008;283:19657–64.

Kehler J, Kilburn JP. Patented PDE10A inhibitors: novel compounds since 2007. Expert Opin Ther Pat. 2009;19:1715–25.

Kotera J, Fujishige K, Yuasa K, Omori K. Characterization and phosphorylation of PDE10A2, a novel alternative splice variant of human phosphodiesterase that hydrolyzes cAMP and cGMP. Biochem Biophys Res Commun. 1999;261:551–7.

Kotera J, Sasaki T, Kobayashi T, Fujishige K, Yamashita Y, Omori K. Subcellular localization of cyclic nucleotide phosphodiesterase type 10A variants, and alteration of the localization by cAMP-dependent protein kinase-dependent phosphorylation. J Biol Chem. 2004;279:4366–75.

Loughney K, Snyder PB, Uher L, Rosman GJ, Ferguson K, Florio VA. Isolation and characterization of PDE10A, a novel human 3′, 5′-cyclic nucleotide phosphodiesterase. Gene. 1999;234:109–17.

Matthiesen K, Nielsen J. Binding of cyclic nucleotides to phosphodiesterase 10A and 11A GAF domains does not stimulate catalytic activity. Biochem J. 2009;423:401–9.

Nishi A, Kuroiwa M, Miller DB, O'Callaghan JP, Bateup HS, Shuto T, Sotogaku N, Fukuda T, Heintz N, Greengard P, Snyder GL. Distinct roles of PDE4 and PDE10A in the regulation of cAMP/PKA signaling in the striatum. J Neurosci. 2008;28:10460–71.

Sano H, Nagai Y, Miyakawa T, Shigemoto R, Yokoi M. Increased social interaction in mice deficient of the striatal medium spiny neuron-specific phosphodiesterase 10A2. J Neurochem. 2008;105:546–56.

Schmidt CJ, Chapin DS, Cianfrogna J, Corman ML, Hajos M, Harms JF, Hoffman WE, Lebel LA, McCarthy SA, Nelson FR, Proulx-LaFrance C, Majchrzak MJ, Ramirez AD, Schmidt K, Seymour PA, Siuciak JA, Tingley 3rd FD, Williams RD, Verhoest PR, Menniti FS. Preclinical characterization of selective phosphodiesterase 10A inhibitors: a new therapeutic approach to the treatment of schizophrenia. J Pharmacol Exp Ther. 2008;325:681–90.

Seeger TF, Bartlett B, Coskran TM, Culp JS, James LC, Krull DL, Lanfear J, Ryan AM, Schmidt CJ, Strick CA, Varghese AH, Williams RD, Wylie PG, Menniti FS. Immunohistochemical

localization of PDE10A in the rat brain. Brain Res. 2003;985:113–26.
Siuciak JA, Chapin DS, Harms JF, Lebel LA, McCarthy SA, Chambers L, Shrikhande A, Wong S, Menniti FS, Schmidt CJ. Inhibition of the striatum-enriched phosphodiesterase PDE10A: a novel approach to the treatment of psychosis. Neuropharmacology. 2006;51:386–96.
Siuciak JA, McCarthy SA, Chapin DS, Martin AN, Harms JF, Schmidt CJ. Behavioral characterization of mice deficient in the phosphodiesterase-10A (PDE10A) enzyme on a C57/Bl6N congenic background. Neuropharmacology. 2008;54:417–27.
Soderling SH, Bayuga SJ, Beavo JA. Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. Proc Natl Acad Sci USA. 1999;96:7071–6.
Threlfell S, Sammut S, Menniti FS, Schmidt CJ, West AR. Inhibition of phosphodiesterase 10A increases the responsiveness of striatal projection neurons to cortical stimulation. J Pharmacol Exp Ther. 2009;328:785–95.
Xie Z, Adamowicz WO, Eldred WD, Jakowski AB, Kleiman RJ, Morton DG, Stephenson DT, Strick CA, Williams RD, Menniti FS. Cellular and subcellular localization of PDE10A, a striatum-enriched phosphodiesterase. Neuroscience. 2006;139:597–607.

Phosphoinositide 3-Kinase

Lomon So and David A. Fruman
Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

Synonyms

Phosphatidylinositol 3-kinase; Phosphatidylinositol 3-OH kinase; PI 3-K; PI 3-kinase; PI3K; PI(3)K

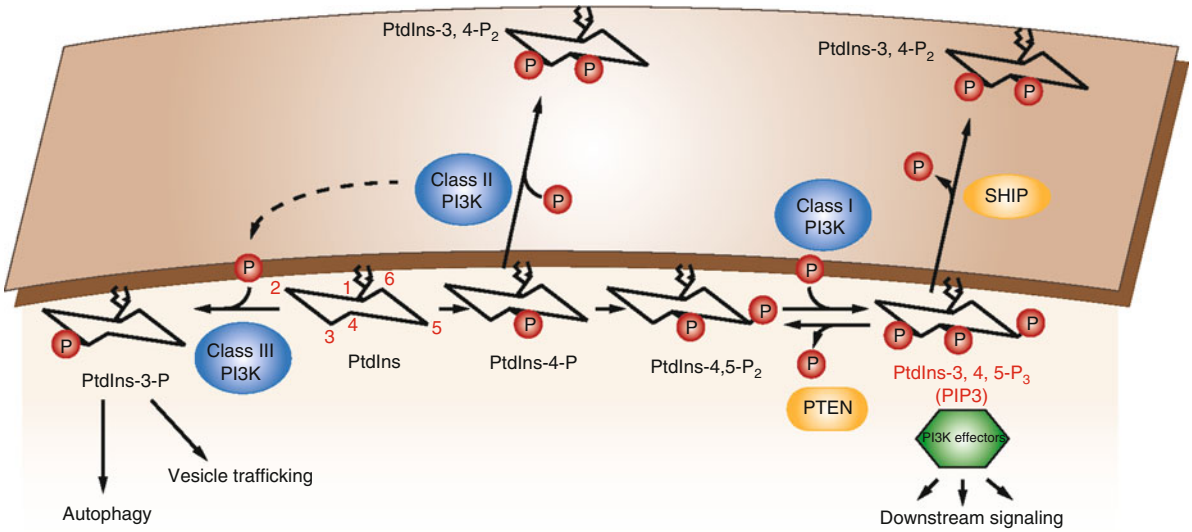
Historical Background

Phosphoinositide 3-kinase, commonly abbreviated PI3K, is one of the most well-studied enzymes in the field of signal transduction (Fruman et al. 1998; Vanhaesebroeck et al. 2010). PI3K actually refers to a family of enzymes encoded by eight genes in mammals. Orthologs of one or more PI3K genes exist in all animals as well as in yeast. These enzymes share the ability to phosphorylate the 3′-hydroxyl of the inositol head group of phosphatidylinositol (PtdIns), generating the lipid PtdIns-3-P (Fig. 1). Some members of the PI3K family can act on phosphoinositides, which are phosphorylated derivatives of PtdIns (such as PtdIns-4,5-P_2). Therefore, the family is properly referred to as phosphoinositide 3-kinases rather than simply phosphatidylinositol 3-kinases. The products of PI3Ks, generally termed 3-phosphorylated inositides (3-PIs), serve as membrane recruitment signals for cytoplasmic proteins with selective 3-PI-binding domains. Since the production of 3-PIs is transient and reversed by lipid phosphatases, PI3K activation provides a means to dynamically control assembly of signaling complexes at cellular membranes.

PI3K was first discovered in the mid-1980s as an enzymatic activity that co-precipitated with activated growth factor receptors and oncoproteins (Cantley et al. 1991). Although controversial at first, it was soon appreciated that activation of PI3K and production of 3-PIs is a signaling response common to a great variety of receptor systems. The genes encoding PI3Ks were cloned starting in the early 1990s. Identification of PI3K inhibitors began at this time and continues to the present day, with pharmaceutical companies and academic laboratories around the world racing to develop the most potent and selective compounds (Marone et al. 2008; Yap et al. 2008; Engelman 2009; Liu et al. 2009; Workman et al. 2010). The reason for this intense activity is that hyperactive PI3K signaling is now recognized as a driving force in human cancer, inflammatory diseases, metabolic disorders, and in many other clinical conditions (Engelman et al. 2006; Fruman and Bismuth 2009). PI3K genes are *bona fide* oncogenes in that gain-of-function mutations are found in a large fraction of human cancers (Samuels and Ericson 2006; Denley et al. 2008). Some of the most common tumor suppressor genes act by opposing PI3K signaling (Salmena et al. 2008). Gene targeting studies have confirmed the central role of PI3K enzymes and regulatory subunits in cell proliferation and in a diverse set of physiological functions (Deane and Fruman 2004; Fruman and Bismuth 2009; Vanhaesebroeck et al. 2010). *Note to reader: The review articles cited in this section are definitive resources that provide references to primary literature. The main text cites additional reviews relevant to specific topics not covered in the above references.*

PI3K Enzymes and Products

The eight PI3K catalytic subunits in mammals are grouped into three classes according to structure and

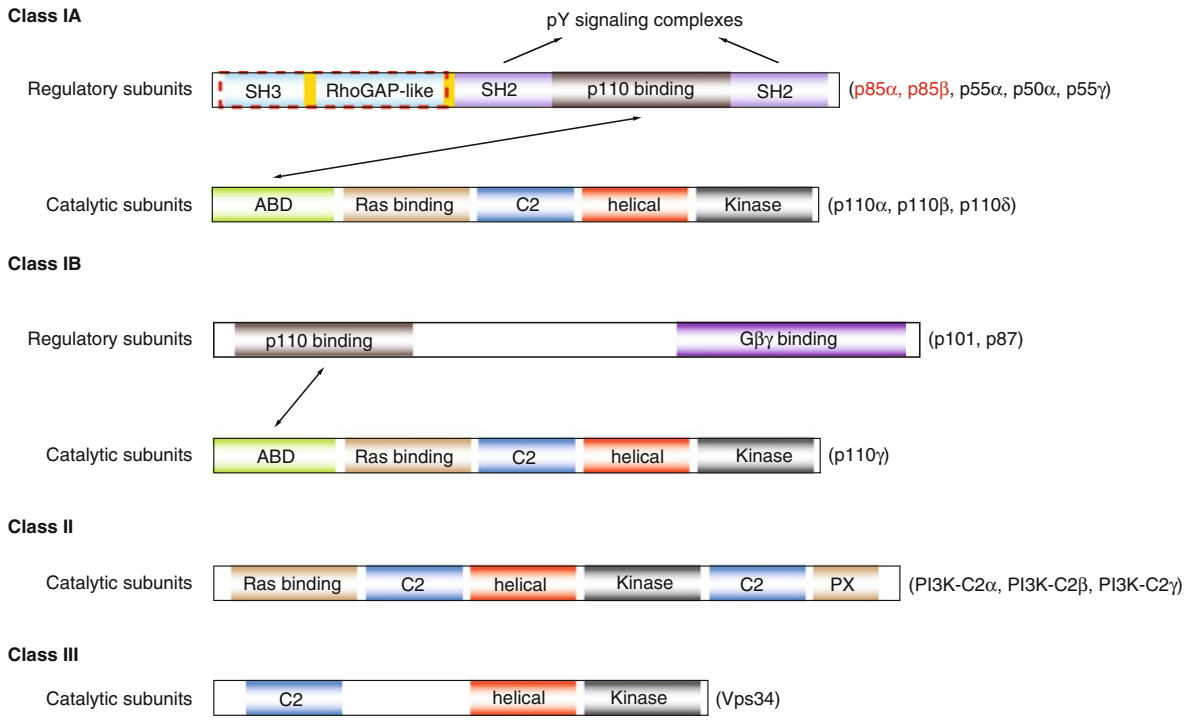


Phosphoinositide 3-Kinase, Fig. 1 Pathways of synthesis and degradation of 3-phosphorylated phosphoinositides (3-PIs). The *brown* area represents the inner leaflet of the membrane bilayer, viewed en face. The three classes of PI3K are indicated in *blue*; major phosphatases are in *orange*. Effectors of class I PI3K (*green*) bind selectively to PIP_3 and/or PtdIns-3,4-P_2. The colors designated in this figure legend and others refer to the electronic versions of the figures

substrate preference (Fig. 2). Class I PI3Ks can utilize various substrates, but it is thought that the primary activity of these enzymes is to convert PtdIns-4,5-P_2 to PtdIns-3,4,5-P_3 (PIP_3) (Fig. 1). PIP_3 is nearly undetectable in unstimulated cells but is rapidly produced following engagement of receptors that activate class I PI3K – which include most receptor tyrosine kinases, tyrosine kinase-coupled receptors, and G-protein-coupled receptors (GPCRs). PIP_3 can be converted back to PtdIns-4,5-P_2 by the phosphatase and tensin homolog (► PTEN), or dephosphorylated on the 5′-phosphate by SH2-containing inositol phosphatases (SHIP1 or SHIP2) to produce PtdIns-3,4-P_2. The four class I enzymes are customarily subdivided into class IA (p110α, β, δ) and class IB (p110γ), based on their distinct regulatory subunits and upstream activators (Fig. 2). Class IA enzymes form obligate heterodimers with one of five regulatory isoforms (p85α, p55α, p50α, p85β, or p55γ) whereas the class IB PI3K associates with distinct regulatory isoforms (p87 or p101). Each class IA regulatory isoform contains Src homology-2 (SH2) domains that mediate binding to tyrosine-phosphorylated signaling complexes. The class IB regulatory subunits link class IB PI3K to GPCR signaling via heterotrimeric G proteins. However, there is increasing evidence that class IA enzymes can be activated downstream of GPCRs, indicating that the class IA-IB distinction is not absolute. Furthermore, both class IA and class IB isoforms possess domains for binding to Ras, a GTPase activated downstream of tyrosine kinases.

Structural studies have provided considerable insight into the domain topology of class I PI3Ks, their mode of regulation by regulatory subunits and Ras, and their interaction with inhibitor compounds (Williams et al. 2009). The p110γ isoform was the first to be crystallized, followed more recently by p110α, p110β and p110δ. The studies of p110α have provided insight into the mechanisms by which oncogenic mutations in the genes encoding p110α (*PIK3CA*) and p85α (*PIK3R1*) cause elevated activity of p85α/p110α dimers.

There are three class II enzymes, each containing a C2 domain and termed PI3K-C2α, PI3K-C2β, and PI3K-C2γ. There is a single class III PI3K that is homologous to the yeast PI3K enzyme Vps34. Class III enzymes associate with the protein Vps15. Class II and III enzymes act on PtdIns to produce PtdIns-3-P, though the class II PI3Ks might also convert PtdIns-4-P to PtdIns-3,4-P_2. It is clear that regulated production of PtdIns-3-P is essential for vesicle trafficking and for the initiation of autophagy. However, compared to the class I PI3Ks, less is known about the function of class II and class III PI3Ks. A recent review article provided



Phosphoinositide 3-Kinase, Fig. 2 Domain structure of PI3K catalytic and regulatory subunits. Among the class IA regulatory subunits, only p85α and p85β possess the domains denoted by the *red* dashed box

a comprehensive review of the available literature on class II and III PI3Ks (Vanhaesebroeck et al. 2010).

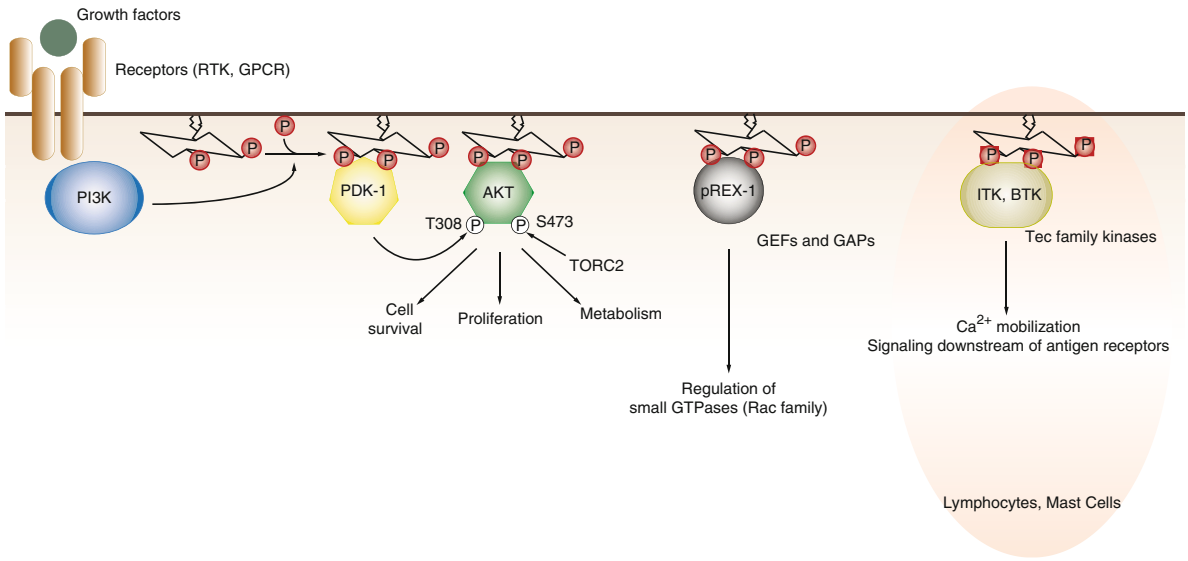
PI3K Effectors

The term "PI3K effector" is applied to any protein that contains a domain with selective affinity for 3-PIs, allowing the protein to be recruited to membranes where a PI3K enzyme is active (Fig. 3). There are several types of 3-PI-binding domain, of which the most common are the pleckstrin homology (PH), phox homology (PX), and FYVE domains (Lemmon 2008). PH domains have diverse lipid preferences, with only a subset binding selectively to 3-PIs and, within this group, differing preference for PIP_3, PtdIns-3,4-P_2, etc. Most PX domains are selective for PtdIns-3-P or PtdIns-3,4-P_2, and FYVE domains mostly bind to PtdIns-3-P.

PH domains selective for PIP_3 or PtdIns-3,4-P_2 are present in a number of important signaling enzymes and small G protein modifiers (Fig. 3). PDK-1 (phosphoinositide-dependent kinase-1) is a constitutively active serine/threonine kinase whose PH domain allows the enzyme to interact with membrane-associated substrates. Most notable of these are the AKT family of serine/threonine kinases (also known as protein kinase B). The three AKT proteins (AKT1, 2, 3) possess PH domains and are phosphorylated by PDK-1-on a threonine residue in the activation loop (T308 in AKT1). Further AKT activation is achieved by phosphorylation of a C-terminal hydrophobic motif (S473 in AKT1). In many cellular contexts, the target of rapamycin (TOR) complex-2 (TORC2) is responsible for AKT-S473 phosphorylation. Active AKT phosphorylates numerous substrates that regulate cell survival, proliferation, nutrient uptake, and metabolism (Manning and Cantley 2007).

PIP_3-selective PH domains are also present in most tyrosine kinases of the Tec family, including ITK and BTK that control Ca^{2+} mobilization and other crucial signaling events downstream of antigen receptors in lymphocytes and mast cells. PH domains in guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) link PI3K activation to regulation of some small GTPases. One well-known example is ▶ pREX-1, a GEF for Rac family GTPases that acts downstream of p110γ in neutrophils.

Many important signaling components and transcription factors are activated (or inactivated)

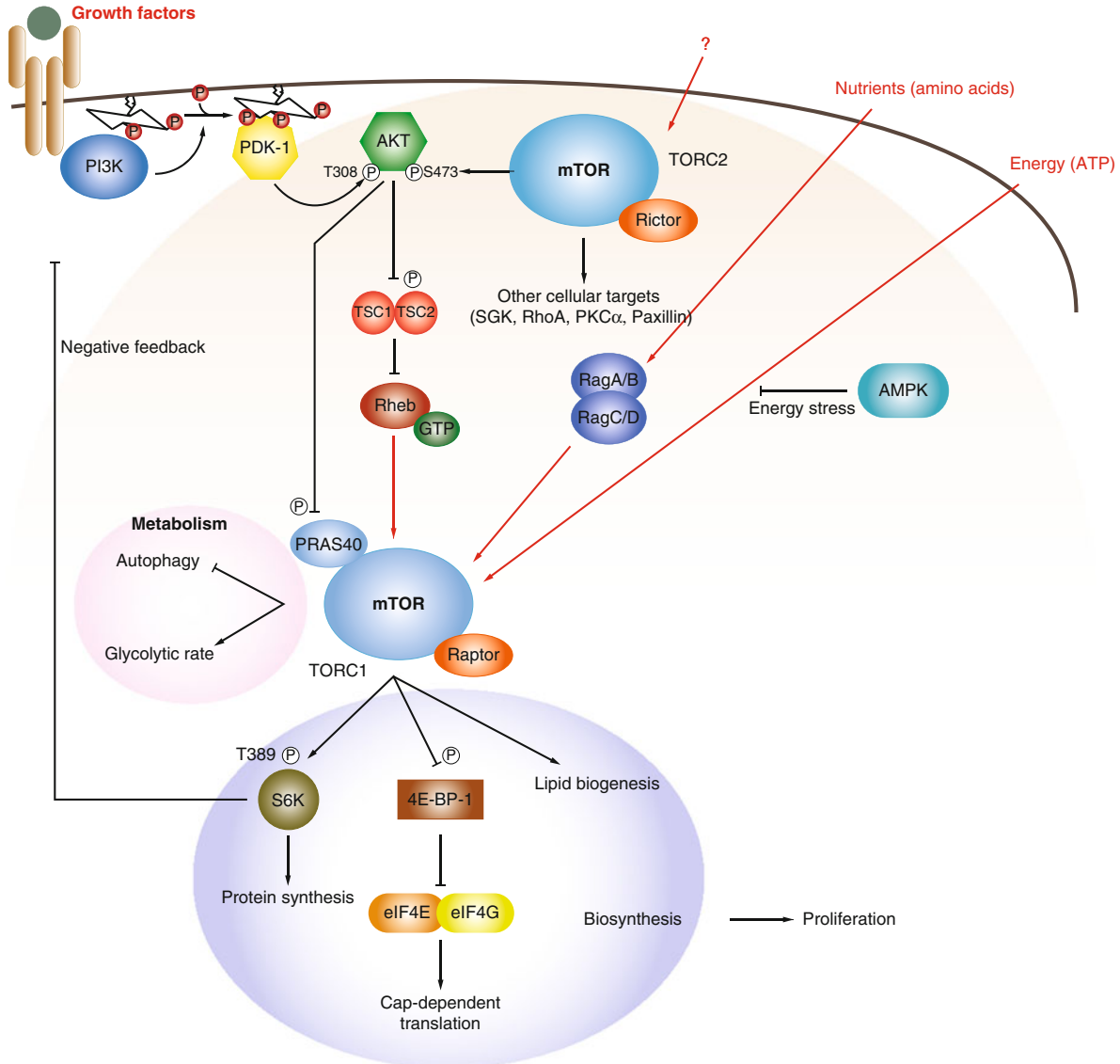


Phosphoinositide 3-Kinase, Fig. 3 Examples of PH domain-containing effectors of class I PI3K. Each of the proteins shown (PDK-1, AKT, pREX-1, Tec family kinases) bind selectively to PIP_3. Key downstream events controlled by these PI3K effectors are shown

indirectly by PI3K signaling, via the direct effectors described in the preceding paragraphs. Space constraints do not allow a comprehensive summary of PI3K-regulated cellular components. The discussion here will focus on one crucial downstream network controlled by TOR complex-1 (TORC1) (Fig. 4). The enzyme TOR (also known as ▶ mTOR) is a serine/threonine kinase that exists in two complexes with distinct components, TORC1 and TORC2 (Foster and Fingar 2010; Sparks and Guertin 2010). While TORC2 acts upstream on AKT as mentioned above, TORC1 acts downstream of AKT. When fully active, TORC1 promotes biosynthetic events that drive cell proliferation, including protein and lipid synthesis; TORC1 also regulates metabolism by increasing glycolytic rates and inhibiting autophagy. TORC1 actually integrates signals from many sources: growth factor signaling pathways (AKT and others), nutrient availability, and stress response pathways. AKT promotes TORC1 activity by phosphorylating and inhibiting two negative regulators of TORC1. One AKT substrate, TSC2, is part of the tuberous sclerosis complex (TSC) that has GAP activity toward the Rheb GTPase upstream of TORC1. Proline-rich AKT substrate of 40kDa (PRAS40) is part of the TORC1 complex and its phosphorylation by AKT relieves PRAS40-mediated suppression of TORC1 activity. Active TORC1 phosphorylates ribosomal S6 kinases (S6K1 and S6K2) and eIF4E-binding proteins (4EBPs) to promote mRNA translation, and other cellular substrates to regulate autophagy and other processes. It has become increasingly clear, however, that compensatory pathways can maintain TORC1 activity in the absence of detectable PI3K/AKT signaling, particularly in cancer cells. The implications of this will be discussed in the section "PI3K Pharmacology."

PI3K Genetics

Gene targeting and transgenesis experiments have greatly added to the understanding of PI3K gene function in model organisms. The discussion that follows will focus on studies of class I PI3K genes in mice. For each of the catalytic subunits, null "knockout" (KO) alleles as well as kinase-dead "knock-in" (KI) alleles have been reported. p110α (*Pik3ca*) KO or KI results in an embryonic lethal phenotype in homozygotes. Mice heterozygous for p110αKI are viable but display severe defects in organismal metabolism, including insulin resistance, hyperglycemia and adiposity. At the molecular level, p110α-deficient cells show reduced signaling downstream of receptors that couple to insulin receptor substrate (IRS) proteins. Together with pharmacological studies, this finding illustrates that p110α has a nonredundant function in cellular



Phosphoinositide 3-Kinase, Fig. 4 Simplified diagram of the TOR signaling network. The target of rapamycin (denoted "mTOR" in this figure, in keeping with previous literature in which the protein is called "mammalian" or "mechanistic" target of rapamycin) is a single kinase that exists in two cellular complexes, TORC1 and TORC2. The subunit composition differs among these complexes, as do the activation mechanisms and downstream functions as indicated. Key points are that TORC2 functions upstream of AKT, TORC1 functions downstream of AKT, and TORC1 also feeds back through S6 kinases to dampen PI3K and AKT activity

responses to insulin and insulin-like growth factor. Other studies of p110αKI mice have revealed a role for this isoform in angiogenesis. A distinct knock-in strain carrying a mutation in the Ras-binding domain of p110α provided evidence that p110α is a Ras effector important for lymphangiogenesis and cellular transformation.

Mice with germline deletion of p110β (*Pik3cb*) die early in embryogenesis, but p110βKI mice are viable. Together with other results this finding establishes kinase-independent functions of the p110β isoform. Kinase-dependent functions of p110β have also been identified through analysis of p110βKI mice as well as strains with conditional p110βKO in specific tissues.

Key observations include the following: (1) p110β plays an important role in the malignant phenotype of cancer cells with loss of PTEN, suggesting that p110β mediates growth factor-independent "basal" production of PIP_3 that is normally hydrolyzed by PTEN. (2) p110β has a key function downstream of GPCRs including receptors for lysophosphatidic acid and chemokines, a finding supported by pharmacological approaches. It remains unclear how p110β activity or localization is modulated by GPCR signaling.

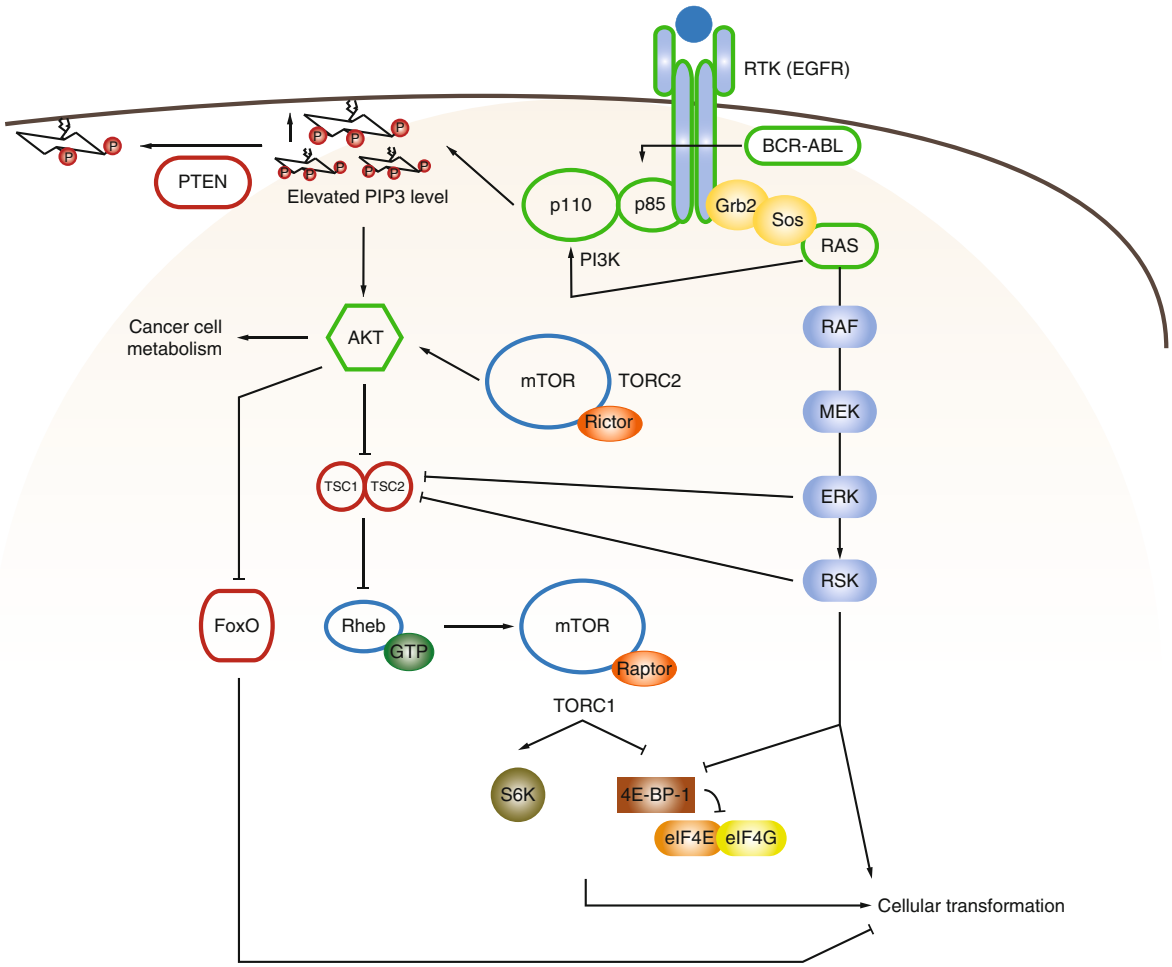
Unlike p110α and p110β, p110δ and p110γ are not ubiquitously expressed, and germline KO mice are viable. The most dramatic phenotypes of p110δ (*Pik3cd*) and p110γ (*Pik3cg*) KO mice are in the immune system. Notable examples include, for p110δKO and KI, impaired B cell development and function, and defective T cell differentiation and trafficking. In p110γKO and KI mice, there are major defects in migration and function of innate immune cells (i.e., macrophages and dendritic cells). Combined inactivation of p110δ and p110γ produces an early block in T cell development and affects natural killer (NK) cell function. Mast cell function and allergic responses are impaired in the absence of either p110δ or p110γ.

There are three genes encoding class IA regulatory subunits: *Pik3r1* encodes p85α, p55α, and p50α; *Pik3r2* encodes p85β; and *Pik3r3* encodes p55γ. Although inactivation of the *Pik3r3* gene has not been reported, the *Pik3r1* and *Pik3r2* genes have been targeted in various ways and in combination. These investigations have shown complex biology of the class IA regulatory subunits. There are unique functions of individual isoforms in some contexts and redundant functions in others. The functions of class IA regulatory subunits in insulin signaling are particularly complex, in that individual deletion of p85α and p85β enhances insulin sensitivity whereas combined deletion reduces insulin sensitivity. p85α and p85β each have diverse functions in immune cells. Studies of insulin-responsive cells and lymphocytes have also provided evidence for "scaffolding" functions of p85α and p85β, independent of the associated catalytic subunit. There are two genes encoding class IB regulatory subunits (*Pik3r5* encodes p101, *Pik3r6* encodes p87). Knockout of *Pik3r5* has shown a nonredundant role for p101 in neutrophil signaling and functional responses to inflammatory stimuli.

Gain-of-function alleles of class I catalytic subunits have been tested in transgenic models and in tissue culture. Increased p110 activity can be accomplished by overexpression of wild-type or oncogenic mutants, including naturally occurring p110α alleles (see below) and engineered mutations that target p110 proteins constitutively to the membrane. Cell culture experiments were the first to demonstrate transforming activity of p110 proteins, and have shown that any of the four class I enzymes can transform fibroblasts when mutated and/or overexpressed. In vivo, activated alleles of p110α and p110β are oncogenic in mice. Analyses of mice with targeted deletion of PTEN have confirmed the tumor suppressor function of this lipid phosphatase and have shown gene dosage-dependent tumor penetrance. In addition, PTEN heterozygous mice develop lymphoproliferative disease. Similarly, mice expressing constitutively active AKT in T cells develop lymphoproliferation and thymic lymphomas. A truncation mutant of p85α (p65) that causes elevated PI3K signaling also disrupts T cell homeostasis and has oncogenic potential.

PI3K in Disease

Many human diseases have been associated with aberrant control of PI3K signaling. The most prominent correlation is between elevated cellular PI3K/AKT activity and cancer. It is now accepted that a large majority of human cancers possess mutations that drive elevated PIP_3 production. This can occur through activated tyrosine kinases (e.g., EGFR, BCR-ABL) or oncogenic Ras upstream of PI3K, loss or inactivation of PTEN, or mutations in PI3K genes themselves (Fig. 5). In epithelial cancers, gain-of-function mutations in *PIK3CA* (p110α) are common. Mutations in *PIK3R1* (p85α, p55α, and p50α) that derepress the associated catalytic subunit are found in glioblastoma at a frequency similar to *PIK3CA* mutations. Elevated p110δ activity has been associated with certain hematologic malignancies. Gene targeting studies support the model that class I PI3K activity is essential for efficient transformation by BCR-ABL or Ras. It is thought that AKT is the major PI3K effector responsible for enhanced proliferation, survival, and altered metabolism in cancer cells. Indeed, some tumor samples display mutation or overexpression of *AKT* genes. The activity of both TOR complexes, TORC1 and TORC2, also appears to be important for the establishment and maintenance of cellular transformation. Notably, some cancer cells can maintain TORC1 signaling in the absence of PI3K/AKT activity through



Phosphoinositide 3-Kinase, Fig. 5 Various mechanisms identified in cancer cells that result in elevated cellular PIP_3 levels. Components in *green* represent protein products of oncogenes that display gain-of-function in cancer. Components in *red* represent tumor suppressors that display loss-of-function in cancer. FOXO proteins are transcription factors that promote cell cycle arrest and apoptosis

inputs from the Ras-ERK pathway and other mechanisms. Also, humans with heterozygous mutations in TSC1 or TSC2 develop benign tumors whose growth is driven by elevated TORC1 activity.

Inflammatory conditions and autoimmune diseases are associated with increased PI3K/AKT/TOR signaling in immune cells. This phenomenon is generally secondary to other environmental or genetic conditions that maintain a heightened state of activation in specific immune cells. Nevertheless, the correlation between immune cell activation and PI3K/AKT/TOR signaling has placed this pathway at the center of worldwide drug discovery efforts for inflammatory diseases.

Insulin-resistance syndromes are a growing health epidemic. By definition, insulin resistance is associated with reduced signaling responses to insulin – including dampened PI3K/AKT activation. Agents that increase PI3K activity or block hydrolysis of PIP_3 could be useful therapies for insulin resistance.

PI3K Pharmacology

As summarized in the previous section, altered PI3K output is involved in the pathogenesis of many human diseases. Consequently, there has been intense and sustained research and development of compounds that modulate PI3K/AKT/TOR signaling for therapeutic benefit. The section that follows will briefly discuss five general strategies (summarized in Table 1) and their potential applicability to human cancer and inflammatory diseases.

Phosphoinositide 3-Kinase, Table 1 Strategies to target PI3K/AKT/TOR activity for human disease therapy

Strategy		Notes	Examples
Pan-class I PI3K inhibition		Has advantage over pan-PI3K inhibitors such as Wortmannin and LY294002 in terms of toxicity. However, may still have toxicity due to inhibition of all class I PI3Ks.	GDC-0941
Isoform-selective PI3K inhibition		Less toxic compared to pan-class I inhibition and may provide better efficacy in diseases driven by a specific isoform of PI3K. On the other hand, the spectrum of applications would be limited.	TGX-221 (p110β), CAL-101 (p110δ)
AKT inhibition		May provide efficacy in cancers driven by elevated AKT activity. Therapeutic window is questionable due to central role of AKT in whole-body glucose homeostasis.	GSK690693
Allosteric TOR inhibition		Currently approved for immunosuppression (rapamycin) and renal cell carcinoma (rapalogs). However, rapalogs have shown overall disappointing efficacy in cancer, correlating with inhibition of negative feedback loop.	Rapamycin, rapalogs (RAD001, CCI-779)
ATP-competitive mTOR inhibition	Nonselective	Have strong anticancer efficacy compared to rapalogs. However, inhibition of PI3K can have toxicity issues such as in the immune system.	BEZ235, XL675
	Selective (asTORi)	Evidence so far shows equivalent anticancer efficacy and less immunosuppression compared to pan PI3K/mTOR inhibitors.	PP242, Torin-1, AZD8055, WYE-354, INK128, OSI-027

One approach is to develop inhibitors of all class I PI3Ks. Until recently, the available PI3K inhibitors were nonselective and suppressed activity of all PI3K classes as well as other cellular enzymes. Now, compounds are available for both preclinical and clinical studies that are highly selective for class I enzymes ("pan-class I inhibitors"). A well-studied example is GDC-0941, a compound developed by Genentech that is in phase I trials for cancer. Other companies and academic laboratories have synthesized compounds with a comparable target profile. A second approach is to develop isoform-selective PI3K inhibitors. An inhibitor of p110δ, CAL-101, has entered trials for blood cancers, and other p110δ or dual p110δ/p110γ inhibitors are under consideration for inflammatory diseases. The relative benefits of pan-class I inhibitors versus isoform-selective inhibitors in cancer have not yet been established, and might vary among tumor subtypes. Inhibitors selective for p110α might be particularly effective against cancers with *PIK3CA* mutations, while avoiding toxicities associated with pan-PI3K inhibition. Likewise, inhibitors of p110β might find utility in cancers with loss of PTEN.

A third approach is to inhibit AKT. Various compounds have been developed that are either ATP-competitive or act through allosteric inhibition of enzyme activity or membrane localization. Considering that AKT inactivation has more profound effects on cellular and organismal metabolism than does PI3K inactivation, there is some concern about the therapeutic window for AKT inhibitors. Another concern about both PI3K and AKT inhibitors in general is that they will not prevent TORC1 activation through other mechanisms.

A fourth approach is to use allosteric inhibitors of TOR, exemplified by rapamycin and its analogs RAD001 and CCI-779. Rapamycin (sirolimus) has been a clinically approved immunosuppressant since 1997. RAD001 (everolimus) and CCI-779 (temsirolimus) are approved for the treatment of advanced renal cell carcinoma. However, the results of oncology trials with rapalogs have been disappointing overall. Rapamycin and rapalogs have mechanistic drawbacks. They do not completely inhibit TORC1 activity and do not bind to TORC2 or inhibit TORC2 activity acutely in cells. Furthermore, rapamycin disables a negative feedback loop by which S6K1 dampens upstream PI3K/AKT and Ras signaling. Therefore, cancer cells treated with rapalogs generally display elevated PI3K/AKT and Ras activity, enhancing survival even when proliferation is suppressed.

A fifth approach is to use ATP-competitive TOR inhibitors. These bind in the active site of the TOR enzyme in both complexes, and fully inhibit phosphorylation of all known TORC1 and TORC2 substrates. There are two classes of competitive TOR inhibitors. One class is not TOR-selective and inhibits

PI3K enzymes (which are structurally related to TOR) at equivalent concentrations. These are termed panPI3K/TOR inhibitors, exemplified by the compounds BEZ235 and XL675. The other class, which was developed only recently, is highly selective active-site TOR inhibitors (asTORi) that do not directly inhibit PI3K. Examples of asTORi are PP242, Torin-1, AZD8055, WYE-354, and INK128. The evidence thus far indicates that asTORi provide anticancer efficacy that is equivalent to panPI3K/TOR inhibitors, and better than rapamycin and rapalogs. asTORi are also less immunosuppressive than panPI3K/TOR inhibitors or rapamycin. Further preclinical and clinical studies are needed to identify the optimal target profile that balances efficacy and tolerability in different cancer patient populations.

Although some genetic events that activate PI3K/AKT are sufficient to transform cells in vitro and promote tumorigenesis in animal models, patient-derived human cancers generally carry multiple additional genetic lesions. Thus, it is likely that inhibitors of this pathway will be most effective when combined with other targeted therapies directed to upstream or parallel pathways. Combined targeting of PI3K/AKT/TOR with Ras/RAF/► MEK/ERK is emerging as a useful strategy in some settings.

Summary

PI3K enzymes generate 3-phosphorylated inositol lipids that coordinate many aspects of cellular physiology. In response to extracellular signals, class I PI3K produces the second messenger PIP_3 that recruits numerous cytoplasmic proteins to the plasma membrane to propagate key signaling pathways. In general, PIP_3 production promotes cell proliferation and survival, and also plays a key role in cellular and organismal metabolism. Most cancer cells carry mutations that trigger constitutive elevation of PIP_3 levels, and activated immune cells depend on PI3K signaling to carry out specialized functions. Gene targeting in mice has revealed unique and complex functions of individual PI3K catalytic and regulatory isoforms. Based on the role of aberrant PI3K activation in human disease, PI3K drug discovery has been a priority and will continue to produce inhibitor molecules of interest to basic and translational researchers.

Cross-References

► Glycogen Synthase Kinase-3

References

Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, et al. Oncogenes and signal transduction. Cell. 1991;64:281–302.

Deane JA, Fruman DA. Phosphoinositide 3-kinase: diverse roles in immune cell activation. Annu Rev Immunol. 2004;22:563–98.

Denley A, Kang S, Karst U, Vogt PK. Oncogenic signaling of class I PI3K isoforms. Oncogene. 2008;27:2561–74.

Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer. 2009;9:550–62.

Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet.. 2006;7:606–19.

Foster KG, Fingar DC. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. J Biol Chem. 2010;285:14071–7.

Fruman DA, Bismuth G. Fine tuning the immune response with PI3K. Immunol Rev. 2009;228:253–72.

Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. Annu Rev Biochem. 1998;67:481–507.

Lemmon MA. Membrane recognition by phospholipid-binding domains. Nat Rev Mol Cell Biol. 2008;9:99–111.

Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov. 2009;8:627–44.

Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129:1261–74.

Marone R, Cmiljanovic V, Giese B, Wymann MP. Targeting phosphoinositide 3-kinase: moving towards therapy. Biochim Biophys Acta. 2008;1784:159–85.

Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. Cell. 2008;133:403–14.

Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. Curr Opin Oncol. 2006;18:77–82.

Sparks CA, Guertin DA. Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. Oncogene. 2010;29(26):3733–44. Epub April 4.

Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol. 2010;11:329–41.

Williams R, Berndt A, Miller S, Hon WC, Zhang X. Form and flexibility in phosphoinositide 3-kinases. Biochem Soc Trans. 2009;37:615–26.

Workman P, Clarke PA, Raynaud FI, van Montfort RL. Drugging the PI3 kinome: from chemical tools to drugs in the clinic. Cancer Res. 2010;70:2146–57.

Yap TA, Garrett MD, Walton MI, Raynaud F, de Bono JS, Workman P. Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. Curr Opin Pharmacol. 2008;8:393–412.

Phospholipase A_2

Makoto Murakami
Lipid Metabolism Project, The Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo, Japan

Synonyms

Arachidonic acid; cPLA2; iPLA2; Leukotriene; Phospholipid; Prostaglandin; sPLA2

Historical Background

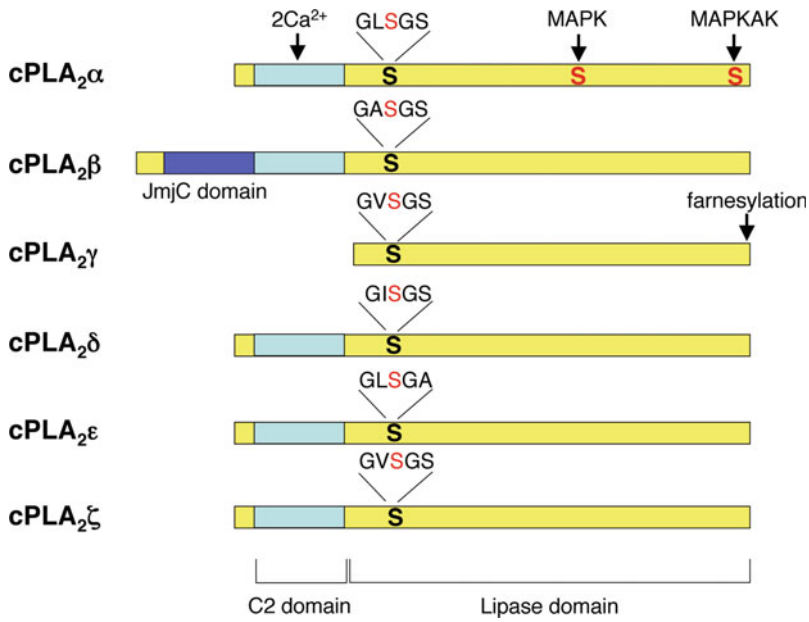
Phospholipase A_2 (PLA_2) hydrolyzes the *sn*-2 position of glycerophospholipids to yield fatty acids and lysophospholipids. In the view of signal transduction, the PLA_2 reaction has been considered to be of particular importance since *arachidonic acid*, one of the polyunsaturated fatty acids released by PLA_2, is metabolized by cyclooxygenases and *lipoxygenases* to the potent lipid mediators *prostaglandins* and ▶ leukotrienes, which are often referred to as *eicosanoids*. In addition, lysophospholipids or its metabolites, such as *lysophosphatidic acid (LPA)* and *platelet-activating factor (PAF)*, also represent another class of lipid mediators. These lipid mediators exert numerous biological actions through their cognate G protein-coupled receptors on target cells. PLA_2 has also been implicated in membrane glycerophospholipid remodeling, thereby contributing to cellular homeostasis.

The PLA_2 enzymatic activity was originally identified more than a century ago in snake venom that potently hydrolyzed egg phosphatidylcholine (PC). By the 1980s, many secreted PLA_2s ($sPLA_2$s) were purified from various venomous animal species including snakes and insects as well as from pancreatic juices in various animals. Pancreatic $sPLA_2$, the first $sPLA_2$ subtype identified in mammals, is now called group IB $sPLA_2$. In the late 1980s, several groups purified and cloned the second mammalian $sPLA_2$ that was markedly induced in inflammatory fluids in human and various animal species. This second type of $sPLA_2$, now called group IIA $sPLA_2$ (also known as an inflammatory $sPLA_2$), was thus supposed to play a key role in the production of *arachidonic acid*-derived lipid mediators in various inflammatory diseases. In the early 1990s, however, the situation became more complex with the identification of the first intracellular PLA_2, i.e., cytosolic PLA_2 group IVA ($cPLA_2\alpha$). Because of its specificity for phospholipids with *arachidonic acid* and its unique properties to undergo stimulus-coupled membrane translocation and phosphorylation, much of the interest was shifted to this enzyme as a central regulator of *arachidonic acid* metabolism. Meanwhile, the late 1990s saw a rapid increase in the discovery of multiple $sPLA_2$s as well as many other intracellular PLA_2s including Ca^{2+}-independent PLA_2s ($iPLA_2$s), PAF acetylhydrolases, lysosomal PLA_2s, and so on, by means of in silico database searches. So far, it is now known that human and mouse genomes encode genes for more than 30 PLA_2 or related enzymes, among which the $cPLA_2$ (6 isoforms), $iPLA_2$ (9 isoforms), and $sPLA_2$ (11 isoforms) families represent three major groups.

$cPLA_2\alpha$-deficient mice, which were first reported in 1997 (Uozumi et al. 1997; Bonventre et al. 1997), have provided unequivocal evidence for the central role of this enzyme in *arachidonic acid* metabolism in many, if not all, biological events. Since the beginning of the twenty-first century, mice with transgenic overexpression and/or targeted disruption of many PLA_2 subtypes have been generated. The phenotypes displayed in individual PLA_2 gene-manipulated mice might not be simply the reflection of changes in lipid mediator signaling or more particularly *eicosanoid* signaling, but could be due to the hydrolysis of one or a combination of various target membranes. In addition, several PLA_2s have been shown to link with human diseases. Here, the properties and functions of PLA_2s in the three major groups ($cPLA_2$, $iPLA_2$ and $sPLA_2$) are overviewed.

Cytosolic PLA_2s ($cPLA_2$s)

Intracellular PLA_2s comprises the $cPLA_2$ (often called group IV PLA_2) and $iPLA_2$ (group VI PLA_2) families, in which six and nine isoforms have been, respectively, identified in mammals. There is structural similarity between $cPLA_2$s and $iPLA_2$s in that the catalytic domain is characterized by a three-layer $\alpha/\beta/\alpha$ architecture employing a conserved Ser/Asp catalytic dyad instead of the classical catalytic triad. A characteristic of all these serine acylhydrolases is their ability to catalyze



Phospholipase A_2, Fig. 1 *The cPLA$_2$ family*. The enzymes belonging to this family typically possess a C2 domain, which binds to two Ca^{2+} ions, near the N-terminus followed by a catalytic domain with the lipase consensus motif GXSXG/A. cPLA$_2$α (group VIA) is a prototypic enzyme in this family, displays arachidonic acid selectivity, and plays a central role in eicosanoid biosynthesis. Phosphorylation at two sites by mitogen-activated protein kinases (MAPK) and MAPK-activated protein kinases (MAPKAPK) is essential for the activation of cPLA$_2$α in cells. cPLA$_2$β (group IVB) has a JmjC domain prior to the C2 domain. Genes for cPLA2β, δ (group IVD), ε (group IVE), and ζ (group IVF) are clustered in the same chromosomal locus, suggesting their latest evolutional relationship. cPLA$_2$γ (group IVC) is unique in that it lacks the C2 domain and that human but not mouse enzyme undergoes farnesylation at the C-terminus. The functions of cPLA$_2$β – ζ in vivo are entirely unknown

multiple reactions (PLA$_2$, PLA$_1$, lysophospholipase, transacylase, or lipase activity) in varying degrees. Therefore, in a general view, these two families seem to be evolved from a common ancestral gene. The cPLA$_2$ family has emerged at the branching point of vertebrates, in correlation with the development of the *eicosanoid* signaling cascades. Enzymes belonging to the cPLA$_2$ family (α, β, γ, δ, ε, and ζ) are characterized by the presence of a C2 domain at their N-terminal region, with an exception of cPLA$_2$γ in which this domain is absent (Fig. 1) (Ohto et al. 2005). cPLA$_2$α, the most extensively studied isoform in the cPLA$_2$ family, is widely expressed in mammalian cells and is the only PLA$_2$ subtype that has specificity for phospholipids containing *arachidonic acid*. Because of its role in initiating agonist-induced release of *arachidonic acid* for the production of *eicosanoids*, cPLA$_2$α activation is important for regulating various pathophysiological processes in a variety of cells and tissues. The biological roles of cPLA$_2$ isoforms other than cPLA$_2$α remain largely obscure.

The overall topology of the C2 domain, which is essential for membrane translocation of cPLA$_2$α in response to calcium signaling, consists of eight antiparallel β-strands interconnected by six loops. Two Ca^{2+} ions bind at one end of the C2 domain. The membrane-binding of cPLA$_2$α is driven by hydrophobic interactions that are achieved by the penetration of hydrophobic residues in the C2 domain into the PC-rich membrane core. The catalytic domain of cPLA$_2$α is composed of 14 β-strands and 13 α-helices. During the nucleophilic Ser^{228} attacks at the *sn*-2 ester bond of glycerophospholipids, Asp^{549} contributes to the activation of this catalytic center. Within the active-site channel, which penetrates one-third of the way into the catalytic domain, the catalytic dyad (Ser^{228} and Asp^{549}) is placed at the bottom of a deep and narrow cleft. The funnel is lined with hydrophobic residues and forms a cradle, to which fatty acyl moieties of phospholipids may bind. Ser^{505}, which represents a phosphorylation site by ▶ MAP kinases, is located near the interdomain linker region (Dessen et al. 1999).

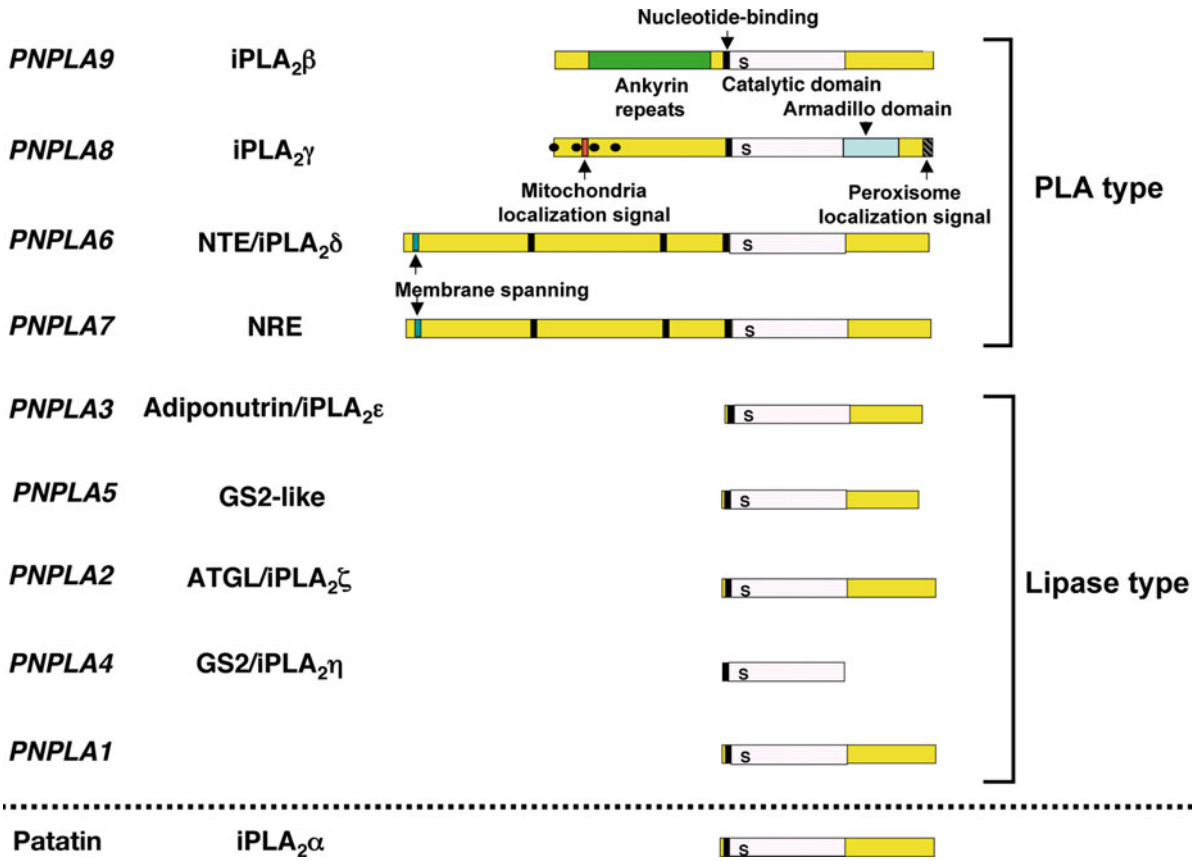
The C2 domain-directed, Ca^{2+}-dependent translocation of cPLA$_2\alpha$ from the cytosol to the Golgi and perinuclear membrane has been thought to be essential for the initiation of *arachidonic acid* release and subsequent *eicosanoid* production in agonist-stimulated cells (Clark et al. 1991). In addition to the C2 domain, the catalytic domain contributes to membrane residence of cPLA$_2\alpha$. Beyond the regulatory role of calcium signaling, the maximal activation of cPLA$_2\alpha$ in cells requires sustained phosphorylation of Ser^{505} by MAP kinases (Lin et al. 1993). The main role of Ser^{505} phosphorylation is to promote membrane penetration of hydrophobic residues in the active-site rim by inducing a conformational change of the protein, and these enhanced hydrophobic interactions allow the sustained membrane interaction of cPLA$_2\alpha$ in response to transient Ca^{2+} increase at a submicromolar level. The full activation of cPLA$_2\alpha$ is achieved by additional phosphorylation at Ser^{727} by MAP kinase-activated protein kinases that disrupts the inhibitory interaction of annexin A2 with cPLA$_2\alpha$. Phosphatidylinositol 4,5-bisphosphate (PIP_2) binds with high affinity and specificity to cPLA$_2\alpha$. Mutations in a PIP_2 binding site (Lys^{488}/Lys^{543}/Lys^{544}) reduce *arachidonic acid* release from cells without alteration in Ca^{2+}-dependent translocation or interfacial binding to membranes, indicating that PIP_2 binding in the catalytic domain principally acts to regulate cPLA$_2\alpha$ hydrolytic activity (Tucker et al. 2009). In addition, ceramide-1-phosphate (C1P) interacts directly with the cationic β-groove of the C2 domain and may contribute to translocation of cPLA$_2\alpha$ to Golgi membranes in response to stimuli.

cPLA$_2\alpha$-deficient mice display a number of phenotypes, most of which can be explained by the reduction of lipid mediators including *eicosanoids* and *PAF* (Uozumi et al. 1997; Bonventre et al. 1997; Nagase et al. 2000). Production of these lipid mediators are markedly if not solely reduced in inflammatory cells such as macrophages, neutrophils, and mast cells in cPLA$_2\alpha$-deficient mice. The null mice are protected from airway disease models such as asthma, acute respiratory distress syndrome and pulmonary fibrosis, brain injury models such as ischemia and amyloid β-induced deficits in learning and memory, autoimmune disease models such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, intestinal cancer, and atherosclerosis. Beyond these roles in pathology, the cPLA$_2\alpha$-dependent *eicosanoid* pathway is also important for tissue homeostasis, as ablation of cPLA$_2\alpha$ perturbs female reproduction, renal function, platelet function, and long-term depression in cerebellar Purkinje cells.

Ca^{2+}-Independent PLA_2s (iPLA$_2$s)

Multiple iPLA$_2$-related genes are encoded in the genomes of yeast, ameba, plants, worms, insects, and vertebrates suggesting that this group of enzymes play fundamental roles in cellular lipid metabolism conserved in the eukaryote kingdom. The human genome encodes 9 iPLA$_2$ enzymes, which are also called patatin-like phospholipase domain-containing lipases (PNPLA1-9) that share a protein domain discovered initially in patatin (iPLA$_2\alpha$), the most abundant protein of the potato tuber. The designation "PNPLA" appears to be more appropriate than "iPLA$_2$," since more than half of these enzymes function mainly as lipases rather than as phospholipases. Briefly, enzymes bearing a large and unique N-terminal region act mainly on phospholipids (phospholipase-type), whereas those lacking the N-terminal domain act on neutral lipids such as triglyceride (lipase-type) (Fig. 2). Herein, the two representative phospholipase-type of iPLA$_2$/PNPLA enzymes, iPLA$_2\beta$ and iPLA$_2\gamma$, are overviewed.

iPLA$_2\beta$ (PNPLA9) is a prototypic iPLA$_2$ enzyme that is ubiquitously expressed in various cells and occurs as several splice variants. iPLA$_2\beta$ shows no strict specificity with respect to *sn*-2 fatty acid and head group of the substrate phospholipids, is fully active in the absence of Ca^{2+}, and also exhibits *sn*-1 lysophospholipase activity and transacylase activity. Like other PNPLA enzymes, iPLA$_2\beta$ has a conserved nucleotide-binding motif proximal to the catalytic site. The N-terminal domain of iPLA$_2\beta$ has eight to nine ankyrin repeats, which contribute to a tetramer formation and give a negative effect on the enzymatic catalysis. Because of its Ca^{2+}-independent property, iPLA$_2\beta$ has long been thought to be involved in homeostatic phospholipid remodeling through deacylation of phospholipids in the land's cycle. However, accumulating evidence suggests that iPLA$_2\beta$ plays fundamental roles in cellular signaling leading to cell activation, proliferation, migration, or apoptosis. Genetic screening of *Drosophila* points Orai1 and STIM1 (components of store-operated Ca^{2+} entry (SOCE) channels) as well as an ortholog of iPLA$_2\beta$



Phospholipase A_2, Fig. 2 *The iPLA$_2$/PNPLA family*. The structures and alternative names of PNPLA1 ~ 9 are illustrated. This family is subdivided into two classes; enzymes acting on phospholipids (PLA type) and on neutral lipids (lipase type). The catalytic center (S) is located in a conserved catalytic domain that shows hology with patatin/iPLA$_2$α from potato. Adjacent to the catalytic center, there is a conserved nucleotide-binding motif. The PLA-type enzymes typically possess a long N-terminal domain, which may be involved in protein-protein interaction, distinct translation, and membrane spanning. The lipase-type enzymes lack the N-terminal domains and are thought to act primarily on triglycerides in lipid droplets. In humans, gene mutations of iPLA$_2$β or iPLA$_2$δ (NTE/PNPLA6) are linked with neurodegeneration, iPLA$_2$ζ (ATGL/PNPLA2) with neutral lipid storage disease, and iPLA$_2$ε (adiponutrin/PNPLA3) with hepatic steatosis

(CG6718) as gene products giving a great impact on SOCE (Vig et al. 2006). iPLA$_2$β has a binding site for calmodulin near the C-terminus, and the association with calmodulin leads to inactivation of iPLA$_2$β. Activation of SOCE channels and capacitative Ca^{2+} influx, which are triggered by depletion of intracellular Ca^{2+} stores, displaces inhibitory calmodulin from iPLA$_2$β, resulting in activation of iPLA$_2$β and generation of lysophospholipids that ultimately activate capacitative Ca^{2+} influx (Bolotina 2008). Given this theory, the SOCE-mediated activation of iPLA$_2$β lies upstream of Ca^{2+}-dependent activation of cPLA$_2$α. In apoptotic cells, iPLA$_2$β is cleaved at one or multiple sites by caspase-3, an event that activates iPLA$_2$β. Inhibition of iPLA$_2$β suppresses phosphatidylserine externalization at an early phase of apoptosis, suggesting that the caspase-truncated form of iPLA$_2$β accelerates apoptotic membranous changes. Strikingly, lysophosphatidylcholine (LPC) produced by the caspase-3-cleaved iPLA$_2$β is released from apoptotic cells and acts as an attracting ("find-me") signal for phagocytes (Lauber et al. 2003). Thus, during the process of inflammation, in which extravasation of neutrophils to the site of inflammation precedes a second wave of emigrating monocytes, the emigrated neutrophils undergo apoptosis leading to iPLA$_2$β-promoted generation of LPC, which in turn attracts monocytes.

Analyses of iPLA$_2$β-deficient and -transgenic mice have revealed broad roles of this enzyme in stimulus-induced *arachidonic acid* release, sperm motility,

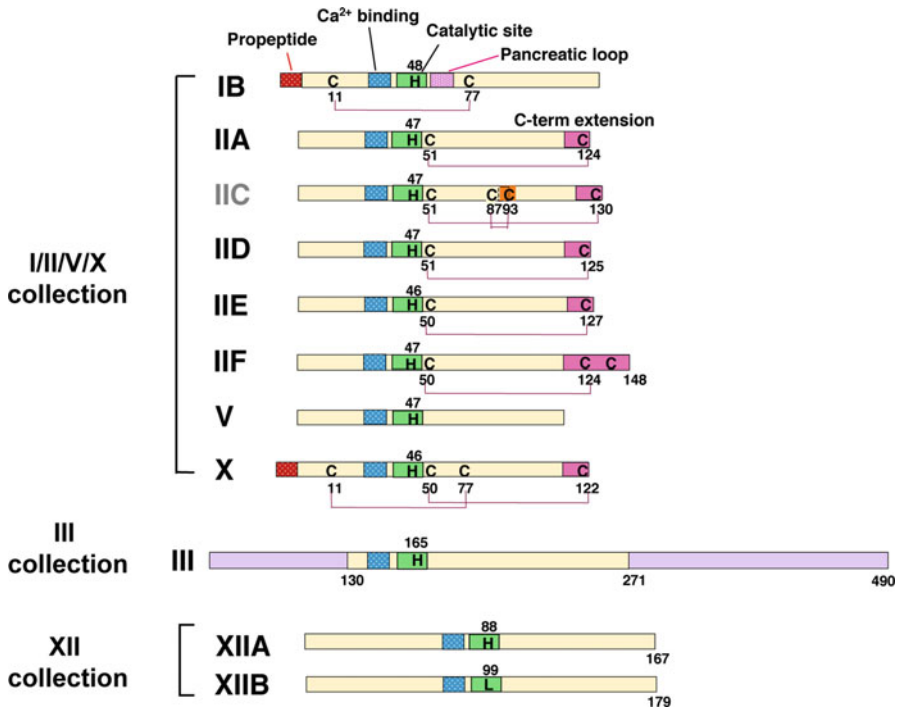
vascular contractility and relaxation, apoptosis induced by endoplasmic reticulum stress, antiviral response, age-related bone loss, glucose-stimulated insulin secretion by pancreatic β-cells, tumorigenesis, and neurodegeneration, among others (Bao et al. 2006; Shinzawa et al. 2008). Because of impaired islet secretory reserve, a high-fat diet induces severe glucose intolerance in $iPLA_2\beta$-null mice, whereas the glucose tolerance is improved in β-cell-specific $iPLA_2\beta$-transgenic mice (Bao et al. 2006). Tumorigenesis and ascites formation are ameliorated in $iPLA_2\beta$-null mice, and the $iPLA_2\beta$ gene haplotypes in humans are strongly associated with a higher risk of colorectal cancer (Hoeft et al. 2010). A locus for the neuroaxonal dystrophies such as infantile neuroaxonal dystrophy (INAD), neurodegeneration with brain iron accumulation (NBIA), and parkinsonism, which harbor the distinctive pathologic feature of axonal degeneration with distended axons (spheroid bodies) throughout the central nervous system, is mapped to the human $iPLA_2\beta$ gene (Morgan et al. 2006). Likewise, mice with $iPLA_2\beta$ deficiency or point mutation display severe motor dysfunction due to widespread degeneration of axons and synapses, accompanied by formation of numerous spheroids and vacuoles (Shinzawa et al. 2008).

$iPLA_2\gamma$ (PNPLA8) has four potential translation initiation sites, which produce distinct sizes of the protein. $iPLA_2\gamma$ catalyzes the cleavage of fatty acids from the *sn*-1 or *sn*-2 position of phospholipids depending upon the substrates. Remarkably, $iPLA_2\gamma$ has a mitochondrial and a peroxisomal localization signal in the N- and C-terminal regions, respectively, and are preferentially distributed in these organelles. Mice null for $iPLA_2\gamma$ display multiple bioenergetic dysfunctional phenotypes, including growth retardation, cold intolerance, reduced exercise endurance, increased mortality from cardiac stress after transverse aortic constriction, skeletal muscle atrophy, abnormal mitochondrial function with a dramatic decrease in oxygen consumption, and hippocampal neurodegeneration with massive autophagy and cognitive dysfunction (Mancuso et al. 2009). $iPLA_2\gamma$-deficient mice are also resistant to diet-induced obesity, hyperlipidemia, and insulin intolerance (Song et al. 2010). Importantly, the reduction in tissue cardiolipin content, accompanied by an altered cardiolipin molecular composition, in $iPLA_2\gamma$-deficient mice indicates that this $iPLA_2$ isoform is involved in mitochondrial cardiolipin remodeling. The phenotypes of $iPLA_2\gamma$-deficient mice are reminiscent of Barth syndrome, an X-linked cardioskeletal myopathy accompanied by exercise intolerance and neutropenia, which is caused by mutations in the gene encoding tafazzin, a mitochondrial phospholipid-lysophospholipid transacylase.

Secreted PLA_2s ($sPLA_2$s)

The $sPLA_2$ family represents structurally related, disulfide-rich, low molecular weight, lipolytic enzymes with a His-Asp catalytic dyad. $sPLA_2$s occur in a wide variety of vertebrate and invertebrate animals, plants, bacteria, and viruses; in mammals, there are 11 $sPLA_2$ isozymes (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA, and XIIB) (Fig. 3). Of these, $sPLA_2$s belonging to the group I/II/V/X collection are closely related, 14–19-kDa secreted enzymes with a highly conserved Ca^{2+}-binding loop (XCGXGG) and a catalytic site (DXCCXXHD). In addition to these elements, there are six absolutely conserved disulfide bonds and up to two additional unique disulfide bonds, which contribute to the high degree of stability of these enzymes. Group III and group XII $sPLA_2$s share homology with the I/II/V/X collection of $sPLA_2$s only in the Ca^{2+}-binding loop and catalytic site, thereby representing the group III and XII collections, respectively. $sPLA_2$s hydrolyze the ester bond at the *sn*-2 position of glycerophospholipids in the presence of millimolar concentrations of Ca^{2+} without showing strict fatty acid selectivity. Since individual $sPLA_2$s display distinct cellular/tissue distributions and substrate head group specificities, they may play nonredundant, isoform-specific roles in vivo. The latest biochemistry and biology of the $sPLA_2$ family have been detailed in recent reviews (Murakami et al. 2010; Lambeau and Gelb 2008).

Group IB $sPLA_2$ has a unique five amino acid extension termed the pancreatic loop in the middle part of the molecule and a group I-specific disulfide between Cys^{11} and Cys^{77}. It is synthesized in the pancreatic acinar cells, and after secretion into the pancreatic juice, an N-terminal heptapeptide of the inactive zymogen is cleaved by trypsin to yield an active enzyme in the duodenum. The main role of this pancreatic $sPLA_2$ is digestion of dietary and biliary phospholipids. Perturbation of this process by gene disruption or pharmacological inhibition of group IB



Phospholipase A_2, Fig. 3 *The sPLA$_2$ family*. Mammalian sPLA$_2$s contain 11 isoforms, which are subdivided into three collections, namely, group I/II/V/X (classical sPLA$_2$s), group III, and group XII. All enzymes have a conserved catalytic center with a His-Asp dyad and a Ca^{2+}-binding loop. sPLA$_2$-IB, a pancreatic sPLA$_2$, is characterized by an N-terminal propeptide whose proteolytic removal gives rise to a functional enzyme, the presence of a Cys11-Cys77 disulfide bond (group I-specific disulfide), and a unique pancreatic loop. The group II subfamily (IIA, IIC, IID, IIE, and IIF) is characterized by the absence of the propeptide and the presence of Cys49-Cys within the C-terminal extension (group II-specific disulfide). sPLA$_2$-IIC is absent in humans (pseudogene). sPLA$_2$-IIF has a longer C-terminal extension, which is Pro-rich. sPLA$_2$-V is evolutionally close to the group II subfamily, but is devoid of the group II-specific disulfide. sPLA$_2$-X possesses an N-terminal propeptide and both group I- and II-specific disulfides, thus having both group I and II properties. sPLA$_2$-III is unique in that the central sPLA$_2$ domain, which is more similar to bee venom PLA$_2$ than to group I/II/V/X sPLA$_2$s, is flanked by unique and highly cationic N- and C-terminal domains. The group XII collection contains two isoforms, XIIA and XIIB, whose overall structures (except for the catalytic domain and Ca^{2+}-binding site) do not show any homology with other sPLA$_2$s. The catalytic center His is replaced by Leu in sPLA$_2$-XIIB, indicating that this enzyme is catalytically inactive

sPLA$_2$ leads to protection from diet-induced obesity and insulin resistance due to decreased lipid digestion and absorption in the gut.

Group IIA sPLA$_2$ has a group II-specific disulfide linking Cys^{50} to the C-terminal Cys and a C-terminal extension of seven amino acids in length. The levels of group IIA sPLA$_2$ in sera or exudative fluids are well correlated with the severity of inflammatory disease: this is the reason why this isozyme is often called inflammatory sPLA$_2$. When overexpressed, group IIA sPLA$_2$ is capable of augmenting *arachidonic acid* release in cytokine-stimulated cells. Despite these facts, the contribution of group IIA sPLA$_2$ to inflammation has remained a subject to debate until recently, since a natural mutation of its gene in C57BL/6 and 129 Sv mice prevents the proper assessment of its functions by a classical gene targeting strategy. A recent study using group IIA sPLA$_2$-deficient BALB/c mice as well as its transgenic mice have provided evidence that the enzyme does play an exacerbating role in inflammatory arthritis. The best-recognized physiologic function of group IIA sPLA$_2$ is the degradation of bacterial membranes, thereby providing the first line of antimicrobial defense of the host. The serum level of group IIA sPLA$_2$ also shows good correlation with the risk of cardiovascular diseases, and group IIA sPLA$_2$-transgenic mice develop atherosclerosis when fed an atherogenic diet. This effect is most likely because sPLA$_2$-mediated hydrolysis of low-density lipoprotein (LDL) phospholipids

leads to generation of small-dense, pro-atherogenic LDL particles that facilitate macrophage foam cell formation.

Group IIC $sPLA_2$ has an additional disulfide bond between Cys^{87} and Cys^{93} in an extended loop region and is expressed in rodent testis. In the human genome, the absence of a portion of one exon indicates that it is a pseudogene and not expressed as a functional protein. Group IID $sPLA_2$ is structurally most similar to group IIA $sPLA_2$, and its transcript is constitutively detected in the lymphoid organs. Group IIE $sPLA_2$, which is another group IIA-related enzyme that is expressed constitutively in several tissues at low levels, has much lower catalytic activity than other group II $sPLA_2$s at least under the standard PLA_2 assay conditions. Group IIF $sPLA_2$ possesses a unique 30-amino acid C-terminal extension that contains an additional cysteine residue, which might contribute to the formation of a homodimer or a heterodimer with a second protein. Group IIF $sPLA_2$ is expressed most abundantly in the skin and seems to play a role in the regulation of skin permeability barrier.

Group V $sPLA_2$ does not possess the group I- and group II-specific disulfides and the group II-specific C-terminal extension. This enzyme potently releases *arachidonic acid* and LPC from cultured cells when overexpressed or added exogenously. Gene ablation of group V $sPLA_2$ in mice results in partial reduction of *eicosanoid* production in zymosan-stimulated macrophages. Group V $sPLA_2$-null mice are protected from fungal infection, since macrophages in the null mice show reduced phagocytosis of fungal particules. Contrary to mice lacking group IIA $sPLA_2$ (see above), those lacking group V $sPLA_2$ are more sensitive to inflammatory arthritis, likely because macrophage phagocytosis of the immune complex, a process that depends on cysteinyl leukotrienes, is hampered in the arthritic joints. The action of group V $sPLA_2$ on LDL phospholipids is rather strong, and LDL receptor-deficient mice transplanted with group V $sPLA_2$-null bone marrow cells are protected from atherosclerosis development. A recent tagging single nucleotide polymorphism analysis has revealed an association of the human group V $sPLA_2$ gene haplotype with LDL levels in patients with type 2 diabetes. Group V $sPLA_2$ is expressed in bronchial epithelium and alveolar macrophages, and its gene ablation in mice leads to protection from airway disorders such as asthma and respiratory distress syndrome. Pulmonary surfactant, a lipid-protein complex that lowers surface tension along the alveolar epithelium and thereby promotes alveolar stability, is one of the best targets of group V $sPLA_2$. Accordingly, transgenic overexpression of this enzyme in mice causes aberrant hydrolysis of surfactant PC, leading to neonatal death.

Group X $sPLA_2$ has both the group I- and II-specific disulfides, the group II-specific C-terminal extension, and the group I-specific propeptide. Like group IB $sPLA_2$, group X $sPLA_2$ is synthesized as a zymogen, and removal of the N-terminal propeptide produces an active mature enzyme. Among mammalian $sPLA_2$s, group X $sPLA_2$ shows the highest binding affinity for PC and thus exhibits the most potent activity to release *arachidonic acid* and LPC from the plasma membrane of target cells. Group X $sPLA_2$ is constitutively expressed in the digestive, respiratory, and genital organs, and is also expressed at trace levels in hematopoietic cells such as neutrophils and eosinophils. Group X $sPLA_2$-null mice are refractory to antigen-induced asthma, with markedly reduced eosinophil and lymphocyte infiltration and reduced cytokine and eicosanoid levels. The null mice are also protected from neutrophil-induced myocardial damage following ischemia-reperfusion. Group X $sPLA_2$ is abundantly stored in acrosomes in the sperm head, and its absence hampers acrosome reaction and fertility of spermatozoa in mice. Although group X $sPLA_2$ is potently active on LDL phospholipids in vitro, its role in atherosclerosis in vivo still remains obscure.

Group III $sPLA_2$ is an unusually large protein (55 kDa) among the $sPLA_2$ family and consists of three domains, in which the central $sPLA_2$ domain that displays all of the features of bee venom $sPLA_2$s, including ten cysteines and the key residues of the Ca^{2+} loop and catalytic site, is flanked by large and unique N- and C-terminal regions. The enzyme is processed to the $sPLA_2$ domain-only form that retains full enzymatic activity in vivo. Transgenic overexpression of group III $sPLA_2$ in mice results in increased atherosclerosis due to accelerated LDL hydrolysis and in increased inflammation due to elevated *eicosanoid* formation. A recent knockout study has revealed an unexplored role of this atypical $sPLA_2$ in epididymal sperm maturation through regulating sperm membrane remodeling.

Group XIIA $sPLA_2$ is a 19-kDa enzyme containing the central catalytic domain with a His-Asp catalytic dyad, yet the locations of cysteines outside the

catalytic domain are far distinct from those of other $sPLA_2$s. Furthermore, in the consensus segment of the Ca^{2+}-binding loop ($X_1CG_1X_2G_2$), the G_2 (glycine) is replaced by proline in group XIIA $sPLA_2$. High expression of this enzyme is found in many tissues, yet its enzymatic activity is very weak compared with others. A study using *Xenopus* suggests the role of this enzyme in early neuronal development. In the ectoderm, gain-of-function of $sPLA_2$-XIIA in embryos leads to ectopic neurogenesis and to the specification of ectopic olfactory sensory structures including olfactory bulb and sensory epithelia. These data identify group XIIA $sPLA_2$ as the factor sufficient to induce anterior sensory neural structures during vertebrate development. However, the roles of $sPLA_2$-XIIA and its catalytically inactive homolog group XIIB $sPLA_2$, in which the catalytic center His is replaced with Leu, in mammals are entirely unknown.

Summary

The understanding of the biological functions of all PLA_2s is now a challenging area of research and will be clarified hopefully in the next decade. The control of particular PLA_2 activities should have advantages over the inhibition of selective lipid mediator pathways and of some other biological events in the treatment of pathological states. Since more than one PLA_2 are likely to be involved in the pathology of various diseases in either a positive or negative way, the understanding of the expression, function, and regulation of each PLA_2 in specific tissues and disease states would be of particular importance. In certain situations, it would be favorable to control the activity of a particular PLA_2 subtype for the treatment of particular disorders. This is indeed true, if we see a particular case where groups IIA and V $sPLA_2$s play opposite (offensive and defensive) roles in inflammatory arthritis. This is probably the main reason why the current application of pan-$sPLA_2$ inhibitors, which broadly inhibits $sPLA_2$s in the group I/II/V/X branch, to rheumatoid arthritis was unsuccessful. Nevertheless, the pan-$sPLA_2$ inhibitor A-002 (valespladib) can decrease the atherosclerotic lesion area by approximately 75% in high-fat diet-fed $apoE^{-/-}$ mice (Rosenson et al. 2009). A-002 is more efficacious when combined with pravastatin. Furthermore, a phase II double-blind, randomized, placebo-controlled trial to assess the effects of A-002 in human patients with stable coronary heart disease demonstrates that the serum concentration of group IIA $sPLA_2$ as well as the levels of vascular (oxidized LDL) and general (C-reactive protein) inflammation markers is progressively decreased to nearly an order of magnitude less than baseline, with no excess of adverse events. A-002 also reduces LDL cholesterol concentrations and particle numbers, mainly by reducing small-dense LDL. These animal and early phase clinical studies highlight that $sPLA_2$s are potential therapeutic targets for atherosclerosis. Likewise, efipladib, an indole inhibitor of $cPLA_2\alpha$ that displays oral efficacy in rat inflammation models, is currently undergoing clinical trials for treatment of inflammatory diseases.

References

Bao S, Song H, Wohltmann M, Ramanadham S, Jin W, Bohrer A, et al. Insulin secretory responses and phospholipid composition of pancreatic islets from mice that do not express Group VIA phospholipase A_2 and effects of metabolic stress on glucose homeostasis. J Biol Chem. 2006;281:20958–73.

Bolotina VM. Orai, STIM1 and $iPLA_2\beta$: a view from a different perspective. J Physiol. 2008;586:3035–42.

Bonventre JV, Huang Z, Taheri MR, O'Leary E, Li E, Moskowitz MA, Sapirstein A. Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A_2. Nature. 1997;390:622–5.

Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, et al. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. Cell. 1991;65:1043–51.

Dessen A, Tang J, Schmidt H, Stahl M, Clark JD, Seehra J, et al. Crystal structure of human cytosolic phospholipase A_2 reveals a novel topology and catalytic mechanism. Cell. 1999;97:349–60.

Hoeft B, Linseisen J, Beckmann L, Müller-Decker K, Canzian F, Hüsing A, et al. Polymorphisms in fatty-acid-metabolism-related genes are associated with colorectal cancer risk. Carcinogenesis. 2010;31:466–72.

Lambeau G, Gelb MH. Biochemistry and physiology of mammalian secreted phospholipases A_2. Annu Rev Biochem. 2008;77:495–520.

Lauber K, Bohn E, Kröber SM, Xiao YJ, Blumenthal SG, Lindemann RK, et al. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell. 2003;113:717–30.

Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ. $cPLA_2$ is phosphorylated and activated by MAP kinase. Cell. 1993;72:269–78.

Mancuso DJ, Kotzbauer P, Wozniak DF, Sims HF, Jenkins CM, Guan S, et al. Genetic ablation of calcium-independent phospholipase $A_2\gamma$ leads to alterations in hippocampal cardiolipin content and molecular species distribution, mitochondrial

degeneration, autophagy, and cognitive dysfunction. J Biol Chem. 2009;284:35632–44.
Morgan NV, Westaway SK, Morton JE, Gregory A, Gissen P, Sonek S, et al. PLA2G6, encoding a phospholipase A_2, is mutated in neurodegenerative disorders with high brain iron. Nat Genet. 2006;38:752–4.
Murakami M, Taketomi Y, Girard C, Yamamoto K, Lambeau G. Emerging roles of secreted phospholipase A_2 enzymes: lessons from transgenic and knockout mice. Biochimie. 2010;92:561–82.
Nagase T, Uozumi N, Ishii S, Kume K, Izumi T, Ouchi Y, Shimizu T. Acute lung injury by sepsis and acid aspiration: a key role for cytosolic phospholipase A_2. Nat Immunol. 2000;1:42–6.
Ohto T, Uozumi N, Hirabayashi T, Shimizu T. Identification of novel cytosolic phospholipase A_2s, murine cPLA$_2$δ, ε, and ζ, which form a gene cluster with cPLA$_2$β. J Biol Chem. 2005;280:24576–83.
Rosenson RS, Hislop C, McConnell D, Elliott M, Stasiv Y, Wang N, et al. Effects of 1-H-indole-3-glyoxamide (A-002) on concentration of secretory phospholipase A_2 (PLASMA study): a phase II double-blind, randomised, placebo-controlled trial. Lancet. 2009;373:649–58.
Shinzawa K, Sumi H, Ikawa M, Matsuoka Y, Okabe M, Sakoda S, Tsujimoto Y. Neuroaxonal dystrophy caused by group VIA phospholipase A_2 deficiency in mice: a model of human neurodegenerative disease. J Neurosci. 2008;28:2212–20.
Song H, Wohltmann M, Bao S, Ladenson JH, Semenkovich CF, Turk J. Mice deficient in group VIB phospholipase A_2 (iPLA$_2$γ) exhibit relative resistance to obesity and metabolic abnormalities induced by a western diet. Am J Physiol Endocrinol Metab. 2010;298:E1097–114.
Tucker DE, Ghosh M, Ghomashchi F, Loper R, Suram S, John BS, et al. Role of phosphorylation and basic residues in the catalytic domain of cytosolic phospholipase A$_2$α in regulating interfacial kinetics and binding and cellular function. J Biol Chem. 2009;284:9596–611.
Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, et al. Role of cytosolic phospholipase A_2 in allergic response and parturition. Nature. 1997;390:618–22.
Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, et al. CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. Science. 2006;312:1220–3.

Phospholipase D

Julian Gomez-Cambronero and Karen M. Henkels
Department of Biochemistry and Molecular Biology, Wright State University School of Medicine, Dayton, OH, USA

Synonyms

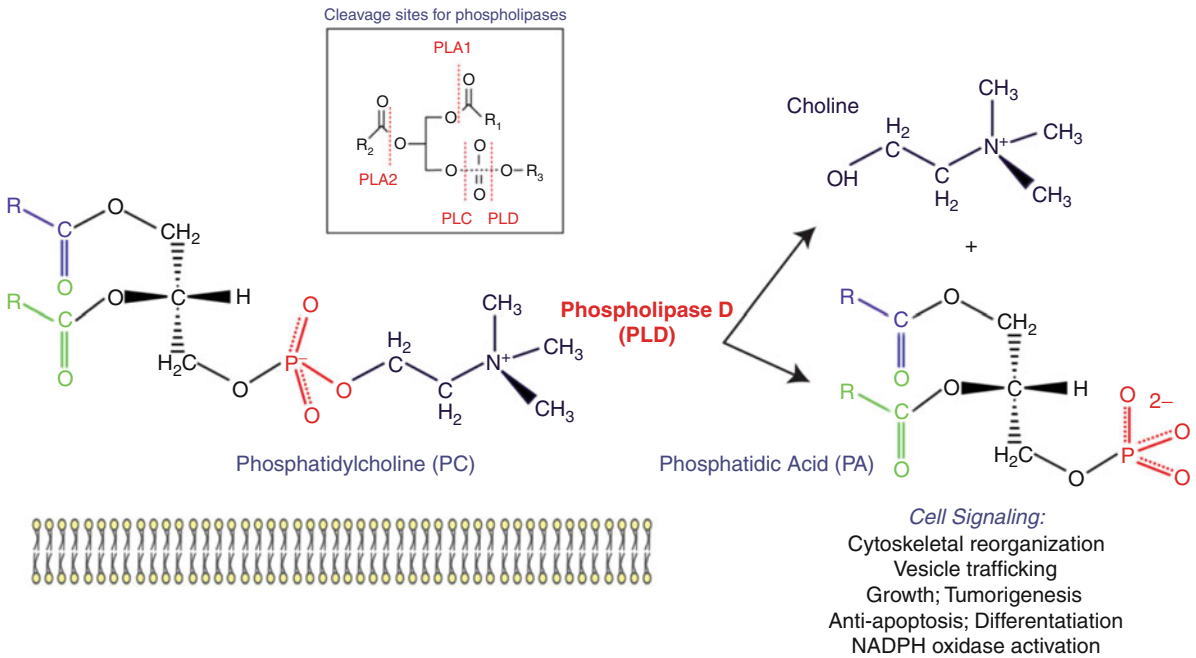
Phospholipase D1; Phospholipase D2; PLD1; PLD2

Historical Background

PLD was first characterized in vegetables by Hanahan and Chaikoff (1947) and changes in its lipase activity have been reported in relation to lipid metabolism in seed germination and lipid turnover and lipid composition during plant development and in membrane deterioration as a result of stress injuries. PLD was first cloned from cabbage (*Brassica oleracea)*, castor bean (*Ricinus communis* L.), rice (*Oryza sativa* L.), corn (*Zea mays* L.), and rockcress (*Arabidopsis*), which code for an 808-amino acid cytosolic protein of ~90 kDa MW, as mentioned in the review by (Cockcroft 1996). Plant PLD is linked to membrane deterioration during plant senescence as a result of decreased membrane phospholipid content. Additionally, PLD is present in large quantities in bacteria (*Streptomyces*), yeast (*Saccharomyces cerevisiae*), and mammalian cells.

Within mammalian sources, PLD has been found in a variety of cell types including neutrophils, promyelocytic leukemia, hepatocytes, platelets, endothelial cells, and spermatozoa, and is predominant in three organs, placenta, brain, and lung. A majority of the primary literature on PLD or PLD activity has been centered on in vivo and in vitro studies referring to changes in cellular distribution, intrinsic molecular alterations, association with other proteins or regulators, and availability of substrate as pertaining to phospholipid turnover in cellular membranes or wherever the substrate might be localized in the cell. PLD is expressed in a wide variety of tissues and cell lines and its activity has been reported predominantly in the plasma membrane, as well as in cytoplasmic locations, the mitochondrial membrane, the Golgi endoplasmic reticulum (ER), the nucleus, the nuclear membrane, and subcellular compartments. Additionally, PLD is palmitoylated on conserved cysteine residues and contributes to localization to membranous environments (Foster and Xu 2003).

As PLD is a protein that breaks down phospholipids, its main mission is directly related to phospholipid turnover and maintenance of the structural integrity of cellular or intracellular membranes (Frohman et al. 1999). In a two-step process, the enzyme PLD first hydrolyzes ester or phosphodiester bonds of lipids in cell membranes such as phosphatidylcholine (PC) that yields two lipid second messengers, phosphatidic acid (PA) and lyso-phosphatidic



Phospholipase D, Fig. 1 The substrate and products generated by the enzymatic action of PLD. The lipase usually utilizes phosphatidylcholine that is hydrolyzed into choline and PA, which is a *bona fide* second messenger involved in many cellular functions (some of which are denoted within this figure). The *inset panel* depicts the cleavage sites of PLD and related other phospholipases in the glycerol backbone of the phospholipid substrate. Copyrighted from Gomez-Cambronero (2010). DOI:10.1100/tsw.2010.116. Reproduced with permission from The Scientific World/Corpus Alienum Oy

acid (lyso-PA), and free choline (the precursor to the common neurotransmitter acetylcholine) and results in the subsequent production of diacylglycerol (DAG) (Gomez-Cambronero 2010) (Fig. 1). PLD degrades the phospholipid substrate to form a phosphohistidine-PLD intermediate and the second step involves the transfer of the phosphatidyl moiety to either H_2O or a primary alcohol, as detailed in the review by Exton (2000). The conversion of PC to PA by PLD in general is dependent on the presence of the cofactor phosphatidylinositol 4,5-bisphosphate (PIP_2) (a lesser anionic lipid localized primarily to the plasma membrane) because (1) neomycin appropriates PIP_2, which inadvertently negatively regulated both GTPγS- and Arf-mediated PLD activity and (2) Mg.ATP increased GTPγS-dependent PLD activity (Cockcroft 1996). PIP_2 plays a role in both Arf-regulated PLD activity, as well as Rho-regulated PLD activity via potential docking of PLD to the plasma membrane (Hammond et al. 1997). As mentioned in the review by Exton (2000), the potential exists for PLD to be directly involved in vesicle trafficking to and through the Golgi via PA generation, which results in membrane budding. Additional evidence of PLD's diverse role in vesicle trafficking is evidenced by its involvement in GLUT4 glucose transporter translocation, EGF-R and FcγRI-R internalization, hepatic very low density lipoprotein assembly, and the release of nascent secretory vesicles from the trans-Golgi network, as described by Powner and Wakelam (2002). Additionally, when endogenous PLD substrates are utilized to measure PLD lipase activity, the fatty acids myristate or palmitate can be used, as well as lyso-PC or choline to label PC (Cockcroft 1996). When the lipase is studied in vitro, short-chain aliphatic alcohols are included in the assay media. PLD can also generate rare phospholipids in the form of phosphatidylalcohols (such as phosphatidylethanol (PEt) and phosphatidylbutanol (PBut)) in the presence of a primary alcohol, such as ethanol or butanol via the transphosphatidylation reaction, which is a unique characteristic marker of PLD and thus is the accepted index of distinguishing PLD activity from that of phospholipase C (PLC). Additionally, incubation of mast cells with a primary alcohol could suppress the antigen-stimulated activation of the lipase without

interfering with translocation of PLD1, which actually facilitated the translocation of PLD1 from vesicles to the plasma membrane (Powner et al. 2002; Powner and Wakelam 2002).

Development of potent isoform-specific small-molecule PLD inhibitors would be integral to the advancement of the PLD field. Until recently, many PLD inhibitors lacked isoform specificity and did not act directly on the lipase. Halopemide and its subsequent derivative 5-fluoro-2-indoyl des-chlorohalopemide (FIPI) have been found to be very effective inhibitors of PLD-mediated F-actin cytoskeleton reorganization, cell spreading, and chemotaxis (Su et al. 2009). Use of iterative analog library synthesis approaches coupled with biochemicals assays and mass spectrometric lipid profiling of cellular responses has given rise to the next generation halopemide derivatives, which have yielded the development of dual PLD1/2, PLD1 selective and PLD2 selective inhibitors (Lewis et al. 2009). Small molecules that either indirectly or directly inhibit PLD1 or PLD2 could represent novel approaches for the treatment of metastatic cancer and inflammatory diseases.

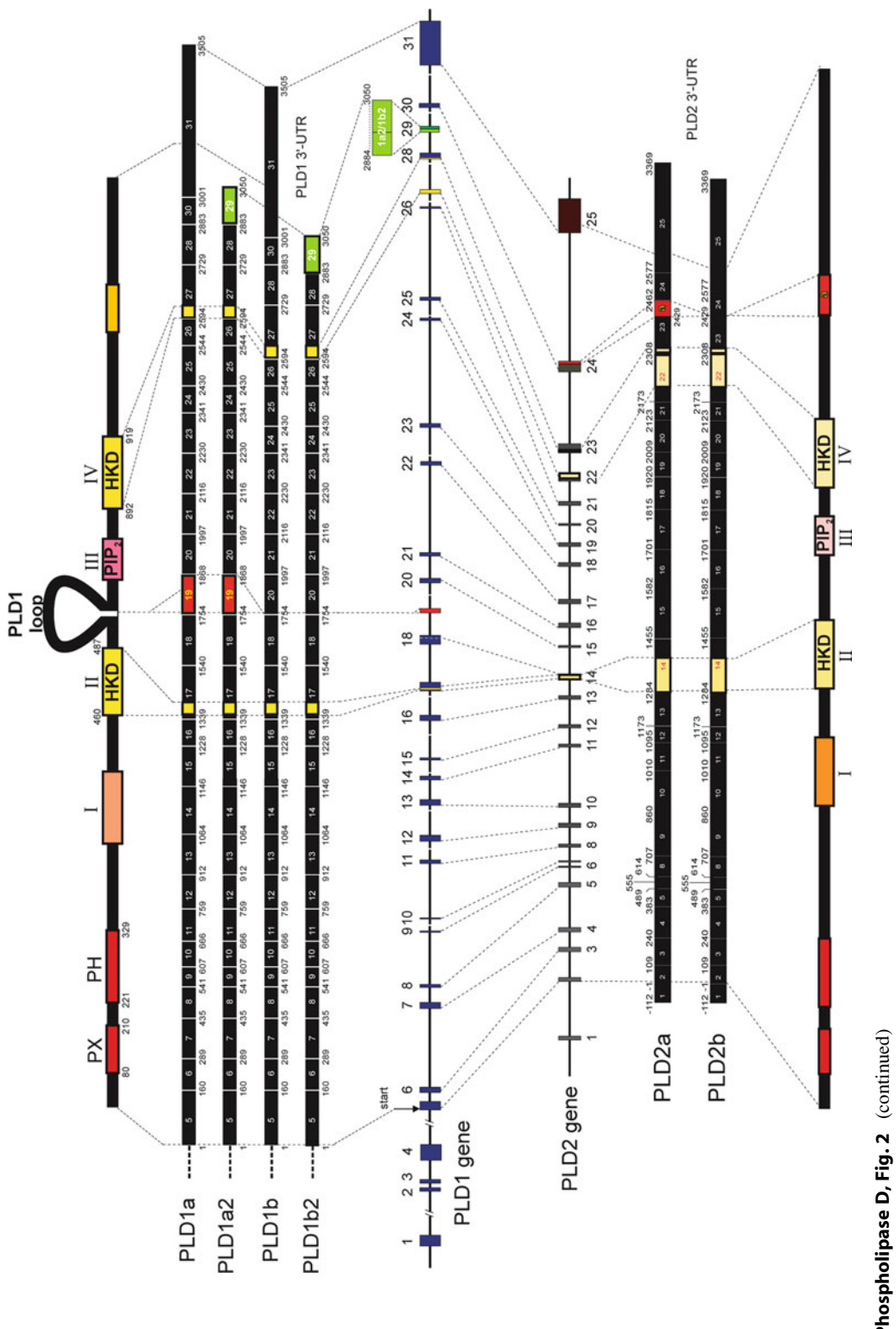
Characterization of PLD

In addition to cloned bacterial PC-specific PLDs from *Corynebacterium pseudotuberculosis*, yeast (SPO14gene), and plant (castor beans, etc.), there are currently two mammalian isoforms of the gene that have been cloned from human and murine sources, *PLD1* and *PLD2*, which yield the PLD1 and PLD2 proteins and four slightly shorter splice variants (Fig. 2). *PLD* genes undergo qualitative/quantitative changes in transcriptional upregulation during granulocytic differentiation of HL-60 cells.

The *PLD1* gene has been localized to the long arm (q) of chromosome 3 (3q26) (Park et al. 1998a), covers 210 kb of genomic DNA that is defined by 31 exons, whereby 27 exons result in the expression of four splice variants (PLD1a, PLD1a2, PLD1b, and PLD1b2) (Hammond et al. 1997; Katayama et al. 1998). PLD1a and PLD1a2 mRNAs express exon 19 (113 bp) and 29 (166 bp), respectively, while PLD1b and PLD1b2 do not express exon 19. PLD1a is the longest PLD1 splice variant at 1,072 amino acids in length and yields a 120 kDa MW protein. PLD1 is for the most part associated with perinuclear, Golgi and heavy membrane fractions, as reiterated in the review by Foster and Xu (2003). PLD1 is PC specific, Mg^{2+} dependent, and Ca^{2+} insensitive (Hammond et al. 1997), inhibited by oleate and has a basal level that is virtually undetectable. Human PLD1 is regulated by the cytosolic GTP binding protein Arf (Arf 1 and Arf3) and by small GTPases (Rac, Cdc42 and RhoA) via GTPγS, while it is also regulated by PKC (α and β isoforms) via Ca^{2+} and DAG/PMA (Cockcroft 1996; Hammond et al. 1997). Evidence of synergy between Arf, Rho, and PKC as related to regulation/activation of PLD1 activity has been reported first in human HL-60 leukemic cells and then in human neutrophils and rat brain, as reiterated by Cockcroft (1996). These facts implicate a widespread and ubiquitous nature to Arf-dependent PLD activity and specifically implicates only one PLD isoform in this process of lipase activation instead of activation by multiple other PLD forms.

The mammalian *PLD2* gene is found on the short arm (p) of chromosome 17 (17p13) (Park et al. 1998b), is defined by 25 known exons of a genomic region spanning 16.3 kb, and encodes for two splice variants (PLD2a and PLD2b) of 933 amino acids in length each (Steed et al. 1998), which yields functionally indistinguishable proteins of 106 kDa MW. PLD2 is for the most part localized on the plasma membrane in light membrane lipid rafts that also associate with caveolin, as restated in the subject review by Foster and Xu (2003). The first PLD2 gene exon (112 bp) encodes for the 5′-untranslated region, the initiation codon ($A^{1}TG$) is located on the second bp of exon 2, whereas the stop codon (TAG^{2803}) is located 568 bp downstream in exon 25. The PLD2b variant is the result of 33 bp being alternatively spliced from exon 23 of the originally described PLD2a. PLD2 requires PIP_2 and is largely insensitive to PKCα, Arf, or Rho (unlike PLD1 which is dependent upon these three cofactors).

Although the DNA sequences of both PLD1 and PLD2 share about 50% homology, all members of the PLD superfamily possess two highly conserved phosphatidyltransferase HKD catalytic domains (HKD1 and HKD2) that are defined by the consensus peptide sequence $HxK(x)4D(x)_6GSxN$, which are vital to the lipase activity, as well as the phox homology (PX) and pleckstrin homology (PH) domains and the phosphatidylinositol 4,5-bisphosphate [PIP_2] binding site (Frohman et al. 1999). As stated in the review by Exton (2000), PLD HKD motifs are requisite for



Phospholipase D, Fig. 2 (continued)

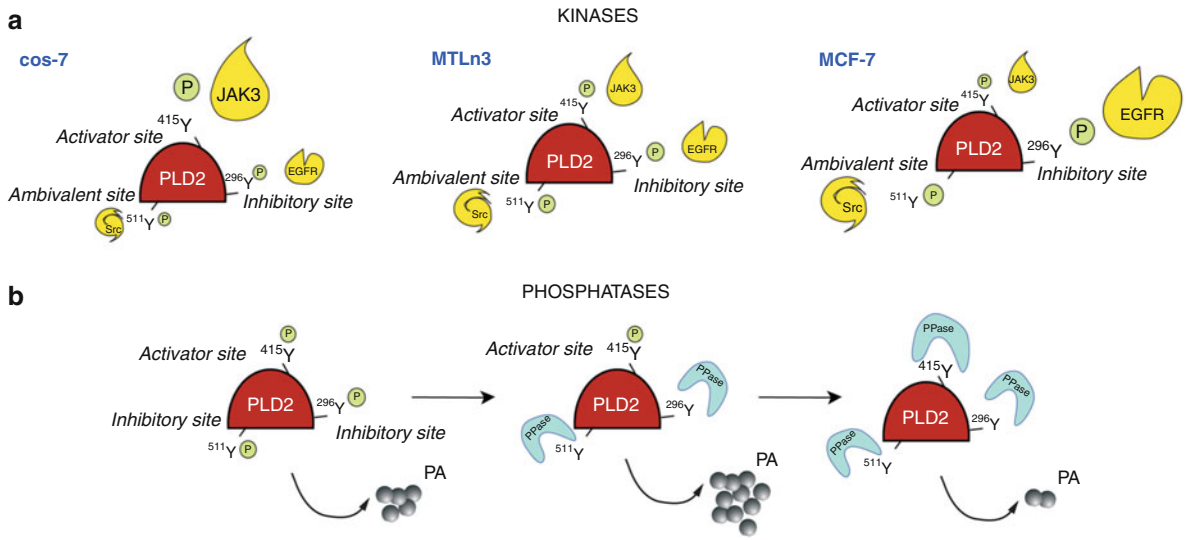
catalytic activity and possibly dimerize to form an active center and are also present in biologically diverse proteins represented by bacterial phospholipid synthases and endonucleases, a pox envelope protein and a Yersinia toxin. Lysine to arginine point mutations of the HKD2 domain of PLD1 at K^{860} or of either the HKD1 or HKD2 domains of PLD2 at K^{444} or K^{758}, respectively, result in lipase-dead enzymes because these K→R mutations yield lipases catalytically incapable of synthesizing PA or PBut as the readout for PLD activity. It has been theorized that the histidine in one of the HKD domains of PLD acts as a nucleophile to degrade the phosphodiester bond and the histidine in the other HKD domain protonates the oxygen of the leaving group, as reiterated by Exton (2000). The PX domain has been heavily implicated in binding to certain regulatory factors (PIP) and proteins (growth factor receptor-bound protein 2 (Grb2) and epidermal growth factor receptor (EGF-R)), while the PH domains of PLD1 and PLD2 have been demonstrated to function as strong modulators of the membrane recycling machinery that results in regulated growth factor receptor endocytosis and also linked to binding to SH2/SH3-containing tyrosine kinases. Deletion of either the PX or the PH domains results in a gross relocalization of PLD from the plasma membrane back to endosomes and in vivo renders the lipase unable to be activated, which ultimately negatively affects the catalytic activity of these isoenzymes.

PLD has been associated with a variety of physiological cellular functions, such as cancer cell progression, intracellular protein trafficking, cytoskeletal dynamics, membrane remodeling and cell proliferation in mammalian cells and meiotic division and sporulation in yeast. PLD regulation in mammalian cells falls into two major signaling categories: growth factors/mitogens that implicate tyrosine kinases (Frohman et al. 1999; Min et al. 1998) and small GTPases (Cockcroft 1996; Hammond et al. 1997; Powner and Wakelam 2002).

Role of Tyrosine Kinases and Phosphatases in PLD Signaling

Although PLD2 can be phosphorylated by the serine/threonine kinase AKT at residue T^{175} which serves to upregulate DNA synthesis, more typically PLD is known as a substrate for many receptor (EGF-R and PDGF-R) and non-receptor tyrosine kinases (Src and JAK3). Reagents like hydrogen peroxide when in the presence of vanadate can activate PLD in many different cells via tyrosine phosphorylation (Exton 2000; Min et al. 1998). Use of phorbol esters (PMA or TPA) or the PKC inhibitor Ro31-8220 to deplete PKC in the cell resulted in a significant loss of PLD activation (Cockcroft 1996). Additionally, evidence of considerable synergy between GTPγS and tyrosine kinase–based mechanisms has been reported using permeabilized cells as mentioned in the review by Cockcroft (1996). The PLD1 isoform is phosphorylated on tyrosine residues, which does not lead to changes in lipase activity (Min et al. 1998). The PLD2 isoform is expressed as a constitutively active enzyme in many different cell types that is detected as a phosphotyrosine protein in vivo and in vitro. These two scenarios heavily implicate a role for phosphorylation/dephosphorylation of PLD by protein tyrosine kinases and phosphatases in the control of PLD activity in response to such signaling mechanisms as osmotic

Phospholipase D, Fig. 2 A genomic and protein map of PLD isoforms. Schematic drawings of the PLD1 (*upper half* of the figure) and PLD2 (*lower half* of the figure) cDNA homologies as related to the specific genomic organization of each PLD gene. Data used to generate the figure were deduced from analysis of the human genome (www.ncbi.nlm.nih.gov). The specific PLD1- or PLD2-spliced exons are depicted in *red* boxes. Exon numerals are indicated beneath the exon boxes (*blue* for PLD1 and *gray* for PLD2 genes); nucleotide positions are in *black* and on *top* of each cDNA representation. Human *PLD1* and *PLD2* genes share ~50% identity and are located on the long arm of chromosome 3 (reverse stand locus 3q26) and on the short arm of chromosome 17 (locus 1p13.1), respectively. The human *PLD1* gene spans ~20.8 kb genomic DNA and is defined by 31 exons, which directs the expression of at least four alternatively spliced variants: PLD1a, PLD1b, PLD1a2, and PLD1b2. PLD1b and the evolutionarily conserved splice variant of PLD1a arise from splicing of a 38-amino acid, codified by the alternate exon 19 (*yellow box*), whereas PLD1a2 is the result of exon 29 splicing (*green box*). In the case of PLD2, ~16.3 kb genomic DNA, located in 25 exons, defines the gene. The *PLD2* gene is responsible for the generation of at least two alternatively spliced transcripts: PLD2a and PLD2b. PX, phox consensus sequence; PH, pleckstrin homology; HKD, HxxxxKxD; PIP2, phosphatidylinositol phosphate; 3′UTR, 3′-untranslated region. Copyrighted from Gomez-Cambronero et al. (2007). DOI:10.1189/jlb.0107033. Reproduced with permission from Journal of Leukocyte Biology/Society for Leukocyte Biology



Phospholipase D, Fig. 3 Schematic representation highlighting the biological significance of phosphorylation/dephosphorylation of PLD2. (**a**) Effect of kinases on PLD2. EGF-R, JAK3, and Src are capable of phosphorylating PLD2 in vitro, and the targets are Y^{296} for EGF-R, Y^{415} for JAK3, and Y^{511} for Src. EGF-R, JAK3, and Src phosphorylate an "inhibitory site," an "activator site," and an "ambivalent site" (one that can yield either effect), respectively. The degree that each site is activated or inhibited depends on the cell type considered. In COS-7 cells, which possess the highest level of PLD2 activity, the Y^{415} is a prominent site that, when phosphorylated by JAK3, compensates for the negative effect by EGF-R on Y^{296}. In MCF-7 cells, which possess the lowest level of PLD2 activity, the opposite is valid, the Y^{296} cannot compensate for the positive effect by Y^{415}. MTLn3 cells, which possess medium or low levels of lipase activity, exhibit an intermediate level of regulation, which is closer to that of MCF-7 cells than to that of COS-7 cells. (**b**) Effect of phosphatases on PLD2. Low concentrations of phosphatases or phosphatases targeting Y^{511} or Y^{296} are positively regulated by the "activator" sites and result in high levels of lipase activity. On the other hand, higher concentrations of phosphatases or phosphatases that specifically target Y^{415} (the activator site) result in the loss of lipase activity to varying extents based on to what degree the cell relies on activator or inhibitory sites. It is proposed that Y^{296}, an inhibitory site in MCF-7 cells, is the reason for the observed low lipase activity in this breast cancer cell line and further emphasizes the importance of this site during PLD2 regulation. Copyrighted from Henkels and Gomez-Cambronero. Mol Cell Biol. 2010; 30(9):2251–63. DOI:10.1128/MCB.01239-09. Reproduced with permission from American Society for Microbiology

stress, de novo DNA synthesis, cell proliferation, differentiation, transformation, and degranulation of mast cells.

Choi et al. (2004) have found that PLD2 is specifically phosphorylated on residues Y^{11}, Y^{14}, Y^{165}, and Y^{470}. Mutation of Y^{470} resulted in a 50% decrease in PLD2 activation and suggests some partial loss of catalytic activity. Additionally, mutation of only Y^{14} and not the other three tyrosine residues yielded mislocalization of PLD2 when using immunofluorescence microscopy. Recently, phosphorylation targets within the PLD2 molecule have been mapped that are vital to its regulation as a lipase and thus correlated in vitro to at least three different tyrosine kinases: EGF-R, Src, and Janus Kinase 3 (JAK3) (Gomez-Cambronero 2010). Using LC-MS analyses to prove the presence of phospho-PLD2-peptides, the specific PLD2 tyrosine residues phosphorylated by these kinases are Y^{296}, Y^{511}, and Y^{415}, respectively, that yield either positive or negative effects on the lipase (Fig. 3a). PLD2 but not PLD1 physically complexes with and interacts with the intracellular part of the EGF-R in a ligand-independent manner following receptor activation. Elevation of either PLD1 or PLD2 has the potential to transform rat fibroblasts and contribute to cancer progression of the malignant phenotype in cells that also have elevated levels of EGF-R or Src tyrosine kinases (Foster and Xu 2003). The potential exists for stimulation of PLD activity to directly contribute to cell proliferation, which further compounds the formation of a fully malignant phenotype (Foster and Xu 2003). Contrarily, it has been hypothesized that PLD2 activity in certain breast cancer cell lines is low compared to non-cancerous cells or other breast cancer cell lines because it is downregulated by tyrosyl phosphorylation at Y^{296} via

EGF-R (Gomez-Cambronero 2010), which can also be correlated to a negative impact on the relative levels of cell invasiveness of these breast cancer cells (Foster and Xu 2003). This low level of PLD activity can be increased by in vitro treatment with either JAK3 or Src. Src participates in the activation of PLD through the Ras pathway and the kinases Fyn and Fgr but not Lyn (Choi et al. 2004). Phosphorylation of PLD2-Y296F at residues Y^{415} or Y^{511} positively affected PLD2 lipase activity in certain breast cancer cell lines, which suggests that when Y^{296} can no longer be utilized by EGF-R, other kinases (possibly JAK3 or Src) are able to use PLD2 more efficiently as a phosphorylation substrate and ultimately yields greater PLD activity.

Phosphorylation/dephosphorylation of PLD2 at certain tyrosine residues dictates whether or not PLD2 is activated or suppressed by certain signaling molecules at other subsequent tyrosine residues. This dichotomy is achieved in part through a complex process of phosphorylation by tyrosine kinases and dephosphorylation by phosphatases, such as CD45 and protein tyrosine phosphatase 1b (PTP1b) (Fig. 3b). PTP1b is already known to dephosphorylate EGF-R substrates and regulate the kinase in vivo. Experiments with phosphatases indicate that both activator and inhibitory sites exist on the PLD2 molecule (Gomez-Cambronero 2010). Low concentrations of phosphatases or phosphatases that target inhibitory or ambivalent sites specifically result in positive regulation of PLD2 and high lipase activity. Contrarily, high concentrations of phosphatases or phosphatases that specifically target activator sites result in loss of lipase activity based on the degree of cellular dependence on activator or inhibitory sites.

Additionally, phosphorylated PLD2 forms a ternary complex with both PTP1b and Grb2 (Gomez-Cambronero 2010), a critical signal transducer of EGF-R, via two SH2 recognition sites (Y^{169} and Y^{179}) expressed within the context of the consensus YXNX in the PX domain of PLD2, which occurs independent of the lipase activity. A recent report indicates that increased cell transformation in PLD2-overexpressing cells occurs as a result of increased de novo DNA synthesis induced by PLD2 with the specific tyrosine residues involved in these functions being Y^{179} and Y^{511} (Gomez-Cambronero 2010). PLD2 residue Y^{169} modulates lipase activity, while PLD2 residue Y^{179} regulates total tyrosine phosphorylation of PLD2 (Gomez-Cambronero 2010). Complete simultaneous removal or phenylalanine replacement of these two sites on PLD2 completely abrogates or reduces binding to Grb2, respectively. Although the kinase that phosphorylates these two PLD2 residues is still unknown, interaction occurs through the C-terminal proline-rich domain of the Ras guanine-nucleotide exchange factor, Sos, and links PLD2 via residue Y^{169} to cellular proliferation and the MAPK and Ras/Erk pathways.

Role of Small GTPases in PLD Signaling

A role for PLD and its product PA has been presumed in the regulation of actin (Porcelli 2002) and leukoctye cell migration (Gomez-Cambronero 2010) because the formation of lamellipodia structures and membrane ruffles can be abolished if PLD is inhibited. It has also been surmised that Rho GTPase stimulation of PLD activity is key to actin stress fiber formation and the ultimate regulation of cell movement because PLD activity has already been shown to aid the formation of stress fibers, as restated by Foster and Xu (2003). These findings indicate a potential signaling feedback mechanism does in fact exist between the activation loop (Switch 1) of the Rho family of small GTPases (RhoA, RhoB, RhoC, Rac1, Rac2, Cdc42, and TC10) and PLD and potentially protein kinase C (PKC) (Cockcroft 1996). These GTPases must be in the active (GTP- or GTPγS-liganded) form to yield PLD stimulation/activation (Exton 2000). Preincubation of plasma membranes from liver cells with RhoGDI (a protein that extracts membrane-associated Rho) led to the removal of both RhoA and Cdc42 concomitant with a decrease in PLD activity, which was reversed in part with the addition of recombinant RhoA and Rac1 (Cockcroft 1996). Rho and Rac activate the synthesis of PIP_2 via the PI4-P5 kinase (PI4-P5K), and PIP_2 controls PLD activity in vivo and in vitro via mediation of nucleotide-binding interactions, such as GTPγS regulation and Mg.ATP. As reported in a mini review elsewhere (Powner et al. 2002), members of the Rho family of small GTPases physically bind to PLD1 between amino acid 984 and 1000. Therefore, taking into consideration these sets of facts, it is likely that Rac, PIP_2, and PLD are involved in the same signaling pathways and collectively regulate a variety of cellular functions.

In neutrophils, Rac1 plays an important role during gradient detections and actin assembly via PI-3K and AKT and has been reported to directly activate PLD1 (Powner and Wakelam 2002). Rho GTPases indirectly regulate PLD1 lipase activity via stimulation of PI(4,5)P_2 kinase, Rho kinase and intracellular translocation of PLD (Powner et al. 2002). RhoA, Rac1, Arf, and Cdc42 also directly interact with and stimulate PLD1 activity in the presence of GTPγS (Cockcroft 1996), because mutation of the Rho-binding site on PLD1 abrogates PLD1–Arf interaction (Du et al. 2000). PLD1 is a downstream target of the Ras/RalA small GTPase cascade that has been associated with mitogenic and oncogenic signaling (Foster and Xu 2003).

PLD2 can be activated in intact cells by agonists and possibly by PLD1 (Foster and Xu 2003) and can be regulated by small GTPases and certain PKC family members (Du et al. 2000). PLD2 and Rac2 physically interact and heterodimerize in vitro, and recently, the biphasic effect of a monomeric GTPase acting as a master switch has been shown to both promote and inhibit phospholipase activity as related to the timeline of chemotaxis (Peng et al. 2011). Macrophages that overexpressed both Rac2 and PLD2 experienced a strong initial response toward the chemoattractant that was significantly decreased at later time points. This initial positive response was attributed to the presence of a PLD2-Rac2 positive feedback loop, while the subsequent negative response of Rac2 on PLD2 was confirmed using cells from $Rac2^{-/-}$ mice that exhibited increased PLD2 enzymatic activity, which was reversed by PIP_2. It has been hypothesized that this Rac2-mediated inhibition of PLD2 function occurs because of Rac2 sterical interference with the PH domain membrane-binding site of PLD2 and ensuing PIP_2 deprivation. Rac2 localized in vivo to the leading edge of leukocyte pseudopodia with PLD2 being physically posterior to this wave of Rac2. Both PLD2 and PA signal to DOCK2, which mediates Rac activation and actin modeling (Nishikimi et al. 2009).

Role of PLD in Leukocyte Cell Adhesion and Migration

Leukocyte adhesion and migration are steps crucial to the antimicrobial and cytotoxic functions of leukocytes. PLD is expressed in monocytes, macrophages, basophils, eosinophils, dendritic cells, lymphocytes and NK cells and a variety of leukemic cells (U937, THP-1, HL-60, and PLD-985) and has been associated with tumor invasion, chemotaxis, adhesion, phagocytosis, degranulation, microbial killing, and leukocyte maturation. PLD is activated in human and murine myeloid-macrophage cell lines following adhesion to various extracellular matrix (ECM) proteins and plastic (Iyer et al. 2006). PLD concentrates at forming phagosomes, which occurs as a result of PA being concomitantly produced (Rossi et al. 1990) and demonstrates that PLD is in fact catalytically active during this process. PLD activation is an early event in neutrophil signal transduction following exposure of adherent cells to GM-CSF and is regulated by tyrosine phosphorylation, which can in turn be inhibited by tyrosine kinase inhibitors.

PLD1 activity is rapidly enhanced following cell adhesion, which serves to regulate the initial stages of neutrophil and macrophage adhesion. If PLD activity is inhibited, then a likewise inhibition in cell adhesion is evidenced. PLD activation plays a vital role in actin cytoskeleton formation, which stimulates the formation of actin stress fibers in cells, and use of lipase-dead mutants suggests this to be a PLD1-mediated process (Powner and Wakelam 2002). Immunofluorescence microscopy of human neutrophils has shown that both PLD isoforms were associated with cell polarity and directionality concomitant with adhesion and F-actin polymerization in response to IL-8 (Gomez-Cambronero 2010). It has been reported elsewhere (Powner and Wakelam 2002) that actin directly binds PLD2 with a concomitant decrease in lipase activity, which can be reversed by Arf1.

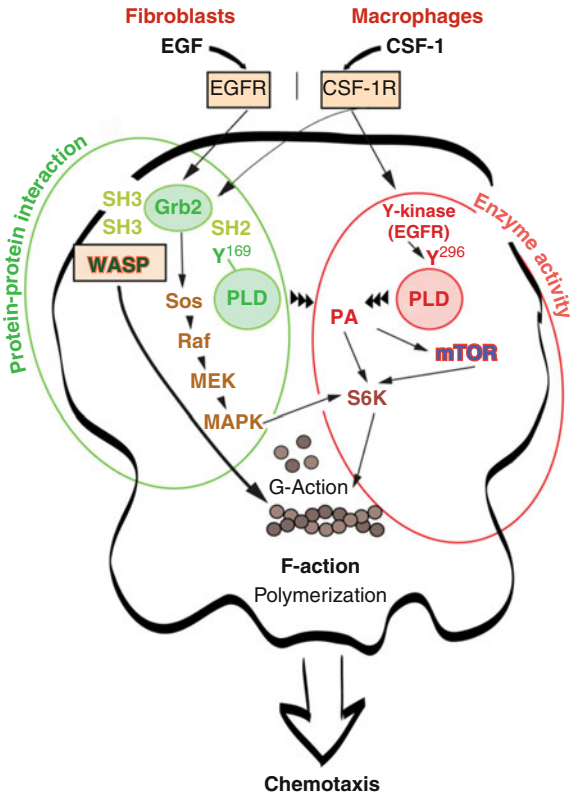
Chemokine receptors differentially regulate PLD, and while PLD1 has been implicated in other migration processes besides chemotaxis (rolling, adhesion, and diapedesis), PLD2 is more directly specialized for chemotactic processes (Gomez-Cambronero et al. 2007). PA is a second messenger in neutrophils that transduces signals to the cell interior upon agonist stimulation and results in development of polarized neutrophil morphology with focused distribution of F-actin, which is also partially dependent on basal PI3K activity and interaction with the C-terminal region of DOCK2 (Nishikimi et al. 2009). PLD-produced PA was able to sequester DOCK2 at the leading egde of migrating neutrophils from the cytocol via interaction with a DOCK2 polybasic amino acid cluster (Ser-Lys-Lys-Arg) that contributed to an

increase in actin polymerization, which was also dependent on an intact lipase activity (Nishikimi et al. 2009). PIP_3 was another cofactor manufactured during this process besides PA, which implicates both phospholipids in chemotactic translocation and stabilization.

Recently, PLD has been implicated as being needed to leukocyte, macrophage, and fibroblast movements. This was demonstrated through use of RNA interference–mediated depletion of PLD1 and PLD2, which resulted in impaired leukocyte adhesion and reduced chemokinesis and chemotaxis toward the chemokine gradient (Gomez-Cambronero 2010; Knapek et al. 2010). Overexpression of either active PLD1 or active PLD2 yielded cell migration capabilities that were elevated well beyond that of chemoattractant only negative controls. The mechanism for this enhancement in lipase activity is complex and involves two different pathways: one pathway is dependent on the lipase activity and signals directly through the product of this reaction, PA, and the other pathway involves protein–protein interactions.

First, PLD-mediated chemotaxis is mediated through extracellular PA, the pleiotropic lipid second messenger derived from PLD hydrolysis, which has been documented to act as a chemoattractant in human neutrophils and dHL-60 cells as membrane-soluble dioleoyl-PA (DOPA) elicited actin polymerization, cell spreading, pseudopodia formation, and chemotaxis (Frondorf et al. 2010). PLD's involvement is directly implicated in these cell migration processes (1) as PC on the outer leaflet of the plasma membrane can be cleaved by PLD action that is secreted by microorganisms following interaction with a phagocyte and (2) via intracellular PLD-derived PA generated by PLD2. It has previously been shown that extracellular PA stimulates PLD and results in the generation of intracellular PA and ultimately amplifies the original signal. Exogenous PA or PA generated in situ by bacterial PLD (*Streptomyces chromofuscus*) enters the cell and results in S6K accumulation in vesicle-like cytoplasmic structures (Frondorf et al. 2010).

Second, PLD-mediated chemotaxis is mediated through specific protein–protein interactions, such as Grb2, which serves as a docking or intermediary protein for PLD2 as detected using co-immunoprecipitation experiments and immunofluorescence microscopy where the PLD2-Grb2 protein complex localizes to actin-rich membrane ruffles during stimulation of murine macrophages via the Y^{169} residue of PLD2, which was dependent on the SH2 domain of Grb2 because use of the SH2-domain-deficient Grb2-R86K mutant impeded chemotaxis (Knapek et al. 2010). Additionally, PLD2/Grb2-mediated chemotaxis of LR5/RAW264.7 macrophages is dependent upon Grb2 interacting with other proteins, especially the ▶ Wiscott-Aldrich syndrome protein (WASP) (Fig. 4).



Phospholipase D, Fig. 4 Model of the role of PLD in the physiological process of chemotaxis. Based on our results and from others, it is proposed that PLD and Grb2 participate in cell chemotaxis, which involves three major pathways: (1) PA, the by-product of PLD2, binds to target proteins mTOR, S6K, or Sos. S6K then stimulates actin polymerization. Data in the present study indicate that Y^{169} is involved in lipase activity (PA production), leading to chemotaxis. (2) PLD can bind to either Grb2 or Sos, whereby residue Y^{179} is required for a PLD2-Grb2 protein–protein interaction that results in the downstream activation of MAPK. MAPK can crosstalk to S6K and provide positive feedback to enhance migration. S6K is heavily implicated in RAW/LR5 macrophage migration via the Y^{296} residue, which is phosphorylated by EGF-R kinase. (3) PLD and PA interact directly via interaction with actin or indirectly via interaction with WASP. Copyrighted from Knapek et al. (2010). DOI 10.1128/MCB.00229-10. Reproduced with permission from American Society for Microbiology

Simultaneous cell transfection of PLD2, Grb2, and WASP has the greatest effect on chemoattractant-mediated chemotaxis than any other limited variation of the three proteins combined.

Additionally, another protein–protein interaction that positively affects PLD-mediated chemotaxis occurs with S6K via the p70 subunit of the ribosomal S6 kinase (p70S6K), which correlates well with immunofluorescent staining of S6K that translocates from perinuclear regions and colocalizes with PLD2 in the cytosol following chemokine stimulation (Gomez-Cambronero 2010). LR5/RAW264.7 macrophages also use a PLD2/S6K-dependent chemotactic pathway that signals through PLD2-Y^{296}, which is already known to be phosphorylated by EGF-R (Knapek et al. 2010) (Fig. 4). Mutation of this tyrosine residue to phenylalanine completely abrogates chemotaxis to basal levels. Overexpression of PLD2 in dHL-60 leukemic cells results in an elevation of S6K activity, phosphorylation of p70S6K and chemokinesis, while both lipase-dead PLD mutants and si-RNA specific for PLD were inhibitory to this type of cell movement. A similar negative effect of the lipase-dead PLD2-K758R mutant on chemotaxis is also evidenced in LR5/RAW264.7 macrophages and through *n*-butanol treatment of cells.

Summary

PLD regulation in cells occurs via two different signaling pathways. One is via growth factors/mitogens, such as EGF, PDGF, insulin, and serum, and implicates tyrosine kinases. This pathway involves interactions with Grb2; Sos; and the kinases EGF-R, JAK3, and Src. The other pathway is via the small GTPases, such as Arf and Rho, and is directly related to chemotaxis, a process in which PLD plays a vital role. Even though the end results of PLD action as related to downstream signaling mechanisms are still currently being elucidated, adhesion and chemotaxis, which are both requisite for the inflammatory actions of leukocytes, are modulated directly by PLD. The functional consequences of receptor activation are not limited to leukocyte movement but also include degranulation, gene transcription, and mitogenic and apoptotic effects and are seen in angiogenesis, organogenesis, inflammation, and tumor development, growth, and metastasis.

References

Choi WS, Hiragun T, Lee JH, Kim YM, Kim HP, Chahdi A, Her E, Han JW, Beaven MA. Activation of RBL-2 H3 mast cells is dependent on tyrosine phosphorylation of phospholipase D2 by Fyn and Fgr. Mol Cell Biol. 2004;24:6980–92.

Cockcroft S. Phospholipase D: regulation by GTPases and protein kinase C and physiological relevance. Prog Lipid Res. 1996;35:345–70.

Du G, Altshuller YM, Kim Y, Han JM, Ryu SH, Morris AJ, Frohman MA. Dual requirement for rho and protein kinase C in direct activation of phospholipase D1 through G protein-coupled receptor signaling. Mol Biol Cell. 2000;11:4359–68.

Exton JH. Phospholipase D. Ann N Y Acad Sci. 2000;905:61–8.

Foster DA, Xu L. Phospholipase D in cell proliferation and cancer. Mol Cancer Res. 2003;1:789–800.

Frohman MA, Sung TC, Morris AJ. Mammalian phospholipase D structure and regulation. Biochim Biophys Acta. 1999;1439:175–86.

Frondorf K, Henkels KM, Frohman MA, Gomez-Cambronero J. Phosphatidic acid (PA) is a leukocyte chemoattractant that acts through S6 kinase signaling. J Biol Chem. 2010;285:15837–47.

Gomez-Cambronero J. New concepts in phospholipase D signaling in inflammation and cancer. Sci World J. 2010;10:1356–69.

Gomez-Cambronero J, Di Fulvio M, Knapek K. Understanding phospholipase D (PLD) using leukocytes: PLD involvement in cell adhesion and chemotaxis. J Leukoc Biol. 2007;82:272–81.

Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q, Cook S, Nozawa Y, Prestwich GD, Frohman MA, Morris AJ. Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha. J Biol Chem. 1997;272:3860–8.

Hanahan DJ, Chaikoff IL. The phosphorous-containing lipids of the carrot. J Biol Chem. 1947;168:233–40.

Iyer SS, Agrawal RS, Thompson CR, Thompson S, Barton JA, Kusner DJ. Phospholipase D1 regulates phagocyte adhesion. J Immunol. 2006;176:3686–96.

Katayama K, Kodaki T, Nagamachi Y, Yamashita S. Cloning, differential regulation and tissue distribution of alternatively spliced isoforms of ADP-ribosylation-factor-dependent phospholipase D from rat liver. Biochem J. 1998;329(Pt 3):647–52.

Knapek K, Frondorf K, Post J, Short S, Cox D, Gomez-Cambronero J. The molecular basis of phospholipase D2-induced chemotaxis: elucidation of differential pathways in macrophages and fibroblasts. Mol Cell Biol. 2010;30:4492–506.

Lewis JA, Scott SA, Lavieri R, Buck JR, Selvy PE, Stoops SL, Armstrong MD, Brown HA, Lindsley CW. Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part I: impact of alternative halogenated privileged structures for PLD1 specificity. Bioorg Med Chem Lett. 2009;19:1916–20.

Min DS, Kim EG, Exton JH. Involvement of tyrosine phosphorylation and protein kinase C in the activation of phospholipase D by H_2O_2 in Swiss 3 T3 fibroblasts. J Biol Chem. 1998;273:29986–94.

Nishikimi A, Fukuhara H, Su W, Hongu T, Takasuga S, Mihara H, Cao Q, Sanematsu F, Kanai M, Hasegawa H, Tanaka Y, Shibasaki M, Kanaho Y, Sasaki T, Frohman MA, Fukui Y. Sequential regulation of DOCK2 dynamics by two phospholipids during neutrophil chemotaxis. Science. 2009;324:384–7.
Park SH, Chun YH, Ryu SH, Suh PG, Kim H. Assignment of human PLD1 to human chromosome band 3q26 by fluorescence in situ hybridization. Cytogenet Cell Genet. 1998a; 82:224.
Park SH, Ryu SH, Suh PG, Kim H. Assignment of human PLD2 to chromosome band 17p13.1 by fluorescence in situ hybridization. Cytogenet Cell Genet. 1998b;82:225.
Peng HJ, Henkels KM, Mahankali M, Bubulya P, Dinauer MC, Gomez-Cambronero J. The dual effect of Rac2 on PLD2 regulation that explains both onset and termination of chemotaxis. Mol Cell Biol. 2011. doi:10.1128/MCB.01348-10.
Porcelli AM, Ghelli A, Hrelia S, Rugolo M. Phospholipase D stimulation is required for sphingosine-1-phosphate activation of actin stress fibre assembly in human airway epithelial cells. Cell Signal. 2002;14(1):75–81.
Powner DJ, Wakelam MJ. The regulation of phospholipase D by inositol phospholipids and small GTPases. FEBS Lett. 2002;531:62–4.
Powner DJ, Hodgkin MN, Wakelam MJ. Antigen-stimulated activation of phospholipase D1b by Rac1, ARF6, and PKCalpha in RBL-2 H3 cells. Mol Biol Cell. 2002;13:1252–62.
Rossi F, Grzeskowiak M, Della Bianca V, Calzetti F, Gandini G. Phosphatidic acid and not diacylglycerol generated by phospholipase D is functionally linked to the activation of the NADPH oxidase by FMLP in human neutrophils. Biochem Biophys Res Commun. 1990;168:320–7.
Steed PM, Clark KL, Boyar WC, Lasala DJ. Characterization of human PLD2 and the analysis of PLD isoform splice variants. FASEB J. 1998;12:1309–17.
Su W, Chen Q, Frohman MA. Targeting phospholipase D with small-molecule inhibitors as a potential therapeutic approach for cancer metastasis. Future Oncol. 2009;5:1477–86.

Phospholipase D1

► Phospholipase D

Phospholipase D2

► Phospholipase D

Phospholipid

► Phospholipase A_2

Phosphoprotein Enriched in Astrocytes 15kDa

► Pea15

Phosphoprotein Enriched in Diabetes

► Pea15

PHRIP

► PHLDA1 (Pleckstrin Homology-like Domain, Family A, Member): Alias: PHRIP; TDAG51; DT1P1B11; MGC131738

PI 3-K

► Phosphoinositide 3-Kinase

PI 3-Kinase

► Phosphoinositide 3-Kinase

PI(3)K

► Phosphoinositide 3-Kinase

PI3K

► Glycogen Synthase Kinase-3
► Phosphoinositide 3-Kinase

PI5P4K

► Phosphatidylinositol 5-phosphate 4-kinase

Pim-1

Christopher T. Cottage[1], Balaji Sundararaman[1], Shabana Din[1], Nirmala Hariharan[1] and Mark A. Sussman[2]
[1]San Diego State Heart Institute, San Diego State University, San Diego, CA, USA
[2]SDSU Heart Institute, San Diego State University Biology Department, San Diego, CA, USA

Synonyms

Provirus insertion site of Moloney murine leukemia virus 1 Pim-1

Historical Background

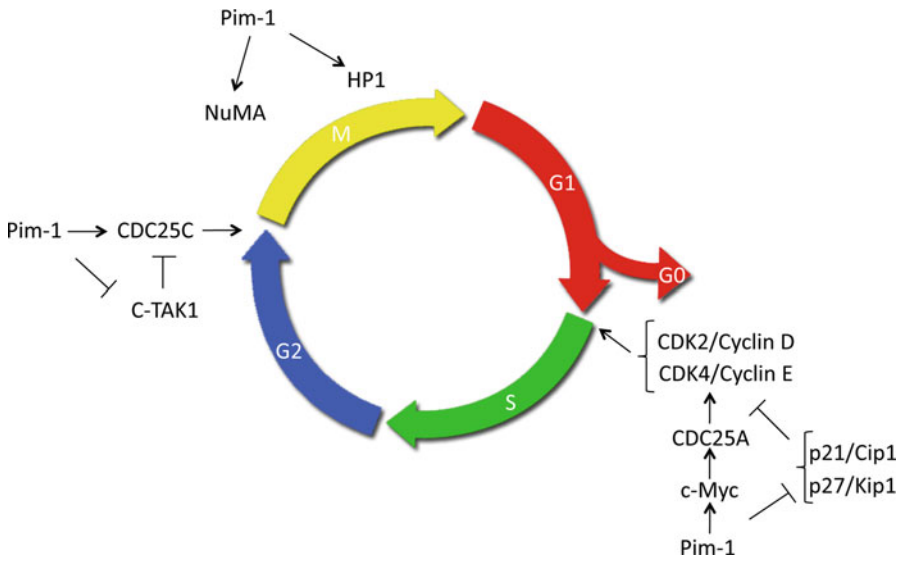
The serine-threonine kinase Pim-1 belongs to the Calmodulin-dependent protein kinase family together with two other highly conserved family members (Pim-2 and Pim-3). Pim-1 is the preferential site of integration for the Moloney murine leukemia virus (*P*roviral *I*ntegration for *M*oloney Virus) discovered over 25 years ago (Selten et al. 1985). Pim-1 plays pivotal roles in cellular proliferation, differentiation, metabolism, and survival by phosphorylating and interacting with many targets. A literature search reveals the dynamic expression and activity of Pim-1 depends upon cell type and response to stimuli, either pathologic or homeostatic. Specifically, Pim-1 is expressed in various hematopoietic sites including thymus, spleen, bone marrow, and fetal liver, but can also be found in the heart, oral epithelia, prostate, hippocampus, vascular smooth muscle, and many tumorigenic cell types (reviewed in Nawijn et al. (2011)). Expression of Pim-1 is preferentially elevated in the hematopoietic system and during fetal development coinciding with periods of increased cell cycling. Upon maturation, Pim-1 is downregulated in most organs until induced by pathologic stimuli to promote survival (Muraski et al. 2007). Presence of Pim-1 in neoplastic cell types can result in a poor prognosis depending on the type of malignancy. For example, the presence of Pim-1 together with a synergistic partner named c-Myc support a good prognosis in prostate adenocarcinoma, yet the opposite is true in the case of mantle cell lymphomas (reviewed in Nawijn et al. (2011)). Ironically, Pim-1 promotes survival and regeneration in heart tissue considered to be resistant to neoplastic transformation (Muraski et al. 2007). Therefore, the same molecular mechanisms that produce relatively bleak outcomes in cancer patients have the potential to produce therapeutic interventions in patients with cardiomyopathy.

Structure and Function

Genomic mapping identified Pim-1 on chromosome 17 in mice and chromosome 6 in humans. Six exons make up the 313 amino acid protein which has two isoforms stemming from alternative start sites; the larger 44 kD and smaller 34 kD isoforms contain a kinase domain, a proton acceptor site, a glycine loop motif, and a phosphate-binding site (Bachmann and Moroy 2005). Both isoforms of Pim-1 are short-lived (~5 min–6 h depending on cell type) and constitutively active. Paradoxically, Pim-1 is known to phosphorylate itself despite the fact that it does not contain the consensus sequence needed for phosphorylation. Selective peptide mapping identified the Pim-1 consensus sequence as either $(K/R)_3$-X-**S**/**T**-X or R/K-R/K-R-R/K-X-**S**/**T**-X where X is an amino acid with a small side chain but neither basic nor acidic (Bachmann et al. 2006). Pim-1 has numerous targets in various cellular compartments, giving rise to speculation that subcellular localization defines effect, with nuclear Pim-1 stimulating proliferation and mitochondrial/cytoplasmic localization promoting survival.

Controlling Pim-1

Regulation of Pim-1 occurs primarily at transcriptional and translational levels. *Pim-1* gene expression is induced by a large array of cytokines: most interleukins, granulocyte macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), and interferon-alpha. Cytokine stimulation results in the activation of multiple pathways such as the Janus kinase-signal transducer and activation of transcription (Jak-STAT) and nuclear factor kB (NFKB)–growth factor signaling pathways. Interleukins 5 and 7 (IL-5 and IL-7) activate

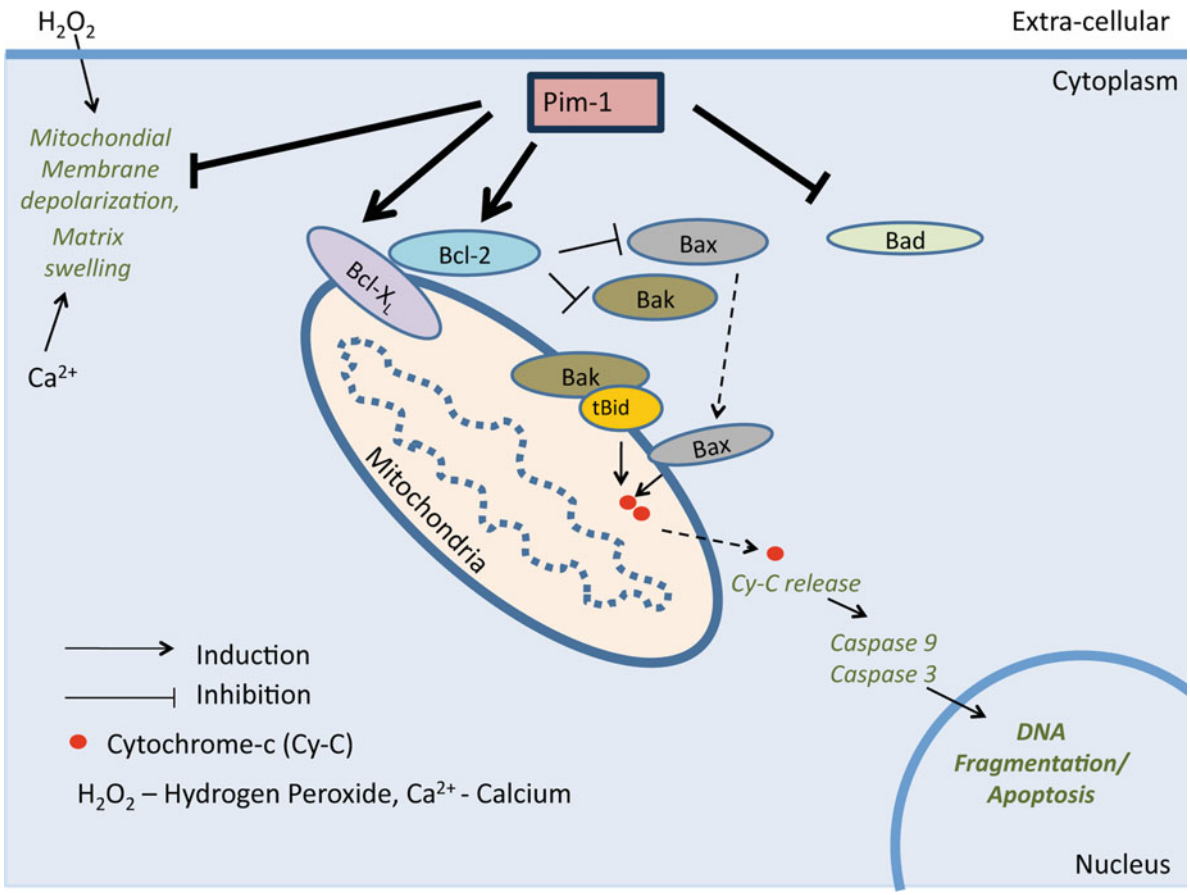


Pim-1, Fig. 1 Cell cycle regulation by Pim-1. Pim-1, together with c-Myc, works synergistically to drive G1/S transition by stabilizing CDK2/Cyclin D and CDK/Cyclin E complexes by phosphorylating CDC25A and cell cycle inhibitors p21/Cip1 and p27/Kip1. Pim-1 phosphorylates c-Tak1, inhibiting c-Tak1 kinase activity, promoting CDC25C driving G2/M transition. During mitosis, Pim-1 interacts with NuMa at the spindle poles, which is thought to promote the segregation of chromosomes. The exact role of Pim-1 during mitosis is currently under investigation but it is known that Pim-1 interacts with and phosphorylates HP-1

STAT5, whereas GM-CSF and EGF activate STAT 3. STAT3/STAT5 are both able to bind to the *Pim-1* promoter and induce transcription (Bachmann and Moroy 2005). Platelet-derived growth factor (PDGF) also increases *Pim-1* transcription in vascular smooth muscle cells resulting in enhanced proliferation that can be blunted with Jak-STAT pathway inhibitors (Willert et al. 2010). The hormone prolactin stimulates *Pim-1* transcription independent of STAT transcription factors. Alternatively, transcription is induced through the PI3K/AKT signaling pathway establishing Akt as an upstream regulator of Pim-1 activity (Krishnan et al. 2003). In addition, pathological stimuli such as DNA damaging agents including peroxide or 5-fluorouracil stimulate *Pim-1* through kruppel-like factor 5 binding to the *Pim-1* promoter activating transcription and subsequently preventing apoptosis (Zhao et al. 2008). Hypoxia elevates levels of Pim-1 mRNA and protein by inhibiting ubiquitin-mediated proteasomal degradation and causes Pim-1 translocation from the cytoplasm to the nucleus (Chen et al. 2009).

Pim1 mRNA has a short half-life due to five copies of the AUUU(A) destabilization motif in the 3' untranslated region (UTR) (Selten et al. 1985). The 5' UTR of Pim-1 contains multiple secondary structures due to repetitive GC rich regions, necessitating a 7-methylguanosine cap. By capping the 5' end of the message, ribosomal assembly is supported resulting in an abundant increase in Pim-1 protein (reviewed in Nawijn et al. (2011).

In addition to transcriptional control, Pim-1 is regulated posttranslationally through protein stabilization. Interactions with several molecules and complexes inhibit ubiquitination and dephosphorylation resulting in accumulation and persistence. Several facets of Pim-1 activity rely on synergistic partners that enhance and sustain kinase activity by preventing degradation. One such partner is heat shock binding partner 90 (Hsp90), association of Pim-1 and Hsp90 prevent ubiquitin-mediated destruction, whereas inhibition or silencing of Hsp90 promote rapid Pim-1 proteolysis (Shay et al. 2005). Conversely, Hsp70 binds ubiquitinated Pim-1 and directs Pim-1 to the proteosome for degradation (Shay et al. 2005). Similar to Akt, Pim-1 is inactivated by protein phosphatases including protein phosphatase 2A (PP2A). Once dephosphorylated, Pim-1 is quickly ubiquitinated and shuttled to the proteosome for degradation (reviewed in Bachmann and Moroy (2005)).



Pim-1, Fig. 2 Mechanisms by which Pim-1 inhibits apoptosis and maintains mitochondrial integrity. Pim-1 inhibits apoptosis by multiple means, involving upregulation of anti-apoptotic proteins, Bcl-2 and Bcl-X_L (in the cytosol and mitochondria respectively), while directly phosphorylating and inhibiting pro-apoptotic protein, Bad. Bcl-2 in turn is known to inhibit pro-apoptotic proteins, Bax and Bak, which translocate into the mitochondria upon stress and induce the release of cytochrome c, triggering apoptotic cell death (in a pathway involving caspase proteins). Pim-1 inhibits tBid-induced cytochrome c release in hearts, thereby inhibiting apoptosis. Pim-1 also maintains mitochondrial integrity by protecting against oxidative stress-induced mitochondrial inner membrane depolarization and calcium-induced matrix swelling

Pim-1 and Cell Cycle

Studies utilizing transgenic mice to overexpress Pim-1 and knockdown techniques to eliminate expression concluded that Pim-1 contributes to cell cycle progression in hematopoietic, cardiovascular, and embryonic stem cells. Subsequent studies revealed that cellular proliferation is achieved by modulating mitotic signals throughout the progression of the cell cycle. Downstream substrates and cell cycle regulators that allow Pim-1 to influence proliferation are depicted in Fig. 1. During G1-S progression, Pim-1 binds and phosphorylates cell division cycle 25 homolog A (CDC25A) increasing its phosphatase activity which in turn activates cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase 4 (CDK4) (Mochizuki et al. 1999). In addition, Pim-1 increases CDK2 activity by phosphorylating cell cycle inhibitors p21/Cip1 (Zhang et al. 2007) and p27/Kip1 (Morishita et al. 2008) resulting in nuclear export and degradation. In a similar fashion Pim-1 enhances progression through G2/M by phosphorylating the N-terminus of Cdc25C enhancing phosphatase activity (Bachmann et al. 2006). To further stimulate transition into M phase, Pim-1 phosphorylates Cdc25C-associated kinase 1 (C-TAK-1), an inhibitor of Cdc25C at multiple sites reducing its kinase activity allowing Cdc25C to promote G2/M transition (Bachmann et al. 2006).

During mitosis Pim-1 is enriched in the nucleus facilitating cell division (Bhattacharya et al. 2002). Pim-1 stabilizes spindle poles by interacting with Nuclear Mitotic Apparatus protein (NuMA) during mitosis, most likely phosphorylating NuMA as cells with kinase dead forms of Pim-1 do not co-localize with NuMA and have a higher frequency of apoptosis (Bhattacharya et al. 2002). Pim-1 phosphorylates NuMA to promote complex formation at the microtubule (−) end and provide a docking site for dynein and dynactin to promote proper chromosome segregation (Bhattacharya et al. 2002). In addition to NuMA, Pim-1 also phosphorylates heterochromatin protein-1 (HP-1) to further contribute to spindle fiber assembly during mitosis.

The transcription factor ▶ c-Myc is a well-characterized partner for Pim-1 known to promote cellular proliferation and differentiation when the two are co-expressed. Pim-1 binds, phosphorylates, and stabilizes ▶ c-Myc, facilitating cell cycle progression by promoting ▶ c-Myc-dependent transcription of target genes (Zhang et al. 2008). Pim-1 contributes to the regulation of around 20% of ▶ c-Myc target genes by promoting heterodimerization between c-Myc and Max that bind to E-box promoter elements present in approximately 15% of all human genes, (Zippo et al. 2007). During mitosis, ▶ c-Myc activates CDC25A, CDK2, and CDK4 driving entry and progression through the cell cycle. Overexpression of Pim-1 in cardiac progenitor cells results in increased cell cycling and elevated ▶ c-Myc expression (Cottage et al. 2010). Pim-1 regulates ▶ c-Myc-dependent transcription by phosphorylating Histone 3 on serine 10, unraveling chromatin providing E box-binding sites for ▶ c-Myc to bind and promote transcription (Zippo et al. 2007).

Pim-1 and Cell Survival

Mitochondrial membrane integrity regulates the release of pro-apoptotic cytochrome C and subsequent caspase cleavage eventually ending in DNA fragmentation and cell death. Multiple Pim-1-dependent cellular survival mechanisms are depicted in Fig. 2. Pim-1 effects cell death at the mitochondria via pro- and anti-apoptotic ▶ Bcl-2 family members. During normal homeostatic conditions ▶ Bcl-2 family members ▶ Bcl-2 and Bcl-XL reside in the outer membrane regulating mitochondrial outer membrane permeabilization. Upon apoptotic stimuli, pro-apoptotic family members, Bax and Bad, associate with ▶ Bcl-2 and Bcl-XL permeabilizing the outer membrane permitting cytochrome C release. Pim-1 phosphorylates BAD on serine 112 resulting in translocation from the mitochondria to the cytoplasm and binding to 14-3-3 scaffold proteins (Zhao et al. 2008). Overexpression of Pim-1 increases levels of both ▶ Bcl-2 and Bcl-XL in mitochondrial fractions protecting cardiomyocytes from hydrogen peroxide-induced stress (Borillo et al. 2010). In addition to forming heterodimers with Bcl-XL, ▶ Bcl-2 interacts with pro-apoptotic family members Bax and Bak to prevent membrane permeabilization and cytochrome C release (Fig. 2). Other non-mitochondrial-associated cell survival–related targets have been proposed using bioinformatic software; however, these are a topic of future research geared at elucidating the role of Pim-1 upon cellular survival.

Conclusions

In summary, Pim-1 is capable of manipulating protective signaling by promoting proliferative and survival signaling in a variety of ways and in a variety of cell types. Oncologists view Pim-1 as an enzyme capable of driving tumorigenesis with transformation promoting partners like ▶ c-Myc. In most hematopoietic malignancies, Pim-1 propels cell proliferation while simultaneously promoting survival creating a "recipe for disaster" and poor prognosis. To slow metastasis, pharmacologists are actively synthesizing new Pim-1 inhibitors in the hope of reversing mitogenic signaling. In contrast, cardiovascular biologists view increased proliferation as a breakthrough capable of resuscitating cardiac cells during times of myocardial damage. Pim-1-engineered cardiac stem cells (CSCs) have been adoptively transferred into damaged myocardium in order to promote repair. Several weeks after transplantation, hearts receiving Pim-1-engineered stem cells contain regenerated cardiomyocytes and possess increased functional output (Fischer et al. 2009). In addition to enhancing stem cell therapy, cardiotropic adenoviruses (AAV-9) overexpressing Pim-1 promote cellular proliferation and survival in cardiac cells. Tail vein injections of Pim-1 overexpressing AAV-9 reversed the effects of diabetic cardiomyopathy without surgery (Katare et al. 2011). In summary,

inhibiting Pim-1 may serve as a powerful therapeutic to halt cancer progression whereas overexpressing Pim-1 empowers stem cells and reverses the deleterious effects of cardiovascular disease.

References

Bachmann M, Moroy T. The serine/threonine kinase Pim-1. Int J Biochem Cell Biol. 2005;37(4):726–30.

Bachmann M, Kosan C, et al. The oncogenic serine/threonine kinase Pim-1 directly phosphorylates and activates the G2/M specific phosphatase Cdc25C. Int J Biochem Cell Biol. 2006; 38(3):430–43.

Bhattacharya N, Wang Z, et al. Pim-1 associates with protein complexes necessary for mitosis. Chromosoma. 2002;111(2):80–95.

Borillo GA, Mason M, et al. Pim-1 kinase protects mitochondrial integrity in cardiomyocytes. Circ Res. 2010;106(7):1265–74.

Chen J, Kobayashi M, et al. Hypoxia-mediated up-regulation of Pim-1 contributes to solid tumor formation. Am J Pathol. 2009;175(1):400–11.

Cottage CT, Bailey B, et al. Cardiac progenitor cell cycling stimulated by Pim-1 kinase. Circ Res. 2010;106(5):891–901.

Fischer KM, Cottage CT, et al. Enhancement of myocardial regeneration through genetic engineering of cardiac progenitor cells expressing Pim-1 kinase. Circulation. 2009;120(21): 2077–87.

Katare R, Caporali A, et al. Intravenous gene therapy with PIM-1 via a cardiotropic viral vector halts the progression of diabetic cardiomyopathy through promotion of prosurvival signaling. Circ Res. 2011;108(10):1238–51.

Krishnan N, Pan H, et al. Prolactin-regulated pim-1 transcription: identification of critical promoter elements and Akt signaling. Endocrine. 2003;20(1–2):123–30.

Mochizuki T, Kitanaka C, et al. Physical and functional interactions between Pim-1 kinase and Cdc25A phosphatase. Implications for the Pim-1-mediated activation of the c-Myc signaling pathway. J Biol Chem. 1999;274(26):18659–66.

Morishita D, Katayama R, et al. Pim kinases promote cell cycle progression by phosphorylating and down-regulating p27Kip1 at the transcriptional and posttranscriptional levels. Cancer Res. 2008;68(13):5076–85.

Muraski JA, Rota M, et al. Pim-1 regulates cardiomyocyte survival downstream of Akt. Nat Med. 2007;13(12):1467–75.

Nawijn MC, Alendar A, et al. For better or for worse: the role of Pim oncogenes in tumorigenesis. Nat Rev Cancer. 2011; 11(1):23–34.

Selten G, Cuypers HT, et al. Proviral activation of the putative oncogene Pim-1 in MuLV induced T-cell lymphomas. EMBO J. 1985;4(7):1793–8.

Shay KP, Wang Z, et al. Pim-1 kinase stability is regulated by heat shock proteins and the ubiquitin-proteasome pathway. Mol Cancer Res. 2005;3(3):170–81.

Willert M, Augstein A, et al. Transcriptional regulation of Pim-1 kinase in vascular smooth muscle cells and its role for proliferation. Basic Res Cardiol. 2010;105(2):267–77.

Zhang Y, Wang Z, et al. Pim-1 kinase-dependent phosphorylation of p21Cip1/WAF1 regulates its stability and cellular localization in H1299 cells. Mol Cancer Res. 2007;5(9): 909–22.

Zhang Y, Wang Z, et al. Pim kinase-dependent inhibition of c-Myc degradation. Oncogene. 2008;27(35):4809–19.

Zhao Y, Hamza MS, et al. Kruppel-like factor 5 modulates p53-independent apoptosis through Pim1 survival kinase in cancer cells. Oncogene. 2008;27(1):1–8.

Zippo A, De Robertis A, et al. PIM1-dependent phosphorylation of histone H3 at serine 10 is required for MYC-dependent transcriptional activation and oncogenic transformation. Nat Cell Biol. 2007;9(8):932–44.

Pin1

Dana Onica[1] and David W. Litchfield[1,2]

[1]Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

[2]Department of Oncology, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada

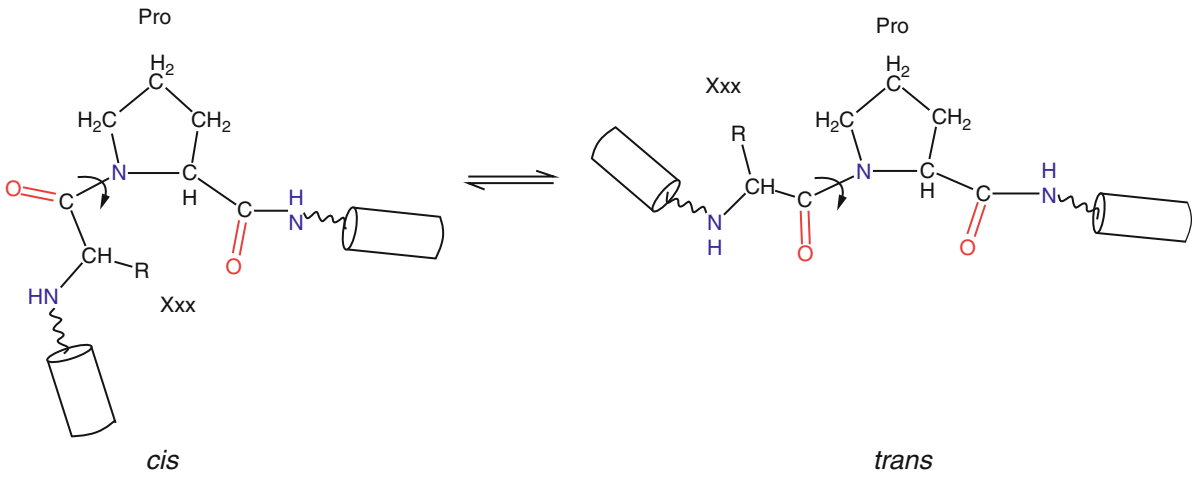
Synonyms

Peptidyl-prolyl cis-trans Isomerase NIMA-interacting 1; Peptidyl-prolyl cis-trans Isomerase Pin1; Rotamase Pin1

Historical Background

Pin1 is a phosphorylation-dependent peptidyl-prolyl isomerase that was first isolated by a yeast two-hybrid screen designed to identify human proteins which interact with the "Never in Mitosis" gene A (NIMA) (Lu et al. 1996). Sequence analysis revealed that human Pin1 exhibits approximately 45% sequence similarity with the product of the ESS1 gene which was previously identified as essential for growth in the budding yeast *Saccharomyces cerevisiae* (Hanes et al. 1989). It has subsequently been found that Pin1-like proteins are highly conserved, found in both eukaryotes and prokaryotes (Maruyama et al. 2004). While it was initially implicated as a regulator of mitosis, it is evident that Pin1 has roles in a number of biological processes.

Pin1 is classified as a peptidyl-prolyl isomerase (PPIase), catalyzing the cis-trans conversion of the peptide bond between a proline and the preceding amino acid (Fig. 1). It is part of a large superfamily of PPIases,



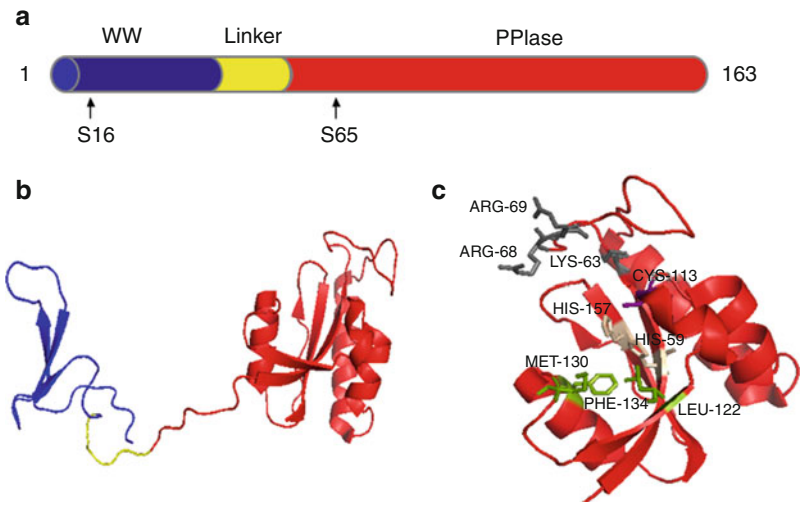
Pin1, Fig. 1 Peptidyl-prolyl isomerization. Cis-trans isomerization of the peptide bond (*arrow*) preceding the proline. Xxx represents any amino acid

which is divided into three families: the cyclophilins, the FK506 binding proteins (FKBPs), and the parvulins (Gothel and Marahiel 1999). One distinguishing characteristic among the three families of PPIases is their substrate specificities, particularly concerning the residue directly preceding the proline. Like all members of the parvulin family, Pin1 has a preference for hydrophobic residues. However, unique to Pin1 is its phosphorylation dependence, requiring that the preceding residue be a phosphorylated serine or phosphorylated threonine (Yaffe et al. 1997). With phosphorylation playing a pivotal role in cell signaling, one can infer that an isomerase with phosphorylated residues in its specificity determinants would add an additional layer of complexity to signaling pathways. This has been shown in multiple cellular processes where Pin1-catalyzed isomerization regulates the conformation of key cellular proteins (Lu and Zhou 2007).

Structural and Enzymatic Features of Pin1

High-resolution structures of Pin1 determined by x-ray crystallography revealed that it consists of two structural domains connected by a relatively short linker, which had originally been predicted from its primary sequence (Fig. 2). The N-terminal WW domain is named for two conserved tryptophan residues and comprises residues 1–39. It consists of a triple-stranded antiparallel β-sheet, with a hydrophobic patch on the surface (Zhou et al. 1999). Generally described as a protein-protein interaction domain, the WW domain of Pin1 binds pSer/Thr-Pro motifs, thus facilitating interactions between Pin1 and its substrates (Lu et al. 1999). The 118 amino acid catalytic PPIase domain (residues 45–163) is found on the C-terminal end of the protein, and consists of four antiparallel β-sheets, and four α-helices. Within this domain are two relatively well-described regions, the proline binding pocket and the phosphate binding loop, which lie on opposite sides of the active site. The hydrophobic proline binding pocket contains three highly conserved residues, Leu122, Met130, and Phe134, which are thought to be responsible for holding the proline in place during catalysis (Ranganathan et al. 1997). The phosphate binding loop contains two positively charged arginine residues at positions 68 and 69, as well as another positively charged amino acid, lysine at position 63, conferring upon Pin1 its preference for phosphorylated residues preceding the proline (Zhou et al. 1999). A short linker connects the WW and PPIase domains, whose flexibility may contribute to the broad substrate specificity of Pin1 (Li et al. 2005). Interestingly, although both domains of Pin1 bind the pSer/Thr-Pro motif, it appears that they may bind differently since the WW domain typically has a higher binding affinity for peptides than the PPIase domain (Lu et al. 1999).

In spite of the evidence confirming the ability of Pin1 to catalyze cis-trans isomerization, there are still questions concerning its precise catalytic mechanism. On the basis of a crystal structure of Pin1, Ranganathan et al. (1997), initially proposed a mechanism that



Pin1, Fig. 2 (**a**) Linear representation of Pin1. Phosphorylation sites are indicated. (**b**) and (**c**) High-resolution structure of Pin1 determined by x-ray crystallography (PDB: 1NMV). (**b**) The WW domain is shown in *blue*, the linker region is *yellow*, and the PPIase domain is shown in *red*. (**c**) Key residues of the PPIase domain are highlighted. See text for more details

involved the formation of a covalent enzyme-substrate intermediate with Cys113, His59, and His157 being key residues involved in catalysis. Since then, however, additional evidence has argued instead for a non-covalent mechanism. In this respect, Lippens et al. (2007) proposed that the role of Cys113 is to destabilize the peptide prolyl bond to allow for its rotation. This hypothesis is supported by data from Behrsin et al. (2007) showing that the Cys113/Asp substitution did not abolish Pin1 function. Additionally, with regard to the histidine residues, it has been shown that they do not directly participate in catalysis, suggesting they instead act structurally to support the integrity of the active site (Bailey et al. 2008).

Physiological Regulation of Pin1

It appears that Pin1 is subject to regulation at a number of levels (Lu and Zhou 2007). For example, its expression is upregulated in response to growth factors through E2F-mediated transcription, an observation consistent with its role in the cell cycle (Lu et al. 2002). Pin1 is also regulated through posttranslational modifications, including phosphorylation and possibly oxidation. Phosphorylation on Ser16 and Ser65 has opposing effects: the former prevents interactions with substrates, while the latter reduces ubiquitylation, thus increasing stability of Pin1. Oxidation of Pin1 may have a relationship to pathologies such as Alzheimer's disease, rather than as a part of normal cell regulation (Sultana et al. 2006).

Cellular Functions of Pin1

Pin1 is primarily localized in the nucleus; however, it can also be detected in the cytoplasm. This pervasive distribution of Pin1 is consistent with its extensive list of target proteins that are localized throughout the cell (Lu et al. 2002). As previously noted, Pin1 was first identified due to its interaction with NIMA, a protein kinase involved in mitotic regulation. This relationship was the first of many which suggested that Pin1 plays an integral role in regulation of the cell cycle and growth. Since then, Pin1 has been shown to be involved in a variety of additional cellular processes, emphasizing its diversity and importance (Fig. 3). Loss of function mutations or deletions of Pin1 in yeast and mammalian cells provides striking evidence for its role in the cell cycle, as these cells undergo mitotic arrest and apoptosis (Lu et al. 2002). Furthermore, Pin1 has a lengthy list of substrates which are known to be involved in the cell cycle, including a number of mitotic regulatory proteins (e.g., ▶ CDC25 and WEE1) which are targets of proline-directed protein kinases, such as CDKs and MAPKs (Lu and Zhou 2007). Pin1-catalyzed isomerization of these phosphorylated sites may be responsible for coordinating the activity of mitotic proteins, thus allowing for progression through the cell cycle (Lu and Zhou 2007). Pin1 has also been shown to coordinate duplication of centrosomes, DNA synthesis (Suizu et al. 2006), and to assist in chromosome condensation (Xu and Manley 2007) further emphasizing its role in the cell cycle.

	SUBSTRATE	CONSEQUENCE OF PIN1 INTERACTION
Cell Cycle Regulation	CDC25	Dephosphorylation
	WEE1	Inhibition of activity
	Cyclin D1	Stabilization
Apoptosis	p53	Stabilization
Neuro-degeneration	Tau	Dephosphorylation
	APP	APP processing

Pin1, Fig. 3 Selected Pin1 substrates and consequences of interaction

Similar to its actions in the cell cycle, Pin1 has been shown to interact with proteins involved in cell signaling events and pathways involving proline-directed protein kinases. One such example is the MAPK pathway, where, following proline-directed phosphorylation by MAPK, the proteins c-Jun and c-Fos are acted upon by Pin1 (Lu et al. 2007).

Briefly adding to the growing list of functions, Pin1 has also been shown to regulate expression of some genes through regulation of their transcription factors (Lu et al. 2007), to assist in the maintenance of telomeres through interactions with TRF1 (Lee et al. 2009), to facilitate DNA repair through interactions with p53 (Takahashi et al. 2008), and finally, to support breast development (Wulf et al. 2003).

Additionally, Pin1 has been shown to have specific roles in the immune and nervous systems. These additional functions provide links to the implication of Pin1 in various pathogenic conditions, which will be discussed in the following section. In short, however, it has been shown that Pin1 is important for regulating transcription of cytokines in T cells, as well as for survival of eosinophils (Lu et al. 2007). The importance of Pin1 in the brain is evident in Pin1 knockout mice, which have progressive and age-related neurodegeneration. This is directly related to the ability of Pin1 to promote normal neuronal cell functioning and survival through the interaction with proteins such as Tau and amyloid precursor protein (APP) (Lu and Zhou 2007).

Pin1 in Pathogenesis

Considering the diversity of its roles and importance as a key regulator of many cellular and biological processes, it is not unexpected that Pin1 appears to be involved in various pathological conditions, including cancer, Alzheimer's disease, and asthma.

In this respect, Pin1 has been implicated in a variety of cancers, including breast, lung, colon, and prostate cancer (Lu and Zhou 2007). This is not surprising given its role as a regulator of the cell cycle. However, the precise role Pin1 plays in cancer is controversial, as levels of Pin1 have been shown to be either positively or negatively related to cancer (Yeh and Means 2007). One of the better understood pathways in which overexpression of Pin1 appears to participate in cancer involves cyclin D1. Not only can Pin1 increase expression of cyclin D1, Pin1 can also directly bind and stabilize cyclin D1 to enhance cyclin D1/CDK activity (Lu et al. 2006). Conversely, loss of Pin1 can suppress transformation by Neu or Ras (Ryo et al. 2002). Additionally, Pin1 has been shown to stabilize ▶ p53, an important tumor suppressor which promotes apoptosis in response to genotoxic stresses (Yeh and Means 2007).

With regard to Alzheimer's disease, the precise role of Pin1 in pathogenesis remains uncertain, although evidence suggests that various mechanisms in Alzheimer's disease downregulate and/or inactivate Pin1 (e.g., through oxidation), suggesting it has a neuroprotective role (Lu and Zhou 2007). The loss of Pin1 function has impacts on two proteins, namely, APP and Tau, both found in senile plaques and neurofibrillary tangles. A current model suggests that without Pin1, the pThr668-Pro motif of APP remains in the cis form and accumulates in plaques. Similarly, the Tau pThr231-Pro motif is also found mostly in the cis form, leading to its hyperphosphorylation and subsequent accumulation (Lu and Zhou 2007).

The association between Pin1 and asthma can be traced back to the role Pin1 plays in immune cell function. By regulating the release of cytokines from eosinophils, and participating in the apoptotic decision of both T-cells and eosinophils, activated Pin1 modulates the allergic inflammatory response in the lungs associated with asthma (Esnault et al. 2008).

Emergence of Pin1 as a Candidate for Molecular-Targeted Therapy

The prevalence of Pin1 in various human diseases, cancer in particular, makes it an obvious candidate for therapies. Additionally, the fact that other PPIase proteins, specifically cyclophilin and FKBP, have been shown to be good therapeutic targets lends support to attempts to achieve the same success with Pin1. The first general inhibitor of parvulins was juglone, and although it has the ability to irreversibly inhibit Pin1, its use as an anticancer therapy is limited by its non-specificity (Wang and Etzkorn 2006). More recently, work has been focused on structure-based design of Pin1 inhibitors. Features that have been targeted by these rationally designed Pin1 inhibitors include its hydrophobic binding pocket, the phosphate binding loop (Potter et al. 2010), and Cys113 within its active site (Dong et al. 2010). Thus far, these Pin1 inhibitors have had varying degrees of specificity, as well as issues with potency, degradation, and cell permeability. In addition to these inhibitors, there have also been efforts to isolate inhibitors in the form of cyclic peptides which are less likely to be subject to proteolysis and may bind Pin1 with a higher affinity due to their reduced flexibility (Liu et al. 2010; Duncan et al. 2011). Although some inhibitors are able to inhibit Pin1 at nanomolar concentrations, their usage currently appears to be more appropriate for further investigations regarding the cellular functions of Pin1, rather than as therapeutic agents. However, this does not preclude the notion of using them as models to guide the design of novel, potentially therapeutic inhibitors of Pin1.

Summary

Since its discovery in 1996, much has been learned regarding the structure, function, and regulation of Pin1. In comparison to other PPIases, one particularly intriguing feature of Pin1 is its phosphorylation dependence which enables Pin1 to introduce an additional level of control in pathways involving proline-directed protein kinases such as CDKs that are central drivers of cell-cycle progression. While Pin1 was initially implicated as a key regulator of mitosis, it has subsequently been shown to be important in a diverse array of cellular processes. In concert with its participation in a broad spectrum of biological events, it is noteworthy that Pin1 has been implicated in a variety of diseases including cancer, neurological disorders such as Alzheimer's disease and asthma. Pin1 has thus emerged as a potential candidate for molecular-targeted therapy. Consequently, it can be anticipated that ongoing efforts to understand its regulation and functions and to elucidate its precise catalytic mechanism will foster efforts to develop new approaches that will harness its promise as a therapeutic target.

References

Bailey ML, Shilton BH, Brandl CJ, Litchfield DW. The dual histidine motif in the active site of Pin1 has a structural rather than catalytic role. Biochemistry. 2008;47:11481–9.

Behrsin CD, Bailey ML, Bateman KS, Hamilton KS, Wahl LM, Brandl CJ, Shilton BH, Litchfield DW. Functionally important residues in the peptidyl-prolyl isomerase Pin1 revealed by unigenic evolution. J Mol Biol. 2007;265:1143–62.

Dong L, Marakovits J, Hou X, Guo C, Greasley S, Dagostino E, Ferre R, Johnson MC, Kraynov E, Thomson J, Pathak V, Murray BW. Structure-based design of novel Pin1 inhibitors (II). Bioorg Med Chem Lett. 2010;20:2210–4.

Duncan KE, Dempsey BR, Killip LE, Adams J, Bailey ML, Lajoie GA, Litchfield DW, Brandl CJ, Shaw GS, Shilton BH. Discovery and characterization of a nonphosphorylated cyclic peptide inhibitor of the peptidylprolyl isomerase, Pin1. J Med Chem. 2011;54:3854–65.

Esnault S, Shen ZJ, Malter JS. Pinning down signaling in the immune system: the role of the peptidyl-prolyl isomerase Pin1 in immune cell function. Crit Rev Immunol. 2008;28:45–60.

Gothel SF, Marahiel MA. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci. 1999;55:423–36.

Hanes SD, Shank PR, Bostian KA. Sequence and mutational analysis of ESS1, a gene essential for growth in *Saccharomyces cerevisiae*. Yeast. 1989;5:55–72.

Lee TH, Tun-Kyi A, Shi R, Lim J, Soohoo C, Finn G, Balastik M, Pastorino L, Wulf G, Zhou XZ, Lu KP. Essential role of Pin1 in the regulation of TRF1 stability and telomere maintenance. Nat Cell Biol. 2009;11:97–105.

Li Z, Li H, Devasahayam G, Gemmill T, Chaturvedi V, Hanes SD, Van Roey P. The structure of the candida albicans Ess1 prolyl isomerase reveals a well-ordered linker that restricts domain mobility. Biochemistry. 2005;44:6180–9.

Lippens G, Landrieu I, Smet C. Molecular mechanisms of the phosphor-dependent prolyl cis/trans isomerase Pin1. FEBS J. 2007;274:5211–22.

Liu T, Liu Y, Kao HY, Pei D. Membrane permeable cyclic peptidyl inhibitors against human peptidylprolyl isomerase Pin1. J Med Chem. 2010;53:2494–501.

Lu KP, Zhou XZ. The prolyl isomerase Pin1: a pivotal new twist in phosphorylation signalling and disease. Nat Rev Mol Cell Biol. 2007;8:904–16.

Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature. 1996;380:544–7.
Lu P-J, Zhou XZ, Shen M, Lu KP. Functions of WW domains as phosphoserine- or phosphothreonine-binding modules. Science. 1999;283:1325–8.
Lu KP, Liou YC, Zhou XZ. Pinning down proline-directed phosphorylation signalling. Trends Cell Biol. 2002;12:164–72.
Lu KP, Suizu F, Zhou XZ, Finn G, Lam P, Wulf G. Targeting carcinogenesis: a role for the prolyl isomerase Pin1? Mol Carcinog. 2006;45:397–402.
Lu KP, Finn G, Lee TH, Nicholson LK. Prolyl cis-trans isomerisation as a molecular timer. Nat Chem Biol. 2007;3:619–29.
Maruyama T, Suzuki R, Furutani M. Archaeal peptidyl prolyl cis-trans isomerases (PPIases) update 2004. Front Biosci. 2004;9:1680–720.
Potter A, Oldfield V, Nunns C, Fromont C, Ray S, Northfield CJ, Bryant CJ, Scrace SF, Robinson D, Matossova N, Baker L, Dokurno P, Surgenor AE, Davis B, Richardson CM, Murray JB, Moore JD. Discovery of cell-active phenyl-imidazone Pin1 inhibitors by structure-guided fragment evolution. Bioorg Med Chem Lett. 2010;20:6483–8.
Ranganathan R, Lu KP, Hunter T, Noel JP. Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. Cell. 1997;89:875–86.
Ryo A, Liou YC, Wulf G, Nakamura M, Lee SW, Lu KP. Pin1 is an E2F target gene essential for Neu/Ras-induced transformation of mammary epithelial cells. Mol Cell Biol. 2002;22:5281–95.
Suizu F, Ryo A, Wulf G, Lim J, Lu KP. Pin1 regulates centrosome duplication andoncogenesis. Mol Cell Biol. 2006;26:1463–79.
Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, Markesbery WR, Zhou XZ, Lu KP, Butterfield DA. Oxidative modification and down-regulation of Pin1 in Alzheimer's disease hippocampus: a redox proteomics analysis. Neurobiol Aging. 2006;27:915–25.
Takahashi K, Uchida C, Shin RW, Shimazaki K, Uchida T. Prolyl isomerase, Pin1: new findings of post-translational modifications and physiological substrates in cancer, asthma and Alzheimer's disease. Cell Mol Life Sci. 2008;65:359–75.
Wang XJ, Etzkorn FA. Peptidyl-prolyl isomerase inhibitors. Biopolymers. 2006;84:125–46.
Wulf G, Ryo A, Liou YC, Lu KP. The prolyl isomerase Pin1 in breast development and cancer. Breast Cancer Res. 2003;5:76–82.
Xu YX, Manley JL. The prolyl isomerase Pin1 functions in mitotic chromosome condensation. Mol Cell. 2007;26:287–300.
Yaffe MB, Schutkowski M, Shen M, Zhou XZ, Stukenberg PT, Rahfeld J-U, Xu J, Kuang J, Kirschner MW, Fischer G, Cantley LC, Lu KP. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science. 1997;278:1957–60.
Yeh ES, Means AR. Pin1, the cell cycle and cancer. Nat Rev Cancer. 2007;7:381–8.
Zhou XZ, Lu PJ, Wulf G, Lu KP. Phosphorylation-dependent prolyl isomerisation: a novel signalling regulatory mechanism. Cell Mol Life Sci. 1999;56:788–806.

PINK1

► PTEN-Induced Kinase 1 (PINK1)

PIP Kinase

► Phosphatidylinositol 5-phosphate 4-kinase

PIP4K

► Phosphatidylinositol 5-phosphate 4-kinase

PIP5K2

► Phosphatidylinositol 5-phosphate 4-kinase

PIPBP

► ADAP1

PIPKII

► Phosphatidylinositol 5-phosphate 4-kinase

PIPkin

► Phosphatidylinositol 5-phosphate 4-kinase

PITSLRE

► CDK11

PK58

► CDK11

PKB

▶ Glycogen Synthase Kinase-3

Pkcs15

▶ Pea15

PKD

Peter Storz
Department of Cancer Biology, Mayo Clinic Comprehensive Cancer Center, Jacksonville, FL, USA

Synonyms

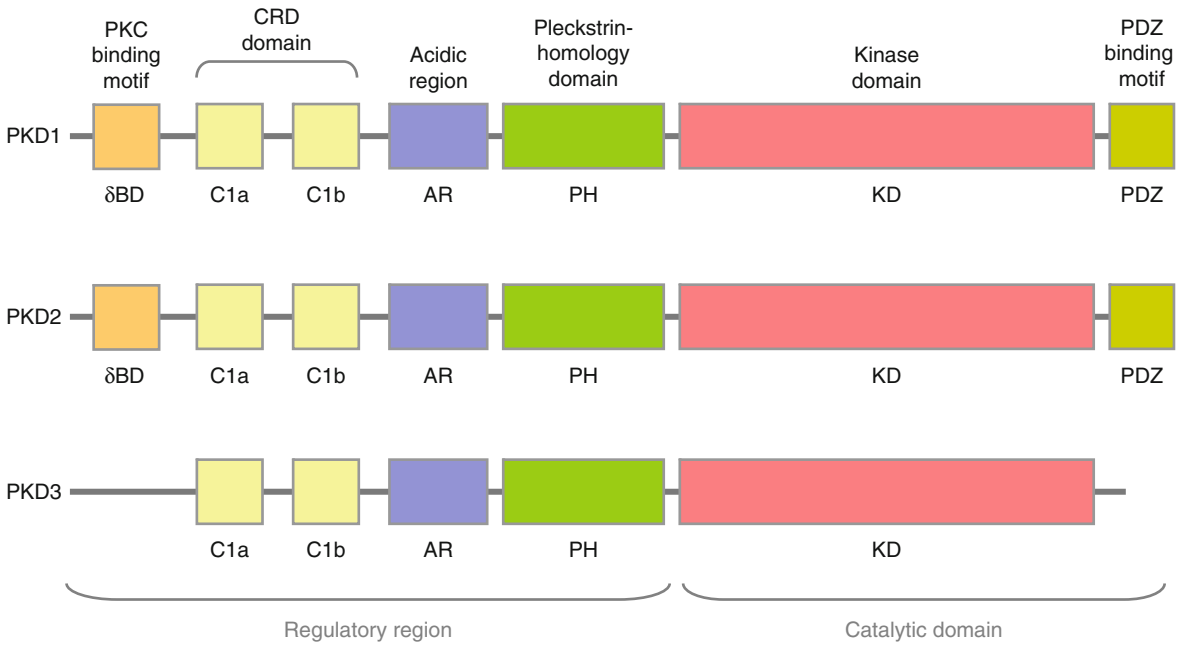
PKD (isoforms: PKD1/PKCμ, PKD2, PKD3/PKCν)

Historical Background

The serine/threonine kinase protein kinase D (PKD) was first described in 1994. Due to some homology in its kinase domain, its ability to bind the lipid diacylglycerol (DAG), and its responsiveness to phorbol ester, PKD was originally classified as a member of the protein kinase C (PKC) family (Johannes et al. 1994). Later it became evident that PKD represents an own group of kinases more closely related to the calcium/calmodulin-regulated (Cam) kinases (Manning et al. 2002). Initially, the PKD field focused on determining the functions of the structural elements and domains of PKD1 as well as the activation mechanisms for this kinase. With the identification of the PKD phosphorylation motif (Hutti et al. 2004) and the subsequent generation of a substrate-specific antibody (Döppler et al. 2005), optimal conditions were provided for the discovery of PKD substrates. This led to an increasing wealth of information on PKD downstream signaling, its role in various cellular processes, as well as its potential roles in health and disease. For example, it was shown that PKD has multiple functions within cells ranging from regulating golgi organization and vesicle transport, cell proliferation, cell survival, to mitochondrial signaling to the nucleus (reviewed in Wang 2006). To date, three highly conserved isoenzymes, PKD1/PKCμ, PKD2, and PKD3/PKCν, have been described. Dependent on the cellular context the three PKD family members may have redundant or unique functions. This is currently under intense investigation using shRNA strategies in cellular settings or knock-out and knock-in animal models.

Structure of PKD Enzymes

PKD enzymes consist of a C-terminal kinase domain and a N-terminal regulatory region (Fig. 1). The regulatory region comprises a binding motif for PKCδ, two cystein-rich C1 domains (also named C1a and C1b or CRD domain), an acidic region (AR), and a pleckstrin homology (PH) domain. The deletion of either of these domains or regions leads to increased PKD activity, suggesting that the N-terminal region acts negative-regulatory for the kinase domain (reviewed in Van Lint et al. 2002). C1 domains are required for binding to the lipid diacylglycerol (DAG), which targets PKD to different intracellular membranes including the plasma membrane, the golgi, or mitochondria (Cowell et al. 2009; Baron and Malhotra 2002). Both C1 domains have distinct roles in targeting and maintaining PKD at the plasma membrane. C1a achieves fast maximal and reversible translocation, while C1b translocates the enzyme more persistent. Persistent localization also requires the binding of a G-protein $G\alpha_q$ subunit to the C1b domain. The C1b domain also includes a nuclear targeting sequence. A role for the acidic region is not known. The PH domain does not bind any particular phosphoinositide with high specificity, but serves as a protein–protein interface. For example, it recruits upstream kinases such as PKCη or binds the G-protein βγ subunit. The PH domain further is required for nuclear export (Rozengurt et al. 2005). Additionally, several phosphorylation sites within this domain control PKD intracellular localization and activity. The C-terminal kinase domain includes an ATP-binding motif, an activation loop motif that requires phosphorylation at two serine residues and an autophosphorylation site within a PDZ-binding motif (Van Lint et al. 2002; Sanchez-Ruiloba et al. 2006).



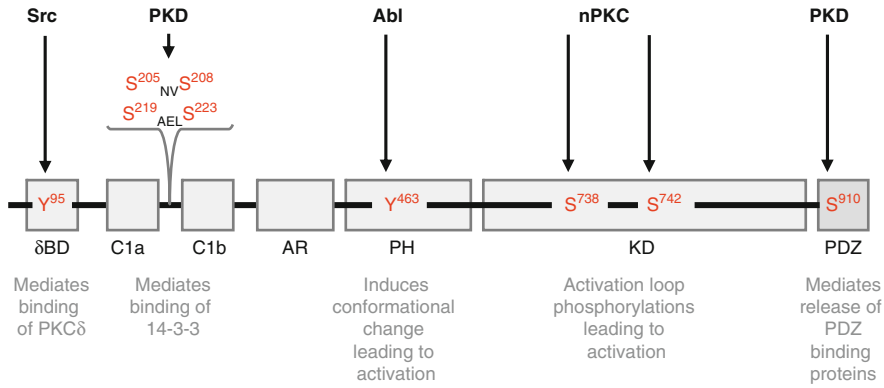
PKD, Fig. 1 Structural organization of PKD enzymes. Structures of the PKD family members PKD1/PKCμ, PKD2, and PKD3/PKCν. PKD enzymes consist of a regulatory region and a kinase domain (KD). The regulatory regions comprise a PKCδ binding motif (δBD; only in PKD1 and PKD2), the CRD region with the two C1 domains C1a and C1b, the acidic region (*AR*), and the pleckstrin homology domain (*PH*). The kinase domain includes a PDZ-binding motif (PDZ; only in PKD1 and PKD2)

Regulation of PKD Activity

Regulation of PKD activity occurs through lipid binding and phosphorylation events (Fig. 2). An initial step leading to PKD activation is its binding to diacylglycerol (DAG) which targets PKD to intracellular membranes. DAG is formed by phospholipases. For example, in response to growth factor receptor signaling PKD1 is activated at the plasma membrane through DAG generated by PLCγ or PLCβ, and in response to mitochondrial oxidative stress PKD1 is activated through binding to mitochondrial DAG that is indirectly (via phosphatidic acid) generated by ▶ PLD1 (Cowell et al. 2009). Membrane targeting as an initial step of PKD activation is followed by phosphorylation events to gain full enzymatic activity (Rozengurt et al. 2005). Dependent on the stimulus and the cellular localization of PKD activating phosphorylations are mediated by members of the novel PKC (nPKC) group and tyrosine kinases such as ▶ Src and Abl.

The canonical activation pathway for PKD is binding to DAG or phorbol ester, which leads to the release of the inhibition through the regulatory domain and subsequent phosphorylation of PKD by nPKC enzymes including PKCε, PKCδ, PKCη, and PKCθ. For example, in response to growth factor signaling, after translocation to the plasma membrane via binding to DAG, PKD is activated through nPKC. PKC enzymes phosphorylate PKD in the activation loop of the kinase domain at serine residues Ser738 and Ser742 (for human PKD1). These phosphorylation steps are required for PKD to gain full activity (Rozengurt et al. 2005). Active PKD can be released from the plasma membrane after this phosphorylation or can stay attached to intracellular membranes through binding to adapter proteins (i.e., golgi localization through binding of G-protein βγ subunits to the PH domain).

Activation of PKD1 and PKD2 in response to oxidative stress requires additional phosphorylation events. For example, at the mitochondria the activation of PKD1 by reactive oxygen species (ROS) also is initiated through binding to DAG. However, PKD1 activation then requires further phosphorylations at several tyrosine residues through the kinases Src and Abl. For example, Abl phosphorylates PKD1 in its PH domain at tyrosine residue Y463 (for human PKD1) and this leads to a release of the inhibitory regulatory domain. Subsequently, Src phosphorylates PKD1 at tyrosine



PKD, Fig. 2 Regulation of PKD1 activity. PKD1 activity is regulated by sequential activation steps. An initial step is the binding of diacylglycerol (DAG). Dependent on the activation mechanism of PKD1 this can be followed by Src- and Abl-mediated tyrosine phosphorylations. Eventually, PKD1 gains full kinase activity through nPKC-mediated phosphorylation of the activation loop serines (S738 and S742 in human PKD1). Other regulatory phosphorylation sites are the serine residues S203, S208, S219, and S223, whose phosphorylation lead to 14-3-3 binding and reduced kinase activity. Moreover, autophosphorylation at S910 regulates the association of a PDZ motif binding protein. The phosphorylation sites shown are for human PKD1

residue Y95, generating a binding motif for PKCδ, which then phosphorylates the two activation loop serines in the kinase domain (Döppler and Storz 2007).

Activation of PKD was also linked to autophosphorylation at serine residue S910 (in human PKD1) (Matthews et al. 1999). However, there is increasing evidence that this site gets phosphorylated independently or prior to PKD1 activation loop phosphorylation, suggesting that in vivo also other kinases may be involved in the phosphorylation of this residue. S910 is localized within a PDZ (Postsynaptic Density-95/Discs Large/Zonula occludens-1-binding motif) motif and its phosphorylation leads to a release of a PDZ-binding protein. This process was functionally linked to the release of vesicles from the TGN (Sanchez-Ruiloba et al. 2006).

Additional in vivo phosphorylation sites in PKD include residues S203, S208, S219, and S223 (for human PKD1), whose phosphorylation regulates binding of PKD1 to 14-3-3, a mechanism that negatively regulates the activity of the kinase (Hausser et al. 1999). The phosphorylation of these sites is most likely mediated through auto- or transphosphorylation.

Cellular Localization and Functions

Shuttling of PKD within cells – Canonical activation of PKD occurs at the plasma membrane. Upon generation of DAG PKD shuttles from the cytosol to the plasma membrane where it obtains activating phosphorylations. Dependent on the stimulus active PKD is then released into the cytosol or shuttled to the nucleus. Cytosolic PKD, for example, mediates activation of the ▶ MAP Kinase cascade. Nuclear targets for PKD are histone deacetylases (HDACs), whose phosphorylation by PKD leads to nuclear export (reviewed in Wang 2006).

PKD at the mitochondria – PKD can be localized to the mitochondria in response to either exogenously or endogenously induced oxidative stress. The mechanism of how PKD1 localizes to the mitochondria is not fully understood, but requires tyrosine phosphorylations and binding to DAG. Substrates for PKD1 at the mitochondria have not been described so far. PKD requires mitochondrial activation to gain the ability to activate anti-apoptotic and anti-oxidant signaling through the transcription factor ▶ NF-κB (Cowell et al. 2009; Storz and Toker 2003). The functions for mitochondrially activated PKD are stress resistance and cell survival.

PKD at the golgi compartment – A subcellular pool of PKD is localized to the *trans*-golgi network (TGN) and regulates the fission of transport carriers specifically destined to the cell surface (reviewed in Ghanekar and Lowe 2005). For example, PKD1 and PKD2 regulate neurotensin secretion in pancreatic cancer cells. A kinase-dead version of PKD1 blocks the detachment of tubes with cargo for the plasma membrane from the TGN. Binding of PKD to the

TGN requires DAG and recruitment to the TGN occurs via the C1a domain (Baron and Malhotra 2002). Further, PKD activation is mediated by PKCη and G-protein βγ subunits. Only few PKD substrates at the golgi were described so far. Phosphatidylinositol 4-phosphate (PI4-P) generated from phosphatidylinositol by PI4 kinases (PI4K) is an important lipid mediator in vesicular trafficking. Phosphorylation of the PI4 kinase PI4KIIIβ by PKD at the golgi complex facilitates its stabilization and increases its kinase activity through binding of 14-3-3 proteins (Hausser et al. 2005). Another PKD substrate is CERT (ceramide transport), which is also somehow involved in PKD activation at the golgi (Fugmann et al. 2007).

PKD at the actin cytoskeleton – Recently it was shown that a subcellular pool of PKD interacts with actin and once active inhibits actin reorganization at the lamellipodium (Eiseler et al. 2009). PKD isoenzymes can be activated by small Rho GTPases including RhoA, Rac, and Cdc42 (Rozengurt et al. 2005). All these Rho GTPases have major roles in regulating responses that lead to cytoskeletal reorganization involved in the regulation of actin stress fibers, focal adhesions, or cell motility, and it was shown that PKD1 regulates some of the RhoA-mediated cytoskeletal changes through phosphorylation of SSH1L and regulation of cofilin activity (Eiseler et al. 2009).

PKD in Human Disease

The in vivo functions for PKD enzymes are not well understood. So far, best studied are the roles in cardiac failure, immune cell function and cancer, although the exact contributions of the three PKD isoforms are still ill-defined.

Role of PKD in coronary heart disease – coronary heart disease is associated with cardiac hypertrophy, which precedes cardiac failure. Cardiac hypertrophy can be mediated by altered expression of genes regulating myocyte contraction, cell metabolism or calcium homeostasis. The transcription of such genes is regulated by histone acetylation processes. PKD1 was shown to negatively regulate the function of histone deacetylase (HDAC) enzymes resulting in increased transcriptional activity of genes that have been linked to mediating hypertrophy in mycocytes (Vega et al. 2004).

Role of PKD in immune cells – PKD is activated after immune cell receptor stimulation. This includes the activation of mast cell receptors and B-cell and T-cell receptors and suggests potential roles for PKD enzymes in mediating diverse immune responses. However, the roles of PKD isoforms in regulating immune functions in vivo are not well defined. Recently, the analysis of knock-in and knock-out animal models demonstrated that PKD2 has a major role in regulating antigen receptor induced cytokine production and T-cell dependent antigen responses in vivo.

Role of PKD in cancer – The functions of PKD enzymes in cancer range from tumor initiating events including increased DNA synthesis, proliferation and survival signaling to events regulating tumor cell progression such as angiogenesis (Wang 2006). Moreover, PKD is activated by tumor-promoting phorbol esters and activating phosphorylations are mediated by PKCs, Src, and Abl, all kinases that previously have been implicated in various functions in cancer. Therefore, on first view PKD represents a *bona fide* oncogene. However, the role of PKD in processes regulating tumor cell migration and invasion are not well understood. Accumulating reports suggest that PKD1/2 activation suppresses and PKD3 increases directed cell migration and tumor cell invasion (Sanchez-Ruiloba et al. 2006; Eiseler et al. 2009). Moreover, converse results were obtained regarding the function of PKD1 in regulating cell migration in epithelial or endothelial cells and it is possible that the effect of PKD1 on cell motility is cell type and cancer type dependent. Additionally, epigenetic silencing of PKD1 occurs in invasive breast or gastric cancers at the transcriptional levels through methylation of a CpG island in its promoter (Kim et al. 2008). This suggests that PKD1 expression may be needed for early tumorigenic events, but is down-regulated during metastatic progression.

Summary

Although intensively investigated since over 15 years, the in vivo roles of PKD enzymes are still unclear. Most of the knowledge on PKD signaling bases on cell culture experiments performed on PKD1. Because of their potential role in human cancer PKD signaling molecules gained increasing interest as chemotherapeutic targets. However, it remains obscure if these strategies will be effective for invasive cancers since PKD enzymes in some cancers may inhibit the

migration and invasion of cancer cells at multiple levels, in other cells may have a promoting function for these processes. In animals adult tissues often show co-expression of the different PKD isoforms and functional redundancy between the family members has been described, but is not always the case. PKD1 and PKD2 are the most closely related mammalian PKD isoforms with an approximately 86% overall identity at the amino-acid level. Within recent years it became apparent that both share common regulatory mechanisms and have redundant functions to some stimuli and unique or even converse functions in response to others. In cancer this becomes most apparent. For example, PKD1 contributes to early events leading to epithelial tumor cell proliferation and survival, but is also a negative-regulator of cell invasion. On the other hand PKD3 seems to facilitate metastatic progression. In other diseases such as cardiac hypertrophy, where all three PKD isoforms seem to have overlapping functions, the use of PKD-specific inhibitors may be more effective.

References

Baron CL, Malhotra V. Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. Science. 2002;295(5553):325–8.

Cowell CF, Döppler H, Yan IK, Hausser A, Umezawa Y, Storz P. Mitochondrial diacylglycerol initiates protein-kinase D1-mediated ROS signaling. J Cell Sci. 2009;122(Pt 7):919–28.

Döppler H, Storz P. A novel tyrosine phosphorylation site in protein kinase D contributes to oxidative stress-mediated activation. J Biol Chem. 2007;282(44):31873–81.

Döppler H, Storz P, Li J, Comb MJ, Toker A. A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. J Biol Chem. 2005;280(15):15013–9.

Eiseler T, Döppler H, Yan IK, Kitatani K, Mizuno K, Storz P. Protein kinase D1 regulates cofilin-mediated F-actin reorganization and cell motility through slingshot. Nat Cell Biol. 2009;11(5):545–56.

Fugmann T, Hausser A, Schoffler P, Schmid S, Pfizenmaier K, Olayioye MA. Regulation of secretory transport by protein kinase D-mediated phosphorylation of the ceramide transfer protein. J Cell Biol. 2007;178(1):15–22.

Ghanekar Y, Lowe M. Protein kinase D: activation for golgi carrier formation. Trends Cell Biol. 2005;15(10):511–4.

Hausser A, Storz P, Link G, Stoll H, Liu YC, Altman A, et al. Protein kinase C mu is negatively regulated by 14-3-3 signal transduction proteins. J Biol Chem. 1999;274(14):9258–64.

Hausser A, Storz P, Martens S, Link G, Toker A, Pfizenmaier K. Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. Nat Cell Biol. 2005;7(9):880–6.

Hutti JE, Jarrell ET, Chang JD, Abbott DW, Storz P, Toker A, et al. A rapid method for determining protein kinase phosphorylation specificity. Nat Meth. 2004;1(1):27–9.

Johannes FJ, Prestle J, Eis S, Oberhagemann P, Pfizenmaier K. PKCu is a novel, atypical member of the protein kinase C family. J Biol Chem. 1994;269(8):6140–8.

Kim M, Jang HR, Kim JH, Noh SM, Song KS, Cho JS, et al. Epigenetic inactivation of protein kinase D1 in gastric cancer and its role in gastric cancer cell migration and invasion. Carcinogenesis. 2008;29(3):629–37.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298(5600):1912–34.

Matthews SA, Rozengurt E, Cantrell D. Characterization of serine 916 as an in vivo autophosphorylation site for protein kinase D/protein kinase Cmu. J Biol Chem. 1999;274(37):26543–9.

Rozengurt E, Rey O, Waldron RT. Protein kinase D signaling. J Biol Chem. 2005;280(14):13205–8.

Sanchez-Ruiloba L, Cabrera-Poch N, Rodriguez-Martinez M, Lopez-Menendez C, Jean-Mairet RM, Higuero AM, et al. Protein kinase D intracellular localization and activity control kinase D-interacting substrate of 220-kDa traffic through a postsynaptic density-95/discs large/zonula occludens-1-binding motif. J Biol Chem. 2006;281(27):18888–900.

Storz P, Toker A. Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. EMBO J. 2003;22(1):109–20.

Van Lint J, Rykx A, Maeda Y, Vantus T, Sturany S, Malhotra V, et al. Protein kinase D: an intracellular traffic regulator on the move. Trends Cell Biol. 2002;12(4):193–200.

Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, et al. Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol. 2004;24(19):8374–85.

Wang QJ. PKD at the crossroads of DAG and PKC signaling. Trends Pharmacol Sci. 2006;27(6):317–23.

PKD (isoforms: PKD1/PKCμ, PKD2, PKD3/PKCν)

► PKD

PKD2

► Polycystin-2

PKD4

► Polycystin-2

PKM (Hamster)

▶ HIPK2

PKNOX

▶ Prep

PKR

Anthony John Sadler
Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Clayton, VIC, Australia

Synonyms

Double-stranded RNA-activated protein kinase; EIF2A protein kinase 2; Interferon-inducible eIF2α kinase; Interferon-inducible RNA-dependent protein kinase; P1/eIF2A protein kinase; p68 kinase, dsRNA-activated inhibitor (DAI); Protein kinase interferon-induced double-stranded RNA-activated (PRKR); Protein kinase RNA-activated (PKR)

Historical Background

Control of protein translation is a universal checkpoint that enables eukaryotic cells to respond to environmental challenge. Accordingly, it had been noted that virus infection halted host protein synthesis, and it was established that viral double-stranded (ds) RNA provoked this effect. A protein kinase, now designated PKR, was identified which phosphorylated the translation initiation factor 2α that accounted for this effect. It was subsequently demonstrated that this kinase was induced by interferon (IFN), establishing this enzyme as an effector molecule for the central antiviral response. It was subsequently shown that stimuli besides dsRNA activate, and conversely, inhibit PKR. The transcript cDNA was cloned and the genetic locus was identified (on human chromosome 2p21 and mouse chromosome 17, band E2), and its regulatory promoter elements described. It was shown that the gene (EIF2AK2) was induced by responses other than IFN. Generation of transgenic mice mutated for the enzyme cemented the function of PKR as an antiviral protein. Moreover, analysis of cells from these transgenic mice identified that PKR acts as a cell-signaling molecule that regulates inflammatory responses. Additional protein substrates, besides eIF2α, have subsequently been identified. The precise mechanisms by which PKR affect cell signaling and the biological consequences of this function are still emerging.

PKR, Table 1 Proteins that interact with PKR as regulators of the kinase's activity, as substrates, or that are not directly phosphorylated but participate in cell-signaling associations are listed. Each protein and their abbreviations are described in the text

Regulators	Substrates	Cell-signaling integrators
PACT	eIF2α	TRAF2
Caspase-3	RHA	TRAF3
Caspase-7	ILR3	TRAF5
Caspase-8	B56α	TRAF6
TARBP2	IRS1	STAT1
MRPL18	MAP2K6	STAT3
METAP2	NPM1	TIRAP
DNAJC3	SPNR	IKK-α/β
PKRKIR		MAP3K5
PPP1C		MAP3K7
HSP70		TAB2
HSP90		FANNC
PKRKIP1		PTEN

The PKR Protein Network

Cellular proteins that have been reported to interact with PKR to convey cell signals are listed in Table 1. These interactions fall into three functional categories in which the interacting proteins modify the activity of PKR, are substrates for the kinase, or are regulated by PKR without being directly phosphorylated. These relationships are not mutually exclusive, as some proteins that modify the activity of PKR are also substrates for the kinase.

PKR Regulators

Three nonprotein ligands for PKR have been identified. Relief of steric hindrance between the N-terminal RNA-binding and C-terminal kinase domains activates

PKR. Accordingly, dsRNA, single-stranded RNA with secondary structures and specific features, such as 5'triphosphate moities, bind to the N-terminal RNA-binding motifs (RBMs) to activate PKR. RNA with these features is characteristic of virus replication. In addition, some endogenous mRNAs are also competent to activate PKR by virtue of specific structural motifs. A second activating ligand is heparin oligosaccharide. Heparin with eight or more sugar residues associates with residues within the protein's kinase domain to relieve autoinhibition. The third PKR non-protein ligand is the nucleotide adenosine triphosphate (ATP). As for other protein kinases, ATP binds PKR within the protein's kinase-fold that is formed between sub-domains (designated the N- and C-lobes) within the kinase domain. PKR has been demonstrated to respond to other nonprotein ligands, for instance lipid molecules such as ceramide, but the mechanisms of these interactions are not well characterized and appear to be indirect. The remaining regulators of PKR are proteins.

PKR is cleaved at the asparagine residue number 251 between the two structured domains of PKR by caspases (caspase-3, -7, and -8) (Saelens et al. 2001). This cleavage relieves autoinhibition by separating the N- and C-termini of the kinase. Caspase cleavage has been demonstrated to generate a constitutively active truncated kinase, as well as advancing activation of the full-length protein.

The PKR protein activator (PACT) binds directly to activate PKR (Patel et al. 1998). Extensive biochemical analysis of this protein interaction has identified residues within the C-terminal RBM as being crucial to disrupt RBM-mediated autoinhibition of PKR. Although not phosphorylated by PKR, PACT must first be phosphorylated by another kinase before activating PKR. Other stress signals lead to activation of PACT that subsequently activates PKR. The activation residue in PACT (serine 18) constitutes a putative phosphorylation motif for mitogen-activated kinases. Accordingly, PKR's response to stress stimuli, such as hydrogen peroxide and the sphingolipid ceramide, appears to be mediated by PACT-dependent activation.

In keeping with the mechanism of RBM-mediated autoinhibition, a number of other proteins containing RBMs interact to inhibit PKR. The transactivation response RNA-binding protein-2 (TRBP2), which encodes three RBMs, inhibits PKR (Park et al. 1994). The adenosine deaminase acting on RNA-1 (ADAR1), which encodes two RBMs, interacts with and inhibits PKR (Toth et al. 2006). Furthermore, the dihydrouridine synthase 2-like (DUS2L) protein, which catalyzes the reduction of uridine residues on the displacement loop of transfer RNA, interacts with PKR to repress its activity (Mittelstadt et al. 2007). Additional proteins that encode RBM have been demonstrated to bind to PKR, such as the protein-coded DRBP120, but the role of these proteins and so the consequence of this interaction is unknown.

Formation of the active dimeric PKR enzyme proceeds by autophosphorylation, and so, the enzyme can be repressed through dephosphorylation. Accordingly, the protein phosphatase-1 (PPP1C) has been demonstrated to inhibit PKR (Tan et al. 2002). Other PKR inhibitors are: the PKR-interacting protein-1 (PKRIP1) (Yin et al. 2003); the mitochondrial ribosomal protein L18 (MRPL18) which sequesters the kinase to the ribosome (Kumar et al. 1999); and in a somewhat similar mechanism, the methionine aminopeptidase-2 (METAP2) also inhibits PKR to prevent translational inhibition (Gil et al. 2000). In addition, two cytosolic chaperones, the heat-shock proteins (HSP) 70 and 90, regulate PKR (Donze et al. 2001). The association with HSP70 mediates an observed interaction between PKR and mutant Fanconi anemia proteins (directly with the complementation group C protein, FANCC). A co-chaperone of HSP70, DNAJC3 (or P58(IPK)), also acts as a PKR inhibitor (Polyak et al. 1996). Interestingly, DNAJC3 is itself regulated by a repressor, PKRIR (or p52(rIPK)) that competes with PKR for binding to DNAJC3 (Gale et al. 1998).

PKR Substrates

PKR is a serine, threonine and, less accepted, a tyrosine protein kinase. The best-described PKR substrate is the α subunit of the eukaryotic initiation factor (eIF2α). Phosphoryation of the serine residue 51 on eIF2α arrests translation of cap-dependent transcripts (Farrell et al. 1977). Importantly, this translational block is not complete, and a number of transcripts escape repression by virtue of specific structures in the 5'untranslated region of their mRNA. Prominent among these escape transcripts are the activating

transcription factors (ATFs), with the best example being the induction of ATF4. Biological processes regulated by ATF4 include redox processes, mitochondrial function, and secretory pathways regulated by protein chaperones and lipid synthesis machinery. In this way, eIF2α phosphorylation, with subsequent ATF4 induction, elicits a manifold response to mitigate stress. Hence, eIF2α phosphorylation is generally considered a protective response. This contradicts many of the proposed functions determined in vitro that supposes a mainly pro-inflammatory role for PKR.

The consequence of PKR's regulation of other substrates is less well established as the functions of the protein substrates themselves, or the consequences of specific modified residues involved are poorly characterized. The various substrates are discussed below.

Nucleoplasmin family member-1 (NPM1) is a PKR substrate (Pang et al. 2003). The specific residues phosphorylated by PKR have not been identified. NPM1 is a highly phosphorylated protein, with at least two other kinases demonstrated to modify the protein. NPM1 has been ascribed a broad range of functions. Relevant to translational control, NPM1 has been demonstrated to mediate ribosomal protein assembly. Aberrant expression of NPM1 has been observed in numerous human malignancies. Although PKR-dependent effects on NPM1 function have not been identified, NPM1 has been shown to inhibit PKR.

Phosphorylation of B56α by PKR has been demonstrated to inhibit this substrate (Xu et al. 2000). As a consequence, PKR promotes the activity of the protein phosphatase 2 catalytic subunit (PP2A) that is controlled by B56α. As PP2A is a significant modulator of global phosphorylation, PKR-dependent control of its regulatory subunit is undoubtedly of biological significance. However, control of PP2A activity is complex with multiple regulatory factors involved besides B56α. Moreover, there are three other cytosolic serine/threonine phosphatases, in addition to PP2A, that have a degree of overlapping substrate specificity. Hence, it is difficult to discern the specific effects of PKR's regulation of B56α.

The mitogen-activated kinase MAP2K6 is reportedly phosphorylated by PKR (Silva et al. 2004). Regulation of MAP2K6 was proposed as the mechanism by which PKR affects the downstream mitogen-activated kinase MAPK14. However, the consequence of PKR-dependent control of MAP2K6 is unclear as the residues phosphorylated did not encompass conserved residues that regulate kinase activity.

PKR has also been reported to phosphorylate the tumor suppressor ▶ p53 at the serine residue 392 (Cuddihy et al. 1999). This is potentially very interesting because of the manifold responses regulated by p53. Phosphorylation of the 392 residue is dispensable for activation of p53 target genes. Consequently, PKR-dependent phosphorylation can only affect transactivation-independent p53 pathways. Modification of this residue has been shown to affect the stability of p53 by enhancing protein oligomerization. This appears to only be of consequence for the mis-folded mutant p53 as modification of the 392 site has not been shown to alter the stability of the wild-type protein. Hence, PKR could regulate nonspecific binding of mutant p53 to DNA structures associated with DNA repair and recombination. Phosphorylation at serine 392 has been correlated with tumor development, and so, PKR-dependent control of p53 may contribute to tumor progression.

It has recently been asserted that the insulin receptor substrate-1 (IRS1) is a substrate of PKR (Nakamura et al. 2010). The IRS1 serine residue 307 is modified by PKR, though this appears at odds with the role of PKR proposed in the study that identified IRS1 as a substrate. It was contended that PKR induces insulin resistance. However, analysis of transgenic mice with a point mutation of the 307 residue of IRS1 demonstrated that phosphorylation control of this site maintains insulin sensitivity. Interestingly, in light of PKR's regulation of PP2A, the okadaic acid inhibitor of PP2A stimulated the mammalian target of rapamycin (▶ mTOR)-dependent phosphorylation of the serine 307 residue on IRS1. Consistent with this, the rapamycin inhibitor of mTOR reduced 307 phosphorylation on IRS1. PKR has been demonstrated to influence the downstream effects of a second phosphatase that influences insulin signaling, the phosphatase, and tensin homolog deleted from chromosome 10 (▶ PTEN). Translational control in response to PTEN is diminished in PKR-null cells. Hence, it appears as if PKR is activated in PTEN-dependent signaling. These effects were independent of PTEN's effects on phosphoinositide 3'-kinase (PI3K). Coincidentally, PTEN has also been shown to dephosphorylate IRS1.

Hence, two other potential mechanisms exist, in addition to the proposed direct phosphorylation, by which PKR might regulate insulin signaling.

Activation of PKR is mediated by autophosphorylation at some 15 residues. Among the autophosphorylation sites identified are residues within the N-terminal RBMs of PKR. This establishes the RBM as a protein substrate. Fittingly, a number of other proteins that share the RBM have been identified as substrates. PKR phosphorylates the RNA helicase A (RHA) within the RBM (Sadler et al. 2009). This phosphorylation inhibits the helicase's association with its nucleic substrates. The interleukin factor-3 (ILF3) is similarly phosphorylated within its RBMs (Langland et al. 1999). The consequence of this phosphorylation has not been identified. In addition, PKR associates with the spermatid perinuclear RNA-binding protein (STRBP). Although not reported as a substrate of PKR, STRBP was identified as being phosphorylated, at serine residues within the two RBMs, in cell culture. All three of these RBM-containing proteins (RHA, ILF3, and STRBP) have been demonstrated to inhibit PKR activity.

Non-substrate Protein Interactions with PKR

Investigation of PKR-dependent cell signaling in vitro has principally been proposed to promote inflammation. PKR participates in cell signals initiated from a variety of receptors that include toll-like, interleukin-1, platelet-derived growth factor (PDGF), IFNγ, and the tumor necrosis factor-α (▶ TNFα) receptors. Through these receptors, PKR modulates the activity of a number of transcription factors. The mechanism(s) by which PKR is integrated into these diverse signaling pathways has not been categorically established. The currently favored mechanism involves association with the TNF receptor-associated factors (TRAFs). PKR encodes two TRAF-binding motifs within each of its structured domains. TRAF factors act as adaptor proteins and ubiquitin ligases. Through this activity, these proteins shape signaling complexes and regulate the stability of the protein components. Dependence upon TRAFs for PKR-dependent control correlates with the transcription factors activated: nuclear factor-κB (▶ NF-κB), c-jun, the signal transducers and activators of transcription (STAT) 1 and 3, and IFN-regulated factor-1 (IRF1). Furthermore, the association with TRAFs could account for other reported associations between PKR and signaling proteins: the I-κB kinases (IKKα and β), mitogen-activated protein kinase kinase kinase-7 (MAP3K7, or TAK1), TAK1-binding protein-2 (TAB2), the toll/interleukin-1 (TIR) domain containing adaptor protein (TIRAP), and the mitogen-activated protein kinase kinase kinase-5 (MAP3K5, or ASK1). Through the association with TRAFs, it is asserted that PKR modifies other components of the signaling complex. Two alternatives have been proposed, by which PKR either modifies signaling components by phosphorylation, or alternatively, may act merely as a scaffold protein independent of its kinase activity. This latter point has support from experiments that have shown that a kinase-dead PKR mutant is competent to activate the NF-κB transcription factor.

Mediation of PKR-dependent cell signaling by TRAFs is attractive as it offers a common explanation for diverse activities of PKR. However, it remains possible that PKR mediates these effects through direct interaction with the separate components, through as yet unidentified interactions, or that alternative adaptors regulate some or all of these effects. As an instance of the latter case, the interaction with HSP90 presents a potential mechanism that could account for many PKR-dependent cell-signaling events. Like TRAFs, HSP90 interconnects with many of the pathways PKR is reported to regulate. HSP90 interacts with the NF-κB regulatory complex, STATs, and p53. As HSP90 has been demonstrated to inhibit PKR, this interaction may support the contention that kinase activity is irrelevant in some PKR-dependent cell-signaling processes. HSP90 has been demonstrated to associate with a large number of other kinases, and so, has been suggested as a general regulator of the kinome.

Summary

In this entry, the endogenous protein interactions and the cell-signaling functions of PKR have been catalogued. The first recognized function of PKR, to combat viral infection, has not been discussed here. It is frequently written that the significance of PKR in the antiviral response is evident by the numerous viral inhibitors that target PKR. It is evident from this discussion that a number of endogenous proteins exist to regulate PKR activity. Hence, this might also be taken

to indicate the significance of PKR-mediated cell signaling. Accordingly, PKR has been shown to respond to stress signals other than dsRNA, and has been shown to phosphorylate additional protein substrates besides eIF2α. Moreover, PKR may modulate cell-signaling pathways independent of its kinase activity. Combined, these observations suggest a more complex regulation and a broader function for PKR than merely in the innate immune response to viral infection that remains to be clarified.

References

Cuddihy AR, Wong AH, Tam NW, Li S, Koromilas AE. The double-stranded RNA activated protein kinase PKR physically associates with the tumor suppressor p53 protein and phosphorylates human p53 on serine 392 in vitro. Oncogene. 1999;18(17):2690–702.

Donze O, Abbas-Terki T, Picard D. The Hsp90 chaperone complex is both a facilitator and a repressor of the dsRNA-dependent kinase PKR. EMBO J. 2001;20(14):3771–80.

Farrell PJ, Balkow K, Hunt T, Jackson RJ, Trachsel H. Phosphorylation of initiation factor elF-2 and the control of reticulocyte protein synthesis. Cell. 1977;11(1):187–200.

Gale Jr M, Blakely CM, Hopkins DA, Melville MW, Wambach M, Romano PR, et al. Regulation of interferon-induced protein kinase PKR: modulation of P58IPK inhibitory function by a novel protein, P52rIPK. Mol Cell Biol. 1998;18(2):859–71.

Gil J, Esteban M, Roth D. In vivo regulation of the dsRNA-dependent protein kinase PKR by the cellular glycoprotein p67. Biochemistry. 2000;39(51):16016–25.

Kumar KU, Srivastava SP, Kaufman RJ. Double-stranded RNA-activated protein kinase (PKR) is negatively regulated by 60S ribosomal subunit protein L18. Mol Cell Biol. 1999;19(2):1116–25.

Langland JO, Kao PN, Jacobs BL. Nuclear factor-90 of activated T-cells: A double-stranded RNA-binding protein and substrate for the double-stranded RNA-dependent protein kinase, PKR. Biochemistry. 1999;38(19):6361–8.

Mittelstadt M, Frump A, Khuu T, Fowlkes V, Handy I, Patel CV, et al. Interaction of human tRNA-dihydrouridine synthase-2 with interferon-induced protein kinase PKR. Nucleic Acids Res. 2007;36(3):998–1008.

Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N, et al. Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. Cell. 2010;140(3):338–48.

Pang Q, Christianson TA, Koretsky T, Carlson H, David L, Keeble W, et al. Nucleophosmin interacts with and inhibits the catalytic function of eukaryotic initiation factor 2 kinase PKR. J Biol Chem. 2003;278(43):41709–17.

Park H, Davies MV, Langland JO, Chang HW, Nam YS, Tartaglia J, et al. TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR. Proc Natl Acad Sci USA. 1994;91(11):4713–7.

Patel RC, Sen GC. PACT, a protein activator of the interferon-induced protein kinase, PKR. EMBO J. 1998;17(15):4379–90.

Polyak SJ, Tang N, Wambach M, Barber GN, Katze MG. The P58 cellular inhibitor complexes with the interferon-induced, double-stranded RNA-dependent protein kinase, PKR, to regulate its autophosphorylation and activity. J Biol Chem. 1996;271(3):1702–7.

Sadler AJ, Latchoumanin O, Hawkes D, Mak J, Williams BR. An antiviral response directed by PKR phosphorylation of the RNA helicase A. PLoS Pathog. 2009;5(2):e1000311.

Saelens X, Kalai M, Vandenabeele P. Translation inhibition in apoptosis: caspase-dependent PKR activation and eIF2-alpha phosphorylation. J Biol Chem. 2001;276(45):41620–8.

Silva AM, Whitmore M, Xu Z, Jiang Z, Li X, Williams BR. Protein kinase R (PKR) interacts with and activates mitogen-activated protein kinase kinase 6 (MKK6) in response to double-stranded RNA stimulation. J Biol Chem. 2004;279(36):37670–6.

Tan SL, Tareen SU, Melville MW, Blakely CM, Katze MG. The direct binding of the catalytic subunit of protein phosphatase 1 to the PKR protein kinase is necessary but not sufficient for inactivation and disruption of enzyme dimer formation. J Biol Chem. 2002;277(39):36109–17.

Toth AM, Zhang P, Das S, George CX, Samuel CE. Interferon action and the double-stranded RNA-dependent enzymes ADAR1 adenosine deaminase and PKR protein kinase. Prog Nucleic Acid Res Mol Biol. 2006;81:369–434.

Xu Z, Williams BR. The B56alpha regulatory subunit of protein phosphatase 2A is a target for regulation by double-stranded RNA-dependent protein kinase PKR. Mol Cell Biol. 2000;20(14):5285–99.

Yin Z, Haynie J, Williams BR, Yang YC. C114 is a novel IL-11-inducible nuclear double-stranded RNA-binding protein that inhibits protein kinase R. J Biol Chem. 2003;278(25):22838–45.

PKS2 (Presumably for Kinase Sequence)

► A-RAF

Plasma Membrane Ca^{2+} ATPase

► Plasma Membrane Calcium-Transporting ATPase

Plasma Membrane Ca^{2+} Pump

► Plasma Membrane Calcium-Transporting ATPase

Plasma Membrane Calcium-Transporting ATPase

Emanuel E. Strehler
Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, USA

Synonyms

Plasma membrane Ca^{2+} ATPase; Plasma membrane Ca^{2+} pump; PMCA

Historical Background

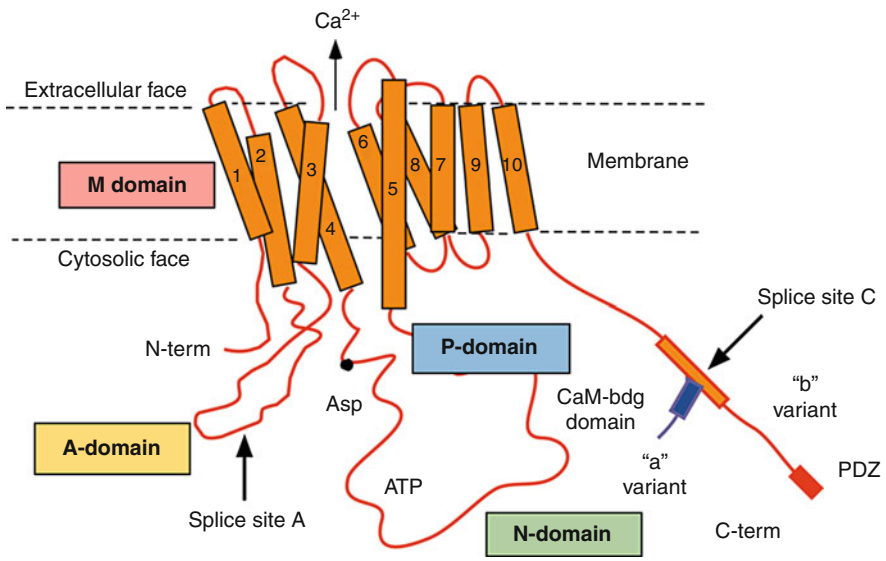
The existence of a plasma membrane calcium-transporting ATPase (PMCA) that actively pumps Ca^{2+} ions out of the cell was first demonstrated in erythrocyte (red blood cell) membranes by Schatzmann (1966). Because of its generally low abundance and difficult biochemical properties, it took over a decade until the PMCA was first isolated in purified form. Crucial for the successful purification was the discovery that the PMCA binds with high affinity, and in a Ca^{2+}-dependent manner, to the Ca^{2+} sensor protein calmodulin (Niggli et al. 1979). Subsequent work showed that at least one type of plasma membrane Ca^{2+} ATPase is found in all eukaryotic cells including those from fungi, animals, and plants (Axelsen and Palmgren 1998; Thever and Saier 2009). It is now well established that active Ca^{2+} expulsion by the PMCAs is an essential component of eukaryotic cellular Ca^{2+} handling. Although PMCAs were originally thought to be required mainly for the "housekeeping" function of maintaining and resetting the low intracellular free $[Ca^{2+}]$ levels, recent studies have shown that these pumps are also active participants in global and local Ca^{2+} signaling.

General Structure and Isoforms

PMCAs belong to the large superfamily of P-type ion-transporting ATPases, where they are classified as IIB subfamily (Axelsen and Palmgren 1998) or family 2 in the IUBMB transporter classification system (Thever and Saier 2009; see www.tcdb.org). The characteristic feature of P-type ATPases is the formation of an obligatory phosphorylated intermediate during the reaction cycle (Pedersen and Carafoli 1987). The PMCA consists of a single polypeptide, which in vertebrates contains about 1,200 amino acids and has a molecular mass of about 140 kDa. The protein has 10 membrane-spanning segments, and both the N- and the C-terminal ends are facing the cytosol. Most of the protein mass is on the cytosolic side of the membrane, only short loops connecting pairs of transmembrane segments are facing the extracellular side. Figure 1 schematically illustrates the domain arrangement of the PMCA in a 2-dimensional model.

The cytosolic loops between transmembrane segments 2 and 3 and between transmembrane segments 4 and 5 are large and contribute the bulk of the mass to the so-called A (actuator) and N/P (nucleotide-binding/catalytic phosphorylation) domains, respectively. In animal PMCAs, the C-terminal tail following the last membrane-spanning segment is about 150 residues long and contains the auto-inhibitory regulatory sequences and the high-affinity calmodulin-binding domain. In contrast, in several plant PMCAs, the calmodulin-binding and auto-inhibitory regulatory domain are instead found in the extended N-terminal tail. The three-dimensional structure of the PMCA has not yet been solved in atomic detail, but based on the high conservation of secondary structure elements (including the 10 transmembrane helices and the functionally important A, N, and P domains), the general structure of the PMCA is thought to be very similar to that of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), for which several high-resolution x-ray structures are available (Toyoshima 2009).

Mammalian PMCAs are encoded by four separate genes, which give rise to PMCA isoforms 1–4. The human genes (gene symbols ATP2B1-ATP2B4) are located on chromosomes 12q21.3, 3p25.3, Xq28, and 1q32.1, respectively, whereas the orthologous mouse genes (Atp2b1-Atp2b4) are on chromosomes 10C3, 6E3, XA7.3, and 1E4. The RNA transcripts of all genes are subject to complex alternative splicing, resulting in over 30 possible PMCA splice variants. The two major sites (sites A and C) where alternative splicing affects the PMCA protein structure are indicated in Fig. 1. Figure 2 shows a scheme of the resulting splice variants found in mammalian PMCAs. The two major splice variants generated at



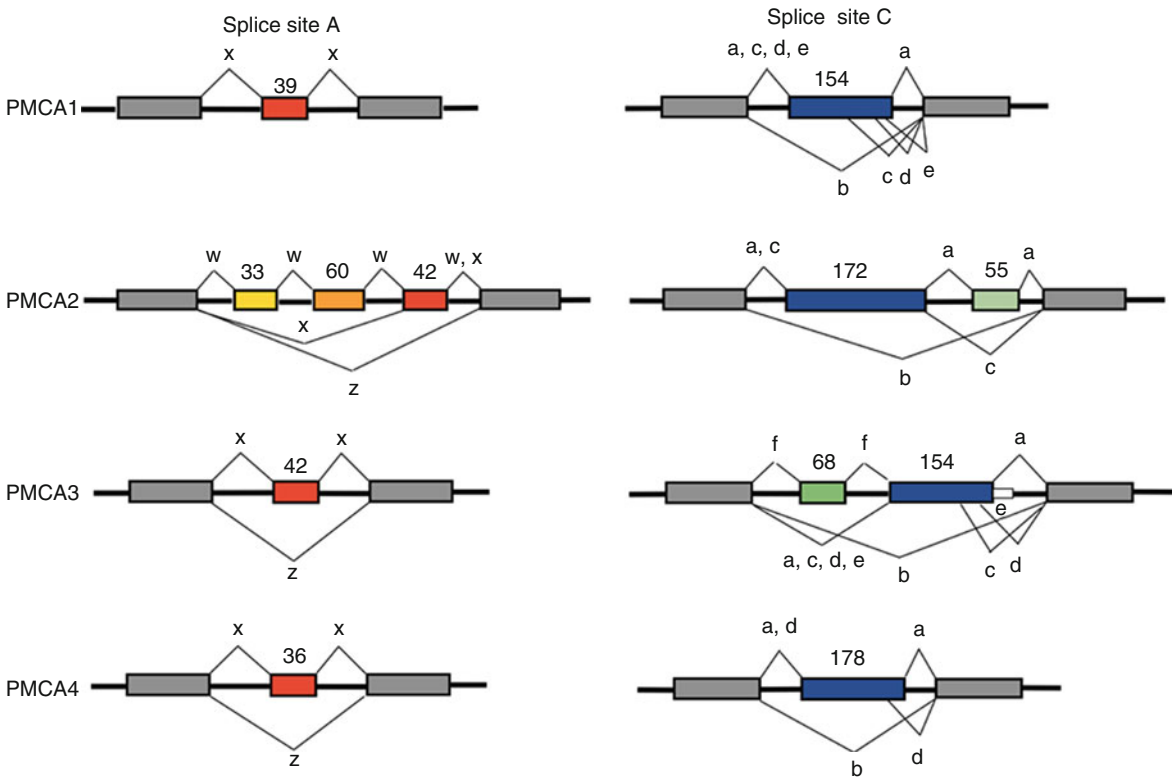
Plasma Membrane Calcium-Transporting ATPase, Fig. 1 Scheme of the plasma membrane Ca^{2+}-transporting ATPase, showing major domains and sites affected by alternative splicing. The 10 membrane-spanning regions are numbered and shown as cylinders forming the M domain. The amino- (N-term) and carboxy-terminal ends (C-term), the conserved aspartate (Asp) residue undergoing phosphorylation during the reaction cycle, and the ATP binding site (ATP) are labeled. The direction of Ca^{2+} transport is indicated by an arrow. The three main cytosolic domains are labeled A (actuator), P (phosphorylation), and N (nucleotide-binding). Splice sites A and C are indicated by *arrows*. Splicing at site C affects the calmodulin-binding (CaM-bdg) domain and results in major splice variants "a" and "b," which differ in their C-terminal amino acid sequence. The "b" splice variants contain a C-terminal PDZ-binding motif, which is missing in the "a" variants

site C are "a" and "b"; they differ significantly in their C-terminal tails because of a change in the open reading frame introduced by the splice. In contrast, splice variants generated at site A ("w," "x," "y," "z") contain different peptide insertions in the first cytosolic loop, but otherwise retain the same reading frame. Alternative splicing affects the cellular targeting and the regulatory and functional properties of the different PMCA isoforms, and thus plays an essential role in the dynamic regulation of Ca^{2+} signaling in cell physiology (Strehler and Zacharias 2001).

Regulation and Functional Properties

The basic function of the PMCA is to catalyze the "uphill" transport of Ca^{2+} ions across the membrane. PMCAs couple the hydrolysis of one molecule of ATP to the transport of 1 Ca^{2+} ion across the membrane. They are high-affinity, but low capacity, Ca^{2+} transporters with a maximal turnover number of ~100/s (Brini and Carafoli 2009). Their general reaction mechanism is conveniently described by the E1–E2 scheme (Fig. 3), in which the pump toggles between two major conformational states E1 and E2. Thus, transport of a Ca^{2+} ion from the intracellular (cytosolic) side to the extracellular side of the membrane is accompanied by large conformational changes in the PMCA. These are a result of the reactions taking place at the intracellular ATP binding and phosphorylation site (where the γ-phosphate of ATP is transferred to an invariant Asp residue), which are coupled to rearrangement of the membrane-spanning domain of the PMCA. The positive charge transfer resulting from the transport of Ca^{2+} across the membrane is at least partially compensated by countertransport of protons (H^+). Accordingly, the PMCA is sensitive to pH, with alkaline pH in the extracellular milieu having an inhibitory effect.

At low intracellular [Ca^{2+}] (<50–100 nM), the PMCA is inactive and present in an auto-inhibited conformation in which the C-terminal tail makes intramolecular contacts with the A- and N-domains. Upon a rise in [Ca^{2+}] the PMCA is activated, and this activation is accelerated and enhanced by Ca^{2+}-calmodulin, which binds to the auto-inhibitory tail and releases

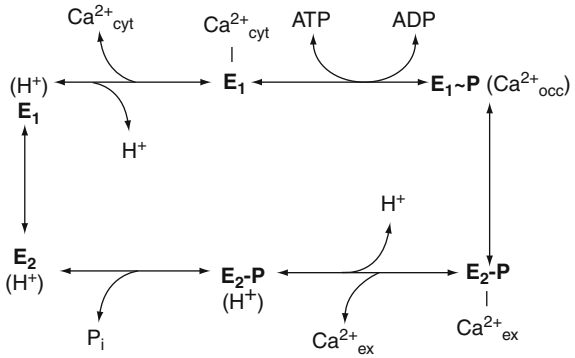


Plasma Membrane Calcium-Transporting ATPase, Fig. 2 *Alternative splicing options of human PMCA isoforms 1–4 at splice sites A and C*. The exon structure of the region involved in alternative splicing is shown for each of the four PMCA genes. Constitutively spliced exons are shown as *gray boxes*. The sizes of alternatively spliced exons (different shades of *gray*) are given in nucleotides, the splice options are indicated by connecting lines, and the resulting splice products are labeled by their lowercase symbol. Note that in PMCA1, only splice option "x" has been detected, and that in PMCA3, splice variant "e" results from a read-through of the 154-nt exon into the adjoining intron (indicated as *thin white box*). Combinatorial use of the splice options at sites A and C results in over 30 possible PMCA splice variants

the inhibition. The effect of calmodulin thus is to shift the apparent Km(Ca^{2+}) for activation of the PMCA to lower [Ca^{2+}] (0.2–0.5 μM). Different isoforms and splice variants of the PMCA vary significantly in their calmodulin affinity and the speed with which they are activated. This is of physiological significance as "fast" PMCAs (such as PMCA2b and PMCA3f) are generally found in "fast" cells (muscle, nerves) requiring rapid Ca^{2+} clearance after a spike. PMCAs are also regulated by acidic phospholipids such as phosphatidylserine and phosphatidylinositol, dimerization (oligomerization), phosphorylation on Ser/Thr residues in the regulatory tail, partial proteolysis of the auto-inhibitory tail by ▶ calpain, as well as by specific protein interactions with regulatory proteins and the cytoskeleton (Strehler et al. 2007; Brini and Carafoli 2009). Multiple other regulatory interactions have also been demonstrated for various PMCA isoforms with proteins such as 14-3-3ε, calcineurin A, RASSF1, α-1 syntrophin and an increasing list of signaling, trafficking, and anchoring proteins containing PDZ (PSD95/Dlg/ZO-1) domains. A summary scheme listing currently known PMCA-interacting proteins is shown in Fig. 4. It should be noted that several of these proteins interact specifically with some, but not all PMCA isoforms and splice variants. For example, the a-splice variants of the PMCA lack a canonical PDZ-binding sequence motif at their C-terminus and are therefore unable to engage in PDZ domain-mediated interactions with other proteins.

Tissue and Subcellular Expression

All tissues and cells express at least one isoform of the PMCA; however, multiple PMCA isoforms and splice variants are often found in the same cell. During



Plasma Membrane Calcium-Transporting ATPase, Fig. 3 *Reaction scheme for the plasma membrane Ca^{2+}-transporting ATPase*. The PMCA assumes two major states E1 and E2. E1 has high affinity for Ca^{2+} on the cytosolic side (Ca^{2+} cyt). Ca^{2+} binding stimulates the ATPase activity of the pump, resulting in the phosphorylation of a conserved Asp residue and formation of the phosphorylated intermediate (E1∼P), as well as occlusion (occ) of the bound transport Ca^{2+} ion. The Ca^{2+} ion is translocated across the membrane and the stored energy is released during the conformational transition from the E1∼P to the E2-P state. The Ca^{2+} affinity of the E2-P state is low and Ca^{2+} dissociates on the extracellular side of the membrane (Ca^{2+} ex). Hydrolysis of the phosphoenzyme E2-P and conformational rearrangement of the E2 to the E1 state complete the cycle. The positive charge movement during Ca^{2+} transport is at least partially compensated by countertransport of protons (H^+)

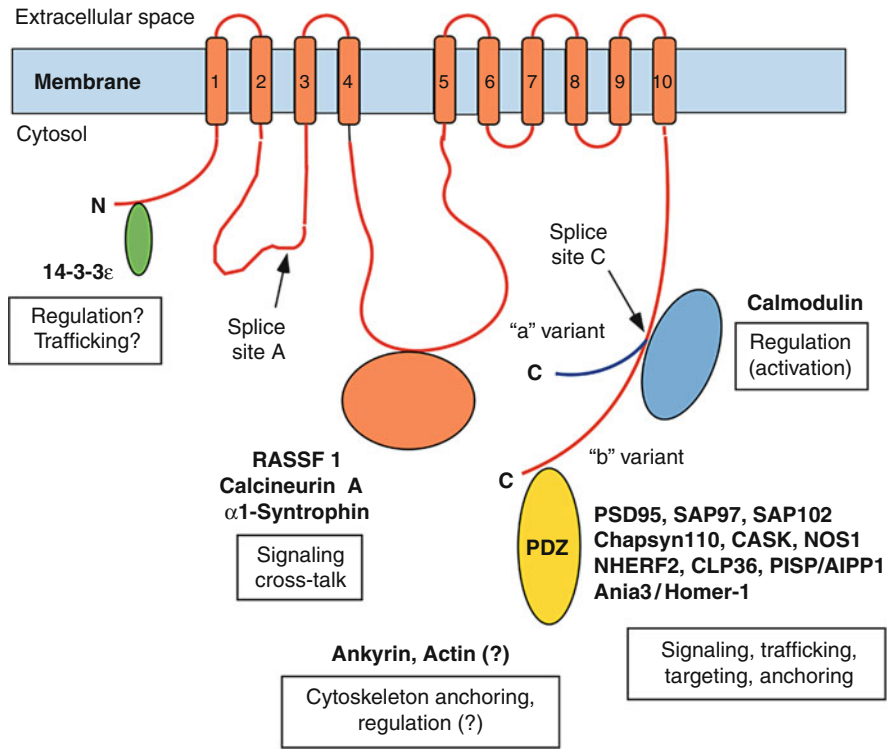
(mouse) embryonic development, PMCA1 (splice variant 1x/b) is detected from the earliest time points studied and is expressed in most tissues throughout life. PMCA1 is thus considered a "housekeeping" PMCA isoform, although this only applies to splice variant 1x/b. PMCA1x/a and 1x/c are much more restricted in their expression and are mainly found in differentiated neurons and skeletal muscle cells. PMCA4x/b is also fairly ubiquitous, although there are large differences in PMCA4b expression among different tissues and cell types. By contrast, PMCAs 2 and 3 are almost exclusively found in excitable tissues including brain and muscle, as well as in secretory cells such as insulin-secreting pancreatic β-cells and lactating mammary epithelial cells (Strehler and Zacharias 2001). Some splice variants are highly specific for particular cells: PMCA2w/a, for example, is specifically and abundantly expressed in auditory and vestibular hair cells of the inner ear (Hill et al. 2006). Different PMCAs are often co-expressed in the same cell, where they are specifically targeted to distinct membrane compartments. This suggests that different PMCA isoforms fulfill distinct roles in global and local Ca^{2+} handling. In cochlear hair cells, for example, PMCA1x/b is expressed in the basolateral membrane where it is involved in maintaining the low resting level of Ca^{2+} in the cell soma. In the same cells, PMCA2w/a is highly concentrated in the apical stereocilia where it plays an important role in regulating $[Ca^{2+}]$ in the endolymph and modulating the function of the mechanotransduction channels.

Functions in Health and Disease

In cells such as human erythrocytes, where the PMCA is the sole Ca^{2+} export system, the pump is obviously essential for normal Ca^{2+} homeostasis and cell physiology. However, because of its low abundance in most plasma membranes and its low capacity (maximal turnover of about 100 Ca^{2+} ions per second), the PMCA is generally not well suited for handling high global Ca^{2+} loads such as those occurring with each heart beat in a cardiac muscle cell. Conversely, due to its very high Ca^{2+} affinity (Kd < 0.2–0.5 μM) in the activated state, the PMCA is the only Ca^{2+} export system capable of lowering intracellular $[Ca^{2+}]$ to the very low resting levels (∼100 nM) normally found in most cells. In terms of global cellular Ca^{2+} homeostasis, the expression of different PMCA isoforms and splice variants allows cells to finely tune and maintain the specific Ca^{2+} set point optimal for their physiological function.

In tissues involved in transcellular Ca^{2+} transport, the PMCA plays an essential role in normal physiology. In kidney and intestinal epithelial cells, PMCA localized in the basolateral membrane is important for the vectorial transport of Ca^{2+} from the apical (lumenal) to the basal (blood) compartment, and thus for Ca^{2+} reabsorption. As may be expected, in these tissues the expression and localization of the PMCA are under hormonal control by vitamin D3. Similarly, in the lactating mammary gland, the PMCA (isoform PMCA2w/b) is concentrated in the apical membrane of secretory epithelial cells and is essential for the export of Ca^{2+} into the (milk) lumen. Accordingly, female mice lacking PMCA2 produce Ca^{2+} deficient milk and their offspring are underweight (Reinhardt et al. 2004).

In addition to their roles in bulk vectorial Ca^{2+} transport, PMCAs are involved in the spatiotemporal control of Ca^{2+} signaling. Specific PMCA isoforms and



Plasma Membrane Calcium-Transporting ATPase, Fig. 4 *Proteins interacting with the PMCA, and their possible roles in PMCA regulation and function.* PMCA-binding proteins are schematically shown as gray ovals near the domain of the PMCA with which they interact. Known or suspected roles of these proteins in PMCA function, regulation, and signaling are indicated in boxes beneath the listed proteins. The PMCA is schematically shown on the top, with the membrane-spanning segments numbered 1–10, and the N- and C-terminal ends labeled N and C, respectively. The two major sites of alternative splicing are also indicated, and the two main splice variants "a" and "b" are shown with separate C-terminal tails to indicate their sequence divergence. Note that PDZ domain-containing proteins (PDZ) can only bind to "b-splice" variants of the PMCA, and that not all PMCA isoforms interact with all of the listed proteins. Ankyrin and actin may also interact directly with the PMCA, although the site of interaction has not yet been established

splice variants are targeted to plasma membrane subcompartments where they form multiprotein signaling complexes that control local Ca^{2+} signals ("Ca^{2+} signalosomes"). By influencing the amplitude and the time to recovery of locally evoked Ca^{2+} spikes, the PMCAs contribute to the decoding of Ca^{2+} signals and affect the frequency of Ca^{2+} oscillations. By reducing the spread of a local increase in Ca^{2+}, they help suppress signaling "noise," thereby increasing the fidelity and spatial resolution of Ca^{2+} signaling. Because of the pronounced differences in their kinetic and regulatory properties, different PMCA isoforms are adapted to handle very different Ca^{2+} signals ranging from slow, solitary Ca^{2+} waves to highly localized, high-frequency Ca^{2+} spikes such as those elicited at neuronal synapses. For example, specific PMCA2 splice variants play important roles in pre- and postsynaptic function such as in the regulation of excitatory synaptic transmission at hippocampal CA3 synapses or in short-term plasticity in cerebellar parallel fiber to Purkinje neuron connections. Deficiency of the corresponding PMCA leads to functional deficits such as impaired motor coordination (Huang et al. 2010). In general, other PMCA isoforms are unable to compensate for the deficiency in a specific isoform, supporting the notion of the highly defined functions that specific PMCAs assume in Ca^{2+} signaling.

Given its early expression during development and ubiquitous presence in all tissues, it is not surprising that PMCA1 ablation is embryonic lethal (Prasad et al. 2007). Heterozygous mice lacking one copy of the PMCA1 gene are phenotypically normal, although they do show physiological differences from the wild type such as an increased stimulated peak tension of

bladder smooth muscles. Deletion of PMCA2 leads to profound deafness, ataxia, and various other deficiencies such as a decrease of milk calcium (as already mentioned above), reduced visual responses from retinal bipolar cells, and spinal cord pathology (Prasad et al. 2007). Even single point mutations affecting the function of PMCA2 can cause significant hearing impairment, underscoring the importance of the unique concentration of PMCA2w/a in auditory hair cell function. Mutations in the PMCA2 (ATP2B2) gene are the only human PMCA mutations linked so far to inherited disease (hearing loss), although polymorphisms or mutations acting as modifiers of other inherited diseases are likely to be discovered in different PMCA genes in the near future (Brini and Carafoli 2009). In mice, knockout of the PMCA4 (Atp2b4) gene results in viable animals without gross abnormalities. However, males are infertile due to a defect in sperm hyperactivated motility, which is apparently dependent on PMCA4 normally concentrated in the sperm tail (Prasad et al. 2007). Lack or altered expression of PMCA4 also leads to changes in cardiac physiology, such as altered response to beta adrenergic stimulation and change in blood pressure, reinforcing the notion of the PMCA4 as an important signaling molecule rather than as general "sump pump" to remove global Ca^{2+} (Holton et al. 2010).

Considering the universal importance of Ca^{2+} signaling, it is no surprise that numerous diseases are characterized by altered expression of specific PMCAs. Major diseases that show distinct changes in PMCA isoform expression and activity (normally a decrease, but in some cases an overexpression) include diverse cancers, neurodegenerative disorders such as Alzheimer's and Huntington's disease, and diabetes (Lehotsky et al. 2002; Brini and Carafoli 2009). However, in most cases it is not clear whether aberrant PMCA expression is causative or reflects a secondary reaction to a different primary insult. Regardless, these findings suggest that targeting specific PMCAs may offer promising new strategies for intervention.

Summary

Plasma membrane Ca^{2+}-transporting ATPases (PMCAs) are essential components of the calcium signaling toolkit of eukaryotic cells. These membrane-embedded transporters couple the expulsion of 1 Ca^{2+} ion to the hydrolysis of 1 ATP, and attain maximal rates of about 100 Ca^{2+} transported per second. PMCAs are the major high-affinity Ca^{2+} export system dedicated exclusively to the export of Ca^{2+} from cells, and are essential for the maintenance of the steep $[Ca^{2+}]$ concentration gradient that is a prerequisite for the high specificity and fidelity of Ca^{2+} signaling. In mammals, four genes encode PMCAs 1–4; alternative RNA splicing augments the number of distinct isoforms to over 30. The PMCAs are highly regulated by multiple mechanisms including Ca^{2+}-calmodulin, acidic phospholipids, phosphorylation, oligomerization, and interactions with numerous signaling, targeting, and anchoring proteins. PMCA isoforms and splice variants show developmental, tissue- and cell type-specific expression, and are targeted to specific cell membrane compartments where they contribute to local Ca^{2+} handling. PMCA isoforms show characteristic differences in kinetic and regulatory properties, providing cells with many options to deploy specific PMCAs to distinct plasma membrane compartments with different Ca^{2+} signaling needs. Consistent with a role in local Ca^{2+} handling, deletion or mutation of specific PMCAs causes characteristic cellular defects. Deletion of the ubiquitously expressed PMCA1 results in embryonic lethality. In contrast, mice lacking PMCA4 are superficially normal but show altered cardiac stress responses and are male-infertile due to impaired sperm hyperactivated motility. Deletion or mutation of PMCA2 results in hearing loss, balance and vision defects, ataxia, spinal cord pathology, as well as reduced milk calcium, with the degree of severity depending on the level of remaining normal PMCA2. PMCAs are rapidly being recognized as major players in spatiotemporal Ca^{2+} signaling with specific involvement in diverse cell functions, and are potential targets for intervention in the treatment of various diseases linked to abnormal Ca^{2+} handling.

References

Axelsen KB, Palmgren MG. Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol. 1998;46:84–101.

Brini M, Carafoli E. Calcium pumps in health and disease. Physiol Rev. 2009;89:1341–78.

Hill JK, Williams DE, LeMasurier M, Dumont RA, Strehler EE, Gillespie PG. Splice-site A choice targets plasma-membrane Ca^{2+}-ATPase isoform 2 to hair bundles. J Neurosci. 2006;26:6172–80.

Holton ML, Wang W, Emerson M, Neyses L, Armesilla AL. Plasma membrane calcium ATPase proteins as novel regulators of signal transduction pathways. World J Biol Chem. 2010;1:201–8.
Huang H, Nagaraja RY, Garside ML, Akemann W, Knöpfel T, Empson RM. Contribution of plasma membrane Ca^{2+} ATPase to cerebellar synapse function. World J Biol Chem. 2010;1:95–102.
Lehotsky J, Kaplán P, Murín R, Raeymaekers L. The role of plasma membrane Ca^{2+} pumps (PMCAs) in pathologies of mammalian cells. Front Biosci. 2002;7:d53–84.
Niggli V, Penniston JT, Carafoli E. Purification of the (Ca^{2+} + Mg^{2+})-ATPase from human erythrocyte membranes using a calmodulin affinity column. J Biol Chem. 1979;254:9955–8.
Pedersen PL, Carafoli E. Ion motive ATPases. I. Ubiquity, properties, and significance for cell function. Trends Biochem Sci. 1987;12:146–50.
Prasad V, Okunade G, Liu L, Paul RJ, Shull GE. Distinct phenotypes among plasma membrane Ca^{2+}-ATPase knockout mice. Ann NY Acad Sci. 2007;1099:276–86.
Reinhardt TA, Lippolis JD, Shull GE, Horst RL. Null mutation in the gene encoding plasma membrane Ca^{2+}-ATPase isoform 2 impairs calcium transport into milk. J Biol Chem. 2004;279:42369–73.
Schatzmann HJ. ATP-dependent Ca^{++} extrusion from human red cells. Experientia. 1966;22:364–8.
Strehler EE, Zacharias DA. Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. Physiol Rev. 2001;81:21–50.
Strehler EE, Caride AJ, Filoteo AG, Xiong Y, Penniston JT, Enyedi A. Plasma membrane Ca^{2+} ATPases as dynamic regulators of cellular calcium handling. Ann NY Acad Sci. 2007;1099:226–36.
Thever MD, Saier MH. Bioinformatic characterization of P-type ATPases encoded within the fully sequenced genomes of 26 eukaryotes. J Membr Biol. 2009;229:115–30.
Toyoshima C. How Ca^{2+}-ATPase pumps ions across the sarcoplasmic reticulum membrane. Biochim Biophys Acta. 2009;1793:941–6.

Platelet Glycoprotein Ia

▶ Integrin α2 (ITGA2)

PLAUR

▶ Structure and Functions of the Urokinase Receptor

PLD1

▶ Phospholipase D

PLD2

▶ Phospholipase D

Plekhg5

▶ SYX/PLEKHG5, A Rhoa Guanine Exchange Factor Involved in Cell Migration and Angiogenesis

PLEKHO1 (Pleckstrin-Homology Domain Containing, Family O Member 1)

▶ CKIP-1

PMCA

▶ Plasma Membrane Calcium-Transporting ATPase

Polycystin-2

Daryl L. Goad, Michael A. Grillo and Peter Koulen
Department of Ophthalmology and Department of Basic Medical Science, University of Missouri - Kansas City School of Medicine, Vision Research Center, Kansas City, MO, USA

Synonyms

APKD2; PC2; PKD2; PKD4; TRPP1

Historical Background

The *pkd2* gene encodes the protein polycystin-2, a member of the ▶ transient receptor potential (TRP) protein family. The genetic locus for *pkd2* was elucidated in 1993 by the Kumar and Spruit groups during the search for genes involved in autosomal dominant polycystic kidney disease (ADPKD) (Kimberling et al. 1993;

Peters et al. 1993). Mutations in *pkd2* account for approximately 15% of the ADPKD patients, with the remainder of ADPKD patients having mutations in *pkd1* or *pkhd1* (Koulen and McClung 2006).

Functionally, polycystin-2 has been implicated in Ca^{2+} release from intracellular stores (Koulen et al. 2002, 2005; Kaja et al. 2011). In conjunction with polycystin-1, polycystin-2 forms a receptor-calcium channel complex that is formed in the cilium of renal epithelial cells (Xia et al. 2010). In *C. elegans* both polycystin-1 and -2 are expressed in the primary cilia of sensory neurons (Barr and Sternberg 1999). These receptor-channel complexes respond to inputs such as mechano-sensation, receptor tyrosine kinases, and G-coupled-protein receptor complexes; and function to regulate diverse cellular processes such as differentiation and morphogenesis (Koulen and McClung 2006; Spirli et al. 2010; Zhou 2009; Montalbetti et al. 2007). Polycystin-2 localizes either to the endoplasmic reticulum (ER) or to the plasma membrane of primary cilia. This localization is dependent on cell type and the subcellular compartment in which polycystin-2 is expressed (Koulen and McClung 2006). However, expression of polycystin-2 has also been detected in the cytoplasm, and the apical and basolateral membranes in the kidney (Zhou 2009). Although mutations in polycystins 1/2 are most commonly associated with ADPKD, recent papers have shown functional genes are required for bone development, cardiac development, and left–right axis patterning (Tsiokas 2009; Xiao and Quarles 2010). Polycystin-2 functions in conjunction with other intracellular Ca^{2+} release channels as part of a positive feedback system during calcium-mediated signaling events (Kaja et al. 2011; Koulen et al. 2002, 2005; Koulen and McClung 2006). In adult mammals polycystin-2 is expressed in kidney, liver, spleen, lung, pancreas, ovary, testicular germ cells, Sertoli cells, corneal epithelium, retinal pigment epithelium, uterus, salivary glands, brain, adrenal cortex, epithelial cells of the GI tract, muscles (cardiac, vascular smooth, and skeletal), and bone (Koulen and McClung 2006; Xiao and Quarles 2010). Polycystins 1/2 function in cilia to sense mechanical shear stress, with bending of the cilia causing increased Ca^{2+} entry into the cell. Additionally, it has been shown that the actin cytoskeleton directly modulates polycystin-2 activity (Tsiokas 2009). Sharif-Naeini et al. showed the dosage dependence of polycystins 1/2 in cilia-mediated pressure sensing, further implicating the polycystin complex as a sensor of pressure and mechanical stress (Sharif-Naeini et al. 2009). In polycystin-2 knockout mice, endothelial cells could not sense fluid sheer stress, and were deficient in the production of nitrous oxide in response to increased blood flow (AbouAlaiwi et al. 2009).

Structure

Polycystin-2 is a membrane spanning protein with six transmembrane domains with both the N and C termini present in the cytoplasm (Tsiokas 2009). The polycystin-2 protein forms a voltage-dependent, calcium-dependent, large conductance calcium permeable nonselective cation channel (Koulen et al. 2002, 2005; Koulen and McClung 2006; Kaja et al. 2011). Polycystin-2 must interact with polycystin-1 to form a functional Ca2+ pore complex. The *Homo sapiens* pkd2 gene consists of 15 exons, that encode the polycystin-2 protein containing 968 amino acid residues (Miyagi et al. 2009). Residues 828–895 of the *H. sapiens* polycystin-2 protein form a coiled coil domain located at the C-terminus, and are required for this protein–protein interaction (Tsiokas 2009; Petri et al. 2010). Additionally the C-terminal tail of polycystin-2 contains an EF-hand domain (residues 720–797), with a linker (residues 798–827) between the coiled coil and EF-hand domains, and the EF-hand domain responsible for the calcium dependence of the pore complex (Koulen and McClung 2006; Petri et al. 2010). The N-terminal tail of polycystin-2 contains an RVxP motif that is responsible for localization of the protein to cilia (Geng et al. 2006).

Interactions with Ligands and Other Proteins

For review of ligand and protein interactions described before 2006, see Koulen and McClung, UCSD Nature Molecule Pages (2006). Polycystin-2 forms complexes with members of other transient receptor potential (TRP) channel families forming heteromultimeric channels with unique properties. The polycystin-2/TRPC1 is a G-protein-coupled activated channel that displays a conductance, ion permeability, and amiloride sensitivity different from channel properties of either protein alone in single channel experiments. The interaction of polycystin-2/TRPV4 in cilium

forms a thermo/mechano-sensitive sensor that effects flow induced Ca^{2+} transients in renal epithelium cells. α-actinin and polycystin-2 colocalize in Madin-Darby canine kidney (MDCK) epithelium cells, medullary collecting duct cells, NIH 3T3 fibroblasts, and human syncytiotrophoblast (hST) vesicles. In vitro electrophysiological recordings show that this interaction stimulates polycystin-2 channel activity (Zhou 2009). Polycystin-2 interacts with the inositol 1,4,5-trisphosphate receptor (IP_3R) through the acidic C-terminal domain of polycystin-2 and the N-terminal ligand domain of the ▶ IP_3R. The consequence of this interaction is increased Ca^{2+} release via IP_3 and other Ca^{2+} agonists (Sammels et al. 2010). Filamin A and polycystin-2 interaction is necessary for polycystin-2's inhibition of stretch activated channels (SACs) which have been implicated in mechano-sensation in kidney epithelium (Sharif-Naeini et al. 2009). Polycystin-2 acts to regulate the cell cycle by interactions between eIF2α, pancreatic ER-resident eIF2α kinase (PERK), and itself. Formation of the complex is necessary for PERK-mediated phosphorylation of eIF2α. Polycystin-2 also interacts with Id2, a member of the helix–loop–helix (HLH) protein family, whose expression suppresses p21 (Zhou 2009; Li et al. 2005). Trafficking of polycystin-2 from Golgi, ER, and the plasma membrane is achieved through phosphorylation of polycystin-2 by casein kinase 2, which regulates the interaction of phosphofurin acidic cluster sorting protein (PACS)-1 and PACS-2 with phosphorylated polycystin-2 protein. Phosphorylation of the N-terminus of polycystin-2 by glycogen synthase kinase 3 (GSK3) has been found to regulate the amount of polycystin-2 resident on the plasma membrane of MDCK cells. Finally degradation of polycystin-2 is regulated through its interaction with ATPase p97, HERP, and glucosidase subunit beta PRKCSH (Gao et al. 2010).

Function

Polycystin-2 is a member of the ▶ TRP channel family and along with polycystin-1(PC1) is linked to cyst formation in polycystic kidney disease. Polycystin-2 is located on the plasma membrane, endoplasmic reticulum membrane, and in cilia (Tsiokas et al. 2007). When located on the plasma membrane, polycystin-2 functions as a nonselective cation channel. Localization of polycystin-2 to the plasma membrane is tightly regulated by ER retention signaling and several chaperone proteins, including PC1. Epidermal growth factor has been shown to activate polycystin-2 in kidney epithelial cell lines, with increases in cytosolic calcium from extracellular, not intracellular, calcium sources (Zhou 2009; Tsiokas 2009). When localized to the ER, polycystin-2 acts as a calcium-induced calcium release channel (Koulen et al. 2002, 2005; Kaja et al. 2011). In addition, it modifies the function of other the ER calcium release channels, ▶ ryanodine receptor (RyR), and inositol trisphosphate receptor (IP_3R) (Zhou 2009; Tsiokas et al. 2007). The C-terminus of polycystin-2 associates with RyR2 and inhibits calcium release in cardiomyocytes and mouse renal epithelial cells. The C-terminus of polycystin-2 associates with the IP_3Rs and enhances the duration of calcium signaling (Sammels et al. 2010; Anyatonwu et al. 2007). Association with several actin and microtubule-associated proteins within the cell changes functional release by polycystin-2 channels, suggesting a role in cytoskeletal remodeling in response to both mechanical and osmotic pressure changes (Zhou 2009; Montalbetti et al. 2007). Cell cycle regulation is also implicated as another possible function of polycystin-2. Association with the Id family directly controls cell cycle regulation, and expression on mitotic spindles has been shown to affect calcium signaling during mitosis. Whether this effect is due directly to polycystin-2 or its interaction with mammalian Diaphanous 1 (mDia1) is not known (Zhou 2009; Tsiokas et al. 2007). In the cilium, association with TRPV4 allows cations to enter the cell in response to mechanical stimulation; and removal of either subunit abolishes this response (Zhou 2009; Tsiokas et al. 2007).

Summary

Mutations of pkd2 are responsible for 15% of APKD cases. However, due to the localization of polycystin complexes to cilia, and the broad expression of these proteins in various tissues, we should not overlook the role that mutations within these complexes may play in other disease processes such as Alzheimer's disease, glaucoma, and hypertension. The polycystin proteins act to integrate multiple inputs such as mechano-stress, G-protein coupled receptors, perturbation of the

cytoskeleton, and kinase cascades into calcium-mediated signaling events. Activation of the polycystin protein complex leads to a positive calcium feedback loop that affects the function and characteristics of other intracellular Ca^{2+} channels. Future research should be directed toward an examination of the regulatory elements that control induction and expression of the pkd2 gene as well as the regulatory posttranslational modifications that control the function, localization, and half-life of the polycystin protein. Researchers should also continue to identify ligands, cofactors, scaffolds, and other regulatory mechanisms in order to elucidate potential pharmacological targets for controlling diseases caused by mutated or mis-regulated polycystin-2 proteins.

References

AbouAlaiwi WA, Takahashi M, Mell BR, Jones TJ, Ratnam S, Kolb RJ, et al. Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades. Circ Res. 2009;104(7):860–869.

Anyatonwu GI, Estrada M, Tian X, Somlo S, Ehrlich BE. Regulation of ryanodine receptor-dependent calcium signaling by polycystin-2. Proc Natl Acad Sci USA. 2007;104(15):6454–6459.

Barr MM, Sternberg PW. A polycystic kidney-disease gene homologue required for male mating behaviour in C. elegans. Nature. 1999;401(6751):386–389.

Gao H, Wang Y, Wegierski T, Skouloudaki K, Putz M, Fu X, et al. PRKCSH/80 K-H, the protein mutated in polycystic liver disease, protects polycystin-2/TRPP2 against HERP-mediated degradation. Hum Mol Genet. 2010;19(1):16–24.

Geng L, Okuhara D, Yu Z, Tian X, Cai Y, Shibazaki S, et al. Polycystin-2 traffics to cilia independently of polycystin-1 by using an N-terminal RVxP motif. J Cell Sci. 2006;119(Pt 7):1383–1395.

Kimberling WJ, Kumar S, Gabow PA, Kenyon JB, Connolly CJ, Somlo S. Autosomal dominant polycystic kidney disease: localization of the second gene to chromosome 4q13-q23. Genomics. 1993;18(3):467–472.

Kaja S, Hilgenberg JD, Rybalchenko V, Medina-Ortiz WE, Gregg EV, Koulen P. Polycystin-2 expression and function in adult mouse lacrimal acinar cells. Invest Ophthalmol Vis Sci. 2011;52(8):5605–5611.

Koulen P, Cai Y, Geng L, Maeda Y, Nishimura S, Witzgall R, Ehrlich BE, Somlo S. Polycystin-2 is an intracellular calcium release channel. Nat Cell Biol. 2002;4(3):191–197.

Koulen P, Duncan RS, Liu J, Cohen NE, Yannazzo JA, McClung N, Lockhart CL, Branden M, Buechner M. Polycystin-2 accelerates Ca2+ release from intracellular stores in Caenorhabditis elegans. Cell Calcium. 2005;37(6):593–601 Epub 2005 Apr 14.

Koulen P, McClung NM. Polycystin 2. [Web Page]: Nature Molecule Pages; 2006 [cited 2006 May].

Li X, Luo Y, Starremans PG, McNamara CA, Pei Y, Zhou J. Polycystin-1 and polycystin-2 regulate the cell cycle through the helix-loop-helix inhibitor Id2. Nat Cell Biol. 2005;7(12):1202–1212.

Miyagi K, Kiyonaka S, Yamada K, Miki T, Mori E, Kato K, et al. A pathogenic C terminus-truncated polycystin-2 mutant enhances receptor-activated Ca^{2+} entry via association with TRPC3 and TRPC7. J Biol Chem. 2009;284(49):34400–34412.

Montalbetti N, Li Q, Wu Y, Chen XZ, Cantiello HF. Polycystin-2 cation channel function in the human syncytiotrophoblast is regulated by microtubular structures. J Physiol. 2007;579(Pt 3):717–728.

Peters DJM, Spruit L, Saris JJ, Ravine D, Sandkuijl LA, Fossdal R, et al. Chromosome 4 localization of a second gene for autosomal dominant polycystic kidney disease. Nat Genet. 1993;5(4):359–362.

Petri ET, Celic A, Kennedy SD, Ehrlich BE, Boggon TJ, Hodsdon ME. Structure of the EF-hand domain of polycystin-2 suggests a mechanism for Ca^{2+}−dependent regulation of polycystin-2 channel activity. Proc Natl Acad Sci USA. 2010;107(20):9176–9181.

Sammels E, Devogelaere B, Mekahli D, Bultynck G, Missiaen L, Parys JB, et al. Polycystin-2 activation by inositol 1,4,5-trisphosphate-induced Ca^{2+} release requires its direct association with the inositol 1,4,5-trisphosphate receptor in a signaling microdomain. J Biol Chem. 2010;285(24):18794–18805.

Sharif-Naeini R, Folgering JH, Bichet D, Duprat F, Lauritzen I, Arhatte M, et al. Polycystin-1 and -2 dosage regulates pressure sensing. Cell. 2009;139(3):587–596.

Spirli C, Okolicsanyi S, Fiorotto R, Fabris L, Cadamuro M, Lecchi S, et al. Mammalian target of rapamycin regulates vascular endothelial growth factor-dependent liver cyst growth in polycystin-2-defective mice. Hepatology. 2010;51(5):1778–1788.

Tsiokas L. Function and regulation of TRPP2 at the plasma membrane. Am J Physiol Ren Physiol. 2009;297(1):F1–F9.

Tsiokas L, Kim S, Ong EC. Cell biology of polycystin-2. Cell Signal. 2007;19(3):444–453.

Xia S, Li X, Johnson T, Seidel C, Wallace DP, Li R. Polycystin-dependent fluid flow sensing targets histone deacetylase 5 to prevent the development of renal cysts. Development. 2010;137(7):1075–1084.

Xiao ZS, Quarles LD. Role of the polycytin-primary cilia complex in bone development and mechanosensing. Ann NY Acad Sci. 2010;1192(1):410–421.

Zhou J. Polycystins and primary cilia: primers for cell cycle progression. Annu Rev Physiol. 2009;71:83–113.

Polycystines, TRPPs

► TRP (Transient Receptor Potential Cation Channel)

PP2C

Hisashi Tatebe[1] and Kazuhiro Shiozaki[1,2]
[1]Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan
[2]Department of Microbiology, University of California, Davis, Davis, CA, USA

Synonyms

Metal-dependent protein phosphatase; PPM; Protein serine/threonine-phosphatase 2C

Historical Background

Protein phosphatase 2C (PP2C) was first defined as magnesium (or manganese)-dependent Ser/Thr-specific dephosphorylation activity in mammalian tissue extract (Cohen 1989). This activity was also found to be resistant to okadaic acid, a potent inhibitor of Ser/Thr-phosphatases. Because of its cation dependency, PP2C is sometimes referred as PPM (protein phosphatase, magnesium or manganese dependent). Genes encoding PP2C were subsequently isolated from yeast to humans, revealing a conserved protein phosphatase family with no apparent sequence similarity to the other Ser/Thr-phosphatases such as PP1, PP2A, and PP2B. It is also notable that eukaryotic species have more genes encoding for PP2C than those for the other Ser/Thr-phosphatase families. For example, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, have seven and six PP2C or PP2C-related phosphatase genes, respectively. The human genome contains at least 16 PP2C genes that express at least 22 isoforms by alternative splicing (Lammers and Lavi 2007). Genome projects in different organisms are rapidly identifying more PP2C genes, ten gens in the fruit fly *Drosophila melanogaster* and eight genes in the nematode *Caenorhabditis elegans*. A record-breaking number, 80, of PP2C-family genes have been identified in the popular plant model system *Arabidopsis thaliana* (Xue et al. 2008).

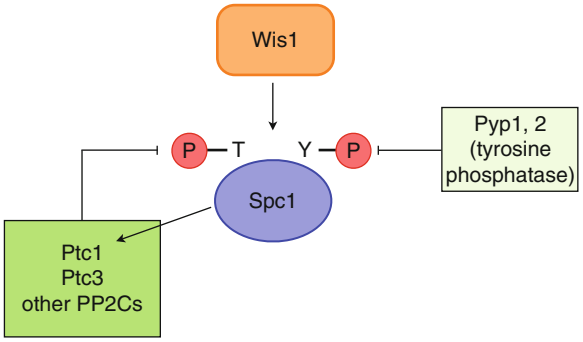
Despite the lack of apparent sequence similarity between PP2C and the PP1, PP2A, and PP2B families, the determined crystal structures of their catalytic cores share significant resemblance (Barford et al. 1998), suggesting a common catalytic mechanism. On the other hand, most PP2C enzymes are found to be monomeric, while PP1, PP2A, and PP2B usually require regulatory subunits for their activity/function. PP2C and PP2C-like enzymes are involved in many different biological processes in diverse organisms; in addition to eukaryotic PP2Cs, several metal ion-dependent phosphatases in bacteria also exhibit sequence similarities to PP2C enzymes (Hecker et al. 2007; Hilbert and Piggot 2004). Some of the well-characterized PP2C functions in eukaryotic signal transduction systems will be reviewed here.

PP2C Negatively Regulating the Stress-Activated MAPK

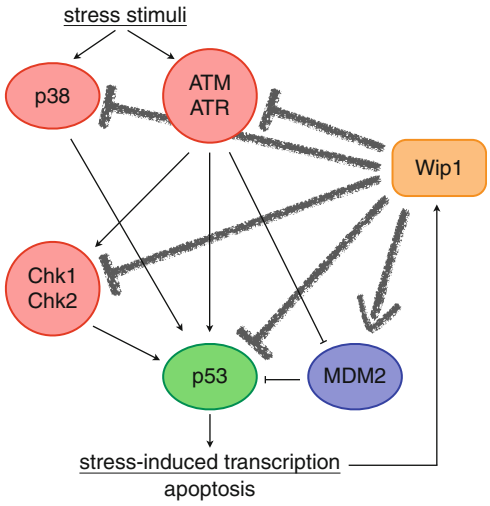
Because of the relatively broad substrate specificity of PP2C enzymes, it is not easy to search cellular substrates of PP2C through biochemical approaches. Genetic screens were instrumental to uncover important roles of PP2C in the negative regulation of the stress-activated MAP kinase (MAPK) cascades.

Two PP2C isoforms in budding yeast, Ptc1 and Ptc3, were identified in a screen for genes whose overexpression suppresses the lethality caused by hyperactivation of Hog1, a MAPK responsive to high osmolarity stress (Hohmann 2002). Studies in the fission yeast *S. pombe* found that loss of Ptc1 and Ptc3 brings about a defective phenotype which can be suppressed by inactivation of a Hog1-like Spc1 MAPK, implying hyperactivation of Spc1 in the absence of PP2C (Hohmann 2002). Indeed, the following biochemical studies in *S. pombe* showed that Ptc1 and Ptc3 inhibit Spc1 MAPK by dephosphorylating its Thr-171, one of the activating phosphorylation sites conserved in the T-loop of all MAPKs (Fig. 1). Furthermore, activation of Spc1 MAPK induces expression of Ptc1, indicating that Ptc1 is part of the negative feedback loop to suppress Spc1 activity. In budding yeast, Thr-174 of Hog1 MAPK, the equivalent of Spc1 Thr-171, is known to be the target of three PP2C enzymes, Ptc1, Ptc2, and Ptc3; Ptc1 dephosphorylates Thr-174 to maintain the low basal activity of Hog1 as well as to inactivate Hog1 during cellular adaptation to osmostress. Ptc2 and Ptc3 appear to limit the maximum activation level of Hog1 during the stress (Martín et al. 2005).

Also in mammals, PP2C enzymes are implicated in the negative regulation of the stress-activated MAPKs called p38 and JNK, through dephosphorylation of



PP2C, Fig. 1 Negative regulation of the stress MAPK by PP2Cs in fission yeast. In response to environmental stress, Wis1 MAP kinase kinase (MAPKK) phosphorylates Thr-171 and Tyr-173 in the T-loop of Spc1 MAPK. Activated Spc1 in turn phosphorylates the downstream transcription factor to induce stress-resistance genes as well as the PP2C gene encoding Ptc1. Dephosphorylation of Spc1 Thr-171 by Ptc1 or by constitutively expressed Ptc3 inactivates Spc1 MAPK. The other activating phosphorylation of Spc1 at Tyr-171 is removed by the Pyp1 and Pyp2 tyrosine-specific phosphatases



PP2C, Fig. 2 Negative regulation of the p53 pathway by Wip1 PP2C. Wip1 negatively regulates the p53 tumor suppressor pathway by dephosphorylating p53 and the protein kinases (*red*) that phosphorylate p53 in response to DNA-damaging stress. In addition, Wip1-dependent dephosphorylation of MDM2 promotes degradation of p53. Expression of Wip1 is induced by active p53, thus forming a negative feedback loop

their T-loop (Lu and Wang 2008). For example, Thr-180 in the T-loop of p38 is dephosphorylated by PP2Cα and PP2Cδ/Wip1 isoforms. Interestingly, p38 MAPK activated by UV stress phosphorylates the tumor suppressor ▶ p53, which induces transcription of Wip1. Thus, as seen with the regulation of the Ptc1 phosphatase in fission yeast, the p38-regulated transcription of Wip1 forms a negative feedback loop (Fig. 2). Moreover, not only MAPKs, but also MAPKKs (MKK3, 4, 6, 7) and MAPKKKs (ASK1, TAK1) that function upstream of p38 and JNK MAPKs are under the negative regulation by PP2Cα, β, and ε isoforms.

In *Arabidopsis*, two PP2C enzymes, AP2C1 and PP2C5, have been identified as negative regulators of the stress-activated MAPKs (Brock et al. 2010; Schweighofer et al. 2007). Environmental stress stimuli activate multiple *Arabidopsis* MAPKs such as MPK3, MPK4, and MPK6, to which AP2C1 and PP2C5 physically bind. Deletion of the *AP2C1* gene in the genome leads to higher activation of MPK4 and MPK6 upon wound stress. Simultaneous deletion of both *AP2C1* and *PP2C5* phosphatase genes induces extremely high activity of MPK3, 4, and 6 in response to the plant hormone abscisic acid (ABA). On the other hand, overexpression of either AP2C1 or PP2C5 represses the stress-activated MAPKs. Expression of AP2C and PP2C5 is significantly induced by wounding and ABA, respectively, implying a negative feedback mechanism. It has not been determined, however, whether the induction is dependent on the stress-activated MAPKs.

Wip1 (PP2Cδ) Negatively Regulating the p53 Tumor Suppressor

Amplification of the PP2Cδ/Wip1 gene is frequently detected in human breast tumors. In addition, PP2Cδ/Wip1 knockout mice are less prone to tumor formation, and fibroblasts derived from the mice are resistant to the transformation activity of oncogenes. Consistent with these observations, recent studies are unveiling the oncogenic activity of the PP2Cδ/Wip1 phosphatase, through inhibition of the p53 tumor suppressor by multiple means (Lu et al. 2008) (Fig. 2). As discussed in the last section, Wip1 dephosphorylates and inactivates p38 MAPK, an activator of p53. p53 is also a direct substrate of the Wip1 phosphatase; ionizing radiation and UV stress activate the ATM and ATR kinases that phosphorylate Ser-15 of p53 to induce apoptosis, while dephosphorylation of this residue by Wip1 suppresses apoptosis. The Ser-15 phosphorylation is also inhibitory to the interaction of p53 with MDM2, the E3 ubiquitin ligase involved in p53 degradation, and therefore, dephosphorylation of Ser-15 by Wip1 can destabilize p53. Moreover, Wip1 indirectly affects the stability and activity of p53

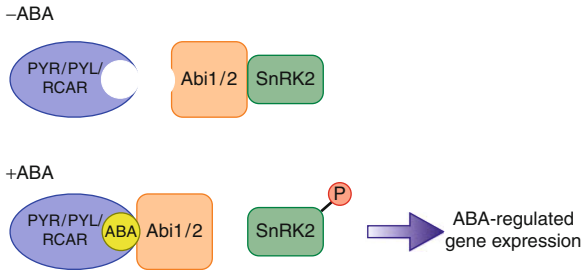
PP2C, Fig. 3 A schematic representation of the PHLPP domain structure

through dephosphorylation of MDM2 as well as inhibition of the ATM kinase that phosphorylates MDM2 (Fig. 2). The ATM-dependent phosphorylation of MDM2 at Ser-395 is removed by Wip1 to stabilize MDM2 and promote its interaction with p53 for increased ubiquitination and degradation of p53.

In the regulation of the p53 pathway discussed above, the Wip1 phosphatase counteracts the ATM and ATR kinases on two different substrates, p53 and MDM2 (Fig. 2). It has also been reported that Wip1 dephosphorylates the protein kinases Chk1 and Chk2 that are phosphorylated by ATR (Fig. 2). Consistent with these observations, Wip1 preferentially dephosphorylates Ser and Thr residues followed by Gln, the consensus sequence motif phosphorylated by the ATM and ATR kinases.

PHLPP, Atypical PP2C with Multiple Regulatory Domains

PHLPP (pleckstrin homology (PH) domain *l*eucine-rich repeat *p*rotein *p*hosphatase) was discovered by genome database search for human PP2C carrying the PH domain (Brognard and Newton 2008). While other PP2C proteins in humans are composed mostly of a catalytic domain, the PHLPP family members possess multiple regulatory domains, an N-terminal PH domain followed by approximately 20 leucine-rich repeats as well as a C-terminal PDZ-binding motif (Fig. 3). PHLPP exhibits PP2C-like protein phosphatase activity, but its catalytic domain lacks the two Asp residues crucial for placing the essential metal ions in the catalytic center of PP2C enzymes. PHLPP dephosphorylates the hydrophobic motif of the protein kinases Akt/PKB and PKC for catalytic inactivation and protein degradation, respectively. Dephosphorylation of Akt by PHLPP promotes apoptosis and suppresses tumor growth. PHLPP also removes the inhibitory phosphorylation of another kinase called Mst1 (*m*ammalian *st*e20-like kinase 1) to induce apoptosis (Qiao et al. 2010). A member of the PHLPP family, called PHLPP1β/SCOP, interacts with K-Ras through the leucine-rich repeats and down-regulates the Ras-Raf-► MEK-ERK pathway (Shimizu et al. 2010). However, it remains to be determined whether the phosphatase activity of PHLPP1β is required for the down-regulation.



PP2C, Fig. 4 Abscisic acid (ABA) signaling through the Abi1/2 PP2C. In the absence of ABA (-ABA), the Abi1/2 PP2C represses SnRK2 by removing the activating phosphorylation on SnRK2. In the presence of ABA (+ABA), the PYR/PYL/RCAR protein bound to ABA forms a complex with the Abi1/2 PP2C to repress its phosphatase activity, resulting in phosphorylation and activation of SnRK2. Active SnRK2 then induces gene expression through activation of the downstream transcription factors

Abi1/2 PP2Cs in Abscisic Acid Signaling in Plants

The *Arabidopsis thaliana* genome contains 80 PP2C genes, which can be classified into 13 subgroups based on the encoded amino acid sequences (Xue et al. 2008). The *Abi1/2* subgroup that functions in the abscisic acid (ABA) signaling (Cutler et al. 2010; Hubbard et al. 2010) has been most extensively characterized. ABA is a plant hormone that has multiple roles in the regulation of plant physiology, including seed dormancy, growth inhibition, and stomatal closure. PP2Cs in the Abi1/2 subgroup negatively regulate the ABA signaling, and mutations to the genes encoding Abi1, Abi2, and some other subgroup members result in ABA-hypersensitive phenotypes.

Recent attempts to discover the ABA hormone receptor successfully identified PYR/PYL/RCAR (*py*rabactin *r*esistance 1/*py*rabactin resistance 1-*l*ike/ *r*egulatory *c*omponent of *A*BA *r*eceptor) family proteins that directly recognize and bind ABA. The PYR/PYL/RCAR proteins belong to the START-domain superfamily, members of which are soluble proteins with a hydrophobic ligand-binding pocket flanked by two loop structures called the "gate" and the "latch." ABA binding to the open pocket closes the gate and locks the latch, creating a new interaction surface for

the Abi1/2 family PP2C (Fig. 4). When the PP2C binds to the ABA-PYR/PYL/RCAR complex, a Trp residue conserved among the Abi1/2 family PP2Cs is pulled to form a contact with ABA via a water molecule, stabilizing the ternary complex of ABA, the ABA receptor, and the Abi1/2 PP2C. The phosphatase activity of Abi1/2 is inhibited in this ternary complex. In the absence of ABA, however, the Abi1/2 PP2C released from the complex can dephosphorylate and inactivate the SNF1-related protein kinases SnRK2s (Fig. 4). Activated SnRK2s in turn phosphorylate and activate downstream transcription factors to induce ABA-regulated genes.

Summary

In eukaryotic species, protein kinases outnumber protein phosphatases. Therefore, PP2C and other protein phosphatases are likely to catalyze dephosphorylation of multiple protein substrates, but our knowledge is still very limited as to the in vivo substrates for each PP2C enzyme. Identification of a complete set of substrates for each PP2C is necessary for a comprehensive understanding of the contribution of the PP2C family enzymes to the cellular signaling network.

Most PP2C enzymes appear to be constitutively active and function as monomer. This is a stark difference from other protein phosphatase families such as PP1 and PP2A, which form multiple different complexes with non-catalytic subunits that determine their substrate specificities (Shi 2009). The PYR/PYL/RCAR family in plants is the first example of regulator proteins for PP2C and may represent a breakthrough in the PP2C field. It will be of great interest to test if other members of the START superfamily also function as regulators of PP2Cs.

References

Barford D, Das AK, Egloff MP. The structure and mechanism of protein phosphatases: insights into catalysis and regulation. Annu Rev Biophys Biomol Struct. 1998;27:133–64.

Brock AK, Willmann R, Kolb D, Grefen L, Lajunen HM, Bethke G, et al. The Arabidopsis mitogen-activated protein kinase phosphatase PP2C5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. Plant Physiol. 2010;153(3):1098–111.

Brognard J, Newton AC. Phlipping the switch on akt and protein kinase C signaling. Trends Endocrinol Metab. 2008;19(6):223–30.

Cohen P. The structure and regulation of protein phosphatases. Annu Rev Biochem. 1989;58:453–508.

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol. 2010;61:651–79.

Hecker M, Pané-Farré J, Völker U. Sigb-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. Annu Rev Microbiol. 2007;61:215–36.

Hilbert DW, Piggot PJ. Compartmentalization of gene expression during *Bacillus subtilis* spore formation. Microbiol Mol Biol Rev. 2004;68(2):234–62.

Hohmann S. Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev. 2002;66(2):300.

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI. Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. Genes Dev. 2010;24(16):1695–708.

Lammers T, Lavi S. Role of type 2C protein phosphatases in growth regulation and in cellular stress signaling. Crit Rev Biochem Mol Biol. 2007;42(6):437–61.

Lu G, Wang Y. Functional diversity of mammalian type 2C protein phosphatase isoforms: new tales from an old family. Clin Exp Pharmacol Physiol. 2008;35(2):107–12.

Lu X, Nguyen TA, Moon SH, Darlington Y, Sommer M, Donehower LA. The type 2C phosphatase wip1: an oncogenic regulator of tumor suppressor and DNA damage response pathways. Cancer Metastasis Rev. 2008;27(2):123–35.

Martín H, Flández M, Nombela C, Molina M. Protein phosphatases in MAPK signalling: we keep learning from yeast. Mol Microbiol. 2005;58(1):6–16.

Qiao M, Wang Y, Xu X, Lu J, Dong Y, Tao W, et al. Mst1 is an interacting protein that mediates phlpps' induced apoptosis. Mol Cell. 2010;38(4):512–23.

Schweighofer A, Kazanaviciute V, Scheikl E, Teige M, Doczi R, Hirt H, et al. The pp2c-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in Arabidopsis. Plant Cell. 2007;19(7):2213–24.

Shi Y. Serine/threonine phosphatases: mechanism through structure. Cell. 2009;139(3):468–84.

Shimizu K, Mackenzie SM, Storm DR. SCOP/PHLPP and its functional role in the brain. Mol Biosyst. 2010;6(1):38–43.

Xue T, Wang D, Zhang S, Ehlting J, Ni F, Jakab S, et al. Genome-wide and expression analysis of protein phosphatase 2C in rice and Arabidopsis. BMC Genomics. 2008;9:550.

PPM

► PP2C

PPMT

► Icmt (Isoprenylcysteine Carboxyl Methyltransferase)

Ppp1r1b

▶ DARPP

PRAK

▶ Map Kinase-Activated Protein Kinase 5 (MK5)
▶ p38 MAPK Family of Signal Transduction Proteins

PRAP (Pyk2/RAFTK-Associated Protein)

▶ SKAP-HOM

Precerebellin

▶ Cbln1

Prep

Giorgio Iotti and Francesco Blasi
IFOM (Fondazione Istituto FIRC di Oncologia Molecolare), Milan, Italy

Synonyms

PKNOX

Historical Background

PREP1 (named PKNOX1, Pbx/KNOX 1) was identified by Chen et al. (1997) in their effort to characterize genes mapping in the human chromosome 21. At the same time, PREP1 was identified as one of the components of the human transcription factor complex UEF3, urokinase enhancer factor 3. Prep1 was found to be a homeodomain-containing DNA-binding protein belonging to the TALE (Three Aminoacid Loop Extension) superclass, most closely related to TGIF (TGF β–induced factor homeobox) and Meis1 (Meis homeobox 1) and recognizing the TGACAG motif. Furthermore, it has been demonstrated that Prep1 heterodimerizes with Pbx, enhancing its affinity and DNA-binding specificity to include both the TGACAG and the TGATNNAT motifs (hence the acronym, for Pbx regulating protein 1) (Berthelsen et al. 1998).

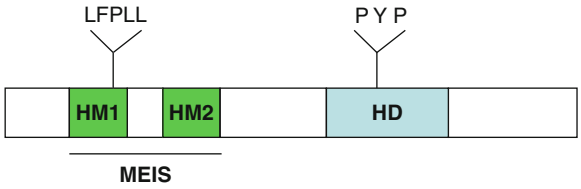
PREP2 was identified by Imoto et al. (2001) as a TALE homeodomain-encoding gene, located at 11q24 in the human genome.

Gene and Protein Structure

The human PREP1 gene maps on chromosome 21 (21q22.3) and covers 102.25 kb, from 43,225,370 to 43,327,616, on the direct strand. The cDNA coding for Prep1 contains a 126 -bp-long 5′-UTR (untranslated region), a 1308 -bp-long ORF (open reading frame), and a 3′-UTR region of more than 1,000 bp. The coding region, when using the canonical ATG translation start, encodes for a protein of 436 amino acids, for a total of 64 kDa (Berthelsen et al. 1998). Murine PREP1 maps on chromosome 17 and covers 42.94 kb, from 31,701,703 to 31,744,640, on the direct strand. The mRNA coding for the murine Prep1 is 4,093 nucleotides long. The protein has the same length and the same characteristics as human Prep1 with only 21 aminoacidic substitutions, 10 of which are conservatives (Ferretti et al. 1999).

Human PREP2 gene maps on chromosome 11 (11q24) and covers 268.73 kb, from 124,539,770 to 124,808,495, on the direct strand. A 4 kb transcript was observed in most tissues, while a longer (7–8 kb long) mRNA is testis-specific. The full length protein is 461 amino acids long (Fognani et al. 2002). Murine PREP2 maps on chromosome 9. Three transcripts (3.8 kb, 1.6 kb, and 0.8 kb long) were identified; the relative accumulation of the longer ones differs among organs, whereas the 0.8 kb form was detected only in testis. Murine Prep2 protein is 462 residues long (Haller et al. 2002). Alternative splicing gives rise to multiple isoforms, including a 25-kDa variant due to retention of intron 4 that lacks the C-terminal half of the protein and the homeodomain (Haller et al. 2004).

The PREP family of proteins (including Prep1 and Prep2 in vertebrates) belongs to the MEIS class of TALE superclass of DNA-binding proteins. Homeoproteins belonging to this superclass contain a divergent homeodomain (HD) with a 3 amino-acid loop extension (TALE, P-Y-P) between the first and



Prep, Fig. 1 Prep-protein structure. The structure of Prep proteins is shown. The homeodomain (HD) contains the TALE motif (Proline-Tyrosine-Proline), which is responsible for contacting the hexapeptide motif of Hox proteins. The bipartite MEIS domain (HM1 and HM2) contains the LFPLL motif, which is essential for Pbx binding

second α-helix. The TALE motif contacts the hexapeptide motif of Hox proteins (Moens and Selleri 2006).

Further, members of the MEIS class (which includes Homothorax in flies and Meis and Prep proteins in vertebrates) encode a bipartite 130-amino-acid-long MEIS domain (also called HM1 and HM2 domains) upstream of the HD (Fig. 1). The PREP family proteins do not contain any well-conserved specific motifs, besides a region downstream the homeodomain that may be the last remnant of a specific motif (Mukherjee and Bürglin 2007). Structure-function analyses demonstrated that a LFPLL motif in the MEIS domain of Prep1 is essential for Pbx1 binding (Díaz et al. 2007).

Sites of Expression

Prep1 is expressed ubiquitously with expression levels modulated in different organs and tissues. Prep1 is expressed in all organs in early zebrafish and mouse embryos. In the adult mouse, highest levels of expression were observed in testis, brain, and thymus (Ferretti et al. 2006). In the hematopoietic system, Prep1 is highly expressed in the stem cell compartment with levels decreasing upon cell differentiation (Di Rosa et al. 2007). Interestingly, Prep1 levels are usually lower in human tumor samples if compared to the normal tissue (Longobardi et al. 2010).

Human Prep2 is highly expressed in heart, brain, skeletal muscle, and ovary (Fognani et al. 2002). Testis-specific transcripts have been detected in both human (7–8 kb) and mouse (0.8 kb) tissues (Fognani et al. 2002; Haller et al. 2002).

Cellular Functions

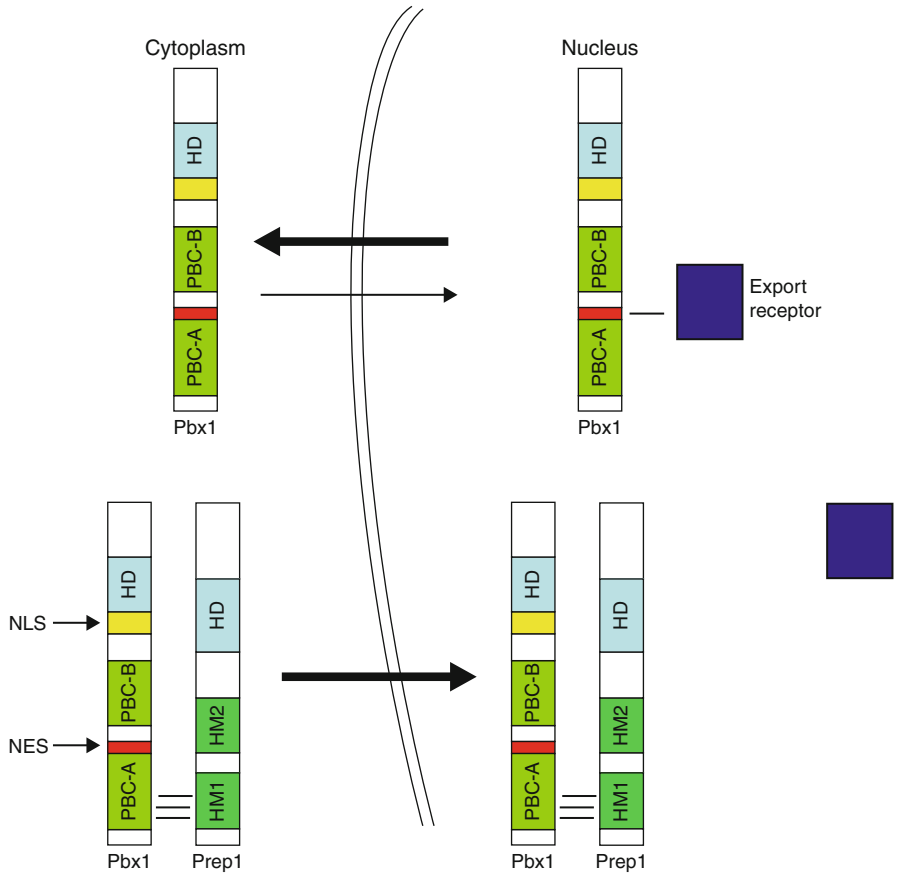
Prep proteins are able to bind the conserved sequence TGACAG. They can bind this sequence on their own, but with low affinity; the interaction with Pbx1a, Pbx1b, or Pbx2 increases the affinity to DNA and allows binding to a TGATNNAT motif (Berthelsen et al. 1998; Fognani et al. 2002). Several targets of the complexes have been identified, including somatostatin (Goudet et al. 1999), HoxB2 (Ferretti et al. 2000), Pax6 (Mikkola et al. 2001), and FSH β-subunit (Bailey et al. 2004).

The interaction between Pbx and Prep proteins is important for their translocation to the nucleus. In its homeodomain, Pbx proteins contain two Nuclear Localization Signals (NLS) that are not present in Prep proteins. Therefore, the interaction with Pbx1 allows nuclear translocation of Prep1 (Berthelsen et al. 1999). Pbx also has a Nuclear Export Signal (NES) in the PBC-A domain that favors its exit from the nucleus. The interaction with Prep (or Meis) masks this signal, allowing nuclear retention of the dimeric complex (Berthelsen et al. 1999) (Fig. 2). Cytoplasmic Prep2 localization is dependent on Crm-1-mediated nuclear export and association with the actin and microtubule cytoskeleton (Haller et al. 2004).

The interaction between Pbx and Hox is compatible with the interaction of Pbx with Prep. Indeed, Prep and Pbx interact through regions in the N-terminal (PBC-A in Pbx and HM1-HM2 in Prep), while the interaction of Hox proteins with Pbx involves the homeodomain. Therefore, ternary complexes between Pbx, Hox and Prep can be formed and are able to bind DNA (Berthelsen et al. 1998). This increases the specificity to the DNA consensus sequences of Hox genes to include both TGACAG and TGATNNAT separated by few bases. As an example, Fig. 3 shows the ternary complex formed between HoxB1, Pbx1, and Prep1 in the rhomobomere 4 enhancer of the *HoxB2* gene (Ferretti et al. 2000).

Prep1-Pbx1 complexes interact also with non-Hox proteins, cooperating in the activation of different promoters. For example, Pbx1 and Prep1 activate the somatostatin promoter when co-expressed with the pancreatic homeodomain factor Pdx1. Also in this context, the cooperative binding of two regulatory elements (UE-A by the Pbx1/Prep1 dimer and TSEI by Pdx1) of the promoter is necessary for the full activation (Goudet et al. 1999).

Prep1 and Pbx1 form trimeric complexes also with non-homeodomain proteins. Indeed, Pbx1 and Prep1 have been identified as Smad partners in a trimeric complex involved in the regulation of FSH β gene by activin (Bailey et al. 2004).

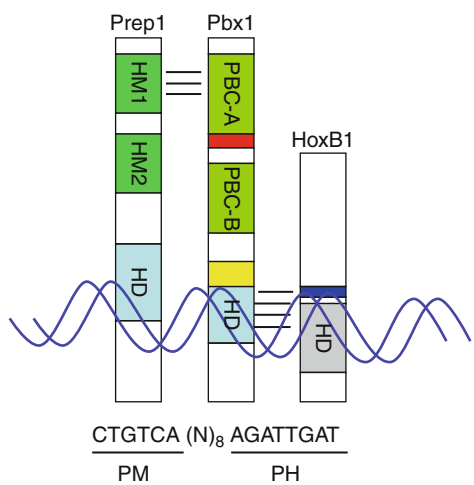


Prep, Fig. 2 Model of Pbx–Prep traslocation to the nucleus. In specific cell contexts, in the absence of Prep/Meis proteins, Pbx1 is actively exported from the nucleus, a process requiring the NES, located within its PBC-A domain, which is recognized by a nuclear export receptor. Pbx1 forms a stable complex with Prep1, when co-expressed, through an interaction surface that coincides with the region required for nuclear export, thereby shielding it. The newly formed complex translocates into the nucleus due to the NLS located within the homeodomain of Pbx1

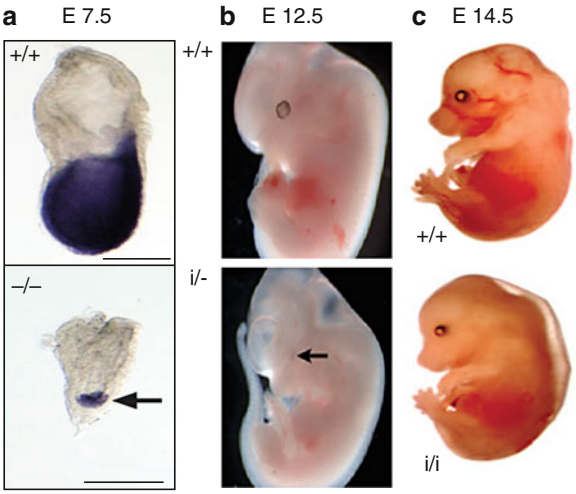
Besides Hox and TALE factors, other Prep1 interactors have been discovered such as Mybbp1A (p160), which may function as an inhibitor of its transcriptional activity (Díaz et al. 2007).

Loss of Function–Associated Phenotypes

Prep1-null embryos die before gastrulation because epiblast cells undergo ▶ p53-dependent apoptosis (Fernandez-Diaz et al. 2010). Mouse embryos carrying a hypomorphic *Prep1i/i* mutation (expressing 2% mRNA, 3–7% protein) show general organ hypoplasia and, in 75% of cases, die at about E17 with major alterations in hematopoiesis, angiogenesis, and eye development (Ferretti et al. 2006; Di Rosa et al. 2007). Interestingly, a further decrease of the Prep1 mRNA level to about 1% (double heterozygous *Prep1i/-* embryos) shows an intermediate phenotype with embryonic lethality around E12.5 (Rowan et al. 2010). A gene dosage effect of Prep1 on embryonic development is evident from these studies (Fig. 4).



Prep, Fig. 3 HoxB1-Pbx1-Prep1 ternary complex in the rhombomere-4 enhancer of HoxB2. In the case of the PM-PH sites of the Hoxb2 enhancer, Hoxb1 forms a dimeric complex with Pbx1 on the PH site (AGATTGAT), while Prep1 binds the PM site (CTGTCA). In this way, three homeodomains are bound to DNA



Prep, Fig. 4 Prep1 loss-of-function phenotypes during embryonic development. (**a**) Whole mount in situ hybridization against Oct4 at E7.5 in *Prep1*+/+ and *Prep1*−/− embryos (Fernandez-Diaz et al. 2010). (**b**) *Prep1*+/+ and *Prep1i*/- embryos at E12.5 (Rowan et al. 2010). (**c**) *Prep1*+/+ and *Prep1i*/*i* embryos at E14.5 (Ferretti et al. 2006)

Remarkably, homozygous *Prep1i*/*i* hypomorphic mice surviving embryonic lethality are prone to develop tumors within 20 months of age, and Prep1 heterozygosity accelerates ▶ Myc-dependent lymphomagenesis (Longobardi et al. 2010).

A loss-of-function mutation for Prep2 has not been described.

Summary

Prep proteins have been extensively studied as critical mediators of the activity of other homeodomain transcription factors (in particular Hox and Pbx proteins). Nevertheless, at least for Prep1, a loss-of-function model has recently demonstrated that this is the only protein of the family which is essential at very early stages of embryonic development (Fernandez-Diaz et al. 2010). This strongly suggests that this protein has other, not yet identified, cellular functions.

Prep1 loss-of-function phenotypes have two other remarkable features:

– Pleiotropy: Prep1 has a pivotal role in several embryonically unrelated cell types, consistently with its ubiquitous expression, suggesting a role in the biology of stem cells of different tissues.
– Gene-dosage dependence: small differences in its expression have great impact on cellular and tissue homeostasis, suggesting that Prep1 levels must be tightly regulated.

Furthermore, recent data demonstrate that (unlike the other TALE factors) Prep1 functions as a tumor suppressor, further prompting for a deep investigation of its mechanisms of action.

Prep2 has not been extensively studied, so far. In particular, a loss-of-function genetic model would be extremely important in order to exploit its functions which, given also its narrower tissue distribution, might be different from Prep1.

References

Bailey JS, Rave-Harel N, McGillivray SM, Coss D, Mellon PL. Activin regulation of the follicle-stimulating hormone beta-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. Mol Endocrinol. 2004;18:1158–70.

Berthelsen J, Zappavigna V, Mavilio F, Blasi F. Prep1, a novel functional partner of Pbx proteins. EMBO J. 1998;17:1423–33.

Berthelsen J, Kilstrup-Nielsen C, Blasi F, Mavilio F, Zappavigna V. The subcellular localization of PBX1 and EXD proteins depends on nuclear import and export signals and is modulated by association with PREP1 and HTH. Genes Dev. 1999;13:946–53.

Chen H, Rossier C, Nakamura Y, Lynn A, Chakravarti A, Antonarakis SE. Cloning of a novel homeobox-containing gene, PKNOX1, and mapping to human chromosome 21q22.3. Genomics 1997;41:193–200.

Díaz VM, Mori S, Longobardi E, Menendez G, Ferrai C, Keough RA, et al. p160 Myb-binding protein interacts with Prep1 and inhibits its transcriptional activity. Mol Cell Biol. 2007;27:7981–90.

Di Rosa P, Villaescusa JC, Longobardi E, Iotti G, Ferretti E, Diaz VM, et al. The homeodomain transcription factor Prep1 (pKnox1) is required for hematopoietic stem and progenitor cell activity. Dev Biol. 2007;311:324–34.

Fernandez-Diaz LC, Laurent A, Girasoli S, Turco M, Longobardi E, Iotti G, et al. The absence of Prep1 causes p53-dependent apoptosis of pluripotent epiblast cells. Development. 2010;137:3393–403.

Ferretti E, Schulz H, Talarico D, Blasi F, Berthelsen J. The PBX-regulating protein PREP1 is present in different PBX-complexed forms in mouse. Mech Dev. 1999;83:53–64.

Ferretti E, Marshall H, Pöpperl H, Maconochie M, Krumlauf R, Blasi F. A complex site including both Pbx-Hox and Prep-Meis-responsive elements and binding a retinoic acid-inducible ternary Hoxb1-Pbx-Prep1 complex is required for HOXB2 rhombomere 4 expression. Development. 2000;127:155–66.

Ferretti E, Villaescusa JC, Di Rosa P, Fernandez-Diaz LC, Longobardi E, Mazzieri R, et al. Hypomorphic mutation of the TALE gene Prep1 (pKnox1) causes a major reduction

of Pbx and Meis proteins and a pleiotropic embryonic phenotype. Mol Cell Biol. 2006;26:5650–62.
Fognani C, Kilstrup-Nielsen C, Berthelsen J, Ferretti E, Zappavigna V, Blasi F. Characterization of PREP2, a paralog of PREP1, which defines a novel sub-family of the MEINOX TALE homeodomain transcription factors. Nucleic Acids Res. 2002;30:2043–51.
Goudet G, Delhalle S, Biemar F, Martial JA, Peers B. Functional and Cooperative Interactions between the Homeodomain PDX1, Pbx, and Prep1 Factors on the Somatostatin Promoter J Biol Chem. 1999;274:4067–73.
Haller K, Rambaldi I, Kovacs EN, Daniels E, Featherstone M. Prep2: cloning and expression of a new prep family member. Dev Dyn. 2002;225:358–64.
Haller K, Rambaldi I, Daniels E, Featherstone M. Subcellular localization of multiple PREP2 isoforms is regulated by actin, tubulin, and nuclear export. J Biol Chem. 2004;279:49384–94.
Imoto I, Sonoda I, Yuki Y, Inazawa J. Identification and characterization of human PKNOX2, a novel homeobox-containing gene. Biochem Biophys Res Commun. 2001;287:270–6.
Longobardi E, Iotti G, Di Rosa P, Mejetta S, Bianchi F, Fernandez-Diaz LC, et al. Spontaneous tumor development and acceleration of EmMyc lymphomas in mice haploinsufficient for the homeodomain transcription factor gene Prep1 (pKnox1) indicates a tumor suppressor function. Mol Oncol. 2010;4:226–34.
Mikkola I, Bruun JA, Holm T, Johansen T. Superactivation of Pax6-mediated transactivation from paired domain-binding sites by DNA-independent recruitment of different homeodomain proteins. J Biol Chem. 2001;276:4109–18.
Moens CB, Selleri L. Hox cofactors in vertebrate development. Dev Biol. 2006;291:193–206.
Mukherjee K, Bürglin TR. Comprehensive analysis of animal TALE homeobox genes: new conserved motifs and cases of accelerated evolution. J Mol Evol. 2007;65:137–53.
Rowan S, Siggers T, Lachke SA, Yue Y, Bulyk ML, Maas RL. Precise temporal control of the eye regulatory gene Pax6 via enhancer-binding site affinity. Genes Dev. 2010;24:980–5.

P-Rex

Daisuke Urano[1] and Hiroshi Itoh[2]
[1]Department of Biology, University of North Carolina, Chapel Hill, NC, USA
[2]Department of Medical Science, Nara Institute of Science and Technology, Ikoma, Nara, Japan

Synonyms

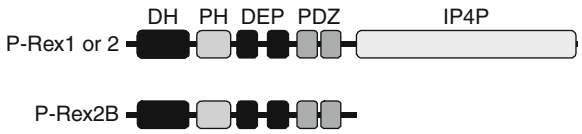
P-Rex1 (BC067047; G630042G04; KIAA1415; mKIAA1415; P-Rex1; Phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger; PREX1), P-Rex2 (DEP domain containing 2; DEP.2; Depdc2; P-Rex2; phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 2; PREX2; PtdIns(3,4,5)-dependent Rac exchanger 2)

Historical Background

A phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger (P-Rex) is an intracellular signaling molecule that regulates leukocyte function and neuronal development by activating a small guanine nucleotide-binding protein, Rac. The *Homo sapiens* genome encodes two P-Rex genes, P-Rex1 and P-Pex2. In 2002, Welch et al. purified a PIP_3-dependent Rac activator from pig neutrophil lysates, identified a 196 kDa Dbl-like GEF (guanine nucleotide exchange factor) protein for Rac, and designated it as P-Rex1 (Welch et al. 2002). They characterized its synergistic activation by Gβγ subunits of heterotrimeric G proteins and PIP_3, and its physiological function in reactive oxygen species (ROS) production in neutrophils (Welch et al. 2002). P-Rex2 and the spliced variant, P-Rex2B, were identified in a search for P-Rex1-homologous genes (Donald et al. 2004; Rosenfeldt et al. 2004). Using P-Rex knockout mice, Welch et al. and the other group clarified physiological roles of P-Rex genes in peripheral white blood cells and in neuronal development (Donald et al. 2008; Dong et al. 2005; Welch et al. 2005). Recently, several studies indicated the involvement of P-Rex genes in proliferation and metastasis of human tumors. Especially, Fine et al. demonstrated that P-Rex2 activates the PI3K (▶ phosphoinositide 3-kinase) pathway by a direct inhibition of ▶ PTEN (phosphatase and tensin homolog) (Fine et al. 2009).

Molecular Structure and Regulation

In vitro, P-Rex catalyzes guanine nucleotide exchange on Rac and Cdc42, but not Rho (Welch et al. 2002). However, P-Rex specifically activates Rac in vivo (Welch et al. 2002). P-Rex activity is positively and negatively regulated by heterotrimeric G protein signals (Mayeenuddin and Garrison 2006; Welch et al. 2002). P-Rex is directly and synergistically activated by Gβγ and PIP_3, which is produced by PI3K in vivo, and is inhibited by direct phosphorylation by cAMP-dependent kinase (Protein kinase A; PKA) (Mayeenuddin and Garrison 2006; Urano et al. 2008).



P-Rex, Fig. 1 Domain structure of P-Rex1, P-Rex2, and P-Rex2B

The human P-Rex1 and P-Rex2 are highly related 1659 and 1606 amino acid proteins containing an amino-terminal DH (Dbl-homology), a PH (Pleckstrin homology), two DEP (Dishevelled, Egl-10, and Pleckstrin), two PDZ (PSD-95, Dlg, and ZO-1), and a carboxyl-terminal IP4P (inositol polyphosphate 4-phosphatase) like domains (Fig. 1). The tandem DH/PH domains are minimal and constitutively active elements that catalyze the exchange of guanine nucleotide on Rac. The DH domain possesses the catalytic core and is thought to be directly stimulated by Gβγ (Hill et al. 2005). The PH domain mediates PIP_3-dependent activation (Hill et al. 2005), promotes translocation of P-Rex from cytosol to plasma membrane (Barber et al. 2007), and confers its substrate specificity (Joseph and Norris 2005). Functions of tandem DEP and PDZ domains have been unclear, but these domains keep the basal GEF activity low (Hill et al. 2005) and mediate Gβγ-dependent activation through their interaction with IP4P-like domain (Urano et al. 2008). The IP4P-like domain shows a significant similarity to inositol polyphosphate 4-phosphatase (Donald et al. 2004; Rosenfeldt et al. 2004; Welch et al. 2002), but its phosphatase activity has not been characterized. Truncation mutants of P-Rex1 indicated that the DH/PH domains act as a constitutively active form and the other domains contribute to keep low basal activity (Hill et al. 2005).

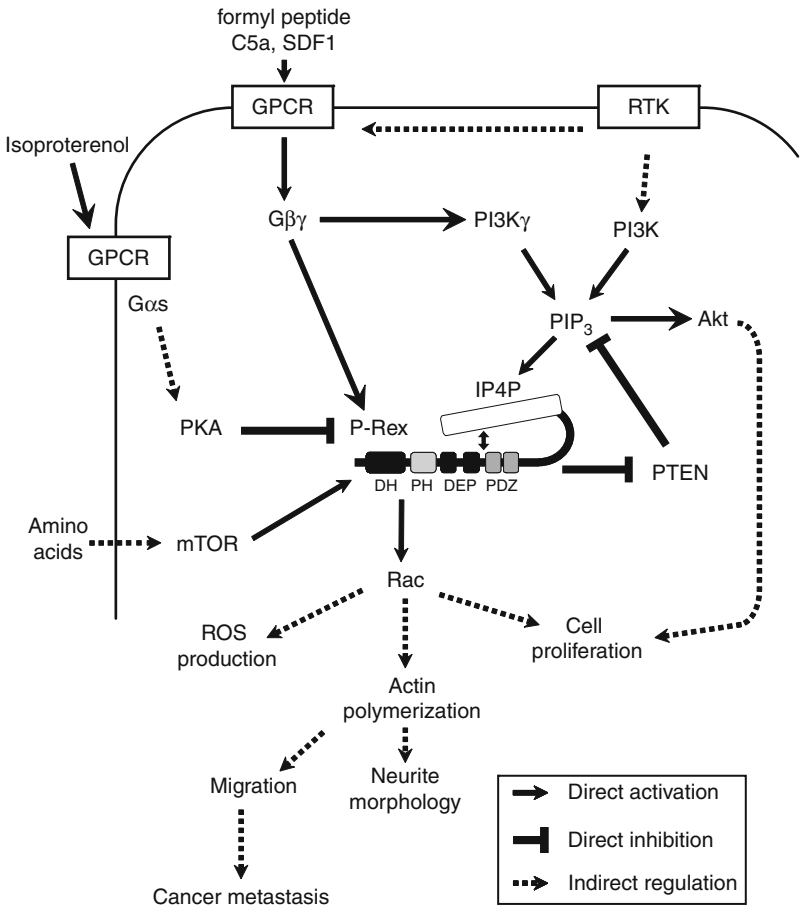
In addition to heterotrimeric G proteins, several receptor tyrosine kinases (RTK) indirectly activate P-Rex1 in a PI3K-dependent manner. The P-Rex activation regulates cell morphology and migration in neuronal cells and human tumor cells (Montero et al. 2011; Qin et al. 2009; Sosa et al. 2010; Yoshizawa et al. 2005) (Fig. 2). In breast cancer cells, heregulins/neuregulins and their receptors, the ErbB subfamily of RTKs, activate P-Rex1 through the transactivation of CXCR (CXC chemokine receptor) and PI3K (Sosa et al. 2010). Neuregulin also regulates P-Rex activity through dephosphorylation of inhibitory residues and phosphorylation of activating residues of P-Rex1 (Montero et al. 2011), although kinases and phosphatases which regulate the phosphorylations have not been clarified yet. Moreover, P-Rex mediates Rac activation and cell migration with ▶ mTOR (mammalian target of rapamycin) (Hernandez-Negrete et al. 2007; Kim et al. 2011), which is a serine-threonine kinase activated by growth factors and nutrient stress. P-Rex1, P-Rex2, and P-Rex2B directly interact with mTOR through the tandem DEP domains (Hernandez-Negrete et al. 2007), but the mechanism of how mTOR modulates P-Rex activity remains unclear.

Physiological Roles

P-Rex plays important roles in Rac-mediated actin polymerization that leads to lamellipodia formation, ROS production, and transcriptional regulation. P-Rex1 is highly expressed in peripheral blood leukocytes and the brain (Donald et al. 2008; Welch et al. 2002; Yoshizawa et al. 2005), and P-Rex2 is abundant in the lung and brain (Donald et al. 2008). In contrast to P-Rex1, expressed widely throughout the whole brain (Donald et al. 2008; Yoshizawa et al. 2005), P-Rex2 expression is more restricted to the cerebellum (Donald et al. 2008). P-Rex2B expression was shown, by northern blots, to be only in the heart (Donald et al. 2004). Knockout mice of P-Rex1 (Donald et al. 2008; Dong et al. 2005; Hill et al. 2005) and P-Rex2 (Donald et al. 2008) revealed their physiological and developmental roles. In peripheral blood leukocytes, P-Rex1 is involved in GPCR (G protein–coupled receptor)-dependent Rac activation, ROS production, cell migration, and cell adhesion (Dong et al. 2005; Lawson et al. 2011; Welch et al. 2002, 2005). P-Rex1 knockout mice also exhibit defects of Rac1 activation, cell migration, and superoxide production in macrophages (Wang et al. 2008). P-Rex2 regulates dendrite structure in mouse cerebellar Purkinje cells (Donald et al. 2008). P-Rex1/P-Rex2 double knockout mice grow up healthy and are fertile, but show morphological defects in cerebellar Purkinje cells and a strong motor coordination defect (Donald et al. 2008).

Roles in Cancer

Overexpression of Rho-family GTPases and GEFs participates in cancer progression and metastasis in



P-Rex, Fig. 2 Signaling pathways mediated through P-Rex1 and P-Rex2. Direct regulation was indicated as *solid lines*. Indirect activation was shown as *dashed line*

various types of tumors. Recently, several studies indicated the involvement of P-Rex genes in proliferation and metastasis in breast and prostate tumors (Fine et al. 2009; Kim et al. 2011; Montero et al. 2011; Qin et al. 2009; Sosa et al. 2010). The human P-Rex1 gene is located on the chromosome 20q13, which is a region frequently amplified in breast cancer, and P-Rex2 gene is on the chromosome 8q13, a region of high amplification in breast, prostate, ovarian, and colorectal cancers. Indeed, it has been recently reported that P-Rex1 mediates Erb2-dependent migration and tumorigenesis in breast cancer cells (Montero et al. 2011; Sosa et al. 2010) and promotes spontaneous metastasis in prostate cancer cells (Qin et al. 2009). P-Rex2 is also highly expressed in several human cancers, and directly inhibits phosphatase activity of PTEN (Fine et al. 2009), which is a tumor suppressor frequently mutated in human cancers and an enzyme that dephosphorylates $PI(3,4,5)P_3$ into $PI(4,5)P_2$. P-Rex2-mediated inhibition of PTEN accumulates PIP_3, and consequently promotes cell proliferation with activation of Akt in breast cancer cells (Fine et al. 2009).

Summary and Perspective

P-Rex proteins function as Rac activators and are involved in ROS production, cell morphology, migration, proliferation, and gene expression in various cells and tissues. P-Rex is activated by heterotrimeric G proteins, RTK and PI3K-pathways, and inhibited by cAMP/PKA signals, allowing it to potentially integrate various hormonal stimuli into a Rac signaling. It is unclear how P-Rex keeps basal activity low and is activated by Gβγ and the molecules. X-ray crystal structure analysis of P-Rex should provide insights into understanding them. Higher expression level of P-Rex1 is linked to malignancy in human breast and prostate tumors, and potentiates RTK signals. However, the mechanisms of how RTK signals activate

P-Rex/Rac pathway have not been clarified. Two potential mechanisms for activation of P-Rex pathways indirectly through transactivation of G proteins or by phosphorylation and dephosphorylation control of P-Rex by RTK signaling have been described, but a more detailed analysis should yield better understanding of multiple P-Rex1 regulations.

References

Barber MA, Donald S, Thelen S, Anderson KE, Thelen M, Welch HC. Membrane translocation of P-Rex1 is mediated by G protein betagamma subunits and phosphoinositide 3-kinase. J Biol Chem. 2007;282:29967–76.

Donald S, Hill K, Lecureuil C, Barnouin R, Krugmann S, John Coadwell W, et al. P-Rex2, a new guanine-nucleotide exchange factor for Rac. FEBS Lett. 2004;572:172–6.

Donald S, Humby T, Fyfe I, Segonds-Pichon A, Walker SA, Andrews SR, et al. P-Rex2 regulates Purkinje cell dendrite morphology and motor coordination. Proc Natl Acad Sci USA. 2008;105:4483–8.

Dong X, Mo Z, Bokoch G, Guo C, Li Z, Wu D. P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils. Curr Biol. 2005;15:1874–9.

Fine B, Hodakoski C, Koujak S, Su T, Saal LH, Maurer M, et al. Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. Science. 2009;325:1261–5.

Hernandez-Negrete I, Carretero-Ortega J, Rosenfeldt H, Hernandez-Garcia R, Calderon-Salinas JV, Reyes-Cruz G, et al. P-Rex1 links mammalian target of rapamycin signaling to Rac activation and cell migration. J Biol Chem. 2007;282:23708–15.

Hill K, Krugmann S, Andrews SR, Coadwell WJ, Finan P, Welch HC, et al. Regulation of P-Rex1 by phosphatidylinositol (3,4,5)-trisphosphate and Gbetagamma subunits. J Biol Chem. 2005;280:4166–73.

Joseph RE, Norris FA. Substrate specificity and recognition is conferred by the pleckstrin homology domain of the Dbl family guanine nucleotide exchange factor P-Rex2. J Biol Chem. 2005;280:27508–12.

Kim EK, Yun SJ, Ha JM, Kim YW, Jin IH, Yun J, et al. Selective activation of Akt1 by mammalian target of rapamycin complex 2 regulates cancer cell migration, invasion, and metastasis. Oncogene. 2011;30:2954–63.

Lawson CD, Donald S, Anderson KE, Patton DT, Welch HC. P-Rex1 and Vav1 cooperate in the regulation of formyl-methionyl-leucyl-phenylalanine-dependent neutrophil responses. J Immunol. 2011;186:1467–76.

Mayeenuddin LH, Garrison JC. Phosphorylation of P-Rex1 by the cyclic AMP-dependent protein kinase inhibits the phosphatidylinositiol (3,4,5)-trisphosphate and Gbetagamma-mediated regulation of its activity. J Biol Chem. 2006;281:1921–8.

Montero JC, Seoane S, Ocana A, Pandiella A. P-Rex1 participates in Neuregulin-ErbB signal transduction and its expression correlates with patient outcome in breast cancer. Oncogene. 2011;30:1059–71.

Qin J, Xie Y, Wang B, Hoshino M, Wolff DW, Zhao J, et al. Upregulation of PIP3-dependent Rac exchanger 1 (P-Rex1) promotes prostate cancer metastasis. Oncogene. 2009;28:1853–63.

Rosenfeldt H, Vazquez-Prado J, Gutkind JS. P-REX2, a novel PI-3-kinase sensitive Rac exchange factor. FEBS Lett. 2004;572:167–71.

Sosa MS, Lopez-Haber C, Yang C, Wang H, Lemmon MA, Busillo JM, et al. Identification of the Rac-GEF P-Rex1 as an essential mediator of ErbB signaling in breast cancer. Mol Cell. 2010;40:877–92.

Urano D, Nakata A, Mizuno N, Tago K, Itoh H. Domain-domain interaction of P-Rex1 is essential for the activation and inhibition by G protein betagamma subunits and PKA. Cell Signal. 2008;20:1545–54.

Wang Z, Dong X, Li Z, Smith JD, Wu D. Lack of a significant role of P-Rex1, a major regulator of macrophage Rac1 activation and chemotaxis, in atherogenesis. Prostaglandins Other Lipid Mediat. 2008;87:9–13.

Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, et al. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell. 2002;108:809–21.

Welch HC, Condliffe AM, Milne LJ, Ferguson GJ, Hill K, Webb LM, et al. P-Rex1 regulates neutrophil function. Curr Biol. 2005;15:1867–73.

Yoshizawa M, Kawauchi T, Sone M, Nishimura YV, Terao M, Chihama K, et al. Involvement of a Rac activator, P-Rex1, in neurotrophin-derived signaling and neuronal migration. J Neurosci. 2005;25:4406–19.

PREX1

► P-Rex

P-Rex1

► P-Rex

PREX2

► P-Rex

P-Rex2

► P-Rex

Prion Protein

▶ Prion Protein (*PRNP*)

Prion Protein (*PRNP*)

Rafael Linden[1], Vilma R. Martins[2] and Marco A. M. Prado[3]
[1]Laboratorio de Neurogenese, Instituto de Biofisica da UFRJ, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[2]International Center for Research and Education Antonio Prudente Foundation, A.C. Camargo Hospital, São Paulo, SP, Brazil
[3]Robarts Research Institute, University of Western Ontario, London, ON, Canada

Synonyms

CD230; Prion protein; Prn-i; Prnp; Prn-p; PrP; PrP^{C}; PrP^{Sc}; Sinc

Historical Background

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases characterized by neuron loss, glial reactions, and tissue spongiosis, which course with motor and/or cognitive symptoms (Knight and Will 2004). The TSEs are associated with conformational conversion of the prion protein (PrP^{C}, the product of the *Prnp* gene), wherein the predominantly α-helical secondary structure of PrP^{C} changes into an aggregation-prone, β-sheet richer structure known as PrP^{Sc}. The latter is believed to coerce PrP^{C} molecules into conformational conversion, thus behaving as a *proteinaceous infectious particle*, or *prion* (Prusiner 1998), which gave TSEs the epithet *prion diseases*.

The much needed development of effective treatment for these still incurable diseases depends on the understanding of functional properties of the prion protein (Soto and Satani 2010). Most studies of physiological functions of PrP^{C} have been directed at its major cell-surface GPI-anchored form, whereas minor transmembrane and cytosolic forms have usually been studied in the context of pathogenesis of prion diseases. The current account, therefore, focuses upon the physiological functions of the GPI-anchored PrP^{C}.

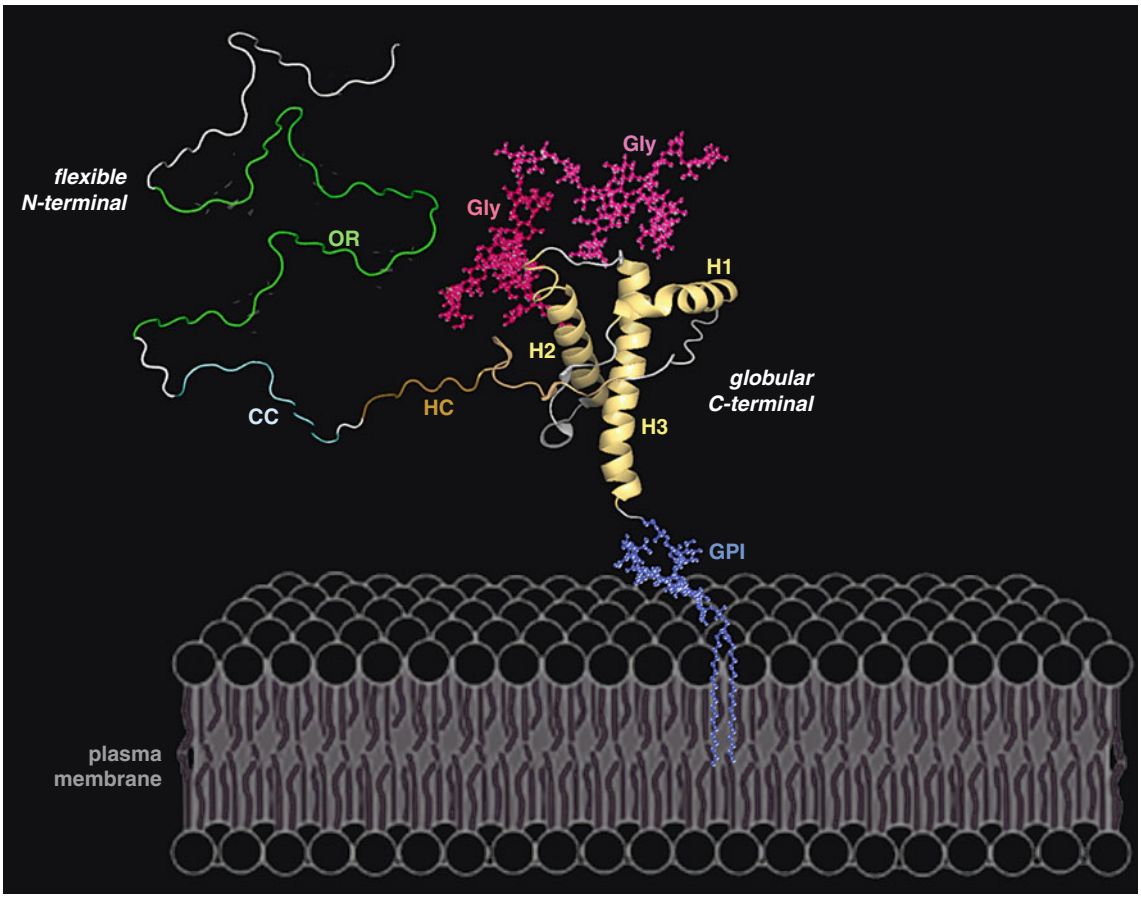
Several dozen distinct molecules have been shown to bind PrP^{C}, albeit on the basis of somewhat variable evidence. The expression and the engagement of PrP^{C} with a variety of ligands activate numerous signal transduction pathways, thus leading to modulation of proliferation, differentiation, and cell death in the nervous and immune systems, as well as in many other organs and cell types. In addition, PrP^{C}-mediated signaling is affected by trafficking of the protein both laterally between distinct plasma membrane domains and along endocytic pathways, as well as by its continuous and rapid recycling. A recent review of these functional properties suggests that the prion protein has a general function analogous to intracellular scaffolding proteins, as a dynamic cell surface platform for the assembly of signaling modules (Linden et al. 2008).

Structure, Expression, and Regulation of the Prion Protein

PrP^{C} is an *N*-glycosylated, glycosyl-phosphatidylinositol (GPI)-anchored protein of 208–209 amino acids, containing an amino (N)-terminal flexible, random coil sequence and a carboxy (C)-terminal globular domain, the major structural features of which are preserved among both mammalian and non-mammalian species (Fig. 1). The globular domain of human PrP^{C} contains 3 α-helices interspersed with an antiparallel β-pleated sheet formed by β-strands at two short stretches, and contains a single disulfide bond. The N-terminal flexible tail spans approximately half of the mature protein, and a short flexible C-terminal domain attaches to the GPI anchor (Wuthrich and Riek 2001).

Full length PrP^{C} is found in non-, mono-, or diglycosylated forms, corresponding to the variable occupancy of two asparagine residues (Rudd et al. 2001). A rather large variety of N-glycans were found attached to both full-length and truncated PrP^{C}, which may be differentially distributed in various areas of the central nervous system (CNS).

A single exon within the *Prnp* gene codes for PrP^{C}. Control of *Prnp* gene expression has been attributed to sequences within the 5′-flanking region, within the first intron, and to 3′-untranslated sequences, as well as to



Prion Protein (*PRNP*), Fig. 1 The structure of the prion protein is drawn at a roughly approximate scale, in a montage of elements taken from various sources: The globular C-terminal domain is a Pymol representation of mouse PrP121-231; the flexible N-terminal domain was freely drawn to scale from segments of unstructured sequences of amino acids in the same Pymol representation, maintaining the number of amino acids ascribed to each individually defined domain (see below); the GPI anchor and the structure of N-linked sugars were taken from (Rudd et al. 2001); the depiction of the plasma membrane is just a rough sketch modified from a drawing in Wikipedia (Abbreviations: *CC* charged cluster, *Gly* N-linked sugars, *GPI* GPI anchor, *H1*, *H2*, *H3* α-helix domains, *HC* hydrophobic core, *OR* octapeptide repeat domain)

interactions between promoter and intronic regions. Differing from the *Prnp* open reading frame, the degree of homology of potential promoter sequences among various mammalian species is quite variable (Mastrangelo and Westaway 2001).

Prnp is often labeled as a housekeeping gene, but evidence that transcription of *Prnp* is modulated by chromatin structure, as well as the identification of potential binding sites for many transcription factors, indicate that expression of *Prnp* likely depends on a variety of cellular factors. Notwithstanding some variation between species, the following elements were reported both in the 5′-flanking region and within the first intron: Sp1, AP1, AP2, MZF-1, MEF2, MyT1, Oct-1, ▶ NFAT, POZ (BCL6); RP58 (ZNF238); NEUROG1; EGR4, Oct-1/Oct-2, NF-IL6, MyoD, ▶ p53, HSE, MRE, MLS (Linden et al. 2008 for review).

Expression of both *Prnp* messenger RNA (mRNA) and prion protein are developmentally regulated, and subject to modulation by growth factors such as NGF, PDGF, and various cytokines. Expression can also be modulated by stressful conditions, inclusive of heat shock, hypoglycemia, oxidative stress and inflammation, as well as copper overload.

The prion protein is highly expressed within the nervous system, although its content varies among distinct brain regions, among differing cell types and

among neurochemically distinct neurons. In addition, substantial amounts of PrP^C are expressed in various cellular components of the immune system, the bone marrow, blood, and peripheral tissues. Other organs and tissues also express PrP^C (Table 1).

Many reports are available on putative ligands of the prion protein, and candidate physiological ligands are listed in Table 2. It can be appreciated that binding domains identified for a number of ligands extend along the entire PrP^C molecule (Fig. 2). It should, however, be noted that the techniques used for those studies were quite variable, and many interactions detected by screening methods have yet to be confirmed by biochemical and cell biological approaches. In particular, some putative ligands appear not to be accessible from the usual topology of PrP^C, which constitutes a critical question to be addressed by future studies (Rutishauser et al. 2009).

Trafficking, Endocytosis, and Recycling of the Prion Protein

An N-terminus peptide (aa. 1–22) is used to drive nascent PrP^C into the endoplasmic reticulum, where its GPI-anchor is added at the C-terminus. Distinct topologies of PrP^C have been described, some of which are of pathological interest. PrP^C follows the classical pathway for its insertion at the plasma membrane, passing through the Golgi and following a Brefeldin A-sensitive pathway to reach the cell surface (Prado et al. 2004 for review).

At the cell surface, PrP^C molecules are found predominantly anchored to low density, detergent insoluble membrane domains, rich in cholesterol and sphingolipids (*lipid rafts*). GPI-anchored proteins located in rafts are thought to recycle between the plasma membrane and intracellular organelles, in particularly to the Golgi. In neurons, endogenous PrP^C appears to internalize as fast as classical membrane receptors, such as the transferrin receptor, with a $T_{1/2}$ of approximately 3–5 min.

Initial data pointed to the possibility that PrP^C, similar to other GPI-anchored proteins, is internalized by a raft-mediated mechanism that is independent from clathrin. Although caveolae anf flotilin-derived vesicles may participate in the internalization of PrP^C in non-neuronal cells and in astrocytes, mounting evidence suggests that, in neurons, clathrin-mediated endocytosis plays an important role on PrP^C internalization. Cell surface biotinylation, live cell microscopy, GFP-tagged PrPs, and electron microscopy support the view that clathrin-coated vesicles and classical endosomal organelles are involved in endocytosis of PrP^C (reviewed by Linden et al. 2008). Dominant negative approaches indicated a role for the activities of dynamin and clathrin in the internalization of PrP^C in distinct cell lines. It was proposed that an N-terminal, positively charged domain of PrP^C (KKRPKP) is responsible for the constitutive endocytosis of PrP^C by clathrin-coated vesicles. Remarkably, a number of reports indicated a role for the N-terminal region of PrP^C upon endocytosis and cellular trafficking, and this basic region of the protein has been previously implicated in the binding of negatively charged proteoglycans, which are thought to modulate PrP^C sequestration (Prado et al. 2004) (Fig. 3).

The hypothesis that GPI-anchored PrP^C may "piggy-back" on an integral membrane protein had long been raised, and recent studies indicated that the low-density lipoprotein receptor-related protein 1 (LRP1) may participate in clathrin-mediated endocytosis of PrP^C, because knock-down of LRP1, but not LRP1b, reduced internalization of PrP^C. LRP1 has also been implicated in PrP fibril entry in cells (Parkyn et al. 2008; Taylor and Hooper 2007).

High extracellular levels of Cu^{2+} induce the endocytosis of PrP^C to intracellular organelles and the Golgi. It was reported that Cu^{2+}-induced endocytosis of PrP^C expressed in neuroblastoma cells caused its movement from raft to non-raft membrane regions. Although the KKRP motif was shown to be important for endocytosis, this motif is not essential for the lateral displacement of PrP^C to non-raft membrane, indicating that this movement occurs prior to PrP^C endocytosis. It was also suggested that Cu^{2+} may destabilize a putative PrP^C interaction within rafts, rather than inducing PrP^C to interact with a non-raft protein. It is not clear yet if the KKRP domain is required for binding to LRP1 or to other membrane proteins that may be accessory in this process. Interestingly, amyloid β peptide 1–42, a major culprit in Alzheimer's disease was recently shown to bind PrP^C. A binding site was identified at aa. 90–110 and, more recently, the endocytic motif 23–27 was also shown to mediate binding to amyloid β peptide. This suggests that amyloid β may be able to regulate PrP^C trafficking. Moreover, one of the ligands of PrP^C, hop/STI1, which

Prion Protein (*PRNP*), Table 1 Expression and distribution of prion protein

Species	Organ/tissue	Cell type/subcellular distribution	Technique	Detection level and regulation
Hamster	Brain	Neuron cell bodies	IHC, WB	
Mouse, hamster	Brain	Neurons, intracellular	IHC	
Hamster, macaque, human	Brain	Pre-synaptic, *not* in cell bodies	WB, IHC (mAb 3F4), EM	
Hamster	Developing brain	Mainly along axon tracts	IHC (mAb 3F4)	Developmentally regulated, remains high in adult olfactory bulb and hippocampus
Mouse	Embryonic brain, spinal cord, PNS	Neurons, non-neuronal cells	ISH	Developmentally regulated.
Mouse	Brain	Neurons, neuronal processes, *not in* glia	IHC (pAbs GAx), ISH	Varied both among and within brain regions, depending on cell type and neurochemical phenotype. Protein often *not* correlated with mRNA.
Hamster, human	Hippocampus	Pre-synaptic	IHC, EM	
Hamster	Cerebellum	Pre- *and* post-synaptic	IHC, EM	
Human	Cerebellum	Neurons	IHC (mAb 3F4)	Low level in cerebellar granule cells of normal brain
Rat	Cerebellum	Neuron and glial cell bodies and processes	IHC (mAb 8H4), EM	
Rat	Neonatal retina	Retinal precursors and differentiating neurons	WB, IHC (mAb 6H4, pAb N10, pAbMo)	
Hamster	Brain (hippocampus, septum, caudate nucleus, thalamus), DRG, blood, heart, skeletal muscle, lung, gut, spleen, testis, ovary, and others	Both in neuronal cell bodies and neuropil	WB, IHC (mAb 3F4 + 2 distinct pAbs), EM	Highest in hippocampus
Mouse	Olfactory bulb, PNS, bone marrow, lymphoreticular system, gut, lung, kidney, testis, skin, *not* liver	Peripheral axons, neuron cell bodies, *not* glia; haemopoietic progenitors, megakaryocytes, monocytes, *not* granulocytes in bone marrow; dendritic cells, pericytes, intraepithelial lymphocytes in various tissues	IHC (pAbs GAx, mAb SAF61), ISH	Varied both among and within distinct organs and tissues. Scattered cells with high expression. Protein correlated with mRNA.

(*continued*)

Table 1 (continued)

Species	Organ/tissue	Cell type/subcellular distribution	Technique	Detection level and regulation
Mouse	Intestine	Submucosa, muscularis mucosa	ISH	
Hamster	Stomach, intestine, lung, kidney	Secretory globules	IHC (pAbs Br-1, R073, P38, mAb 3F4), EM	
Human	Stomach, kidney, spleen	Secretory globules	IHC (pAbs Br-1, R073, P38, mAb 3F4), EM	
Bovine	Ovary	Ovarian follicles	Microarray, real time PCR, WB (mAb HumP)	Up regulated in theca cells of dominant, as compared with subordinate follicles
Sheep	Spleen, lymph node, lung, heart, kidney, skeletal muscle, uterus, adrenal gland, parotid gland, intestine, mammary gland, *not* liver, *not* pancreas.		WB (home-made pAbs and mAbs), NB	
Mouse, hamster, human	Muscle	Subsynaptic sarcoplasm, *not* postsynaptic plasma membrane	IHC (pAb R254, R073), EM	
Mouse	Muscle	Myoblast cell lines	WB (pAb Ra5)	Up regulated with differentiation of myotubes from myoblasts
Human	Blood	Lymphocytes and lymphoid cell lines, monocytes, *not* erythrocytes, *not* mature granulocytes	FC (mAb 3F4), NB	Downregulated with differentiation in granulocytes
Human	Blood	Monocytes, T cells, NK cells, B cells	FC (mAbs 3F4, 3F5)	Up regulated in activated T cells and monocytes, not uniform among NK cells, low in B cells

Human	Blood	Monocytes, T cells, B cells, DC	FC (4 distinct mAbs)	Upregulated in activated T cells
Human	Blood	$CD34^+$ cells, megakaryocytes, platelets	WB, FC, IHC (mAb 6H4), EM (pAb P3), RT-PCR	Surface PrP^C increases upon platelet activation
Mouse	Bone marrow, thymus, fetal liver, *not* spleen, *not* peritoneum	Haemopoietic stem cells, immature thymocytes, *not* peripheral blood leukocytes, *not* gut intraepithelial lymphocytes	FC (mAb 6H4)	
Mouse	Skin, thymus, spleen, lymph nodes	DC	FC (mAb SAF83)	Heterogeneous distribution among DC subtypes; absent in B220+ DCs; up regulated with DC maturation
Bovine	Spleen, lymph nodes, blood	Follicular DC, B cells, lymphocytes, monocytes, PMN	IHC, FC (mAb 6H4)	Monocytes and PMN immunoreactivity low, B cells high
Sheep	Blood	B cells, T cells, monocytes, *not* granulocytes, *not* erythrocytes	FC (mAbs 8H4, 5B2, 7A12), RT-PCR	Platelet fraction contained PrP mRNA, *not* surface PrP^C
Sheep	Blood	PBMC, platelets, *not* granulocytes	WB, FC (mAbs FH11, 4F2. 8G8, 6H4)	PrP^C in platelets intracellular only; Level of PrP^C expression in B cells higher in scrapie-susceptible than in scrapie-resistant genotype

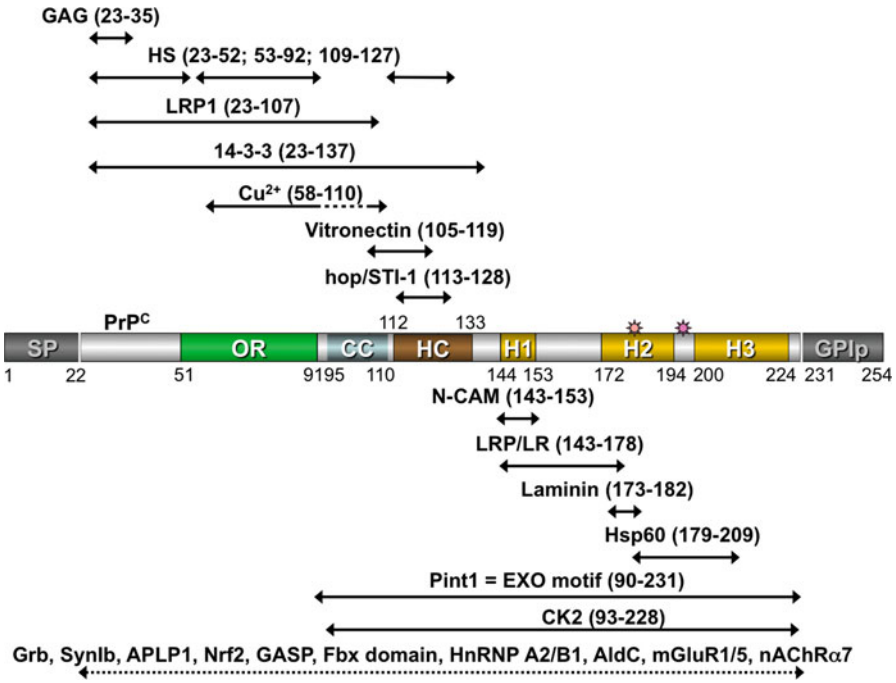
Modified from Linden et al. (2008)

Abbreviations: *DC* dendritic cell, *EM* electron microscopy, *FC* Flow Cytometry, *GAx* glutaraldehyde cross-linked epitopes, *IHC* immunohistochemistry, *ISH* in situ hybridization, *mAb* monoclonal antibody, *NB* Northern Blot, *pAb* polyclonal antiserum, *pAbMo* polyclonal antiserum raised in PrP-null mouse, *PMN* polmorphonuclear, *WB* Western Blot, *3F4, 3F5, 4F2, 5B2, 6H4, 7A12, 8G8, 8H4, FH11, HumP, SAF61, SAF83* designations of monoclonal antibodies, *Br-1, N10, p3, P38, Ra5, R254, R073* designations of polyclonal antisera

Prion Protein (*PRNP*), Table 2 Putative physiological ligands of the prion protein

Ligand	Description	Method
αB-crystalline	Stress-induced, small heat shock protein	Two-hybrid screen, N-PAGE, optical biosensor
β-Dystroglycan	Transmembrane protein	coIP, detergent-sensitivity
14-3-3	Intracellular scaffolding protein	Overlay, MS
50, 56, 64, 72, 110 kDa proteins	Unidentified	Overlay
Aldolase C/zebrin II	Glycolytic pathway enzyme	Overlay, coIP, MS
APLP1	Amyloid precursor-like protein	*Lambda-gt11* mouse brain cDNA library
▶ Bcl-2	Anti-apoptotic protein	Two-hybrid screen
BiP/Grp78	Endoplasmic reticulum chaperone	coIP
▶ CK2	Protein kinase	Overlay, SPR
Cu^{+2} ions	Metal	
DNA	Nucleic acid	Biophysical measurements
Fbx6/Fbxo2	Substrate recognition unit of ubiquitin ligase complex (Fig. 2c)	*Lambda-gt11* mouse brain cDNA library
Fyn, ▶ ZAP-70	Protein tyrosine kinases, soluble	coIP
GASP	G protein-coupled receptor-associated sorting protein (Fig. 2b)	*Lambda-gt11* mouse brain cDNA library
GFAP	Intermediate filament protein	Overlay
Glycosaminoglycans	Glycosaminoglycans	
Grb	Adaptor protein for tyrosine kinase receptors	Two-hybrid screen
Heparin/heparan sulfate	Glycosaminoglycans	SPR, ELISA
hnRNP A2/B1	RNA-binding protein	Overlay, coIP, MS
Hop/STI1	Co-chaperone	Complementary hydropathy, binding assays
Hsp60	Chaperone	Two-hybrid screen
Laminin	Extracellular matrix component	Binding assay
LRP/LR	Laminin receptor precursor/laminin receptor	Two-hybrid, cell binding
LRP1	Scavenger receptor	Cross-linking, coIP, binding assay
mGluR1/R5	Glutamate receptor	Phage display; coIP
nAChRα7	Acetylcholine receptor	Phage display; coIP
▶ N-CAM	Cell adhesion molecule	Cross-linking, coIP
NMDAR	Glutamate receptor	coIP
▶ Nrf2	Transcription factor	*Lambda-gt11* mouse brain cDNA library
Pint1	Exonuclease motif (Fig. 2a)	Two-hybrid screen
PSD-95	Post-synaptic density scaffolding protein	*Lambda-gt11* mouse brain cDNA library
PTPD1	Protein tyrosine phosphatase, soluble	*Lambda-gt11* mouse brain cDNA library
RNA	Nucleic acid	EMSA
Synapsin Ib	Synaptic vesicle release regulator	Two-hybrid screen
Vitronectin	Extracellular matrix component	Binding assay

Modified from Linden et al. (2008)
Abbreviations: *ELISA* enzyme-linked immunosorbent assay, *EMSA* Electrophoretic mobility shift assay, *coIP* coimmunoprecipitation, *MS* mass spectrometry, *SPR* surface plasmon resonance, *N-PAGE* non-denaturing polyacrylamide gel electrophoresis, *n.d.* not determined



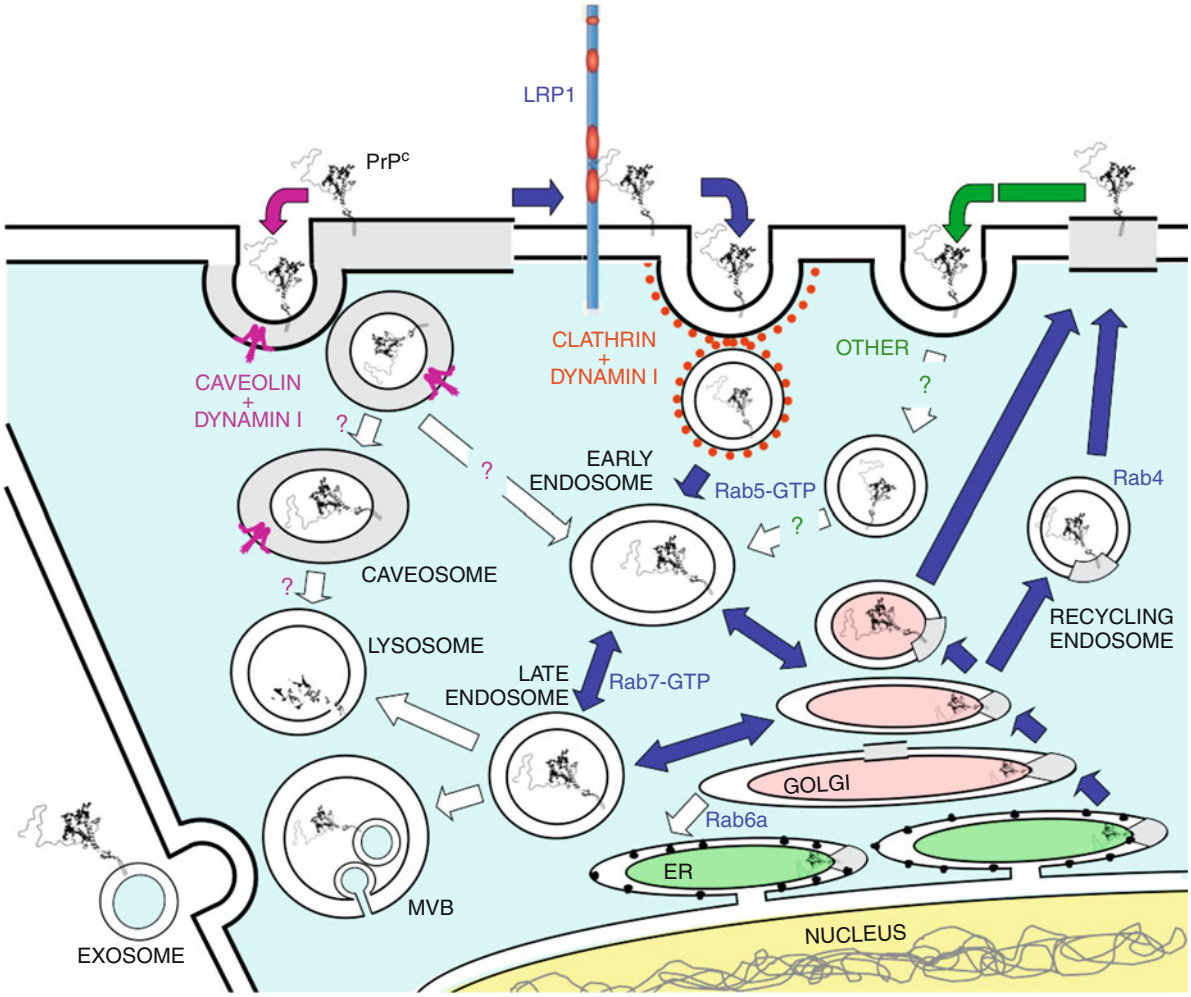
Prion Protein (*PRNP*), Fig. 2 The translated sequence of the mouse prion protein is depicted as a rod-like shape, with major domains shown in color such as in Fig. 1. Each binding partner is indicated together with the stretch of aa. residues that contain their binding domain in mouse PrP^C. *Pink stars* indicate the position of the glycosylation residues. The *double arrow* straddling the whole mature molecule represents undetermined binding site for the partners listed at the bottom of the figure. Abbreviations as in Fig. 1, and Table 2, plus: *SP* signal peptide, *GPIp* PI anchor-signaling peptide (Modified from Fig. 3 in Linden et al. 2008)

signals in cells by coupling PrP^C to the alpha7 nicotinic acetylcholine receptor, triggers the internalization of PrP^C, which regulates the extent of PrP^C modulated neuronal signaling.

Systemic Functions of the Prion Protein

The systemic functions of the prion protein have been addressed in PrP-null mice, in transgenic mice overexpressing PrP^C, or in transgenic mice expressing deletion mutants of PrP^C (Linden et al. 2008; Weissmann et al. 1998; Wadsworth and Asante 2010; Martins et al. 2010 for reviews). Although no overt phenotypic changes were described in the first generated PrP-null animals, later studies showed altered patterns of sleep, enhanced locomotor activity, and increased anxiety, possibly because of changes in the glutamatergic system. Impairment of both short- and long-term memory is found in old PrP-null mice. On the other hand, spatial memory is impaired in young PrP-null mice, which can be rescued upon PrP^C expression in neurons. Impairment of memory formation and retention in rats also followed the blockade of PrP^C interaction with its ligands, by direct hippocampal infusion of anti-PrP^C antibodies or competitor peptides.

At the synaptic level, impairment in long-term potentiation (LTP) was found in the CA1 area of the hippocampus of PrP^C-null mice when experiments were done at physiological temperature. These results were, however, not reproduced when LTP was examined at room temperature. LTP in hippocampal CA1 is at the root of memory formation of one-trial inhibitory (passive) avoidance in the rat. Thus, changes in LTP in PrP-null mice may explain at least in part their memory impairment, as may be the case following PrP^C blockade by specific antibodies. Changes in the afterhyperpolarization potential (AHP) were also detected both in constitutive PrP^C-null mice, and in conditional



Prion Protein (*PRNP*), Fig. 3 Subcellular trafficking of PrP^C. The plasma membrane is represented by *thick lines*, intracellular membranes by *thinner lines*, and lipid rafts by a *gray*-shaded wider bilayer. The cytoplasm is colored *light blue*. PrP^C is depicted similar to Fig. 1, including a loose representation of the N-terminal flexible domain. The major vesicular systems involved in both the synthesis (*bottom right*), the endocytic cycle of PrP^C, and release of PrP^C-containing exosomes (*bottom left*) are indicated with *black* lettering, and the major pathways of trafficking are indicated in *colored* letters. Molecules involved in the clathrin-dependent pathway are indicated in *dark blue* lettering. *Arrows* indicate trafficking, the most likely pathways based on current experimental evidence are shown with *dark blue arrows*. Clathrin is represented by *red circles*, caveolin is depicted in *purple*. Note that PrP^C is associated with rafts early in the secretory pathway and that cell surface PrP^C leaves the rafts and associated with LRP1 on its way to internalization via clathrin. Abbreviations: *MVB* multivesicular bodies, *ER* endoplasmic reticulum, *LRP1* Low density lipoprotein receptor like protein-1 (Modified from Linden et al. 2008)

knockouts in which the expression of PrP^C is abolished at 12 weeks of age. This indicates that this phenotype was caused by neural dysfunction, rather than by a developmental deficit.

Although the changes in the central nervous system are subtle, and do not compromise the life of the PrP-null mice, the expression of some deletion mutants such as PrPΔ32-121, PrPΔ32-134, PrPΔ94-134 in knockout mice causes neurodegeneration. In particular, the deletion of amino acids 105–125 causes cerebellar atrophy, loss of cerebellar granule cells, gliosis, and astrocytic hypertrophy, with decreased body size, immobility, myoclonus, and death within one month after birth. This suggests that compensatory

mechanisms effective in PrP-null mice do not function upon expression of specific deletion mutants. Remarkably, these deleted domains contain sites for binding of ligands that promote specific responses at the cellular level (Linden et al. 2008; Martins et al. 2010 for reviews).

PrP^C has also been implicated in protection against brain insults. In PrP-null mice the threshold for seizures is lower than in wild-type. In addition, PrP-null mice suffered more extensive damage in the brain than wild-type following either hypoxic-ischemic insult or administration of ethanol. Remarkably, overexpression of PrP^C reduced infarct volume and improved neurobehavioral signals after cerebral ischemia in rats.

In transgenic mouse models of amyotrophic lateral sclerosis, the expression of human mutated superoxide dismutase 1 (SOD1) in the absence of PrP^C causes significantly reduced life span, an earlier onset, and accelerated progression of disease when compared with control transgenic mice expressing PrP^C. In this case, PrP^C has a pivotal function in the control of neuronal and/or glial factors associated with antioxidant defenses (Steinacker et al. 2010). Recently, it was also shown that the expression of the prion protein in axons is required for maintenance of peripheral myelin (Bremer et al. 2010).

The absence of PrP^C is associated with altered sensitivity to injury not only in the central nervous system, but also in other tissues. PrP-null mice showed impairment of locomotor activity under extreme exercise conditions. PrP^C is also a relevant regeneration factor in acutely damaged muscle (Stella et al. 2010). Moreover, a uniform pattern of increased PrP^C expression was described in a series of muscular disorders, as well as in an experimental model of chloroquine-induced myopathy, which suggests a role for PrP^C in muscle physiology (Linden et al. 2008 for review).

The expression of PrP^C is variable both across species and among subsets and states of maturation of immune cells. Although PrP-null mice do not present gross defects in the immune system, PrP^C modulates the ability of long-term hematopoietic stem cells (HSC) to sustain self-renewal under stress. PrP-null mice also showed altered inflammatory responses, and it has been shown that PrP^C in dendritic cells is a positive regulator of the immunological synapse (Linden et al. 2008; Nitta et al. 2009; Isaacs et al. 2006 for reviews).

Cellular Functions of the Prion Protein

Studies of the expression of PrP^C during development, and also its cellular distribution in neuronal cell body, dendrites, and axons provided conflicting results, highlighting the difficulties in attributing function to PrP^C on the basis of its distribution. Nonetheless, a large number of studies have identified roles of PrP^C in cell proliferation, differentiation, and cell death in both neural precursors and central neurons. In addition, functions were also attributed to PrP^C in peripheral neurons, lymphoid cells, and some tumor cells (Linden et al. 2008; Martins et al. 2010; Mehrpour and Codogno 2010) (Table 3).

PrP^C in a cell can modulate cellular functions of either the same (in *cis*) or of a distinct cell (in *trans*). The *trans* effects were demonstrated in experiments with recombinant, Fc-bound soluble PrP^C or its fragments, GPI-anchorless recombinant forms, or using a feeder layer of PrP^C expressing cells. The evidence that PrP^C can be secreted by exosomes from various cell types extends the possibilities of *trans* effects at both the tissue and system levels (Porto-Carreiro et al. 2005).

The rate of proliferation of neuronal precursors correlated with PrP^C content in both the subventricular zone and dentate gyrus of the hippocampus of adult mouse brain, but PrP^C expression in proliferating zones was restricted to postmitotic neurons. Thus, the effect of PrP^C upon proliferation of neuronal precursors are probably indirect, a possibility that must be considered when dealing with complex tissues. One hypothesis, not yet explored, is the potential effect of exosomes containing PrP^C from postmitotic neurons. PrP^C can also affect the proliferation of splenic lymphocytes, splenocytes, and enterocytes.

The role of PrP^C in neurite and axon outgrowth has been demonstrated both in human and mouse neurons from hippocampus, cerebellum, cortex, dorsal root ganglia, as well as in neuronal derived cell lines. These effects are mediated by PrP^C acting in *cis* upon binding to specific ligands, such as hop/STI1, laminin, and NCAM, and in *trans* together with either NCAM or other unidentified ligands at the cell surface. PrP^C also modulates astrocyte differentiation, secretion of neurotrophic factors, and metabolism. These results are consistent with PrP^C functions at system levels (see Sect. Systemic functions of the prion protein).

Prion Protein (*PRNP*), Table 3 Cellular functions of PrP^C

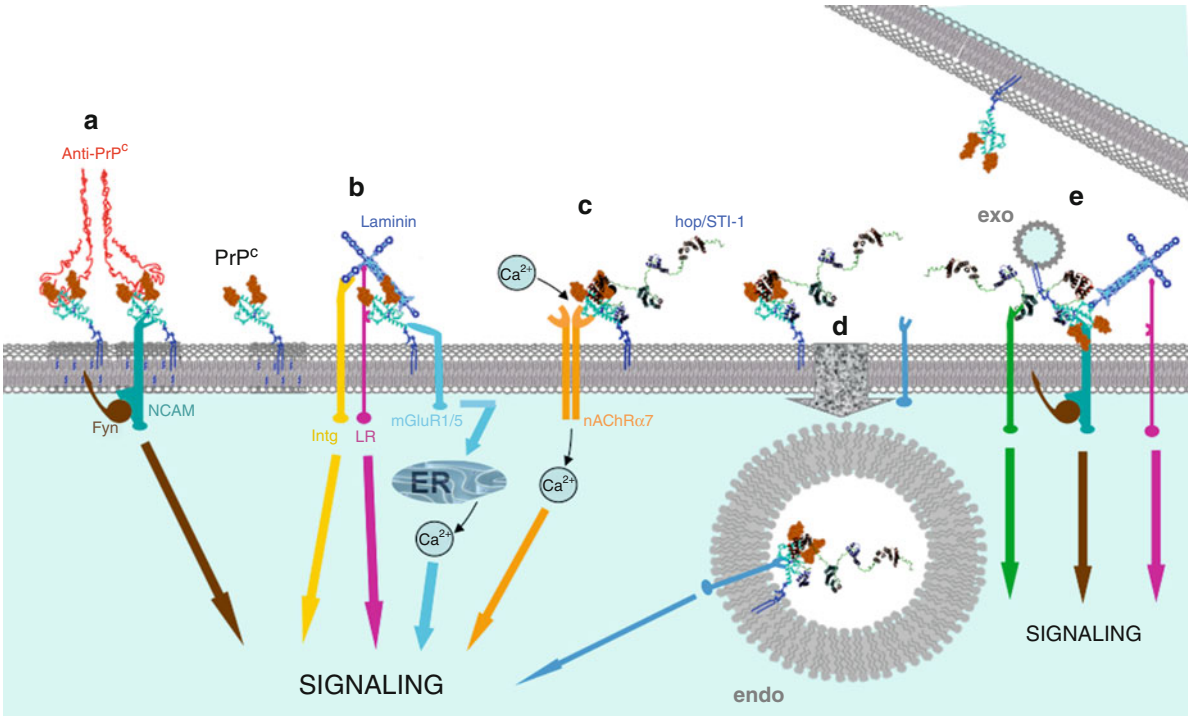
Cell type/organism	Function	Type of interaction/ligand
Neuronal precursors; subventricular zone; dentate gyrus from PrP-null; and wt mice	↑Proliferation	*Cis*-acting PrP
Splenic lymphocytes from PrP-null and wt mice	↑ Mitogen-induced proliferation	*Cis*-acting PrP
Splenocytes from PrP-null and wt mice	↑ or ↓ Mitogen-induced proliferation	*Cis*-acting PrP
Human glioblastoma cell line	↑Proliferation	*Cis*-acting/PrP-hop/STI1 binding
Cultured human enterocytes	Balance between proliferation and polarization – differentiation	*Cis*-acting/interacts with cytoskeleton and desmosome-associated proteins
Multipotent neuronal precursors from PrP-null, wt and PrP overexpressor mice	↑Differentiation	*Cis*-acting PrP
Neurons from hamster developing brain	↑Expression and localization suggest involvement in axon growth, guidance, and synaptogenesis	*Cis*-acting PrP
Hippocampal neurons from PrP-null and wt mice	↑ Neurite outgrowth	*Cis*-acting PrP
Cerebellum granule cells and hippocampal mouse neurons from PrP-null, wt mice, and PrP overexpressor mice	↑Neurite outgrowth	*Trans*-acting: PrP-Fc or PrP expressed by monolayers of CHO cells
Embryonic rat hippocampal neurons	↑Neurite outgrowth, dendrite like and single axon like process, enhanced synaptogenesis	*Trans*-acting recombinant PrP
Embryonic hippocampal mouse neurons from PrP-null and wt mice	Adhesion and neuritogenesis	PrP-laminin
Dorsal root ganglia neurons from PrP-null and wt mice	Axonal growth	PrP-vitronectin
PC-12	↑Neurite outgrowth	PrP-laminin
PC-12	↑Neurite outgrowth	PrP-NCAM
Bone marrow derived granulocytes and HL60 leukemia cells	↓Expression	*Cis*-acting PrP
	↑Differentiation	
Murine peritoneal macrophages from wt and PrP-null mice	↓Phagocytosis	*Cis*-acting PrP
Bone marrow-derived macrophage cell line	↑Phagocytosis	*Cis*-acting PrP
Murine erythroleukemia, neuroectodermal and myeloid cell lines	↑Expression	*Cis*-acting PrP
	↑Differentiation	
myc and *ras* transformed fibroblasts from wt and PrP-null mice	↑Aggregation, embolization, and metastasis	*Cis*-acting
Tissue samples from gastric adenocarcinoma	↑Expression	*Cis*-acting PrP
	↑Histopathological	
	Differentiation and tumor progression	

Embryonic hippocampal neurons from PrP-null and wt mice	↑Differentiation and survival	*Cis*-acting binding to hop/STI1
Embryonic astrocytes from PrP-null and wt mice	↑Differentiation and survival	*Cis*-acting binding to hop/STI1 or laminin
Embryonic astrocytes from PrP-null and wt mice	Regulation of glutamate-dependent lactate transport	*Cis*-acting PrP, complex of GluR2, α2/β2-ATPase, basigin, and MCT1
Immortalized hippocampal neurons from PrP-null and wt mice	Higher sensitivity to apoptosis upon serum deprivation	*Cis*-acting PrP
Fetal human neurons	Protection against bax- induced apoptosis	*Cis* and *Trans*-acting: GPI-anchored, cytosolic and anchorless PrP
Retinal explants from PrP-null and wt mice	↑Survival of undifferentiated postmitotic cells subject to inhibition of protein synthesis	*Cis*-acting
Human neurons, mouse neuronal cell lines	↑Survival, delays conformational change of Bax	*Cis*-acting
Cerebellar granule neurons from PrP-null and wt mice	↑Survival	*Trans*-acting soluble PrP
Undifferentiated post mitotic retinal neurons from PrP-null and wt mice	↑Survival	*Cis*-acting binding to Hop/STI1
Retinal ganglion cells	↑Survival in hypoxia-induced apoptosis	*Trans*-acting, soluble, alpha-secretase-derived N-terminal PrP
Embryonic primary hippocampal neurons or immortalized neuronal cell lines from PrP-null and wt mice	↑Survival	*Cis*-acting PrP:hop/STI1 binding
Cell line derived from neocortical neuronal precursors expressing SV40 large-T antigen v-Src and v-Myc or HEK293	Pro-apoptotic, ↑ sensitivity to staurosporine-induced cell death	Ectopic overexpression of PrP
1 C11 Neuroectodermal cell line	Survival, proliferation, and neuronal plasticity	*Cis*-acting
HEK293 and rabbit kidney epithelial cell line	Pro-apoptotic, ↑ sensitivity to staurosporine-induced cell death	Ectopic overexpression of PrP
Gastric cancer cells	↑Protection against apoptosis	Overexpression of PrP
Human breast cancer cell lines	↑Resistance to TNF-α induce cell death	Overexpression of PrP in resistant cells
Human breast cancer cell line	No effect upon staurosporine-induced cell death	Overexpression of PrP
Pancreatic tumors and melanona	↑Expression of unprocessed signal peptide PrP^{C} ($Pro\text{-}PrP^{C}$) associated with tumorigenesis	Pro-PrP binding to filamin A
Yeast	Survival against Bax-induced cell death	Ectopic expression of mammalian PrP

Abbreviations: *wt* wild-type, *Fc* Fc chain of IgG, *N-CAM* neural cell adhesion molecule, *GluR2* glutamate receptor 2, *MCT1* monocarboxylate transporter 1

Prion Protein (*PRNP*), Table 4 Examples of signaling mediated by the prion protein

Signaling pathway	Cell type	Method	Intracellular messenger	Transmembrane signal transfer
Tyrosine phosphorylation	Embryonic carcinoma 1C11 transformed cell line	Antibody cross-linking	Fyn tyrosine kinase	Caveolin, N-CAM?
Tyrosine phosphorylation	Central nervous system neurons	Overexpression, antibody cross-linking, ligand binding assays, co-IP	Fyn	N-CAM, receptor type protein phosphatase α
Protein kinase A	Central nervous system neurons	Peptide or hop/STI1 binding to PrP^C, PKA antagonist	cAMP	Unknown
Erk MAP kinase	Central nervous system neurons	Peptide or hop/STI1 binding to PrP^C, ► MEK inhibitor	Unknown	Unknown
Erk MAP kinase	Embryonic carcinoma 1C11, neurohypothalamic GTI-7, lymphoid BW5147, and Jurkat T cell lines	Antibody cross-linking	Fyn? ROS?	NADPH oxidase, EGFR?
Erk MAP kinase	Macrophage-like P388D-1 cell line	Soluble PrP^C-Fc fusion protein, Src and PI3-K antagonists	► Src family soluble tyrosine kinases Syk and Pyk2	PI3-kinase
Calcium influx	Central nervous system neurons	Comparison PrP-null vs. wild type; recombinant soluble PrP^C.		Voltage-gated calcium channels
Calcium influx	CEM-T and Jurkat cell lines	Antibody cross-linking		Unknown
Calcium influx	Hippocampal neurons; transfected HEK293 cells	Ligand binding		Alpha-7 nicotinic cholinergic receptor
Calcium homeostasis	Hippocampal neurons; transfected HEK293 cells	Ligand binding		Group I metabotropic ► glutamate receptors
Calcium homeostasis	Chinese hamster ovary cells; neuron-derived cell lines	Overexpression	ER release and mitochondrial uptake	store-operated calcium channels?
Protein kinase C	Splenocytes	Comparison PrP-null vs. wild type;	Calcium-dependent PKC forms	Unknown
► PI 3-kinase	Brain; neural cell lines; hippocampal neurons	Comparison PrP-null vs. wild type; transfection of cell lines; recombinant soluble PrP^C		Unknown
PI3-kinase	P388D-1 macrophage-like cell line	Recombinant soluble PrP^C; PI3-K inhibitor	Erk (reciprocal effect with Akt)	Unknown
PI3-kinase	Hippocampal neurons	Ligand binding	Akt; ► mTOR; p70S6 kinase; 4E-BP1	Unknown



Prion Protein (*PRNP*), Fig. 4 Schematic representation of cell surface signaling modules scaffolded by the prion protein. (**a**–**d**) represent *cis*-acting PrP^C. (**a**) NCAM-Fyn interaction induced by cross-linking of the prion protein; (**b**) laminin receptors, including integrin, the laminin receptor and PrP^C itself may interact in various combinations, and binding of the laminin γ-chain to PrP^C induces calcium release from intracellular stores, transduced by PrP^C-interacting, group 1 metabotropic glutamate receptors, via phospholipase C; (**c**) interaction of hop/STI1 with PrP^C leads to calcium influx mediated by the α7 nicotinic acetylcholine receptor; (**d**) certain signals induced by the interaction of hop/STI1 with PrP^C require endocytosis, and are probably mediated by a hitherto unidentified transmembrane protein; (**e**) *Trans*-acting PrP^C may be located in either neighboring cells or exosomes, and may scaffold various signaling modules. Molecular structures are used only for illustration purposes, and are not to scale. The N-terminal flexible domain of PrP^C was omitted for clarity. Abbreviations as in Table 2, plus: *exo* exosome, *endo* signaling endosome, *ER* endoplasmic reticulum. Lipid rafts are represented by a slightly thicker membrane depiction containing *blue* cholesterol molecules, only where there is evidence for the location therein of assembled complexes at the cell surface (Modified from Linden et al. 2008)

One of the clearest roles of PrP^C is upon cell survival. This is particularly relevant, since PrP^C is associated with TSEs, and, more recently was suggested to play a role in Alzheimer's disease. Although the importance of PrP^C loss-of function in neurodegeneration is still debatable, most studies using primary neuronal brain and retina cultures, immortalized neuronal cells, tumor cell lines, and even yeast support a cytoprotective function for PrP^C. Nonetheless, the expression of PrP^C may also be associated with increased cell death in certain circumstances. Remarkably, in recent years PrP^C has been implicated in tumor proliferation, progression, invasiveness, and resistance to drug treatment (Mehrpour and Codogno 2010 for review).

Together these results suggest that PrP^C may be a therapeutic target to increase neuronal survival in either acute or chronic neurodegenerative diseases. In addition, this ubiquitously expressed molecule may also represent both a new biomarker and a therapeutic target in cancer.

To explain all these possible functions of PrP^C we have raised the possibility that this molecule acts in a much more fundamental level coordinating signaling pathways at the cell surface. Proteins with unstructured domains can bind multiple partners, and are critical as intracellular scaffolds to regulate intracellular signaling. The unstructured N-terminal region of PrP^C may play a similar role and position the protein to organize signaling with multiple partners.

Mechanisms of Prp^{C}-Mediated Signal Transduction

Roles for the prion protein in signal transduction have been unraveled by various approaches. The main procedures involve the use of antibodies, modulation of protein content via either the knockout or overexpression of the *Prnp* gene, the use of anchorless, soluble recombinant forms of PrP^{C}, and engagement of PrP^{C} with one of its ligands.

In certain cases, modulation of signaling was shown by direct measurements, whereas in others, the effect of either pharmacological or molecular inhibitors was used to infer a role of PrP^{C} upon cellular responses. Both approaches may, in fact, unravel downstream responses networked to signaling pathways, instead of direct activation by the PrP^{C}. Also, when antibodies were used, some caution is necessary to interpret the results, since antibodies may have either a blocking or agonist effect, as well as either cross-linking or non-cross-linking activity. Nonetheless, a variety of signaling pathways have been shown to be modulated by the prion protein (Table 4).

Thus, current data strongly support the hypothesis that the prion protein may be physiologically engaged by a variety of extracellular and cell surface ligands, and mediates signal transduction through interaction with various transmembrane partners. The PrP^{C}-dependent signaling complexes are likely to vary among distinct cell types, depending on: the level of expression and distribution of PrP^{C}, as indicated in Table 1; the availability of ligands amongst, at least, those indicated in Table 2; structural rearrangements caused by multiple ligands; and kinetics of endocytosis/recycling. Indeed, the resulting signals likely depend on a complex interplay of allosteric effects caused by the binding to PrP^{C} of multiple partners with varying kinetics. Interactions are likely to be of significance both *in cis* and *in trans*. The latter are inferred mostly from results unraveled with the use of the soluble recombinant forms of PrP^{C}, which may correspond to PrP^{C} located at the surface of either neighboring cells, exosomes, or other exocytic particles (Linden et al. 2008).

Further analysis of signaling mediated by the prion protein is likely to unravel the mechanisms by which modulation of expression, engagement, or exposure to soluble PrP^{C} trigger proliferative, differentiating, or death/survival responses, as well as other effects upon cell metabolism, such as modulation of responses to oxidative stress, synaptic modulation, and immunomodulation. These, in turn, will likely explain the systems-level functions of PrP^{C}. An integrated systems approach may help defining signaling patterns generated by multiple pathways.

Summary and Future Directions

The currently available data suggest that the prion protein plays a significant role in signal transduction. PrP^{C} likely works as a cell surface scaffold protein, with the ability to organize multi-component complexes at the cell surface, which include other proteins, glycosaminoglycans, and free ions (Fig. 4). This role of PrP^{C} probably involves dynamic changes along its path of trafficking among distinct plasma membrane domains and endosomes. The resulting signals contribute to biological responses such as cell proliferation, differentiation, and modulation of cell death and responses to oxidative stress, synaptic modulation, and immunomodulation.

The relative lack of a spontaneous phenotype reported after deletion of the *Prnp* gene, which, for many years, has driven research interest off the physiological roles of the prion protein, may indeed invite one or more among several explanations, such as: a compensatory role of other members of the prion family; complex regulatory changes among networked signaling pathways; or a more subtle role of PrP^{C} as a modulator of signal transduction, particularly in the processing of either systemic or cellular stress and danger signals. Further investigation of these hypotheses, as well as of the proposed role of PrP^{C} as a cell surface scaffold protein, may contribute to a better understanding of its physiological functions, as well as to the establishment of effective therapeutic options for the still incurable transmissible spongiform encephalopathies.

Acknowledgments The authors' research groups have been supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Programa Institutos Nacionais de Ciência e Tecnologia (CNPq/MCT), Ludwig Institute for Cancer Research, and PrioNet-Canada. V.R.M. is an International Scholar of the Howard Hughes Medical Institute.

References

Bremer J, Baumann F, Tiberi C, Wessig C, Fischer H, Schwarz P, Steele AD, Toyka KV, Nave KA, Weis J, Aguzzi A. Axonal prion protein is required for peripheral myelin maintenance. Nat Neurosci. 2010;13(3):310–318.

Isaacs JD, Jackson GS, Altmann DM. The role of the cellular prion protein in the immune system. Clin Exp Immunol. 2006;146(1):1–8.

Knight RS, Will RG. Prion diseases. J Neurol Neurosurg Psychiatry. 2004;75(Suppl 1):i36–i42.

Linden R, Martins VR, Prado MA, Cammarota M, Izquierdo I, Brentani RR. Physiology of the prion protein. Physiol Rev. 2008;88(2):673–728.

Martins VR, Beraldo FH, Hajj GN, Lopes MH, Lee KS, Prado MM, et al. Prion protein: orchestrating neurotrophic activities. Curr Issues Mol Biol. 2010;12(2):63–86.

Mastrangelo P, Westaway D. Biology of the prion gene complex. Biochem Cell Biol. 2001;79(5):613–628.

Mehrpour M, Codogno P. Prion protein: from physiology to cancer biology. Cancer Lett. 2010;290(1):1–23.

Nitta K, Sakudo A, Masuyama J, Xue G, Sugiura K, Onodera T. Role of cellular prion proteins in the function of macrophages and dendritic cells. Protein Pept Lett. 2009;16(3):239–246.

Parkyn CJ, Vermeulen EG, Mootoosamy RC, Sunyach C, Jacobsen C, Oxvig C, et al. LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein. J Cell Sci. 2008;121(Pt 6):773–783.

Porto-Carreiro I, Fevrier B, Paquet S, Vilette D, Raposo G. Prions and exosomes: from PrPc trafficking to PrPsc propagation. Blood Cells Mol Dis. 2005;35(2):143–148.

Prado MA, Alves-Silva J, Magalhaes AC, Prado VF, Linden R, Martins VR, et al. PrPc on the road: trafficking of the cellular prion protein. J Neurochem. 2004;88(4):769–781.

Prusiner SB. Prions. Proc Natl Acad Sci USA. 1998;95(23):13363–13383.

Rudd PM, Wormald MR, Wing DR, Prusiner SB, Dwek RA. Prion glycoprotein: structure, dynamics, and roles for the sugars. Biochemistry. 2001;40(13):3759–3766.

Rutishauser D, Mertz KD, Moos R, Brunner E, Rulicke T, Calella AM, et al. The comprehensive native interactome of a fully functional tagged prion protein. PLoS One. 2009;4(2):e4446.

Soto C, Satani N. The intricate mechanisms of neurodegeneration in prion diseases. Trends Mol Med. 2010;17(1):14–24.

Steinacker P, Hawlik A, Lehnert S, Jahn O, Meier S, Gorz E, et al. Neuroprotective function of cellular prion protein in a mouse model of amyotrophic lateral sclerosi. Am J Pathol. 2010;176(3):1409–1420.

Stella R, Massimino ML, Sandri M, Sorgato MC, Bertoli A. Cellular prion protein promotes regeneration of adult muscle tissue. Mol Cell Biol. 2010;30(20):4864–4876.

Taylor DR, Hooper NM. The low-density lipoprotein receptor-related protein 1 (LRP1) mediates the endocytosis of the cellular prion protein. Biochem J. 2007;402(1):17–23.

Wadsworth JD, Asante EA, Collinge J. Contribution of transgenic models to understanding human prion disease. Neuropathol Appl Neurobiol. 2010;36(7):576–597.

Weissmann C, Fischer M, Raeber A, Bueler H, Sailer A, Shmerling D, et al. The use of transgenic mice in the investigation of transmissible spongiform encephalopathies. Rev Sci Tech. 1998;17(1):278–290.

Wuthrich K, Riek R. Three-dimensional structures of prion proteins. Adv Protein Chem. 2001;57:55–82.

PRKMK1/2

► Mek

Prkmk3

► Mek3

PRKMK6

► MKK6

PrLZ (Human)

► TPD52 (Tumor Protein D52)

Prn-i

► Prion Protein (*PRNP*)

Prnp

► Prion Protein (*PRNP*)

Prn-p

► Prion Protein (*PRNP*)

PRO2706

► Rab7a in Endocytosis and Signaling

Proliferation-Inducing Gene 17

▶ TMEM85 (Transmembrane Protein 85)

Proline-Rich Tyrosine Kinase 1

▶ ACK1

Prostaglandin

▶ Phospholipase A_2

Prostate Specific G-Protein-Coupled Receptor

▶ PSGR

Protein Farnesyltransferase

Lai N. Chan and Fuyuhiko Tamanoi
Molecular Biology Institute, Los Angeles, CA, USA
Department of Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles, CA, USA

Synonyms

CAAX farnesyltransferase; Farnesyltransferase; PFT

Historical Background

Protein prenylation is a posttranslational modification of proteins involving the addition of isoprenyl lipids (Tamanoi and Sigman 2001). It is a post-translational modification that occurs in the cytosol and is essential for the proper localization and functions of many proteins. There are three kinds of prenyltransferases: protein farnesyltransferase (FTase), protein geranylgeranyltransferase-I (GGTase-I), and Rab geranylgeranyltransferase (RabGGTase). No additional prenyltransferases have been described to date. Farnesyltransferase (FTase) catalyzes the addition of a 15-carbon farnesyl group to proteins such as Ras proteins, Rheb proteins, nuclear lamins, and Hdj2 that end with the Cys-A_1-A_2-X (CA_1A_2X) motif, where A is an aliphatic amino acid and X is usually serine, methionine, glutamine or alanine. The lipid is covalently attached to the Cys via a thioether linkage between C1 of the farnesyl group and the sulfur of Cys. Some CA_1A_2X-ending proteins such as K-Ras and N-Ras undergo geranylgeranylation catalyzed by GGTase-I when farnesylation is inhibited, in a process known as alternative prenylation (Whyte et al. 1997). Interestingly, RhoB protein can be either farnesylated or geranylgeranylated (Lebowitz et al. 1997). See chapters on Ras and Rho for the function of these proteins. A recent study identified novel peptide substrates for FTase (Hougland et al. 2009). In their study, Hougland J.L. et al. selected and screened a library of small peptides representing the C-termini of more than 200 human proteins for reactivity with FTase. They identified two peptide substrate classes with distinct sequence selectivities – one class that exhibits multiple-turnover (MTO) reactivity, and another class that exhibits single-turnover (STO) reactivity, presumably due to slow dissociation of the prenylated peptide substrate under steady-state reaction conditions. In addition, they examined the amino acid composition of the two peptide substrate classes. While the STO peptides vary significantly at both the A_2 and X residues, the MTO peptides are enriched in a non-polar amino acid at the A_2 position and a Phe, Met, or Gln at the terminal X residues (Hougland et al. 2009). Some of the STO peptide substrates identified include CVLL (R-Ras), CAKS (Rab 38), CYSN (Ubiquitin-conjugating enzyme E2 variant 1), and CPLG (Ras association domain family 1) (Hougland et al. 2009).

Protein Farnesyltransferase

Enzyme Structure

FTase is a heterodimeric, zinc metalloenzyme that consists of the α-subunit (48 kDa) and the β-subunit (46 kDa). The α-subunit is composed of fewer amino acids than the β-subunit (377 vs 437 residues)

(Tamanoi and Sigman 2001; Zhang and Casey 1996). FTase and GGTase-I share the same α-subunit; the cDNA cloning of FTase and GGTase-I confirmed that the α-subunits of both prenyltransferases are encoded by the same gene. The β-subunit of FTase is distinct from that of GGTase-I, but they share an overall amino acid similarity of about 30%. Crystal structures have revealed that both α and β subunits are composed primarily of α helices (Park et al. 1997). They are arranged in a crescent-shaped superhelix (α-subunit) that wraps around an α-α barrel (β-subunit) which has a deep central cleft that forms the active site of FTase (Park et al. 1997). Although the majority of the active site residues are derived from the β-subunit, both subunits are important for substrate binding and catalysis.

Crystal structures of FTase indicate the presence of a single Zn^{2+} ion bound to the β subunit near the α/β subunit interface (Taylor et al. 2003). The Zn^{2+} ion is required for catalytic activity and coordinates the Cys thiol of the CA_1A_2X substrate. Mg^{2+} ions have also been found to bind to the active site of FTase and can accelerate the protein farnesylation reaction by up to 700-fold (Pickett et al. 2003). Recently, Yang Y. et al. reported a computational study regarding the Mg^{2+} binding site in FTase. Their calculations support the idea that D352β plays a critical role in Mg^{2+} binding and Mg^{2+} is important for the conformational transition step of the reaction (Yang et al. 2010).

In an earlier study, crystallographic analysis of FTase and GGTase-I complexed with substrate peptides, including those that were derived from the C termini of K-Ras, H-Ras, and TC21, was performed to define rules of protein substrate selectivity for both prenyltransferases (Reid et al. 2004). They showed that residues Trp102β, Trp106β, and Tyr361β of FTase bind to the A_2 residue of the CA_1A_2X. Interestingly, their findings suggested that the X residue of the CA_1A_2X of FTase substrates can bind in one of two different sites, one found by residues Tyr131α, Ala98β, Ser99β, Trp102β, His149β, Ala151β, and Pro152β of FTase, the other formed by residues Leu96β, Ser99β, Trp102β, Trp106β, and Ala151β of FTase (Reid et al. 2004).

Mutagenesis studies and crystal structures have provided an insight into the structure of FTase in complex with the isoprenoid of farnesyl diphosphate (FPP), the prenyl donor (Reid et al. 2004; Bowers and Fierke 2004). FPP reaches to the bottom of the hydrophobic, deep central cleft of the β-subunit. The diphosphate moiety of FPP interacts with residues Lys164α, Arg291β, His248β, Lys294β, and Tyr300β at the top of the α-α barrel. Residues Lys164α, Arg291β, His248β, Lys294β, and Tyr300β form the diphosphate binding pocket. Crystal structures of inactive FTase•FPP•peptide complexes illustrate that Lys164α interacts with the α-phosphate of FPP, while His248β and Tyr300β form hydrogen bonds with the β-phosphate of FPP. Consistent with previous reports, molecular dynamic simulations of the ternary structure of FTase•FPP•acetyl-capped tetrapeptide (acetyl-CVIM) showed that residues Arg291β, Lys164α, Lys294β, and His248β contributed to the binding of the diphosphate group (Cui et al. 2005). In addition, the first isoprene unit (C1-C5) of FPP binds in an aromatic pocket consisting of residues Tyr251β, Tyr166α, Tyr200α, His248β, and His201α.

Regulation of Farnesyltransferase Activity

In one line of investigation, it was shown that insulin stimulates phosphorylation of the α-subunit by four-fold and enhances FTase activity in 3T3-L1 fibroblasts and adipocytes. In another study, phosphorylation of the α-subunit was shown to affect FTase activity. This conclusion was supported by experiments showing that insulin-stimulated vascular smooth muscle cells (VSMC) expressing a non-phosphorylatable mutant FTase-α (S60A, S62A) exhibited reduced phosphorylation and decreased FTase activity (Solomon and Goalstone 2001). Furthermore, expression of the non-phosphorylatable mutant FTase-α (S60A, S62A) in MCF-7 cells blocked IGF-1 and insulin-stimulated BrdU incorporation and cell count. Interestingly, phosphorylation of the α- and β-subunits of FTase was detected in rat adrenal medulla pheochromocytoma (PC12) cells (Kumar and Mehta 1997).

The potential regulation of prenyltransferase activity by glucose, which regulates insulin secretion in the pancreatic β-cell, was investigated (Goalstone et al. 2010). They showed that an insulinotropic concentration of glucose [20 mM] stimulated the expression of the α-subunit of FTase/GGTase-I by about threefold in insulin-secreting INS 832/13 cells and by about fourfold in isolated rate pancreatic islets, but not the β-subunit of FTase or GGTase-I. Moreover, an insulinotropic concentration of glucose stimulated FTase activity in INS 832/13 cells and rat islets by about 2.75- and 3.5-fold, respectively. Likewise,

GGTase-I activity was increased by about 3.5 fold in INS 832/13 cells and by about fourfold in rat islets following exposure to high glucose [20 mM] (Goalstone et al. 2010).

In addition, cleavage of the α-subunit of FTase by caspase-3 during apoptosis was reported (Kim et al. 2001). Serial N-terminal deletions and site-directed mutagenesis showed that residue Asp59 of the α-subunit was cleaved by caspase-3. The cleavage resulted in the inactivation of FTase and GGTase-I. In another line of investigation, it was reported that JNK is involved in the C-terminal processing of Ras proteins (Mouri et al. 2008). Inhibition of JNK was shown to prevent C-terminal processing of H-Ras and its subsequent plasma membrane localization. Interestingly, the C-terminal processing of H- and N-Ras but not K-Ras was sensitive to JNK inhibition. Further study is needed to elucidate the biological significance of the involvement of JNK in C-terminal processing of Ras proteins.

In a recent study, Zhou J. et al. (2009) have shown that FTase forms a protein complex with microtubules and a histone deacetylase HDAC6 in vitro and in cells. FTase was shown to bind microtubules via its α-subunit, and that microtubules are required for the interaction between FTase-HDAC6 (Zhou et al. 2009). Furthermore, treatment with an FTI removes FTase from the protein complex and abrogates the deacetylase activity of HDAC6, suggesting that FTase regulates the function of HDAC6 in a microtubule-dependent manner (Zhou et al. 2009).

Biological Significance of FTase in Tumor Development

Mijimolle et al. (2005) addressed the biological significance of FTase by generating mice with knockout alleles for the gene encoding the β-subunit of FTase. They showed that FTase is essential for embryonic development, but dispensable for adult homeostasis. They reported that mouse embryonic fibroblasts (MEFs) deficient in FTase-β displayed a flat morphology, and reduced motility and proliferation rates. Surprisingly, H-Ras remained associated with the membrane fraction of FTase-β knockout cells, and the development of K-Ras induced tumors was not affected by FTase-β deficiency (Mijimolle et al. 2005). In a more recent study, it was shown that the FTase-β knockout allele generated by Mijimolle et al. yielded a transcript with an in-frame deletion, raising the possibility that this mutant transcript still yielded a protein with some residual FTase activity (Yang et al. 2009).

Conditional knockout of the β-subunit of GGTase-I has been shown to reduce tumor formation and increase survival in mice expressing K-Ras-induced lung cancer (Sjogren et al. 2007). Knockout of GGTase-I-β resulted in disrupted actin cytoskeleton, reduced cell migration and proliferation in fibroblasts expressing oncogenic K-Ras (Sjogren et al. 2007). More recently, Liu et al. (2010) created a conditional knockout allele for FTase-β and reevaluated the effects of FTase-β deficiency on protein prenylation, cell proliferation, and growth of K-Ras induced tumors. Furthermore, they assessed the effect of simultaneous inactivation of both FTase and GGTase-I on the development of K-Ras-induced lung cancer by breeding mice homozygous for conditional knockout alleles in both FTase-β and GGTase-I-β. They showed that FTase-β deficiency blocked proliferation of primary and oncogenic K-Ras-expressing fibroblasts, and that inactivation of FTase-β in mice inhibited K-Ras-induced lung cancer growth and improved survival (Liu et al. 2010). Moreover, simultaneous inactivation of both FTase-β and GGTase-I-β was shown to markedly reduce tumors and improve survival without apparent pulmonary toxicity (Liu et al. 2010). These findings suggested that targeting both prenyltransferases could be useful in cancer therapeutics.

Inhibitors of Farnesyltransferase (FTIs)

There has been considerable clinical interest in developing inhibitors of farnesyltransferase (FTIs) as anticancer agents because FTase catalyzes the processing of the Ras family members. In previous preclinical studies, FTIs were shown to block the growth of numerous tumors, including H-, K-, and N-Ras transgenic mouse models (Kohl et al. 1995). However, K- and N-Ras both undergo alternative prenylation when farnesylation is inhibited (Whyte et al. 1997), suggesting that the effects of FTIs on tumor growth might be due to FTI targets other than Ras. Clinical studies of FTI have been reported. For example, a phase I study to evaluate the tolerance and benefice of a combination of FTI tipifarnib (R115777/Zarnestra®, Janssen Research Foundation) and sorafenib (a multi-kinase inhibitor) in patients with advanced cancer has been reported (Chintala et al. 2008).

Recently, a nonpeptidic FTI, LB42708, has been shown to suppress vascular endothelial growth factor-induced angiogenesis by inhibiting Ras-dependent MAPK and PI3K/Akt signaling pathways (Kim et al. 2010). FTIs have also been shown to inhibit mammalian target of rapamycin complex 1 (mTORC1) signaling (Gau et al. 2005). See chapters on MAP kinases, Akt(PKB) and mTOR for signaling pathways. Moreover, it has been shown that FTIs preferentially inhibit mTORC1 signaling in non-small cell lung cancer cells (Zheng et al. 2010).

In addition, FTIs were evaluated for the treatment of Hutchinson Gilford Progeria Syndrome (HGPS), a rare condition that arises from accumulation of farnesylated prelamin A (Fong et al. 2006). Costello syndrome (CS) is a congenital disorder that is characterized by mental retardation, distinctive facial appearance, cardiovascular abnormalities, tumor predisposition, and skin and musculoskeletal abnormalities (Lin et al. 2005). Interestingly, about 80% of CS patients have *H-Ras* mutations, primarily $H\text{-}Ras^{G12S}$ (34G → A), suggesting that the development of FTIs as therapeutics for the treatment of CS could be promising as H-Ras is a farnesylated protein.

To elucidate the mechanism of FTI selectivity, Reid T.S. and Beese L.S. examined the crystal structures of FTIs, R115777 (tipifarnib/Zarnestra®) and BMS-214662 (Bristol-Myers Squibb), complexed with mammalian FTase (Reid and Beese 2004). Both FTIs are selective toward FTase, with almost no activity against GGTase-I. It was shown that both FTIs bind to the active site of FTase, and that drug binding does not induce a change in the structure of the active site of the enzyme. Furthermore, they showed that both FTIs bind as a ternary complex with farnesyl diphosphate (FPP) and coordinate the catalytic zinc ion (Reid and Beese 2004).

Summary

Protein farnesyltransferase is a heterodimeric enzyme catalyzing farnesylation of proteins ending with the CA_1A_2X motif found at the C-termini of proteins such as Ras, Rheb, nuclear lamins and the γ-subunit of some heterotrimeric G-proteins. The enzyme consists of the α- and β-subunits and its crystal structure has been determined. Regulation of the enzyme activities has been reported. Finally, a variety of small molecule inhibitors have been developed against the enzyme with the intention to inhibit membrane association of signaling proteins such as Ras.

References

Bowers KE, Fierke CA. Positively charged side chains in protein farnesyltransferase enhance catalysis by stabilizing the formation of the diphosphate leaving group. Biochemistry. 2004;43:5256–65.

Chintala L, Kurzrock R, Fu S, Naing A, Wheler JJ, Moulder SL, Newman R, Gagel R, Sebti S, Wright JJ, Hong DS. Phase I study of tipifarnib and sorafenib in patients with biopsiable advanced cancer. J Clin Oncol. 2008;26:3595 (NCI protocol 7156).

Cui G, Wang B, Merz KM. Computational studies of the farnesyltransferase ternary complex part I: substrate binding. Biochemistry. 2005;44:16513–23.

Fong LG, Frost D, Meta M, Qiao X, Yang SH, Coffinier C, Young SG. A protein farnesyltransferase inhibitor ameliorates disease in a mouse model of progeria. Science. 2006;311:1621–3.

Gau CL, Kato-Stankiewicz J, Jiang C, Miyamoto S, Guo L, Tamanoi F. Farnesyltransferase inhibitors reverse altered growth and distribution of actin filaments in Tsc-deficient cells via inhibition of both rapamycin-sensitive and -insensitive pathways. Mol Cancer Ther. 2005;4:918–26.

Goalstone M, Kamath V, Kowluru A. Glucose activates prenyltransferases in pancreatic islet beta-cells. Biochem Biophys Res Commun. 2010;391:895–8.

Hougland JL, Hicks KA, Hartman HL, Kelly RA, Watt TJ, Fierke CA. Identification of novel peptide substrates for protein farnesyltransferase reveals two substrate classes with distinct sequence selectivities. J Mol Biol. 2009; 395:176–90.

Kim KW, Chung HH, Chung CW, Kim IK, Miura M, Wang S, Zhu H, Moon KD, Rha GB, Park JH, Jo DG, Woo HN, Song YH, Kim BJ, Yuan J, Jung YK. Inactivation of farnesyltransferase and geranylgeranyltransferase I by caspase-3: cleavage of the common alpha subunit during apoptosis. Oncogene. 2001;20:358–66.

Kim CK, Choi YK, Lee H, Ha K-S, Won M-H, Kwon Y-G, Kim Y-M. The farnesyltransferase inhibitor LB42708 suppresses vascular endothelial growth factor-induced angiogenesis by inhibiting ras-dependent mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signal pathways. Mol Pharmacol. 2010;78:142–50.

Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ, Giuliani EA, Gomez RP, Graham SL, Hamilton K, et al. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nat Med. 1995;1:792–7.

Kumar A, Mehta KD. p21ras farnesyltransferase alpha- and beta-subunits are phosphorylated in PC-12 cells: TGF-beta signaling pathway independent phosphorylation. Neurosci Lett. 1997;231:143–6.

Lebowitz PF, Casey PJ, Prendergast GC, Thissen JA. Farnesyltransferase inhibitors alter the prenylation and

growth-stimulating function of RhoB. J Biol Chem. 1997;272:15591–4.
Lin AE, Gripp KG, Kerr BK. Management of genetic syndromes. 2nd ed. Hoboken: Wiley; 2005.
Liu M, Sjogren AK, Karlsson C, Ibrahim MX, Andersson KM, Olofsson FJ, Wahlstrom AM, Dalin M, Yu H, Chen Z, Yang SH, Young SG, Bergo MO. Targeting the protein prenyltransferases efficiently reduces tumor development in mice with K-RAS-induced lung cancer. Proc Natl Acad Sci USA. 2010;107:6471–6.
Mijimolle N, Velasco J, Dubus P, Guerra C, Weinbaum CA, Casey PJ, Campuzano V, Barbacid M. Protein farnesyltransferase in embryogenesis, adult homeostasis, and tumor development. Cancer Cell. 2005;7:313–24.
Mouri W, Tachibana K, Tomiyama A, Sunayama J, Sato A, Sakurada K, Kayama T, Kitanaka C. Downregulation of Ras C-terminal processing by JNK inhibition. Biochem Biophys Res Commun. 2008;371:273–7.
Park H-W, Boduluri SR, Moomaw JF, Casey PJ, Beese LS. Crystal structure of protein farnesyltransferase at 2.25 angstrom resolution. Science. 1997;275:1800–4.
Pickett JS, Bowers KE, Fierke CA. Mutagenesis studies of protein farnesyltransferase implicate aspartate beta 352 as a magnesium ligand. J Biol Chem. 2003;278:51243–50.
Reid TS, Beese LS. Crystal structures of the anticancer clinical candidates R115777 (Tipifarnib) and BMS-214662 complexed with protein farnesyltransferase suggest a mechanism of FTI selectivity. Biochemistry. 2004;43:6877–84.
Reid TS, Terry KL, Casey PJ, Beese LS. Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. J Mol Biol. 2004;343:417–33.
Sjogren AK, Andersson KM, Liu M, Cutts BA, Karlsson C, Wahlstrom AM, Dalin M, Weinbaum C, Casey PJ, Tarkowski A, Swolin B, Young SG, Bergo MO. GGTase-I deficiency reduces tumor formation and improves survival in mice with K-RAS-induced lung cancer. J Clin Invest. 2007;117:1294–304.
Solomon CS, Goalstone ML. Dominant negative farnesyltransferase alpha-subunit inhibits insulin mitogenic effects. Biochem Biophys Res Commun. 2001;285:161–6.
Tamanoi F, Sigman DS, editors. The enzymes, vol. 21. San Diego: Academic Press; 2001.
Taylor JS, Reid TS, Terry KL, Casey PJ, Beese LS. Structure of mammalian protein geranylgeranyltransferase type-I. EMBO J. 2003;22:5963–74.
Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, Bishop WR, Pai JK. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem. 1997;272:14459–64.
Yang SH, Bergo MO, Farber E, Qiao X, Fong LG, Young SG. Caution! Analyze transcripts from conditional knockout alleles. Transgenic Res. 2009;18:483–9.
Yang Y, Chakravorty DK, Merz KM. Finding a needle in the haystack: computational modeling of Mg2+ binding in the active site of protein farnesyltransferase. Biochemistry. 2010;49:9658–66.
Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem. 1996;65:241–70.
Zheng H, Liu A, Liu B, Li M, Yu H, Luo X. Ras homologue enriched in brain is a critical target of farnesyltransferase inhibitors in non-small cell lung cancer cells. Cancer Lett. 2010;297:117–25.
Zhou J, Vos CC, Gjyrezi A, Yoshida M, Khuri FR, Tamanoi F, Giannakakou P. The protein farnesyltransferase regulates HDAC6 activity in a microtubule-dependent manner. J Biol Chem. 2009;284:9648–55.

Protein Kinase B/Akt

▶ Glycogen Synthase Kinase-3

Protein Kinase CK2

▶ Casein Kinase II

Protein Kinase Interferon-Induced Double-Stranded RNA-Activated (PRKR)

▶ PKR

Protein Kinase RNA-Activated (PKR)

▶ PKR

Protein Kinase, Mitogen-Activated Kinase 3

▶ Mek3

Protein Phosphatase 1 DARPP-32 Inhibitor Protein

▶ DARPP

Protein Phosphatase 1, Regulatory (Inhibitor) Subunit 1B

▶ DARPP

Protein Serine/Threonine-Phosphatase 2C

▶ PP2C

Protein Tyrosine Kinase-6 (PTK6)

Amanda Harvey
Brunel Institute for Cancer Genetics and Pharmacogenomics, Brunel University, West London, UK

Synonyms

*Br*east Tumor *K*inase (Brk)

Historical Background

The intracellular protein tyrosine kinase, PTK6 (also known as the breast tumor kinase, Brk), has been implicated in the development and progression of a number of different tumor types. First identified in three separate studies in the early 1990s, it was initially found in a study to determine which tyrosine kinases were expressed in human melanocytes and then the full-length sequence was subsequently cloned from metastatic breast cancer using a screen to identify novel kinases that were expressed in tumors, but not normal breast tissue. Identification of the murine orthologue, Sik, in mouse intestinal cells was achieved by the generation of a library of kinase catalytic domains.

PTK6 is related to the Src family of protein kinases and belongs to a distinct class of enzymes which includes Frk and SRMS (reviewed in Brauer and Tyner 2010). Under normal physiological conditions, PTK6 expression is highly controlled and is usually limited to differentiating cells of epithelial origin. Several studies have reported that, pathologically (i.e., in disease tissue), PTK6 expression is increased in a number of different cancers compared to normal cells of the same tissue type (reviewed in Brauer and Tyner 2010; Ostrander et al. 2010). The apparent disparity in PTK6 expression and the difference in proliferation status between cellular differentiation in normal tissues, where cells tend not to be dividing; and the fact that tumors are highly proliferative (and cells are dividing rapidly) makes PTK6 an intriguing molecule for study, as there are likely to be both context- and function-specific differences that could be exploited in the development of antitumor therapies conferring benefit to patients.

Cellular Localization

PTK6 has been reported to have different functions in different tissue types; for example, in normal tissues PTK6's role appears to be related to regulating the differentiation process, whereas in tumors PTK6 promotes proliferation and cell survival. Variations in cellular localization are thought to be associated with PTK6's opposing roles in both differentiation and proliferation. Altered cellular localization will no doubt affect the variety of substrates and binding partners that are available for PTK6, thereby contributing to the different functions/effects that have been ascribed to PTK6 expression.

Myristoylation is a post- or co-translational protein modification, whereby a fatty acid-derived group is attached to an N-terminal amino acid. Such modifications allow proteins to associate with membrane structures. Although PTK6 is structurally related to Src, it lacks the amino-terminal myristoylation site (Mitchell et al. 1994). Without a myristoylation site, PTK6 is not able to associate directly with the plasma membrane, and therefore cellular localization is not tightly regulated. Originally PTK6 was thought to be solely a cytoplasmic kinase, however, it is now known to be in different cellular compartments, including at the membrane via association with its binding partners as well as in the nucleus (reviewed in Brauer and Tyner 2010). Association of a protein to the plasma membrane can be mimicked by experimental inclusion of a myristoylation site. Adding a myristoylation site to the N-terminus, enhanced PTK6's oncogenic role by promoting cell proliferation, survival, and migration of human embryonic kidney cells. Trapping PTK6 in the nucleus with synthetic nuclear localization signal abrogated these effects (Kim and Lee 2009); these in vitro experimental effects are supported by evidence from human tumors demonstrating that normal prostate epithelial cells and well-differentiated prostate carcinomas

had nuclear PTK6, whereas as poorly differentiated prostate cancers were found to have cytoplasmic PTK6 (Derry et al. 2003). These studies suggest that PTK6's oncogenic role may be dependent on its cellular localization.

As discussed, PTK6 contains neither myristoylation sites nor nuclear localization signals so it is unclear how PTK6 delocalizes from one subcellular compartment to another and which cellular signals are controlling this transition. It is possible that PTK6 is held in the cytoplasm by an as yet unidentified protein (Brauer and Tyner 2010).

PTK6 Substrates and Binding Partners

As a tyrosine kinase with amino acid sequence homology to Src, PTK6 also has a similar domain structure consisting of SH2 and SH3 domains (which typically interact with phosphorylated tyrosines and proline-rich sequences, respectively) as well as a kinase domain (which phosphorylates its target substrates on tyrosine). It is therefore capable of phosphorylating a number of target molecules and there is a rapidly expanding list of known interacting proteins (summarized in Table 1). To date, 29 PTK6-associated interactions have been identified (Irie et al. 2010; reviewed in Brauer and Tyner 2010), but whether all of these interactions result in phosphorylation has not yet been determined. PTK6 is proposed to have some kinase independent function (Harvey and Crompton 2003), suggesting that a functional kinase domain is not required for all of PTK6's activities. It has been suggested that it may function as an adaptor molecule, and therefore it is highly likely that not all the interacting proteins will be substrates of PTK6's kinase activity. Therefore it is possible that one of PTK6's functions is to stabilize signaling complexes to allow phosphorylation of some of its interacting proteins and/or additional molecules within the complex by other kinases. This association in a large signaling complex, as an adaptor or scaffolding molecule, may also contribute to PTK6's cellular localization.

PTK6 and Signaling Pathways

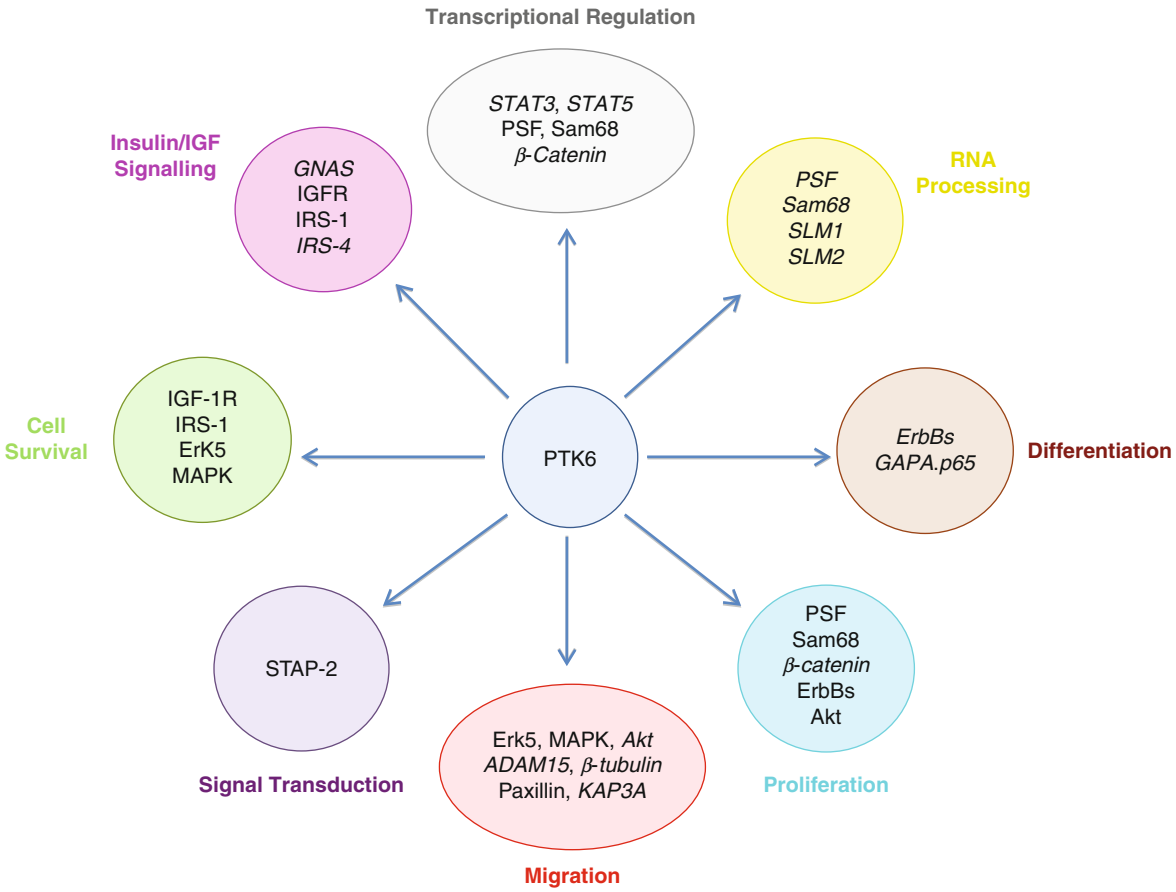
From the variety of interacting proteins that have already been identified (Table 1), it is clear that PTK6 plays a role in a number of different cellular processes and signaling pathways (Fig. 1). This is also apparent from the range of signaling molecules that activate PTK6, which include epidermal growth factor (EGF), Heregulin (HRG), insulin-like growth factor (IGF), Hepatocyte growth factor (HGF), osteopontin (OPN), and calcium ions.

Although still incomplete, the PTK6-ErbB receptor signaling "picture" is the most comprehensive in term of our current knowledge relating to PTK6's role in tumor cells. PTK6 has been shown to associate with all members of the ErbB receptor family (reviewed in Ostrander et al. 2010) and potentiates the proliferative effects of EGF by activating the phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway (Kamalati et al. 2000). PTK6 is known to play a role in cell migration through the phosphorylation of paxillin, which is a "molecular scaffold" that interacts with signaling molecules and a number of proteins that are involved in cell motility. In response to EGF, activated PTK6 phosphorylates paxillin resulting in the activation of Rac1 and an increase in cell migration

Protein Tyrosine Kinase-6 (PTK6), Table 1 Summary of the localization of the major identified PTK6-interacting proteins

Membrane Proteins	Intracellular Cytoplasmic Proteins	Nuclear-Protein
EGFR	ARAP-1	β-Catenin*
HER2	IRS-1	Sam68
ErbB3	IRS-4	SLM-1
ErbB4	Erk5	SLM-2
IGF-1R	β-Catenin*	PSF
ADAM-15A	Akt	KAP3A*
ADAM-15B	Erk	STAT3*
β-Catenin*	MAPK	STAT5b*
	PTEN	
	Paxillin	
	KAP3A*	
	STAT3*	
	STAT5b*	
	STAP-2 (BKS)	
	GNAS	
	FL139441	
	GapA-p65	

*These proteins are reported to have functions in different cellular compartments; however, it has not yet been determined whether PTK6 affects the function of these proteins in all their localizations. A further two interactions have been reported; however, the proteins involved (which are 23 and 100 kDa in size) have yet to be identified.



Protein Tyrosine Kinase-6 (PTK6), Fig. 1 Potential biological effects of PTK6-mediated interactions. PTK6-substrate interactions result in a number of known biological effects. Known effects of the interactions are represented by normal text font, whereas italic text represents effects based on current knowledge of the PTK6-binding protein

(Chen et al. 2004). Treatment of breast cancer cells with the ErbB ligand HRG also increased cell migration, via activation of both Erk5 and an Erb-PTK6-Rac-p38 MAPK signaling pathway (Ostrander et al. 2007).

PTK6's interactions with the nuclear STAR (Signal Transduction and Activation of RNA) proteins (Sam68 and SLMs) and polypyrimidine tract-binding protein-associated splicing factor (PSF) are induced in response to EGF treatment. Phosphorylation of PSF and Sam68 (as well as the Sam68-like mammalian proteins, SLM1 and SLM2) by PTK6, results in inhibition of their RNA-binding activities. Reducing their RNA binding capability is one mechanism of reducing the function of proteins that bind RNA. As these proteins regulate a number of RNA processing events, including alternative splicing, PTK6 activation could result in the posttranscriptional regulation of gene expression. In addition EGF-mediated activation of PTK6 induced phosphorylation of Sam68 which increased cell proliferation. This presumably occurred through suppressing the antiproliferative properties of Sam68 (Lukong et al. 2005; reviewed in Ostrander et al. 2010).

PTK6 expression has also been linked to the potential regulation of IGF signaling. PTK6 interacts with the IGF-1R/IRS-1 complex resulting in increased activation of IGF-1R as well as Akt. An increase in IGF-1-mediated, anchorage-independent cell survival was also observed in both breast and ovarian cancer cells (Irie et al. 2010). Similar effects on cell survival have also been shown in serum stimulated breast cancer cells, where PTK6 again protected against cell death in suspension culture (Harvey et al. 2009). Interestingly, in IGF-1 stimulated cells PTK6 protected cells from classical apoptosis/anoikis

(Irie et al. 2010), whereas the Harvey study showed that PTK6 protected breast cancer cells from cell death via autophagy. Taken together, these studies indicate that PTK6 can protect cells from different types of programmed cell death, and that this protection could feasibly be through different mechanisms.

Furthermore, treatment of breast cancer cells with the Met receptor ligand, HGF, induced both activation of Erk5 and cell migration (Castro and Lange 2010). The PTK6/Erk5 interaction and the increased migration that was induced in response to HGF was not dependent on the kinase activity of PTK6, providing further evidence that some aspects of PTK6 function are not reliant on its kinase domain. This implies that PTK6 could coordinate the large signaling complexes that are required for cell migration, without directly phosphorylating components of the complex.

Most of the PTK6-mediated interactions and activation events result in altered cell behaviors, such as increased proliferation, cell survival, migration, and the secretion of angiogenic factors, all of which are traits that are characteristic of tumor cells and are required for tumor development (Reviewed in Hanahan and Weinberg 2000). It would appear from the majority of studies carried out in tumor cell lines, that PTK6 could play a central role in tumor progression, especially in breast cancer. In support of this hypothesis Chakraborty and colleagues showed that treating cells with OPN resulted in PTK6 activation and an increase in vascular endothelial growth factor (VEGF) production. Combined with in vivo models, they were able to demonstrate that OPN triggers VEGF-dependent angiogenesis (formation of new blood vessels) and tumor growth as a result of PTK6 activation (Chakraborty et al. 2008).

In cells where PTK6 is physiologically expressed, i.e., normal differentiating cells, the effects of PTK6 activation are distinct to those seen in tumor cells. When keratinocytes (skin cells) are treated with calcium, PTK6 is transiently activated and cell differentiation is induced, in contrast to the proliferation or migration seen in tumor cells (Wang et al. 2005; reviewed in Brauer and Tyner 2010). Recent evidence suggests that PTK6, the EGF receptor (EGFR) and a marker of differentiation, may be co-regulated during differentiation, and that the differentiation of normal primary human keratinocytes could be influenced by altered PTK6 expression (Tupper et al. 2011).

It is clear that PTK6 signaling is diverse (Fig. 1), and that PTK6's function with respect to signaling is different in normal differentiating cells to that in tumor progression. PTK6 has also been shown to associate directly with Akt (Zhang et al. 2005) and, in conjunction with results from several other studies, has lead to the hypothesis that one of PTK6's roles in normal cells could be to constrain Akt activation, possibly to allow differentiation to occur. In tumor cells, the constraint is lost and the PI3-K/Akt pathway subsequently becomes activated (Ostrander et al. 2010), thereby upregulating the processes that are known to be involved in tumor growth.

PTK6 Expression Profile

PTK6 expression is increased in many tumors compared to normal epithelial cells (reviewed in Ostrander et al. 2010; Brauer and Tyner 2010) and this is most notable and most well characterized in breast tissue. The murine homologue of PTK6, Sik, is absent in normal adult mammary gland and during the various developmental stages. PTK6 is not found to be present in normal human mammary cells or in benign lesions (reviewed in Harvey and Crompton 2004), however, separate studies have now demonstrated that up to 86% of breast cancers have elevated PTK6 expression and that the increase in expression correlated with an increase in tumor grade (Ostrander et al. 2007; Harvey et al. 2009). PTK6 expression has also been shown to correlate with HER2 overexpression (Aubele et al. 2007) and, the PTK6 gene is known to be co-amplified with HER2 in some tumors (Xiang et al. 2008). This is important from a tumor development stand-point, as interaction of PTK6 protein with HER2 induces and prolongs the activation of the MAPK pathway which would result in increased cell cycle progression and tumor cell proliferation. In addition, PTK6 is proposed to contribute to resistance to the new more specifically targeted anticancer therapy such as the EGFR/HER2 inhibitor lapatinib (Xiang et al. 2008).

These findings, taken together alongside the current knowledge of PTK6's multiple signaling roles in tumor cells, suggest that PTK6 is strongly implicated in tumor development. It could therefore be expected that PTK6 expression would have a negative impact on breast cancer patient survival. Aubele and colleagues,

however, suggest that PTK6 is a positive prognostic marker of metastasis-free survival (Aubele et al. 2007). One explanation for this arises from previous reports that PTK6 expression may also be correlated with expression of the estrogen receptor, a known positive prognostic indicator (discussed in Harvey et al. 2009). In addition, it is possible that driving tumor cells to proliferate may make them more susceptible to the effects of conventional chemotherapy agents. These are known to target actively dividing rather than "resting" cells, so increasing susceptibility to these agents could enhance the benefits of such therapy thereby aiding patient survival.

Summary

There is little doubt that the role of PTK6 is complex. It depends on a number of factors including cellular localization, accessibility of binding partners, availability of extracellular signaling molecules and potentially, at least in tumor cells, the extent to which PTK6 expression is elevated. There is the possibility that there are tumor specific modifications beyond changes in gene expression, and that there are tumor type and stage specific mechanisms of PTK6 function that need to be considered (Ostrander et al. 2010; Harvey and Crompton 2004). Both Brauer and Tyner, and Ostrander and colleagues (reviewed in Brauer and Tyner 2010; reviewed in Ostrander et al. 2010) suggested that further studies are fundamental to unraveling the complex and distinct roles that PTK6 plays in both normal differentiating cells and in tumor development. Understanding the distinctions between a pro-differentiation role in normal cells, and a potentially oncogenic role in tumor progression, is key to the development of novel therapeutic agents that could target either context-specific or cell-specific molecular functions and/or interactions with minimal effects in other tissues.

References

Aubele M, Auer G, Walch AK, Munro A, Atkinson MJ, Braselmann H, et al. PTK (protein tyrosine kinase)-6 and HER2 and 4, but not HER1 and 3 predict long-term survival in breast carcinomas. Br J Cancer. 2007;96(5):801–7.

Brauer PM, Tyner AL. Building a better understanding of the intracellular tyrosine kinase PTK6 - BRK by BRK. Biochim Biophys Acta. 2010;1806(1):66–73. doi:10.1016/j.bbcan.2010.02.003.

Castro NE, Lange CA. Breast tumor kinase and extracellular-signal-regulated kinase 5 mediate Met receptor signaling to cell migration in breast cancer cells. Breast Cancer Res. 2010;12(4):R60. doi:10.1186/bcr2622.

Chakraborty G, Jain S, Kundu GC. Osteopontin promotes vascular endothelial growth factor-dependent breast tumor growth and angiogenesis via autocrine and paracrine mechanisms. Cancer Res. 2008;68(1):152–61. doi:10.1158/0008-5472.CAN-07-2126.

Chen HY, Shen CH, Tsai YT, Lin FC, Huang YP, Chen RH. Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin. Mol Cell Biol. 2004;24(24):10558–72.

Derry JJ, Prins GS, Ray V, Tyner AL. Altered localization and activity of the intracellular tyrosine kinase BRK/Sik in prostate tumor cells. Oncogene. 2003;22(27):4212–20.

Hanahan D, Weinberg RA. Hallmarks of cancer. Cell. 2000;100:57–70.

Harvey AJ, Crompton MR. Use of RNA interference to validate Brk as a novel therapeutic target in breast cancer: Brk promotes breast carcinoma cell proliferation. Oncogene. 2003;22:5006–10.

Harvey AJ, Crompton MR. The Brk tyrosine kinase as a novel therapeutic target in breast cancer: opportunities and challenges. Anticancer Drugs. 2004;15(2):107–11.

Harvey AJ, Pennington C, Porter S, Burmi RS, Edwards DR, Court W, et al. Brk protects breast cancer cells from autophagic cell death induced by loss of anchorage. Am J Pathol. 2009;175(3):1226–34.

Irie HY, Shrestha Y, Selfors LM, Frye F, Iida N, Wang Z, et al. PTK6 regulates IGF-1-induced anchorage-independent survival. PLoS One. 2010;5(7):e11729. doi:10.1371/journal.pone.0011729.

Kamalati T, Jolin HE, Fry MJ, Crompton MR. Expression of the Brk tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signaling to PI 3-kinase and Akt, via erbB3 phosphorylation. Oncogene. 2000;19(48):5471–6.

Kim HI, Lee S-T. Oncogenic functions of PTK6 are enhanced by its targeting to plasma membrane but abolished by its targeting to nucleus. J Biochem. 2009;146(1):133–9. doi:10.1093/jb/mvp050.

Lukong KE, Larocque D, Tyner AL, Richard S. Tyrosine phosphorylation of sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression. J Biol Chem. 2005;280(46):38639–47.

Mitchell PJ, Barker KT, Martindale JE, Kamalati T, Low PN, Page MJ, et al. Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours. Oncogene. 1994;9(8):2383–90.

Ostrander JH, Daniel AR, Lofgren K, Kleer CG, Lange CJ. Breast tumor kinase (protein tyrosine kinase 6) regulates heregulin-induced activation of ERK5 and p38 MAP kinases in breast cancer cells. Cancer Res. 2007;67(9):4199–209.

Ostrander JH, Daniel AR, Lange CA. Brk/PTK6 signaling in normal and cancer cell models. Curr Opin Pharmacol. 2010;10(6):662–9. doi:10.1016/j.coph.2010.08.007.

Tupper J, Crompton MR, Harvey AJ. Breast tumor kinase (Brk/PTK6) plays a role in the differentiation of primary keratinocytes. Arch Dermatol Res. 2011. doi:10.1007/s00403-010-1118-4.

Wang TC, Jee SH, Tsai TF, et al. Role of breast tumor kinase in the in vitro differentiation of HaCaT cell. Br J Dermatol. 2005;153(2):282–9.
Xiang B, Chatti K, Qiu H, Lakshmi B, Krasnitz A, Hicks J, et al. Brk is coamplified with ErbB2 to promote proliferation in breast cancer. Proc Natl Acad Sci USA. 2008;105(34):12463–8.
Zhang P, Ostrander JH, Faivre EJ, Olsen A, Fitzimmons D, Lange CA. Regulated association of protein kinase B/Akt with breast tumor kinase. J Biol Chem. 2005;280(3):1982–91.

Protein Tyrosine Phosphatase 1 C (PTP1C)

▶ PTPN6

Protein Tyrosine Phosphatase Receptor Type C

▶ CD45 (PTPRC)

Protein Tyrosine Phosphatase, Non-receptor Type 6 (PTPN6)

▶ PTPN6

Protocadherins

▶ Cadherins

Provirus Insertion Site of Moloney Murine Leukemia Virus 1 Pim-1

▶ Pim-1

PrP

▶ Prion Protein (*PRNP*)

PrP^C

▶ Prion Protein (*PRNP*)

PrP^{Sc}

▶ Prion Protein (*PRNP*)

PSGR

Mingyao Liu[1] and Melissa Rodriguez[2]
[1]Department of Molecular and Cellular Medicine, Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, TX, USA
[2]Center for Cancer and Stem Cell Biology, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, TX, USA

Synonyms

OR51E2; OR51E3P; OR52A2; Prostate specific G-protein-coupled receptor

Historical Background

The G-protein-coupled receptor (GPCR), PSGR, was first identified by three distinct laboratories using different experimental approaches (Xu et al. 2000; Xia et al. 2001; Yuan et al. 2001). GPCRs constitute the largest gene family in the genome capable of influencing multiple signal transduction pathways; at the same time, they are the target of over half of the medications currently available in the pharmaceutical industry, demonstrating the importance of these receptors in maintaining tissue homeostasis. Research on this molecule has focused on its expression pattern and probable role as a potential biomarker for prostate cancer as well as in the possibility of a role in influencing prostate cancer initiation and progression, and the mechanisms through which this is done.

PSGR as a Molecule: Structural Analysis

Protein sequence analysis of PSGR predicted the presence of seven transmembrane domains with high homology to the olfactory subfamily of GPCRs (Xia et al. 2001). The full-length cDNA of PSGR contains an open reading frame of 320 amino acids (estimated cDNA size of 1.4 kb). Chromosomal analysis showed PSGR to be located at the GPCR cluster region in chromosome 11p15 (Xu et al. 2000). PSGR is also highly conserved among different species. The rat homolog to PSGR, RA1c, was found to have a high level of homology (~93%) with this gene even within the 5′and 3′untranslated regions. Cloning of the mouse PSGR using degenerate PCR amplification showed it is also highly homologous with 93% sequence similarity to the human gene, and 99% sequence similarity to the rat gene.

PSGR expression analysis was performed through multiple methods. First, the distribution of PSGR mRNA in 23 different normal human tissues was analyzed through Northern blot experiments. Results showed that human PSGR mRNA-specific transcripts could be detected only in the prostate (Xu et al. 2000). This data was confirmed by the other two laboratories also working on PSGR (Xia et al. 2001 and Yuan et al. 2001), with the notion of very faint signals for human PSGR in spleen and liver (Yuan et al. 2001). This expression pattern, however, does not remain true for other organisms. Northern blot analysis of mouse RNA showed expression in both brain and colon but no expression in prostate tissues. Rat tissue analysis showed PSGR expression in the brain and liver but none in prostate. These results were further confirmed by RT-PCR.

In an effort to understand the role of PSGR in prostate tissue and disease, PSGR expression levels were analyzed in prostate tumor samples through PCR and Southern blot analysis. Results show that PSGR expression is significantly increased in prostate tumor samples when compared to normal prostate tissue (Xia et al. 2001). Comparison between normal and tumor tissues showed overexpression in approximately 62% of tumor specimens. In situ RNA hybridization showed a significantly higher PSGR expression level in transformed prostate epithelial cells when compared to normal adjacent cells (Xu et al. 2000). Subsequently, a large cohort of human normal and tumor tissue samples were analyzed for PSGR expression levels. The results showed a significant overexpression of PSGR in both prostate intraepithelial neoplasia (PIN) and prostate carcinoma (~76%) when compared to normal tissues and samples with benign prostatic hyperplasia. This study suggested the possibility of a biomarker function for PSGR due to its specificity of expression and overexpression (Weng et al. 2005a).

In an in vitro setting, different prostate cancer cell lines were analyzed for PSGR expression where a weak signal was found only in LNCaP cells, leading researchers to believe that PSGR expression might be tissue context specific or that its expression in cell lines may be lost through continuous cell culture (Xu et al. 2000).

PSGR as a Biomarker for Prostate Cancer

To study the potential function of PSGR as a biomarker, pure target cells, either from normal prostate epithelium or tumor samples, were microdissected using laser capture microdissection (LCM) technology, and then amplified and analyzed. Expression levels for PSGR were graded as fold change between normal and tumor cells. PSGR overexpression was found in 67.2% of tumor samples. Also, Pearson correlation coefficients analysis showed a correlation between PSGR and PSA levels ($P = 0.03$), suggesting a role for PSGR in prostate cancer progression (Xu et al. 2006).

The possibility of a biomarker function for PSGR was further analyzed in conjunction with the already known diagnostic marker AMACR and PSGR2. AMACR or a-methylacyl-CoA racemase is also highly overexpressed in prostate cancer when compared to normal tissue. When used in conjunction with immunohistochemistry (IHC) for basal cell specific markers, this marker is very useful in solving diagnostic problems during a biopsy. Quantitative real-time PCR analysis showed that AMACR expression was related to a Gleason score of 7 but was lower in poorly differentiated tumors than in moderately differentiated tumors. Of all the three markers analyzed, however, AMACR was overexpressed in 78% of the samples, with a 30-fold increase in expression, PSGR was overexpressed in 64% of the samples, with a 10-fold increase, and PSGR2 was overexpressed in 60% of the tissues, with a 20-fold increase. PSGR was not a stronger biomarker than AMACR, according to statistical analysis, nor was there any correlation between overexpression of all three genes. There were, however, some cases where PSGR overexpression was

significantly higher than AMACR, suggesting PSGR may be useful as a biomarker for disease only in specific clinical cases (Wang et al. 2006).

PSGR was also studied as a biomarker for urine analysis diagnosis of prostate cancer. Prostate cancer gene 3 (PCA3) is the current gold standard of prostate cancer diagnosis in urine. It was found that the specificity of each biomarker alone, while fixing the sensitivity at 95%, is somewhat low (15% and 17% respectively). However, measuring both biomarkers increased the specificity to 34%, improving the probability of adequate decision making in a clinical scenario. So overall, PSGR detection in urine was able to aid PCA3 detection and decrease the possibility of false-negative diagnosis of prostate cancer patients.

Regulation of PSGR Gene Expression

In an effort to understand the mechanisms that regulate the specific expression for PSGR in prostate tissues and tumors, Weng and colleagues (Weng et al. 2005b) characterized the PSGR promoter and analyzed its expression. They found that PSGR had two distinct regulating promoters. The first promoter, located within exon 1 and its upstream region, with a TATA box at −31 bp, and the second promoter, located upstream of exon 2, with no TATA box or GC-rich sequences. First, they verified the presence of PSGR in human prostate cancer cell lines, finding the highest expression levels in LNCaP cells. As mentioned before, PSGR has two exons, separated by a 14.9-kb intron. Exon 1 is non-coding; the entire protein coding from exon 2. Bioinformatics analysis showed that aside from the TATA box, exon 1 contains multiple DNA *cis*-element binding sites, including ▶ STAT3, NFkB, CACCC-binding factor, and AP1. For the upstream region of exon 1, a 40-bp sequence previously described (Yuan et al. 2001) was found to be the transcription start site for PSGR. For exon 2, another 40-bp sequence was found upstream of the exon. This sequence lacks a TATA box and is non-GC rich but has *cis*-element binding sites including GATA factors, Lom2 and a forkhead recognition sequence, Freac-6. Luciferase assays characterizing different regions of the potential promoters show that transcriptional activity from sequences outside of exon 1 is not as strong as sequences containing the exon, suggesting that exon 1 has transcription factor–binding sites with a positive transcriptional activity. The activity was also much higher in LNCaP cells, suggesting tissue specificity for these factors. It was concluded that the basic promoter for PSGR is within the first −123 bp before exon 1, including the TATA box at −31 bp. For the second region found before exon 2, a luciferase assay analyzing promoter activity from different orientations showed that its activity is in fact orientation dependent, suggesting it acts as a promoter and not as an enhancer for PSGR transcription. Serial deletion constructs showed that the highest promoter activity was at −331 bp. The transcription start site for this second promoter was determined by RPA protection assay, finding a very strong start site at −311 bp with two other weak ones at −175 bp and −118 bp upstream from the ATG in exon 2. Because of the presence of two transcription initiation sites, the authors propose the presence of two different sizes of PSGR transcripts in prostate tissues, the longer variant being the most commonly expressed one. Finally, both promoter regions were stimulated with a variety of growth factors and cytokines to analyze for PSGR expression regulation. Out of all the factors, including R1881, PSGR promoter activity was mostly increased by stimulation with IL-6. IL-6 has been found to be increased in patients with hormone refractory prostate cancer, and the specific relationship between IL-6 and PSGR remains to be found.

Mechanisms of Action

As mentioned before, PSGR is a member of the olfactory family of GPCRs. Recent research on PSGR has focused on the discovery of a potential ligand capable of activating the PSGR signaling pathway. In order to find a ligand for PSGR, Neuhaus and colleagues (Neuhaus et al. 2009) transiently expressed PSGR in HEK293 cells, which do not normally express the receptor, and stimulated the cells with a mixture of 100 steroid hormones from different structural classes and measured the cell responses with ratiofluorometric Ca^{2+} imaging. Using this technique, this research group identified androgen-related compounds as the active ligands for PSGR; the presence of an aldehyde group at position 3 together with at least two double bonds at positions 4 and 6 (Neuhaus et al. 2009) was the key determinant for compounds being

effective PSGR ligands. Testing another mixture which includes 100 aromatic and short chain aliphatic hydrocarbon compounds, b-ionone was found to be a potential ligand for PSGR. To prove that the effect of b-ionone was specific for PSGR, LNCaP cells were transfected with siRNA for PSGR linked to a GFP marker. Cells were then stimulated with b-ionone and within the same cell culture, siRNA-transfected cell calcium response was compared to non-siRNA-transfected adjacent cells still expressing PSGR. The calcium response in cells still expressing PSGR compared to cells with siRNA for PSGR was significantly higher. No effect was seen with transfection of a scrambled control siRNA as well. They also tested for the functional effect of b-ionone activation on LNCaP and PC3 cells, showing that the suggested PSGR ligand has antiproliferative effects on cell proliferation, possible through activation of the SAPK/JNK signaling pathway.

Recently, a study on the mechanisms behind the induction of a Ca^{2+} cytosolic increase related to PSGR was also published. It was found that PSGR activation by b-ionone in LNCaP cells is able to open Ca^{2+} conducting channels in the plasma membrane and that the behavior of these channels is highly Ca^{2+} concentration dependent, behaving in a time course previously described for TRPV6 channels. To prove activation of the PSGR by b-ionone functions through TPRV6 channels, siRNA linked to a GFP protein was used to knock down TPRV6 channels and were then stimulated with b-ionone. None of the cells showed the typical response described previously, suggesting that the b-ionone-induced Ca2+ response in LNCaP cells is dependent on TRPV6 cells. G protein signaling, however, does not seem to be involved in the b-ionone-induced calcium response, since locking G proteins in their active or inactive states does not change the b-ionone-induced current nor does it seem to be linked to the olfactory receptor canonical pathway. The G_{olf}-mediated activation of adenylyl cyclase, which ultimately leads to a Ca^{2+} influx through cAMP-gated ion channels suggests that PSGR is capable of activating an alternate signaling pathway. The possible mechanism for this alternate pathway is through direct interaction between PSGR and Src kinases, without the involvement of G proteins. The b-ionone-induced current was abolished with the introduction of Src family inhibitors, confirming the involvement of Src kinase family in this pathway.

Summary

PSGR is a G-protein-coupled receptor member of the olfactory subfamily of GPCRs that is specifically expressed in prostate and overexpressed in prostate cancer. Because of this behavior, it was proposed to have possible potential as a prostate cancer biomarker. Several studies performed on prostate and prostate cancer tissues, however, did not find a very strong correlation between PSGR expression and prostate cancer progression. It did increase the specificity of other biomarkers such as *AMACR* and *PCA3*, when analyzed in conjunction with them. It was suggested, however, that PSGR may work as a good biomarker for certain specific clinical scenarios, though which scenarios remains to be found. PSGR expression regulation analysis found two promoter sequences active in the PSGR gene. The first is located upstream of exon 1, with a TATA box as the transcription starting site, while the second is located 311 bp upstream of the ATG site in exon 2, suggesting the existence of two PSGR transcripts. It was also found that PSGR expression is induced by stimulation with IL-6, not R1881 or other growth factors or cytokines. PSGR was also found to respond to b-ionone, an aromatic compound that induces a Ca^{2+} response that inhibits proliferation of prostate cancer LNCaP cells. Furthermore, the b-ionone response seems to be related to the calcium-conducting channels TPRV6, which seem to interact directly through Src kinase family members, independently of G proteins. What does future research on this molecule look like? Current research is focused on elucidating the potential signaling pathway through G proteins that is activated by this receptor, thereby describing its canonical pathway and finding what potential ligands are capable of activating this pathway. Also, there are several mouse models currently in development and characterization that will help understand the mechanisms through which PSGR functions and the role it plays in prostate cancer progression.

References

Neuhaus EM, Zhang W, Gelis L, Deng Y, Noldus J, Hatt H. Activation of an olfactory receptor inhibits proliferation of prostate cancer cells. J Biol Chem. 2009;284(24):16218–25.

Rigau M, Morote J, Mir MC, Ballesteros C, Ortega I, Sanchez A, et al. PSGR and PCA3 as biomarkers for the detection of prostate cancer in urine. Prostate. 2010;70(16):1760–7.

Spehr J, Gelis L, Osterloh M, Oberland S, Hatt H, Spehr M, Neuhaus EM. G-protein-coupled receptor signaling via Src kinase induces endogenous human transient receptor potential vanilloid type 6 (TRPV6) channel activation. J Biol Chem. 2011;286(15):13184–92.

Wang J, Weng J, Cai Y, Penland R, Liu M, Ittmann M. The prostate-specific G-protein coupled receptors PSGR and PSGR2 are prostate cancer biomarkers that are complementary to alpha-methylacyl-CoA racemase. Prostate. 2006; 66(8):847–57.

Weng J, Wang J, Cai Y, Stafford LJ, Mitchell D, Ittmann M, Liu M. Increased expression of prostate-specific G-protein coupled receptor in human prostate intraepithelial neoplasia and prostate cancers. Int J Cancer. 2005a;113(5):811–18.

Weng J, Ma W, Mitchell D, Zhang J, Liu M. Regulation of human prostate-specific G-protein coupled receptor, PSGR, by two distinct promoters and growth factors. J Cell Biochem. 2005b;96(5):1034–48.

Xia C, Ma W, Wang F, Hua Shao-bing, Liu M, et al. Identification of a prostate-specific G protein coupled receptor in prostate cancer. Oncogene. 2001;20:5903–7.

Xu LL, Stackhouse BG, Florence K, Zhang W, Shanmugam N, Sesterhenn IA, et al. PSGR, a novel prostate-specific gene with homology to a G protein-coupled receptor, is overexpressed in prostate cancer. Cancer Res. 2000;60(23): 6568–72.

Xu LL, Sun C, Petrovics G, Makarem M, Furusato B, Zhang W, et al. Quantitative expression profile of PSGR in prostate cancer. Prostate Cancer Prostatic Dis. 2006;9(1):56–61.

Yuan TT, Toy P, McClary JA, Lin RJ, Miyamoto NG, Kretschmer PJ. Cloning and genetic characterization of an evolutionarily conserved human olfactory receptor that is differentially expressed across species. Gene. 2001; 278(1–2):41–51.

PSMD4/Rpn10/Proteasome (Prosome, Macropain) 26S Subunit, Non-ATPase, 4 (AGS9)

► Activators of G-Protein Signaling (AGS)

PSMD7

► RPN8

PtdIns(3,4,5)-Dependent Rac Exchanger 2

► P-Rex

PtdIns(5)*P* 4-Kinase

► Phosphatidylinositol 5-phosphate 4-kinase

PtdIns*P* kinase Type II

► Phosphatidylinositol 5-phosphate 4-kinase

PTEN

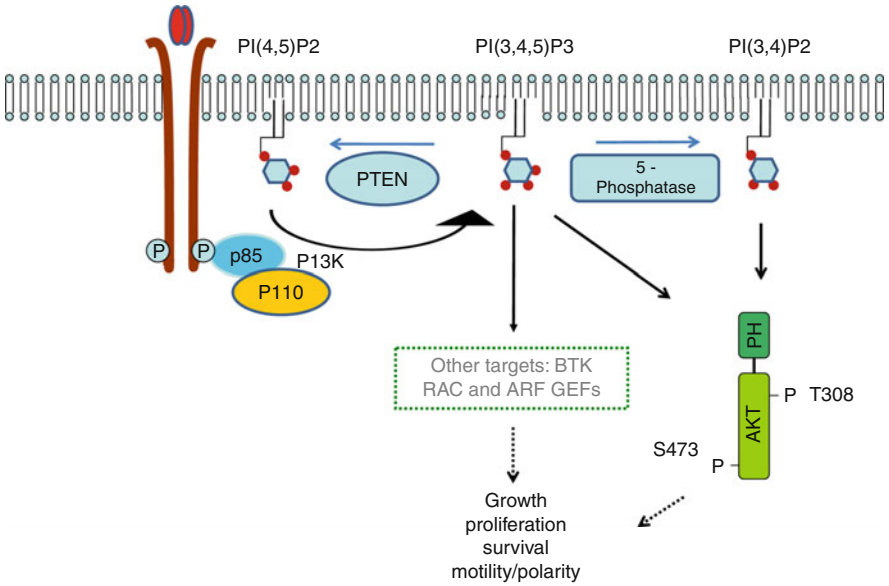
Nicholas R. Leslie, Laura Spinelli, Georgios Zilidis, Nimmi R. Weerasinghe and Priyanka Tibarewal
Division of Cell Signalling and Immunology, College of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dundee, UK

Synonyms

MMAC; TEP1

Historical Background

Widespread interest was generated in 1997 when PTEN was identified almost simultaneously by three research groups as a candidate tumor suppressor in cancers of the brain, prostate, and breast (Li and Sun 1997; Li et al. 1997; Steck et al. 1997). As a member of the large and diverse protein tyrosine phosphatase superfamily, it was expected that PTEN would act to oppose oncogenic tyrosine kinase signaling pathways by dephosphorylating specific tyrosine phosphorylated substrates. However, it soon emerged that PTEN is primarily a lipid phosphatase and that by dephosphorylating the lipid second messenger PtdIns $(3,4,5)P_3$, it acts to suppress signaling through the PI3K signaling pathway (Maehama and Dixon 1998) (Fig. 1). Here the substantial insight that has been provided in the last 13 years by the intensive study of this important tumor suppressor will be discussed.



PTEN, Fig. 1 *PTEN and the PI3K signaling pathway*. A schematic diagram of PI3K-dependent signaling pathway is shown. Upon receptor-driven activation of Class I PI3K enzymes, these lipid kinases phosphorylate PtdIns(4,5)P_2 to form the lipid second messenger PtdIns(3,4,5)P_3. This lipid in turn activates downstream responses, generally including cellular growth, proliferation, survival, and motility through direct effector proteins including the AKT and BTK groups of protein kinases and activating exchange factors (GEFs) for the RAC and ARF families of small GTPases. PtdIns(3,4,5)P_3 is in turn metabolized either by the 3-phosphatase PTEN or by members of a family of phopshoinositide 5-phosphatases

PI3K Signaling

The class I ▶ phosphoinositide 3-kinases (PI3K) are a family of lipid kinases that play a central role in an evolutionarily conserved signal transduction pathway/network of which PTEN is part (Hawkins et al. 2006). Their tightly controlled activity phosphorylates the relatively abundant phosphoinositide PtdIns(4,5)P_2 on the D3 position of the inositol ring, to form the lipid second messenger PtdIns(3,4,5)P_3 (Fig. 1). Class II and Class III PI3K enzymes also exist in mammalian cells, but although these phosphoinositide kinases also phosphorylate the D3 position of the inositol ring, they act upon different substrates and will not be further considered here.

The class I PI3Ks exist in cells as heterodimers, comprising a catalytic subunit of 110 kD in size and a regulatory subunit, which ranges in size from approximately 55 kD to 100 kD. The human and mouse genomes each encode four different catalytic p110 subunits, which display some differences in their expression patterns, regulatory partners, and signaling inputs (Hawkins et al. 2006). The p110 α, β, and δ enzymes bind with regulatory subunits, most commonly of 85 kD in size, that contain paired SH2 domains, and result in activation of PI3K activity through tyrosine kinase–based signaling mechanisms, in particular via receptor tyrosine kinase (RTK) signaling. In contrast, the p110 γ PI3K catalytic subunit binds to one of two unrelated regulatory subunits, p84 or p101, that allow regulation of kinase activity through G-protein coupled receptor (GPCR)-based signaling. It has been shown more recently that the p110 β isoform is also activated by many GPCRs, although the mechanism of this activation is currently unclear. Although these differences exist in the inputs into the PI3Ks via their regulatory subunits, each of the four catalytic subunits has a binding site for the activated form of the RAS small GTPases. Thus, PI3K activity appears to be tightly controlled, with low activity in the absence of stimulation that can be provided through mechanisms that include RTK, GPCR, and RAS signaling (Vanhaesebroeck et al. 2010).

The direct PI3K lipid product PtdIns(3,4,5)P_3 propagates the effects of PI3K activation through a large and diverse group of effector proteins that are able to bind directly to this lipid with high selectivity (Fig. 1). The best studied downstream targets for PI3K/PIP_3 activation

are the AKT family of serine/threonine kinases that have an N-terminal PIP_3 binding Pleckstrin Homology (PH) domain and a C-terminal kinase domain. Experiments in flies and worms have shown that the AKT kinases are evolutionarily conserved and key functional mediators of PI3K signaling. The three human AKT kinases have many substrates and are important regulators of cell growth, proliferation, survival, and metabolism (Manning and Cantley 2007). However, estimates are that there are between 25 and 50 selective PIP_3 binding proteins encoded in the human genome and in many cases the relative contributions of these proteins to the PI3K signaling network is unclear (Hawkins et al. 2006).

Once synthesized by PI3K, the PIP_3 signal can be removed either by one of two routes of metabolism. A family of phosphoinositide 5-phosphatases, the best characterized being the ▶ SHIP and SHIP-2 enzymes, dephosphorylate the 5-position of PIP3 to form the alternate phosphoinositide signal PtdIns(3,4)P_2, which shares some selective binding partners with PIP3, including the AKT kinases, and is believed to contribute directly to the outcomes of PI3K activation (Fig. 1). PtdIns(3,4)P_2 in turn appears to be removed at least in part, by the action of the INPP4 phosphoinositide 4-phosphatase enzymes.

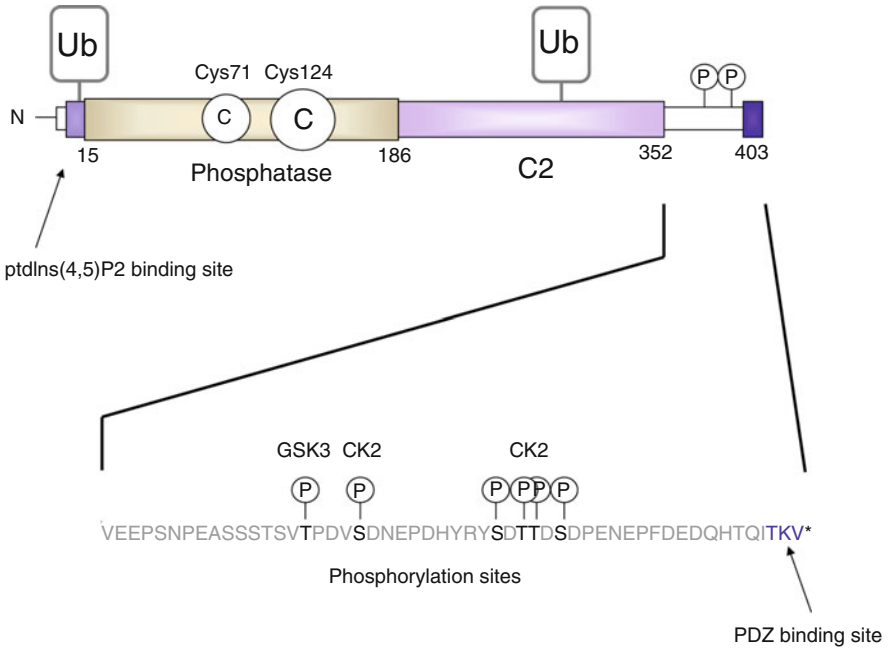
The other main route of metabolism of PIP_3 is by removal of its 3-phosphate, acting to terminate PI3K signaling by reproducing PtdIns(4,5)P_2. It is this reaction that is performed by PTEN and which forms the basis for its tumor suppressor activity.

The PTEN Protein

The human *PTEN* gene appears to encode a single 403 amino acid protein, with an N-terminal phosphatase domain, a more C-terminal C2 domain, and a 50 amino acid regulatory C-terminal tail (Fig. 2). One crystallographic 3D structure has been determined for the minimal phosphatase/C2 core of PTEN, which along with the phosphatase domain amino acid sequence, identifies PTEN as a member of the protein tyrosine phosphatase (PTP) superfamily (Lee et al. 1999). This structure also revealed a broader active site pocket than most other PTP family members, capable of accommodating the inositol lipid headgroup, and containing the conserved PTP family catalytic residues, including the nucleophilic cysteine, Cys124. This would seem to fit with PTEN's substrate selectivity for PtdIns(3,4,5)P_3, over the other 3-phosphorylated phosphoinositides such as PtdIns(3,4)P_2 and the ability of the phosphatase to dephosphorylate peptide substrates, certainly in vitro, with a preference for phospho-tyrosine substrates over phosphorylated serine or threonine substrates (Myers et al. 1997). Although several substrates have been proposed for PTEN's protein phosphatase activity, such as FAK, SHC, and ▶ beta-catenin, further supporting data confirming any protein substrate is currently lacking. Detailed interfacial kinetic analysis also shows that PTEN strongly prefers PtdIns(3,4,5)P_3 (and other phosphoinositide lipid substrates) presented within acidic lipid surfaces over the same phosphorylated inositol headgroup (Ins(1,3,4,5,)P_4) presented as a soluble molecule. This enhanced interfacial activity appears to be mediated by the correct positioning of the active site on lipid surfaces in part by the C2 domain and to be accompanied by conformational activation on such surfaces (Leslie et al. 2008).

In almost all cell types investigated, PTEN is found located throughout the cytosol, and in many cells also in the nucleus. The nuclear localization of PTEN, which appears to be regulated in part by monoubiquitination, will be discussed with more detail below. In a very few tissues, strong subcellular enrichment of PTEN has been demonstrated, most notably localization to apical junctions in the polarized epithelia of the chick epiblast and murine retinal pigment epithelium. However, the reported clear subcellular localization of PTEN with strong validating controls is very rare.

To access its membrane substrate, PTEN associates transiently with the plasma membrane, with an estimated average residence time of a few hundred milliseconds, but any membrane enrichment is not normally evident using normal epifluorescence microscopy. Basic surfaces on the phosphatase domain and the calcium-independent C2 domain appear to be involved in mediating interactions with acidic non-substrate membrane lipids and correctly positioning the active site relative to membrane surfaces. There also appears to be a specific PtdIns(4,5)P_2 interacting motif at the N-terminus of PTEN that mediates a conformational change within the phosphatase and leads to activation. With emerging evidence for rapid regulated changes in the electrostatic surface charge of cellular membranes and PtdIns(4,5)P_2 levels, it will be interesting to see whether such changes play an important role in PTEN regulation.



PTEN, Fig. 2 *The PTEN protein*. The PTEN protein is represented, including an expansion of its regulatory C-terminal tail. As discussed in the text, PTEN can be regulated through phosphorylation of its C-terminal tail, ubiquitination of lysine residues at positions 13 and 289, and oxidation of its active site Cysteine 124, which can form a disulfide bridge to Cysteine 71

PTEN Regulation

Several mechanisms of PTEN regulation have been identified, but it seems worth noting in advance that most of the mechanisms of PTEN regulation are through inhibition of the enzyme. Accordingly, unphosphorylated bacterially expressed PTEN has robust lipid phosphatase activity in vitro that can be inhibited by such mechanisms as phosphorylation, oxidation, and ubiquitination.

PTEN has two well-documented groups of phosphorylation sites: a cluster of serines and threonines surrounded by acidic residues between amino acids 380 and 385 that appear to be phosphorylated by the protein kinase, ▶ CK2, and also threonine 366, which seems to be phosphorylated by Glycogen Synthase Kinase 3 (GSK3) after priming phosphorylation of serine 370 by CK2 (Fig. 2).

It has been shown by many studies that phosphorylation of the 380–385 cluster sites stabilizes a "closed" conformation of PTEN due to a strengthened interaction between the flexible C-terminal tail of PTEN and the phosphatase and C2 domains (Ross and Gericke 2009). This closed conformation interferes with membrane binding, so reduces lipid phosphatase activity, but also enhances PTEN stability, it seems as an indirect consequence of this reduced membrane localization (Maccario et al. 2010). It should be noted that although a small fraction of cellular PTEN has been identified in an unphosphorylated state (at these 380–385 sites) and incorporated in high molecular weight complexes, most cellular PTEN appears to be found in the phosphorylated "closed" state correlating with its cytosolic localization. A similar picture emerges in studies of the 366/370 phosphorylation sites, which have been shown capable of affecting PTEN stability in some, but not all, cells, and also controlling the phosphospecific binding of a nucleolar protein, MSP58 to PTEN. However, as with the 380–385 sites, although at least a little is known about the consequences of phosphorylation, there is no clear picture of how regulated changes in phosphorylation fit into any system of controlling PTEN function.

The catalytic mechanism of PTEN, shared with the rest of the PTP family, makes it potentially sensitive to regulation by oxidation of the reactive catalytic cysteine. The oxidation of a fraction of cellular PTEN occurs in several circumstances in which cells

stimulated by growth factors and other receptor ligands are driven to produce endogenous reactive oxygen (ROS) species and is accompanied by a ROS-dependent activation of downstream signaling (Leslie et al. 2008). Although studies in both cardiac and skeletal muscle have found a correlation between PTEN oxidation and Akt activation in vivo, as with many areas of redox signaling, compelling evidence for the functional significance of any observed oxidation is currently lacking (Leslie et al. 2008).

PTEN, like many proteins, has its degradation controlled in large part through ubiquitination and subsequent proteolysis by the proteasome. Two lysine residues have been proposed to be sites for ubiquitination, Lys13 and Lys289, located within the N-terminal PtdIns(4,5)P_2 binding site and within the C2 domain, respectively (Fig. 2), and two different E3 ubiquitin ligases have been proposed to act on PTEN, the HECT domain ligase NEDD4.1, and the RING domain protein XIAP. PTEN ubiquitination, and in particular, monoubiquitination of Lys289, appears to enhance nuclear localization (Salmena et al. 2008). Significantly, ubiquitination of PTEN in vitro leads to a substantial loss of phosphatase activity, indicating that ubiquitination may act to directly inhibit catalytic activity, in addition to other effects on PTEN function (Maccario et al. 2010). Given the current data, it seems appealing to speculate that there may be significant complexity in the area of PTEN ubiquitination and that individual ubiquitination events on multiple sites, mediated by different regulated ubiquitin ligases, may have distinct functional consequences, with ubiquitination differentially controlling activity, stability, and nuclear localization. The functional consequences of nuclear localization are currently rather unclear, with several PtdInsP_3-independent nuclear functions having been proposed, along with a simple model that nuclear PTEN may be a stable protein pool that cannot access its membrane substrate.

PTEN Functions in Health and Disease: The Significance of PTEN as a Tumor Suppressor

As discussed above, PTEN is a core component and critical regulator of a signaling pathway that controls many diverse cellular processes. As such, PTEN plays a role in a vast array of physiological processes such as neuronal development and function, central control of adiposity, and cardiac hypertrophy and contractility (Chang et al. 2007; Oudit and Penninger 2009; Plum et al. 2006). However, by far the best recognized role for PTEN in human disease is as an important tumor suppressor in many forms of cancer. Analyses of the PTEN coding regions in tumor samples indicate that it is the second most frequently mutated tumor suppressor across all cancers after ▶ p53 (Salmena et al. 2008; Keniry and Parsons 2008). It is also very common that tumors carry deletions of one, or less frequently, both copies of the *PTEN* gene. The pathological significance of loss of the *PTEN* gene in different tumor types, including the loss of just one allele, has been supported by the generation of many different lines of transgenic mice lacking PTEN, throughout the body or in selected tissues, most of which have greatly increased risks of tumor formation (Salmena et al. 2008; Suzuki et al. 2008). It is outside the scope of this entry to describe the wealth of research over the last decade or more that has provided increasing evidence for the significance and mechanisms of PTEN functional loss in cancer, a deeper understanding of the downstream pathways through which PTEN loss affects tumor development and has described the development of therapies aimed at treating the many tumors lacking PTEN. However, these topics have been recently reviewed by others (Keniry and Parsons 2008; Chalhoub and Baker 2009; Garcia-Echeverria and Sellers 2008).

Summary/Future Directions

PTEN's status as one of the most important tumor suppressors in human cancer has driven a great deal of research into its function. In particular, the discovery that PTEN appears to act in a dose-dependent, haploinsufficient manner as a tumor suppressor has broad implications for the potential importance in cancer of pathways that regulate PTEN activity and expression. Also, great efforts are going into understanding what can be learned from a tumor's PTEN status in terms of predicting outcome and response to treatments. It is to be hoped that our developing understanding of PTEN function and the increasing number of drugs targeting the PI3K pathway will lead to an improvement in the outcome for this patient group.

References

Chalhoub N, Baker SJ. PTEN and the PI3-kinase pathway in cancer. Annu Rev Pathol. 2009;4:127–50.
Chang N, El-Hayek YH, Gomez E, Wan Q. Phosphatase PTEN in neuronal injury and brain disorders. Trends Neurosci. 2007;30:581–6.
Garcia-Echeverria C, Sellers WR. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. Oncogene. 2008;27:5511–26.
Hawkins PT, Anderson KE, Davidson K, Stephens LR. Signalling through Class I PI3Ks in mammalian cells. Biochem Soc Trans. 2006;34:647–62.
Keniry M, Parsons R. The role of PTEN signaling perturbations in cancer and in targeted therapy. Oncogene. 2008;27:5477–85.
Lee JO, Yang H, Georgescu MM, Di Cristofano A, Maehama T, Shi Y, et al. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell. 1999;99:323–34.
Leslie NR, Batty IH, Maccario H, Davidson L, Downes CP. Understanding PTEN regulation: PIP2, polarity and protein stability. Oncogene. 2008;27:5464–76.
Li DM, Sun H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. Cancer Res. 1997;57:2124–9.
Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997;275:1943–7.
Maccario H, Perera NM, Gray A, Downes CP, Leslie NR. Ubiquitination of PTEN (phosphatase and tensin homolog) inhibits phosphatase activity and is enhanced by membrane targeting and hyperosmotic stress. J Biol Chem. 2010;285:12620–8.
Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 1998;273:13375–8.
Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129:1261–74.
Myers MP, Stolarov JP, Eng C, Li J, Wang SI MHW, et al. PTEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci USA. 1997;94:9052–7.
Oudit GY, Penninger JM. Cardiac regulation by phosphoinositide 3-kinases and PTEN. Cardiovasc Res. 2009;82:250–60.
Plum L, Ma X, Hampel B, Balthasar N, Coppari R, Munzberg H, et al. Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. J Clin Invest. 2006;116:1886–901.
Ross AH, Gericke A. Phosphorylation keeps PTEN phosphatase closed for business. Proc Natl Acad Sci USA. 2009;106:1297–8.
Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. Cell. 2008;133:403–14.
Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet. 1997;15:356–62.
Suzuki A, Nakano T, Mak TW, Sasaki T. Portrait of PTEN: messages from mutant mice. Cancer Sci. 2008;99:209–13.
Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol. 2010;11:329–41.

PTEN-Induced Kinase 1 (PINK1)

Laura M. Westrate[1,2] and Jeffrey P. MacKeigan[3]
[1]Van Andel Graduate School, Grand Rapids, MI, USA
[2]Van Andel Research Institute, Grand Rapids, MI, USA
[3]Center for Cancer Genomics and Quantitative Biology, Van Andel Research Institute, Grand Rapids, MI, USA

Synonyms

BRPK; PARK6; PINK1

Historical Background

The discovery of PTEN-induced kinase 1 (PINK1) was first described in 2001 by Valente et al. as a novel locus for autosomal recessive Parkinson's Disease (PD). Termed PARK6, the new locus was identified in a genome-wide homozygosity screen performed in a Sicilian family with multiple PD-affected members (Valente et al. 2001, 2004). Localized to the mitochondria, PINK1 was the first nuclear-encoded mitochondrial protein to be implicated in PD pathogenesis and strongly suggests the role of mitochondrial pathomechanisms in PD (Valente et al. 2004). The precise mechanism by which PINK1 contributes to mitochondrial dysfunction, however, remains unclear as there remains a significant gap in knowledge in our understanding of the role of PINK1 in human disease.

First identified as a serine/threonine kinase with reported homology to the Ca2+/calmodulin family, PINK1 does not fall within any of the previously identified kinase families as determined by sequence similarity of their kinase domains (Manning et al. 2002), indicating the PINK1 substrates may be unique. Studies examining the cellular role of PINK1 have shown that the serine-threonine kinase protects cells from oxidative stress and, thus, is proposed to serve an important role in protecting the cell from mitochondrial crisis

(Jendrach et al. 2009). Recent publications have suggested several intriguing hypothesis addressing the cellular functions of PINK1. This review will highlight the role of PINK1 in mitochondrial fission and fusion, in clearance of damaged mitochondria through mitophagy, and in mitochondrial trafficking. Additionally, insight gained from studying PINK1 in model organism or through the identification of PINK1 specific substrates will also be discussed.

Localization and Structure

Located on chromosome 1p35–p36, PINK1 is composed of three main domains: an *N*-terminal mitochondrial targeting sequence (MTS), a small transmembrane domain (TM), and a highly conserved serine/threonine kinase domain that has been reported to exhibit autophosphorylation activity in vitro (Zhou et al. 2008; Thomas and Cookson 2009). Analysis of PINK1's kinase domain led to the recognition of highly conserved subdomains involved in either anchoring and orienting the ATP molecule (subdomains I–IV) or binding of the peptide substrate (subdomains VIa–XI) (Silvestri et al. 2005). PINK1 is made up of eight exons spanning 1.8 kB, corresponding to an ubiquitously expressed 581 amino acid protein. The highest expression levels are observed in the heart, skeletal muscle, and testis (Silvestri et al. 2005), all mitochondrial dense tissue. In the brain, PINK1 expression is primarily neuronal and localized to the hippocampus, *Substantia nigra*, and the cerebellar Purkinje cells (Thomas and Cookson 2009). The 581 amino acids (63 kDa protein) of PINK1 are further processed upon mitochondrial import to generate two mature isoforms, 54 and 45 kDa, respectively (Deas et al. 2009).

It is well established that PINK1 localizes to the mitochondria as the N-terminal mitochondrial target sequence is sufficient for mitochondrial import of PINK1 (Valente et al. 2001; Silvestri et al. 2005). Specific subcellular localization of PINK1, however, remains somewhat controversial as it has been reported to be located on the outer mitochondrial membrane (OMM), the mitochondrial intermembrane space (IMS), the inner mitochondrial membrane (IMM), and even the cytoplasm (Thomas and Cookson 2009). Recent work on the topology of PINK1 has concluded that PINK1 localization depends on the transmembrane domain, which embeds itself into the outer mitochondrial membrane leaving the kinase domain exposed to the cytosol (Zhou et al. 2008). This would imply that PINK1 specific substrates are located in the cytosol or localized to the outer mitochondrial membrane. Interestingly, two of the most widely reported interactors or substrates of PINK1, TRAP1, and Omi/HtrA2 reside within the mitochondria. Therefore, PINK1 interactors (TRAP1 and Omi/HtrA2) and PINK1 localization need to be rectified through additional mechanistic studies.

Substrates and Interactors of PINK1

Identified as a serine/threonine kinase, PINK1 has been shown to exhibit autophosphorylation activity in vitro. However, despite robust phosphorylation activity, the in vivo substrates remain unknown making it difficult to ascertain the biochemical function and physiological substrates of PINK1 (Geisler et al. 2010). One of the first specific PINK1 substrates to be identified was the mitochondrial chaperone tumor necrosis factor type 1 receptor associated protein 1 (TRAP1). Through a combination of both in vitro and in vivo assays, Pridgeon et al. showed that the phosphorylation of TRAP1 by PINK1 played a critical role in protecting cells from oxidative-stress-induced cell death (Pridgeon et al. 2007). The protective action of PINK1 through suppression of cytochrome c release was impaired by PD-linked mutations including G309D, L347D, and W437X, which resulted in a reduced capability for PINK1 to phosphorylate TRAP1. Reported to be a direct substrate for PINK1, this work by Pridgeon et al. provided some of the first insight into the mechanism by which loss of PINK1 leads to neurodegeneration.

Further confirmation that the kinase activity of PINK1 is required for its in vivo function came with the identification of the PD relevant substrate, Parkin. Parkin, also known as PARK2, is one of the most commonly affected PD genes and encodes for an E3 ubiquitin ligase that is also associated with autosomal recessive forms of PD. Shown to mediate both classical and nonclassical ubiquitin linkages, Parkin has been implicated in both protein degradation and inclusion-body formation (Kim et al. 2008). The first indication that Parkin and PINK1 may be linked in a common pathway came from initial work in *Drosophila*, where it was determined that Parkin and

PINK1 function in a linear pathway with PINK1 upstream of Parkin (described below). However, the mechanism by which the two proteins interacted together remained unknown until Kim et al. elegantly performed studies in both mammalian and *Drosophila* systems, that they clearly demonstrated that PINK1's kinase activity regulates the translocation of Parkin to the mitochondria (Kim et al. 2008). Given the role of Parkin in protein degradation and PINK1's proposed role in mitochondrial dynamics, the identification of Parkin as a PINK1 specific substrate has important implications into PD-related mechanisms in which PINK1 and Parkin work together to provide a mitochondrial quality control system. This was further confirmed by Geisler et al., demonstrating that PINK1 kinase activity and its mitochondrial localization sequence are required for the recruitment of Parkin to depolarized mitochondria. Parkin localization results in selective degradation of the damaged mitochondria through selective autophagy of the mitochondria, a process termed mitophagy (Geisler et al. 2010).

Supporting a role of PINK1 in protecting cells from mitochondrial dysfunction, Plun-Favreau et al. reported that the serine protease Omi/HtrA2 is an interactor of PINK1 (Plun-Favreau et al. 2007). Loss of function of Omi/HtrA2 has been reported in the literature to result in a neurodegenerative disorder with a similar phenotype to PD. Classically, Omi/HtrA2 is thought to be released from mitochondria during apoptosis upon permeabilization of the outer mitochondrial membrane. Once in the cytosol, the serine protease binds to inhibitor of apoptosis (IAP) proteins and thus contributes to induction of apoptosis (Plun-Favreau et al. 2007). While proposed to be a proapoptotic protein, the parkinsonian neurodegenerative phenotype observed in Omi/HtrA2 knockout mice has lead to the hypothesis that the serine protease may also serve to protect mitochondrial homeostasis. Further work indicated that PINK1 and Omi/HtrA2 are both components of the same regulatory pathway involved in the maintenance of mitochondria homeostasis. They provided evidence that p38-mediated phosphorylation of Omi/HtrA2 occurs in a PINK1-dependent manner (Plun-Favreau et al. 2007). The authors concluded that PINK1-dependent phosphorylation of HtrA2 may modulate the protease's proteolytic activity and thereby serve to provide a mechanism by which PINK1 phosphorylation of HtrA2 results in increased resistance of cells to mitochondrial stress. It remains unclear, however, whether Omi/HtrA2 is a direct substrate of PINK1 and whether differences in cell viability due to PINK1 inactivation are indirectly affecting other kinases that are responsible for direct phosphorylation of Omi/HtrA2 activity.

Investigating PINK1 Function Using Model Organisms

Several model organisms have been used to help researchers elucidate the roles of PINK1. Specifically, given the role of PINK1 in PD, many of the studies in model organisms have been focused toward determining the mechanism by which loss of PINK1 contributes to the pathogenesis of PD. Using *C. elegans* as a model organism, loss of function PINK1 mutations result in mutant worms demonstrating reduced mitochondrial cristae lengths (Samann et al. 2009). Additionally, worms harboring the mutant form of PINK1 also display an increased sensitivity to the reactive oxygen species catalyst, paraquat, which could be rescued upon expression of transgenic PINK1. The reported mitochondrial phenotype coinciding with an observable sensitivity to oxidative stress supports findings in other model organisms and the hypothesis that PINK1 functions to preserve mitochondrial homeostasis in the cell (Samann et al. 2009).

A second model organism that has been used extensively to gain insight into the function of PINK1 in the cell has been *Drosophila*. Interestingly, these fruit fly models for PD resemble many of the characteristics of the human form of PD including the loss of dopaminergic (DA) neurons along with the presentation of various locomotive defects (Park et al. 2009). In 2006, three papers came out reporting that loss of PINK1, either by loss of function mutations or through the removal of the *Drosophila PINK1* homologue results in male sterility, indirect flight muscle, and DA neuronal degeneration accompanied by locomotive defects. Additionally, loss of PINK1 resulted in defects in mitochondrial morphology (increased mitochondrial cristae fragmentation and mitochondrial swelling observed in tissues with high energy demands) and demonstrated an increased sensitivity to multiple stressors including oxidative stress (Park et al. 2009).

Interestingly, *Drosophila* PINK1 fly mutants phenocopy almost all of the observed phenotypes of the

Parkin knockout, including indirect flight muscle/DA neuronal degeneration, impaired flight, and slow climbing ability. Subsequent genetic studies probing for the mechanism behind the phenotype similarity observed with Parkin and PINK1 mutants resulted in the discovery of an epistatic relationship between the two genes. Further studies revealed that PINK1 and Parkin are linked in a linear pathway involved in protecting the integrity and function of the mitochondria with Parkin acting downstream of PINK1 (Park et al. 2009). Together, these two models strongly implicate the role of mitochondrial dysfunction in PINK1 or Parkin-related PD pathogenesis.

To further elucidate the physiological role of PINK1 in mammalian cells and to gain insight into the disease mechanism of PINK1's involvement in PD pathogenesis, several labs have worked to understand novel phenotypes of PINK1-deficient mice. PINK1$^{-/-}$ mice have functional defects in the dopaminergic system, including a decrease in dopamine release in striatal slices. This functional defect could be rescued by treatment with dopamine receptor agonists or other agents that increase dopamine release (Kitada et al. 2007). These results suggest a critical role for PINK1 in providing a functional link between mitochondria and regulation of dopamine release and thus may provide insight into the pathogenesis of PINK1 in playing a role in causing PD-associated dopaminergic dysfunction. Unlike studies in both *Drosophila* and *C. elegans* models, loss of murine PINK1 resulted in no gross changes in the structure of mitochondria; however, the authors did report that the overall size of mitochondria increased in the PINK1-deficient mice compared to control (Gautier et al. 2008). While no gross changes in mitochondrial structure were observed, significant defects in mitochondrial respiration were detected in complexes I, II, III, and IV. At 3–4 months of age, this respiration defect was specific for the striatum; however, mitochondrial respiration activity was decreased in other areas, including the cerebral cortex, at 2 years of age compared to control. Similar mitochondrial defects in other regions outside of the striatum could also be induced following induction of ROS formation through the treatment of H_2O_2 or mild heat shock, suggesting that ROS accumulation as a consequence of aging may exasperate the mitochondrial dysfunction (Gautier et al. 2008).

Mitochondrial Function of PINK1

Mitochondrial Morphology

Mitochondria are dynamic organelles that require active fission and fusion to maintain a distinct morphology, not only to maintain mitochondrial homeostasis but also to maintain changing cellular energy demands (Thomas and Cookson 2009). Work performed in *Drosophila* and mouse models have found that loss of PINK1 results in enlarged/swollen mitochondria, suggesting a potential role for PINK1 in regulating mitochondrial morphology. The distinct morphology of the mitochondria is tightly regulated through two opposing process: mitochondrial fission and fusion. These two processes have also been shown to play important roles in maintaining mitochondrial maintenance and function, along with regulating the stabilization of the mitochondrial genome in response to the changing cellular environment (Okamoto and Shaw 2005). This regulation will be described briefly below, but for a more detailed review behind the mechanism of mitochondrial fission and fusion, readers are encouraged to refer to Chan et al. for a comprehensive overview of the mechanism of fission and fusion (Chan 2006).

Capable of undergoing several fission/fusion events, mitochondria are frequently changing shape in response to their surrounding cellular energy demands. Conserved dynamin-related GTPases are responsible for maintaining the balance between mitochondrial fission and fusion. Two highly conserved proteins, the outer mitochondrial membrane GTPases Mitofusins (MFN 1 or MFN 2) and the inner membrane GTPase OPA1, are responsible for controlling mitochondrial fission. Mitofusins are large GTPases embedded in the outer mitochondrial membrane (OMM) that initiate interaction between the two mitochondria. Fusion of the inner mitochondrial membrane (IMM) is predicted to be regulated by OPA1, a dynamin-related GTPase that localizes to the inner mitochondrial space (IMS) and associates with the IMM (Chan 2006).

Mitochondrial fission is controlled by two proteins: Drp1 and hFIS1. The small protein, hFIS1, uniformly localizes to the OMM and is responsible for recruiting Drp1 to sites of intended mitochondrial fission. Drp1 is a large dynamin-related GTPases found in the cytosol that localizes to the mitochondria to initiate mitochondrial fission. Current theories suggest that upon

recruitment to the mitochondria, Drp1 forms a ring around the mitochondria and acts as a mechanochemical enzyme that uses GTP hydrolysis to drive mitochondrial constriction and fission. Interestingly, Drp1 has been reported to be recruited to the mitochondria in the absence of hFIS1, and thus hFIS1 is not required for Drp1 localization to the mitochondria (Chan 2006).

While it has been proposed that PINK1's function in the cell is to regulate mitochondrial dynamics, the exact mechanism of PINK1's involvement with mitochondrial fission and fusion remains to be elucidated. Knockdown of PINK1 in *Drosophila* models have demonstrated that loss of PINK1 results in elongated mitochondria, while studies in *C. elegans* resulted in heavily fragmented mitochondria. Studies in mammalian cell models have been even more controversial where loss of PINK1 has been reported to result in either excess fusion, excess fission, or result in no effect on mitochondrial morphology, depending on the cell model and the method of knockdown (Thomas and Cookson 2009; Jendrach et al. 2009). Mitochondrial fission and fusion are important processes which allow the mitochondria to control the structure and distribution of the mitochondria network. Given the proposed role of mitochondrial defects in the pathology of PD, a better understanding of the involvement of PINK1 in mitochondrial biology and homeostasis may provide important insight into the specific pathogenic pathways that leads to mitochondrial dysfunction in PD.

Mitophagy

PINK1 and Parkin have previously been reported to interact genetically and function within the same linear molecular pathway (Park et al. 2009). The mechanistic details of how a serine/threonine kinase and an E3 ligase function to protect mitochondrial dynamics has remain largely unknown, until recently, with the discovery that PINK1 may play a direct role in the elimination of mitochondria through interaction with Parkin (Geisler et al. 2010). In an elegant set of experiments, Geisler et al. demonstrated that PINK1's kinase activity and its mitochondrial localization sequences are both required to translocate Parkin to depolarized mitochondria. From there, Parkin subsequently mediates the formation of two distinct polyubiquitin chains, and degradation of the damaged organelle is further promoted through the addition of the autophagic adaptor p62 (SQSTM1), whose presence is essential for the clearance of mitochondria (Geisler et al. 2010).

Following mitochondria depolarization, indicative of mitochondrial damage, PINK1 interaction with Parkin is significantly enhanced. Furthermore, modulations of PINK1 expression levels either through siRNA knockdown or expression of kinase deficient PINK1 results in a reduction of Parkin translocation to depolarized mitochondria and thus the subsequent degradation of the damaged mitochondria. Therefore, PINK1 appears to serve as an important sensor for mitochondria damage upstream of Parkin (Geisler et al. 2010). Many questions still remain to further elucidate the recently identified novel role for PINK1's involvement in mitophagy. In particular, it is still unclear how the kinase activity of PINK1 is regulated following mitochondrial damage and whether PINK1 can mediate phosphorylation of Parkin to stimulate its E3 ligase activity. Given the role of both Parkin and PINK1 in autosomal recessive PD, this model provides an attractive hypothesis to provide a common pathomechanism in which loss of either protein leads to the onset of hereditary PD.

Mitochondrial Trafficking

Mitochondrial trafficking is a critical process to ensure proper distribution of the mitochondria within the cell. Mitochondrial transport occurs in both anterograde and retrograde directions within the cell and is controlled by a combination of cytoskeleton and mitochondrial proteins, including the atypical GTPase Miro and the adapter protein Milton (Weihofen et al. 2009). Miro and Milton complex on the outer mitochondrial membrane, facing the cytosol where they link to the heavy chain of a kinesin to drive anterograde transport of the mitochondria along microtubules within the cell. Work published by Weihofen et al. showed that PINK1 forms a multiprotein complex through interactions with the two cytoplasmic proteins Miro and Milton, and this suggests a novel role for PINK1 in mitochondrial trafficking (Weihofen et al. 2009). This model depends on the topology report of Zhou et al., as it requires the kinase to be anchored to the OMM with its kinase domain facing the cytoplasm to ensure interactions with Miro and Milton. Mitochondrial transport is also tightly regulated by mitochondrial dynamics, including fission and fusion (Okamoto and Shaw 2005). PINK1, therefore, in addition to direct interactions with Miro and Milton, may also regulate mitochondrial transport

through its roles in mitochondrial fission and fusion. The proposed role of PINK1 in regulating mitochondrial transport provides yet another means by which PINK1 can protect the cell from oxidative stress or other forms of mitochondrial crisis.

PINK1 and Parkinson's Disease

Parkinson's disease is a progressive neurodegenerative disorder in which dysregulation of mitochondrial structure and function has come to the forefront as one of the central factors in the pathogenesis of PD (Chu 2010). Characterized by a loss in dopaminergic neurons in the *Substantia nigra*, as well as the presence of ubiquitin-positive Lewy neuritis and Lewy bodies, PD is progressive disease with no current therapy to slow progression of the disease. Understanding the molecular pathways that result in PD pathology remains an important goal to facilitate development of more effective therapeutic strategies. While the majority of PD cases appear to be sporadic, there is a significant amount of PD cases that are caused by specific genetic defects linked to familial forms of PD, which have provided important insight into the pathology underlying PD. Loss of function of PINK1 has been implicated in autosomal recessive PD as most of the identified pathogenic mutations occur within the kinase domain and result in decreased kinase activity.

Neurons are highly specialized cells that have very specific energy demands, and thus proper mitochondrial function and distribution play a critical role in maintaining neuronal function. As previously discussed, mitochondrial fission and fusion play a critical role in both mitochondrial homeostasis and trafficking (Okamoto and Shaw 2005). Therefore, modulation of the fission and fusion process due to PINK1 mutants found in autosomal recessive PD may result in an inability to properly traffic mitochondria along the length of the neuron to the synapse. This would result in an energy deficit throughout the neuron and could culminate in the degeneration of the DA neurons. Additionally, given the interaction between PINK1 and characterized mitochondrial trafficking proteins, Miro and Milton, loss of PINK1 may have a more direct means to modulate mitochondrial trafficking (Weihofen et al. 2009).

PINK1 also appears to play a critical role in maintaining mitochondria function as it may serve as an important role in ensuring that the mitochondria population remains maximally functional (Geisler et al. 2010). Given recent insight into the potential role of PINK1 and Parkin in mitophagy, as described in the section above, PINK1 may also be directly involved in the clearance of damaged mitochondria. Loss of PINK1 may therefore result in an inability to segregate and remove damaged mitochondria, thus leading to accumulation of mitochondrial dysfunction within the neuron (Geisler et al. 2010). The inability to maintain energetic demands of the neuron would therefore result in neuronal degradation and may provide a molecular pathway to explain the mitochondrial dysfunction observed as one of the classic pathologies of PD (Thomas and Cookson 2009; Chu 2010). It will be interesting to see if the molecular pathways by which Parkin mutations lead to PD are the same as PINK1 given the linear epistatic relationship between the two proteins.

Summary

Several recent publications have implicated PINK1 in playing a role in regulating mitochondrial morphology, mitochondrial homeostasis, and mitochondrial trafficking. Insights from the Parkinson's field have also demonstrated that PINK1 appears to function to protect the cell from stresses, such as oxidative stress, that would otherwise compromise the mitochondria and lead to cellular distress or death. Given the role of the mitochondria in cellular metabolism, it could be proposed that altered mitochondrial bioenergetics through the disruption of PINK1 function may play an important role in PD pathology. However, there remains much to be done to further understand the role of PINK1 in PD. More mechanistic studies focusing on the localization and processing of PINK1 remain to be elucidated. Most likely, the reason behind the controversy related to the localization of PINK1 is due in some part to the inability to detect PINK1 endogenously with commercially available antibodies. Therefore, researchers are often forced to tag the protein or overexpress it to such a level that is no longer biologically relevant. Similarly, PINK1 interactors and substrates remain largely undetermined, probably somewhat due for the same reason as it is difficult to study protein interactions and kinase activity at the endogenous single-cell level. Likewise, the exact mechanism by which PINK1

regulates mitochondrial dynamics is beginning to become clear; however, more work is required to further identify the specific substrates and interactors of this serine/threonine kinase. Clearly, PINK1 is involved in several pathways aimed at maintain mitochondrial homeostasis. A better understanding of how PINK1 is modified or processed, along with an awareness of how these changes alter the function of the kinase both under normal and stressed conditions, will provide important clues into the mechanism by which PINK1 protects cells from mitochondrial crisis.

References

Chan DC. Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol. 2006;22:79–99.

Chu CT. A pivotal role for PINK1 and autophagy in mitochondrial quality control: implications for Parkinson disease. Hum Mol Genet. 2010;19(R1):R28–37.

Deas E, Plun-Favreau H, Wood NW. PINK1 function in health and disease. EMBO Mol Med. 2009;1(3):152–65.

Gautier CA, Kitada T, Shen J. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proc Natl Acad Sci USA. 2008;105(32):11364–9.

Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol. 2010;12(2):119–31.

Jendrach M, Gispert S, Ricciardi F, Klinkenberg M, Schemm R, Auburger G. The mitochondrial kinase PINK1, stress response and Parkinson's disease. J Bioenerg Biomembr. 2009;41(6):481–6.

Kim Y, Park J, Kim S, Song S, Kwon SK, Lee SH, et al. PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. Biochem Biophys Res Commun. 2008;377(3):975–80.

Kitada T, Pisani A, Porter DR, Yamaguchi H, Tscherter A, Martella G, et al. Impaired dopamine release and synaptic plasticity in the striatum of PINK1-deficient mice. Proc Natl Acad Sci USA. 2007;104(27):11441–6.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298(5600):1912–34. New York, NY.

Okamoto K, Shaw JM. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu Rev Genet. 2005;39:503–36.

Park J, Kim Y, Chung J. Mitochondrial dysfunction and Parkinson's disease genes: insights from Drosophila. Dis Model Mech. 2009;2(7–8):336–40.

Plun-Favreau H, Klupsch K, Moisoi N, Gandhi S, Kjaer S, Frith D, et al. The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. Nat Cell Biol. 2007;9(11):1243–52.

Pridgeon JW, Olzmann JA, Chin LS, Li L. PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1. PLoS Biol. 2007;5(7):e172.

Samann J, Hegermann J, von Gromoff E, Eimer S, Baumeister R, Schmidt E. Caenorhabditits elegans LRK-1 and PINK-1 act antagonistically in stress response and neurite outgrowth. J Biol Chem. 2009;284(24):16482–91.

Silvestri L, Caputo V, Bellacchio E, Atorino L, Dallapiccola B, Valente EM, et al. Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. Hum Mol Genet. 2005;14(22):3477–92.

Thomas KJ, Cookson MR. The role of PTEN-induced kinase 1 in mitochondrial dysfunction and dynamics. Int J Biochem Cell Biol. 2009;41(10):2025–35.

Valente EM, Bentivoglio AR, Dixon PH, Ferraris A, Ialongo T, Frontali M, et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. Am J Hum Genet. 2001;68(4):895–900.

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 2004;304(5674):1158–60. (New York, NY.)

Weihofen A, Thomas KJ, Ostaszewski BL, Cookson MR, Selkoe DJ. Pink1 forms a multiprotein complex with Miro and Milton, linking Pink1 function to mitochondrial trafficking. Biochemistry. 2009;48(9):2045–52.

Zhou C, Huang Y, Shao Y, May J, Prou D, Perier C, et al. The kinase domain of mitochondrial PINK1 faces the cytoplasm. Proc Natl Acad Sci USA. 2008;105(33):12022–7.

PTP Epsilon

► PTPe (RPTPe and Cyt-PTPe)

PTPe

► PTPe (RPTPe and Cyt-PTPe)

PTPe (RPTPe and Cyt-PTPe)

Ari Elson and Liat Rousso-Noori
Department of Molecular Genetics, Arnold R. Meyer Institute of Biological Sciences, The Weizmann Institute of Science, Rehovot, Israel

Synonyms

cyt-PTPe; PTP Epsilon; PTPe; PTPeC; PTPeM; RPTPe; tm-PTPe

Historical Background

Phosphorylation of tyrosine residues in proteins is one of the better-studied molecular mechanisms for regulating protein structure and function and with it – the function of cells and organisms. Tyrosine phosphorylation is a reversible process that is controlled by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The numbers of PTKs and PTPs are similar and small relative to the numbers of their potential substrates; there are 81 and 85 genes that yield active, protein-specific PTPs and PTKs, respectively (Alonso et al. 2004). As a result, individual PTPs and PTKs each target multiple substrates and fulfill distinct roles in different physiological systems. Although PTPs were first described in molecular terms in 1988, about a decade after PTKs, both protein superfamilies are now recognized as critical regulators of protein phosphorylation and cellular physiology (Alonso et al. 2004).

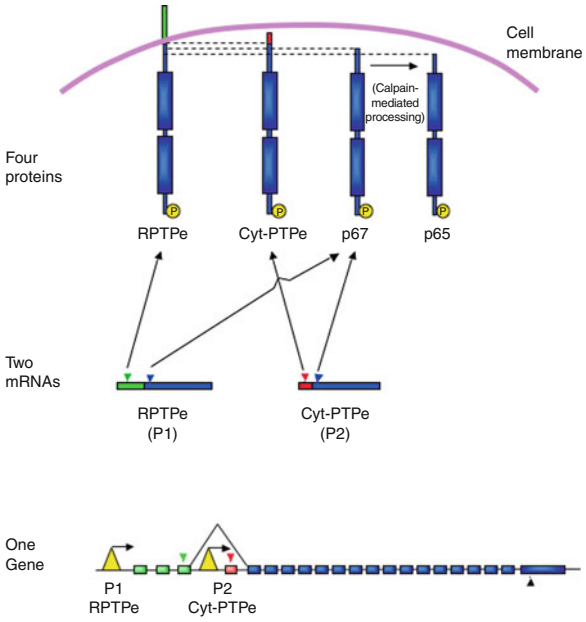
The several dozen members of the PTP superfamily can be subdivided into smaller groups based on their structures and substrate specificities (Andersen et al. 2001b; Alonso et al. 2004). The first subfamily of PTPs that was identified and characterized was the "classical" tyrosine-specific subfamily of PTPs. This subfamily is now known to contain 38 genes; 21 of these encode receptor-type products that are integral membrane proteins, while the remaining 17 encode non-receptor-type proteins. Use of alternative promoters, alternative splicing, and posttranslational processing are fairly common among PTPs, hence the 38 "classical" PTP genes give rise to a larger number of protein products. All members of this family contain one or two PTP domains that contain the PTP signature motif "(I/V)HCSXGXGR(S/T)G"; in PTPs that contain two PTP domains, typically only the N-terminal of the two is catalytically active (Andersen et al. 2001b; Alonso et al. 2004). Studies conducted during the past two decades have shown that PTPs participate in regulating many distinct physiological processes, that they target specific substrates, and that they can activate or inhibit signaling processes in a context-dependent manner (Tonks 2006; Hendriks et al. 2008).

Protein tyrosine phosphatase Epsilon (PTPe), which is the focus of this review, was first identified in 1990 as a receptor-type PTP within the so-called type IV subfamily of the "classical" family of PTPs (Krueger et al. 1990). Subsequent work revealed that there is a single PTPe gene that gives rise to at least two mRNA and four protein species. More recent work, which is outlined below, has described roles for PTPe in regulating various processes at the levels of the cell and the organism.

PTPe – The Gene and its Protein Products

Humans and similar higher organisms possess a single gene for PTP Epsilon, which maps to the distal part of chromosome 10 (band 10q26.2; chromosome 7 in mouse). The *PTPRE* gene contains two known promoters, each of which gives rise to a distinct major protein product. The proximal P1 promoter gives rise to the mRNA for the receptor-type form of PTPe noted above, RPTPe, while the distal P2 promoter produces the mRNA for the non-receptor form, cyt-PTPe ((Elson and Leder 1995a; Nakamura et al. 1996; Tanuma et al. 1999); Fig. 1). Since both mRNAs are derived from the same gene, they share most of their sequences; differences exist only at their 5′ ends. A similar situation exists at the protein level: RPTPe and cyt-PTPe share a large segment of their protein sequence, which includes the two PTP catalytic domains. The N termini of both forms are, however, distinct: the membrane-spanning domain and the short (27 AA in humans) and heavily-glycosylated extracellular domain of RPTPe are replaced by a 12 AA-long hydrophilic sequence in cyt-PTPe (Fig. 1). As a result, RPTPe is an integral membrane protein; in contrast, cyt-PTPe is predominantly cytosolic, although about 20% of cyt-PTPe molecules are found in association with the cell membrane and a further 10% are detected in the cell nucleus (Elson and Leder 1995a; Nakamura et al. 1996). Two additional shorter protein forms of PTPe exist. These are p67, which is produced from the mRNAs for RPTPe and for cyt-PTPe by initiation of translation at an internal initiation codon located in the sequence identical in both mRNAs, and p65, which is produced from RPTPe, cyt-PTPe, or p67 proteins by calpain-mediated proteolytic processing (Gil-Henn et al. 2000; Gil-Henn et al. 2001). Finally, a fifth form of PTPe (cyt-PTPePD1) has been suggested to exist. This form, which is believed to be produced by alternative splicing of cyt-PTPe mRNA, contains only the first (D1) PTP domain followed by a unique C-terminal tail (Wabakken et al. 2002).

The cell- and tissue-specificity of the two promoters of the *PTPRE* gene differ significantly to the point where RPTPe and cyt-PTPe are rarely co-expressed



PTPe (RPTPe and Cyt-PTPe), Fig. 1 PTPe: gene, mRNAs and proteins. *Gene*: The single *PTPRE* gene (*bottom*) contains two promoters (P1, P2; *yellow* triangles) and 21 exons. The distal P1 promoter produces the RPTPe transcript that links the first three unique exons (1–3, *green*) directly to exon 5 (*blue*). The proximal P2 promoter produces the cyt-PTPe transcript that starts at exon 4 (*red*), linking it to exon 5 and beyond (*blue*). Exons 5–21 (*blue*) are common to both transcripts. *Green* and *red* triangles denote the respective initiator ATG codons of both mRNAs; the black triangle in exon 21 denotes the termination codon. Exons and introns are not drawn to scale. *mRNAs*: the RPTPe and cyt-PTPe mRNAs are shown. The *green* and *red* regions depict sequences unique to RPTPe or cyt-PTPe that are derived from genomic exons 1–3 or 4, respectively. Regions marked in *blue* are derived from exons 5–21. Triangles mark the initiation codons used for producing RPTPe (*green*), cyt-PTPe (*red*) or p67 (*blue*). *Proteins*: Four major protein forms of PTPe are shown. The *green* and *red* regions of RPTPe and cyt-PTPe, respectively, are unique to each form; regions drawn in *blue* are common to all forms and are derived originally from exons 5–21 in the *PTPRE* gene. The C-terminal phosphorylation site (Y695 in RPTPe = Y638 in cyt-PTPe) is marked in all forms by a *yellow* circle. p67 is produced from both RPTPe and cyt-PTPe mRNAs by initiation of translation at an ATG codon located in the sequence common to both (*blue* triangle, mRNA level). p65 is produced by calpain-mediated proteolytic processing of RPTPe, cyt-PTPe or p67

in the same cell type. RPTPe is expressed predominantly in neurons, testes, and lungs, while cyt-PTPe is found mainly in hematopoietic cells (B- and T-lymphocytes, macrophages, erythrocytes), osteoclasts, Schwann cells, and muscle cells. Along with their distinct subcellular localization, their different expression patterns among cell types argue strongly that RPTPe and cyt-PTPe are physiologically nonequivalent.

Regulation of PTPe Activity

In general, PTPs are active enzymes. Many of the mechanisms that regulate PTP activity do so therefore either by inhibiting the activity of the enzyme or by directing it to specific substrates or to particular subcellular regions (den Hertog et al. 2008). In the case of PTPe, several regulatory mechanisms have been described:

A. Expression: The divergent expression patterns of the two promoters of the *PTPRE* gene among cells types and tissues and their distinct localization patterns within cells ensure that RPTPe and cyt-PTPe are present at specific locations and encounter particular substrates. Importantly, the two promoters also differ in their ability to respond to physiological signals. Little is known about how the activity of the P1 promoter, which drives expression of RPTPe, is controlled. In contrast, the P2 promoter is readily activated by growth factors and activators of mitogenic signaling, such as serum, TPA, EGF, and basic FGF. Differentiation of human promyelocytic leukemia HL60 cells with TPA causes a massive increase in cyt-PTPe expression, while treatment of M1 mouse myeloid leukemia cells with IL6 increases cyt-PTPe expression and reduces expression of RPTPe (Elson and Leder 1995a; Tanuma et al. 1999).

B. Oxidation: The signature motif (I/V) HCSXGXGR(S/T)G present in PTPs contains at its core a cysteine residue, which is essential for dephosphorylation to occur. Oxidizing this residue by, for example, reactive oxygen species that are produced during the normal course of signaling processes is sufficient to abolish PTP catalytic activity (den Hertog et al. 2008). All forms of PTPe are susceptible to oxidation, which inhibits their activity (Toledano-Katchalski et al. 2003).

C. Dimerization: Dimerization of PTPs has been suggested to inhibit their catalytic activity; this sets PTPs apart from PTKs, where dimerization typically stabilizes the active form of the enzyme (den Hertog et al. 2008). RPTPe and cyt-PTPe undergo spontaneous inhibitory dimerization and higher-order aggregation in cells. In the case of cyt-PTPe, dimerization can be induced by physiological signals, such as increased oxidative stress and following activation of the EGF receptor (Toledano-Katchalski et al. 2003).

D. Phosphorylation: A major phosphorylation site exists at the C-terminus of PTPe (Y695 in RPTPe = Y638 in cyt-PTPe). This site undergoes phosphorylation in various physiological situations, including in the presence of Neu in mouse mammary tumor cells (Berman-Golan and Elson 2007) and following activation of integrins in osteoclasts (Granot-Attas et al. 2009). Phosphorylation most likely does not affect the specific activity of PTPe (Berman-Golan and Elson 2007). It does, however, drive the phosphatase to activate ▶ Src in osteoclasts and in mammary tumor cells, probably by allowing phosphorylated PTPe to bind the Src SH2 domain, thus releasing the inhibitory interaction between this domain and Y527 of the kinase (Berman-Golan and Elson 2007; Granot-Attas et al. 2009). C-terminal phosphorylation of PTPe also promotes its association with other molecules, as in its inhibitory association with tubulin (Sines et al. 2007).

E. Cleavage: RPTPe, cyt-PTPe, and p67 can undergo proteolytic processing by calpain to generate p65, an N-terminally-truncated form of PTPe that is catalytically active and entirely cytosolic (Gil-Henn et al. 2001). Processing of RPTPe or of cyt-PTPe, which are entirely or significantly membrane-associated proteins, effectively removes PTPe from the cell membrane and may control its access to substrates.

F. Other mechanisms: RPTPe possesses an extracellular domain, which suggests that its activity or physiological role may be affected by binding of extracellular ligands. However, as is the case with most RPTPs, no extracellular ligands of RPTPe are known, and the functional role of the extracellular domain of this PTP is not clear. RPTPe also undergoes massive glycosylation, but the roles of this modification, if any, are also unknown.

Physiological Roles of PTPe

Cancer

PTKs often activate signaling processes, and many are *bona fide* oncogenes. As a result, PTPs are often perceived as inhibitors of signaling processes and of malignant transformation. While this is true in many instances, in several cases, including that of PTPe, PTPs can activate signaling processes and support malignant transformation. Moreover, such roles are often specific for a given physiological system; a particular PTP may support signaling and transformation in one context but inhibit it in another. Along these lines, RPTPe is expressed at high levels in mouse mammary tumors initiated in vivo by transgenic Neu or Ras proteins (Elson and Leder 1995b), suggesting that RPTPe participates in the transformation processes induced by these two oncogenes. In support of this conclusion, overexpression of RPTPe in mouse mammary glands leads to massive mammary hyperplasia and associated tumorigenesis (Elson 1999). At the molecular level, RPTPe activates the Src PTK in mammary tumors initiated by Neu, by dephosphorylating Src at its inhibitory Y527. Activation of Src is important for these cells to manifest their full malignant properties; in mammary tumors initiated by Neu in mice lacking PTPe (EKO mice), Src is less active and the transformed phenotype of the resulting mammary tumor cells is weakened (Gil-Henn and Elson 2003; Berman-Golan and Elson 2007). On the other hand, overexpression of cyt-PTPe (but not of RPTPe) in M1 murine leukemia cells inhibited JAK-STAT signaling that was induced by interleukins 6 or 10, and led to reduced tumorigenicity of these cells following their implantation in SCID or in nude mice (Tanuma et al. 2001; Tanuma et al. 2003). The various protein forms of PTPe can therefore either support or inhibit signaling events in a context- and system-specific manner.

Myelination in the Nervous System

Experimental evidence has shown that PTPe helps regulate myelination in the nervous system. Cyt-PTPe is expressed in Schwann cells, which are responsible for axon myelination in the peripheral nervous system. In mice that genetically lack PTPe, myelination of axons in the sciatic nerve is significantly delayed, indicating that this form of PTPe supports the myelination process (Peretz et al. 2000). Molecular studies have established that the delayed-rectifier, voltage-gated potassium channels Kv2.1 and Kv1.5 are substrates of cyt-PTPe in Schwann cells, and that dephosphorylation by cyt-PTPe downregulates channel activity. In agreement, Kv2.1 and Kv1.5 are hyper-phosphorylated, and the activity of Kv channels in general is elevated in EKO Schwann cells, suggesting that loss of cyt-PTPe in Schwann cells affects their function by dysregulating Kv channel activity (Peretz et al. 2000). In separate studies, expression of PTPe, presumably RPTPe, was significantly up-regulated during differentiation of CG4 progenitor cells into oligodendrocytes, the cells that drive

myelination in the central nervous system. Expression in mice of an inactive mutant of RPTPe under the direction of the myelin protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) promoter delayed myelination of the optic nerve, most likely due to dominant-negative effects of the transgene (Muja et al. 2004). The limited scope of the phenotype in this case suggests that lack of RPTPe does not significantly affect the myelination capability of most oligodendrocytes.

Macrophages, Osteoclasts, and Bone Degradation

Cyt-PTPe is expressed in cells of various hematopoietic lineages, including in cells of the monocyte lineage that gives rise to macrophages and osteoclasts. Macrophages from mice lacking PTPe are defective in their ability to mount a respiratory burst in response to exposure to lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF alpha). Bone marrow cells from these mice also produce more interleukin-10 and less TNF alpha in response to LPS treatment (Sully et al. 2001). These findings indicate that cyt-PTPe plays an important role in regulating macrophage activity, although the molecular basis for this remains unknown at present.

Cyt-PTPe also plays a major role in regulating the function of osteoclasts, the resident cells that degrade bone. Bone mass is regulated by the opposing activities of osteoblasts, which produce bone matrix, and osteoclasts, which degrade it. Both cell types coexist and function in close proximity, ensuring that the resulting bone is of the proper mass and physical properties. Mice lacking PTPe exhibit increased amounts of bone that are secondary to reduced activity of their osteoclasts. Osteoclasts lacking cyt-PTPe do not adhere to bone well in vivo and display significant defects in the structure, organization, and stability of podosomes, the adhesion structures of these cells (Granot-Attas et al. 2009). At the molecular level, cyt-PTPe helps dephosphorylate and activate Src downstream of integrins, which are activated when osteoclasts make physical contact with bone or matrix. Lack of cyt-PTPe results in reduced Src activation in osteoclasts; normal Src activity can be restored by expression of cyt-PTPe in the cells. Importantly, the defects in podosomal organization and stability can be rescued in PTPe-deficient osteoclasts not only by expressing cyt-PTPe but also by expressing Src, which functions in this system downstream of cyt-PTPe. This finding demonstrates that the integrin-cyt-PTPe-Src axis is critical for proper structure and function of osteoclasts (Granot-Attas et al. 2009).

Insulin Receptor Signaling, Glucose Homeostasis and Body Weight Regulation

A number of studies have suggested that PTPe negatively regulates the activity of the insulin receptor PTK. Accordingly, RPTPe, but not cyt-PTPe, has been shown to down-regulate the insulin receptor in baby hamster kidney (BHK) cells (Andersen et al. 2001). Later studies showed that expression of exogenous RPTPe down-regulates the insulin receptor in primary hepatocytes, possibly by targeting the insulin receptor itself. Expression of the phosphatase also reduced the extent of activation by insulin of downstream signaling molecules, such as ERK, AKT, and GSK3 (Nakagawa et al. 2005). A third study showed that cyt-PTPe negatively regulates the insulin receptor in muscle cells. Treating L6 skeletal muscle cells with insulin induced association of the insulin receptor with cyt-PTPe, while expression of exogenous cyt-PTPe resulted in decreased phosphorylation of the insulin receptor, IRS-1, AKT and GSK3, and decreased glucose uptake. In agreement, opposite results were obtained when expression of endogenous cyt-PTPe was inhibited in these cells. Phosphorylation of the insulin receptor and or IRS-1 were also increased in primary muscle cells from PTPe-deficient mice (Aga-Mizrachi et al. 2008). Examination of mice genetically lacking PTPe revealed improved glucose clearance in both lean and obese mice, particularly in males, indicating that PTPe plays a role in regulating glucose homeostasis on the level of the intact organism (Rousso-Noori et al. 2011). RPTPe and cyt-PTPe are therefore inhibitors of insulin receptor signaling, although more studies are required to determine whether organ-specific roles exist for various isoforms of PTPe.

Female mice lacking PTPe are leptin hypersensitive and are resistant to weight gain that normally follows physiological challenges, such as a high-fat diet. This is most likely caused by down-regulation of leptin receptor signaling by RPTPe in the hypothalamus; RPTPe performs this role by directly dephosphorylating and inhibiting Jak2, a tyrosine kinase that is activated downstream of the leptin receptor and which plays a key role in leptin signaling (Rousso-Noori et al. 2011).

Additional Roles

PTPe is expressed in endothelial cells (Thompson et al. 2001; Nakagawa et al. 2004). The role of PTPe in these cells appears to be isoform-specific, since expression of RPTPe activated Src and stimulated migration and survival of porcine aortic endothelial cells, while expression of cyt-PTPe had the opposite effect (Thompson et al. 2001; Nakagawa et al. 2004). RPTPe is expressed in erythrocytes; erythrocytes of EKO mice exhibit abnormal morphology, increased calcium-activated K+ channel activity, and increased activity of the Src family PTKs Fyn and Yes. These findings indicate that RPTPe plays an important role in erythrocyte physiology and provide an example where this PTP down-regulates, instead of activating, PTKs of the Src family (De Franceschi et al. 2008). Finally, RPTPe is expressed in bone marrow–derived mast cells (BMMC), where it participates in downregulating FceRI-mediated mast cell function (Akimoto et al. 2009). RPTPe most likely targets the Syk PTK downstream of the FceRI receptor, leading ultimately to decreased calcium mobilization and MAPK activation. Accordingly, EKO mice display elevated levels of passive systemic anaphylaxis induced by antigen and IgE. EKO mice contain normal numbers of mast cells, indicating that lack of PTPe affects the function, but not production, of these cells (Akimoto et al. 2009).

Summary

Like other PTPs and PTKs, PTPe is a multifaceted participant of signaling processes. The single PTPe gene produces several distinct protein isoforms, which act in different cell types, in distinct areas within a given cell, and target various substrates. It is therefore not surprising that PTPe supports the transduced signal in some cases while inhibiting it in others. It is very well established that dysregulation of protein phosphorylation is a cause for disease in human beings; indeed, increasing numbers of novel drugs are becoming available, which treat a particular disease by specifically targeting molecules that play key roles in its etiology. Design of reagents that target a particular PTP for medical gain remains a worthy, albeit elusive, goal. For this to occur, continued studies that will characterize in full the functions of PTPe and of other PTPs at the molecular, cellular, and whole-organism levels are required. Studies of this type should also address functional redundancies between PTPs, making it clear which other PTPs should also be targeted to obtain a particular outcome, and which PTPs perform opposite roles in this context and should not be targeted. A related challenge is to fully decipher the repertoire of proteins that PTPe acts upon in cells. Traditional biochemical methods address this issue at the level of the individual substrate protein, while advanced proteomic techniques, such as mass spectrometry, may provide unbiased data on a cell-wide basis.

References

Aga-Mizrachi S, Brutman-Barazani T, Jacob AI, Bak A, Elson A, Sampson SR. Cytosolic protein tyrosine phosphatase-epsilon is a negative regulator of insulin signaling in skeletal muscle. Endocrinology. 2008;149(2):605–614.

Akimoto M, Mishra K, Lim KT, Tani N, Hisanaga SI, Katagiri T, et al. Protein tyrosine phosphatase epsilon is a negative regulator of FcepsilonRI-mediated mast cell responses. Scand J Immunol. 2009;69(5):401–411.

Alonso A, Sasin J, Bottini N, Friedberg I, Osterman A, Godzik A, et al. Protein tyrosine phosphatases in the human genome. Cell. 2004;117(6):699–711.

Andersen JN, Elson A, Lammers R, Romer J, Clausen JT, Moller KB, et al. Comparative study of protein tyrosine phosphatase-epsilon isoforms: membrane localization confers specificity in cellular signalling. Biochem J. 2001;354(Pt 3):581–590.

Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, et al. Structural and evolutionary relationships among protein tyrosine phosphatase domains. Mol Cell Biol. 2001b;21(21):7117–7136.

Berman-Golan D, Elson A. Neu-mediated phosphorylation of protein tyrosine phosphatase epsilon is critical for activation of Src in mammary tumor cells. Oncogene. 2007;26(49):7028–7037.

De Franceschi L, Biondani A, Carta F, Turrini F, Laudanna C, Deana R, et al. PTPepsilon has a critical role in signaling transduction pathways and phosphoprotein network topology in red cells. Proteomics. 2008;8(22):4695–4708.

den Hertog J, Ostman A, Bohmer FD. Protein tyrosine phosphatases: regulatory mechanisms. FEBS J. 2008;275(5):831–847.

Elson A. Protein tyrosine phosphatase epsilon increases the risk of mammary hyperplasia and mammary tumors in transgenic mice. Oncogene. 1999;18(52):7535–7542.

Elson A, Leder P. Identification of a cytoplasmic, phorbol ester-inducible isoform of protein tyrosine phosphatase epsilon. Proc Natl Acad Sci USA. 1995a;92(26):12235–12239.

Elson A, Leder P. Protein-tyrosine phosphatase epsilon. An isoform specifically expressed in mouse mammary tumors initiated by v-Ha-ras OR neu. J Biol Chem. 1995b;270(44):26116–26122.

Gil-Henn H, Elson A. Tyrosine phosphatase-epsilon activates Src and supports the transformed phenotype of Neu-induced mammary tumor cells. J Biol Chem. 2003;278(18):15579–15586.

Gil-Henn H, Volohonsky G, Toledano-Katchalski H, Gandre S, Elson A. Generation of novel cytoplasmic forms of protein

tyrosine phosphatase epsilon by proteolytic processing and translational control. Oncogene. 2000;19(38):4375–4384.
Gil-Henn H, Volohonsky G, Elson A. Regulation of protein-tyrosine phosphatases alpha and epsilon by calpain-mediated proteolytic cleavage. J Biol Chem. 2001;276(34):31772–31779.
Granot-Attas S, Luxenburg C, Finkelshtein E, Elson A. PTP epsilon regulates integrin-mediated podosome stability in osteoclasts by activating src. Mol Biol Cell. 2009;20(20):4324–4334.
Hendriks WJ, Elson A, Harroch S, Stoker AW. Protein tyrosine phosphatases: functional inferences from mouse models and human diseases. FEBS J. 2008;275(5):816–830.
Krueger NX, Streuli M, Saito H. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. EMBO J. 1990;9:3241–3252.
Muja N, Lovas G, Romm E, Machleder D, Ranjan M, Gallo V, et al. Expression of a catalytically inactive transmembrane protein tyrosine phosphatase epsilon (tm-PTP epsilon) delays optic nerve myelination. Glia. 2004;48(4):278–297.
Nakagawa Y, Yamada N, Shimizu H, Shiota M, Tamura M, Kim-Mitsuyama S, et al. Tyrosine phosphatase epsilonM stimulates migration and survival of porcine aortic endothelial cells by activating c-Src. Biochem Biophys Res Commun. 2004;325(1):314–319.
Nakagawa Y, Aoki N, Aoyama K, Shimizu H, Shimano H, Yamada N, et al. Receptor-type protein tyrosine phosphatase epsilon (PTPepsilonM) is a negative regulator of insulin signaling in primary hepatocytes and liver. Zool Sci. 2005;22(2):169–175.
Nakamura K, Mizuno Y, Kikuchi K. Molecular cloning of a novel cytoplasmic protein tyrosine phosphatase PTP epsilon. Biochem Biophys Res Commun. 1996;218:726–732.
Peretz A, Gil-Henn H, Sobko A, Shinder V, Attali B, Elson A. Hypomyelination and increased activity of voltage-gated K(+) channels in mice lacking protein tyrosine phosphatase epsilon. EMBO J. 2000;19:4036–4045.
Rousso-Noori L, Knobler H, Levy-Apter E, Kuperman Y, Neufeld-Cohen A, Keshet Y, et al. Protein tyrosine phosphatase epsilon affects body weight by downregulating leptin signaling in a phosphorylation-dependent manner. Cell Metab. 2011;13(5):562–572.
Sines T, Granot-Attas S, Weisman-Welcher S, Elson A. Association of tyrosine phosphatase epsilon with microtubules inhibits phosphatase activity and is regulated by the epidermal growth factor receptor. Mol Cell Biol. 2007;27(20):7102–7112.
Sully V, Pownall S, Vincan E, Bassal S, Borowski AH, Hart PH, et al. Functional abnormalities in protein tyrosine phosphatase epsilon-deficient macrophages. Biochem Biophys Res Commun. 2001;286:184–188.
Tanuma N, Nakamura K, Kikuchi K. Distinct promoters control transmembrane and cytosolic protein tyrosine phosphatase epsilon expression during macrophage differentiation. Eur J Biochem. 1999;259:46–54.
Tanuma N, Shima H, Nakamura K, Kikuchi K. Protein tyrosine phosphatase epsilonC selectively inhibits interleukin-6- and interleukin- 10-induced JAK-STAT signaling. Blood. 2001;98:3030–3034.
Tanuma N, Shima H, Shimada S, Kikuchi K. Reduced tumorigenicity of murine leukemia cells expressing protein-tyrosine phosphatase, PTPepsilon C. Oncogene. 2003;22(12):1758–1762.
Thompson LJ, Jiang J, Madamanchi N, Runge MS, Patterson C. PTP-epsilon, a tyrosine phosphatase expressed in endothelium, negatively regulates endothelial cell proliferation. Am J Physiol Heart Circ Physiol. 2001;281:H396–403.
Toledano-Katchalski H, Tiran Z, Sines T, Shani G, Granot-Attas S, den Hertog J, et al. Dimerization in vivo and inhibition of the nonreceptor form of protein tyrosine phosphatase epsilon. Mol Cell Biol. 2003;23(15):5460–5471.
Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. Nat Rev Mol Cell Biol. 2006;7(11):833–846.
Wabakken T, Hauge H, Funderud S, Aasheim HC. Characterization, expression and functional aspects of a novel protein tyrosine phosphatase epsilon isoform. Scand J Immunol. 2002;56(3):276–285.

PTPeC

▶ PTPe (RPTPe and Cyt-PTPe)

PTPeM

▶ PTPe (RPTPe and Cyt-PTPe)

PTPH1

▶ PTPN3/PTPN4

PTPMEG/PTPMEG1

▶ PTPN3/PTPN4

PTPN3/PTPN4

Timothy J. Bauler[1] and Philip D. King[2]
[1]Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT, USA
[2]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA

Synonyms

PTPH1; PTPMEG/PTPMEG1

Historical Background

A common event in cellular signal transduction pathways is the phosphorylation of proteins on tyrosine residues. Tyrosine phosphorylation is reversible. The forward reaction is mediated by protein tyrosine kinases. By contrast, the reverse reaction is performed by protein tyrosine phosphatases (PTP). The PTP family consists of 107 genes whose protein products are diverse in form and specificity (Alonso et al. 2004). PTPN3 and PTPN4 constitute two members of this family that were initially identified by PCR amplification using primers specific to conserved regions of the catalytic domain of canonical PTP. PTPN3 was initially cloned from a HeLa cell cDNA library, whereas PTPN4 was cloned from a megakaryoblastic cell line, (Gu et al. 1991; Yang and Tonks 1991). PTPN3 and PTPN4 are 50% identical and 67% homologous at the amino acid level.

Structure and Expression Studies

PTPN3 and PTPN4 are cytosolic proteins that localize to the plasma membrane. Structurally, each PTP comprises of an amino-terminal FERM (band 4.1, Ezrin, Radixin, and Moesin) domain, a central PDZ (PSD-95, Dlg, ZO-1) domain, and a carboxy-terminal PTP domain. FERM and PDZ domains are protein–protein interaction domains that commonly bind the cytosolic tail of transmembrane proteins and can also interact with the phospholipid, phosphatidylinositol 4,5 bisphosphate, $PI(4,5)P_2$. The FERM domains of PTPN3 and PTPN4 are required for plasma membrane association (Gjorloff-Wingren et al. 2000).

PTPN3 and PTPN4 are expressed ubiquitously (Pilecka et al. 2007; Bauler et al. 2008). Both PTP are expressed at high levels in the thalamus. In addition, PTPN4 is highly expressed in testes. A recent comprehensive examination of the PTP transcriptome in the murine immune system reported expression of PTPN3 in all immune cell types examined, with elevated transcript levels in immature dendritic cells (DC), NKT cells, activated CD4 cells, and intestinal intraepithelial CD8 cells (Arimura and Yagi 2010). The same study reported elevated transcript levels for PTPN4 specifically in immature DC, NK cells, and B cells, and also found that PTPN4 transcript levels in DC were increased following LPS stimulation. Alterations in PTPN3 expression levels have been associated with esophageal cancer, and mutations of PTPN3 have been found in colorectal cancer cell lines (Wang et al. 2004). However, a subsequent examination of primary colorectal cancer samples did not detect mutations in PTPN3 (Wood et al. 2007).

Enzymatic Substrates and Protein–Protein Interactions

PTPN3 has been shown to associate with and dephosphorylate several different target proteins. In COS-7 cells, PTPN3 was demonstrated to dephosphorylate cotransfected T cell antigen receptor (TCR) ζ chain (Sozio et al. 2004). Furthermore, in a T cell line, overexpressed PTPN3 was shown to inhibit TCR-induced activation of the promoter of the gene for the cytokine interleukin-2 (Han et al. 2000). Physical association of PTPN3 with TCRζ has also been described (Sozio et al. 2004). PTPN3 interacts with phosphorylated growth hormone receptor in vitro, and overexpression of PTPN3 in cell lines modulates signaling from this receptor (Pasquali et al. 2003; Pilecka et al. 2007). Expression of PTPN3 in fibroblasts can inhibit cellular growth. This effect has been attributed to the ability of PTPN3 to dephosphorylate p97/valosin-containing protein, an established regulator of the cell cycle (Zhang et al. 1999). In contrast to this, PTPN3 binding to and dephosphorylation of p38γ mitogen-activated protein kinase has been recently demonstrated to promote cellular proliferation (Hou et al. 2010). Oncogenic human papillomavirus E6 protein binds PTPN3 and targets it for degradation by the proteasome (Jing et al. 2007). Other proteins that PTPN3 has been shown to interact with include tumor necrosis factor alpha-convertase, the cardiac sodium channel $Na_v1.5$, and 14-3-3β protein (Zhang et al. 1997; Jespersen et al. 2006).

PTPN4 has also been characterized as having multiple binding partners. Like PTPN3, PTPN4 has been demonstrated to bind and dephosphorylate TCRζ in vitro, and when over-expressed in a T cell line, it inhibits TCR signaling (Young et al. 2008). PTPN4 has been shown to bind the δ and ε subunits of the glutamate receptor, signaling from which is required for learning and coordination (Hironaka et al. 2000). Interaction of PTPN4 with attenuated rabies virus glycoprotein is associated with apoptotic death of infected cells (Prehaud et al. 2010).

Generation and Characterization of PTPN3- and PTPN4-Deficient Organsims

So as to understand the importance of PTPN3 and PTPN4 in normal physiological processes, organisms deficient in expression of these PTP were generated. *Drosophila* has a single homologue of PTPN3 and PTPN4, termed PTPMEG. Flies that lack expression of full length PTPMEG were observed to become trapped alive in their food, suggesting a neuronal defect. Subsequent examination of neural connectivity patterns in the brain revealed roles for PTPMEG in the establishment and maintenance of axon projections in *Drosophila* (Whited et al. 2007).

PTPN3-deficient mice have been shown to be grossly normal, although subtle phenotypes have been observed. One group has reported that male PTPN3-deficient mice have a higher body mass and reduced working memory compared to wild-type littermates, whereas female PTPN3-deficient mice exhibit motor learning deficiencies (Pilecka et al. 2007; Patrignani et al. 2008). These mild phenotypes have not been replicated in PTPN3-deficient mice generated by an independent group (Bauler et al. 2008). A function for PTPN3 in TCR signal transduction has not been observed in primary murine T cells, contrary to the aforementioned evidence generated in cell lines (Bauler et al. 2007). Potential subtle roles for PTPN3 in spontaneous pain perception and the positive regulation of LPS-induced cytokine release in mice have been recently reported (Patrignani et al. 2010).

PTPN4-deficient mice have also been shown to be largely normal. Two independent groups demonstrated that T cell function remained intact in PTPN4-deficient mice (Bauler et al. 2008; Young et al. 2008). One other group reported that motor learning and cerebellar synaptic plasticity were impaired in PTPN4-deficient mice, although altered motor learning was not confirmed by an independent group (Kina et al. 2007; Bauler et al. 2008).

Owing to the high degree of homology between PTPN3 and PTPN4 and broad expression patterns of both PTP, it remained possible that loss of either protein was functionally compensated for by the other, thus accounting for the lack of discernible phenotypes in single PTP-deficient mice. However, double PTPN3-PTPN4-deficient mice are also indistinguishable from wild-type mice when considering T cell function, body mass, and motor learning (Bauler et al. 2008).

Summary

PTPN3 and PTPN4 are homologous, ubiquitously expressed PTP that have been implicated as regulators of diverse cellular signaling cascades through interactions with a variety of protein substrates. However, definitive physiological roles for PTPN3 and PTPN4 have not yet been described in higher organisms. Further study of these PTP may reveal nonredundant functions that can be readily demonstrated in gene-targeted mice. Given the high degree of similarity and between PTPN3 and PTPN4, both in terms of structure and expression, it is likely that such functions will be revealed only in double PTP-deficient animals.

References

Alonso A, Sasin J, Bottini N, Friedberg I, Osterman A, Godzik A, et al. Protein tyrosine phosphatases in the human genome. Cell. 2004;117(6):699–711.

Arimura Y, Yagi J. Comprehensive expression profiles of genes for protein tyrosine phosphatases in immune cells. Sci Signal. 2010;3(137):rs1.

Bauler TJ, Hughes ED, Arimura Y, Mustelin T, Saunders TL, King PD. Normal TCR signal transduction in mice that lack catalytically active PTPN3 protein tyrosine phosphatase. J Immunol. 2007;178(6):3680–7.

Bauler TJ, Hendriks WJ, King PD. The FERM and PDZ domain-containing protein tyrosine phosphatases, PTPN4 and PTPN3, are both dispensable for T cell receptor signal transduction. PLoS ONE. 2008;3(12):e4014.

Gjorloff-Wingren A, Saxena M, Han S, Wang X, Alonso A, Renedo M, et al. Subcellular localization of intracellular protein tyrosine phosphatases in T cells. Eur J Immunol. 2000;30(8):2412–21.

Gu MX, York JD, Warshawsky I, Majerus PW. Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. Proc Natl Acad Sci USA. 1991;88(13):5867–71.

Han S, Williams S, Mustelin T. Cytoskeletal protein tyrosine phosphatase PTPH1 reduces T cell antigen receptor signaling. Eur J Immunol. 2000;30(5):1318–25.

Hironaka K, Umemori H, Tezuka T, Mishina M, Yamamoto T. The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor delta 2 and epsilon subunits. J Biol Chem. 2000;275(21):16167–73.

Hou SW, Zhi HY, Pohl N, Loesch M, Qi XM, Li RS, et al. PTPH1 dephosphorylates and cooperates with p38gamma MAPK to increase ras oncogenesis through PDZ-mediated interaction. Cancer Res. 2010;70(7):2901–10.

Jespersen T, Gavillet B, van Bemmelen MX, Cordonier S, Thomas MA, Staub O, et al. Cardiac sodium channel Na(v) 1.5 interacts with and is regulated by the protein tyrosine phosphatase PTPH1. Biochem Biophys Res Commun. 2006;348(4):1455–62.

Jing M, Bohl J, Brimer N, Kinter M, Vande Pol SB. Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. J Virol. 2007;81(5):2231–9.
Kina S, Tezuka T, Kusakawa S, Kishimoto Y, Kakizawa S, Hashimoto K, et al. Involvement of protein-tyrosine phosphatase PTPMEG in motor learning and cerebellar long-term depression. Eur J Neurosci. 2007;26(8):2269–78.
Pasquali C, Curchod ML, Walchli S, Espanel X, Guerrier M, Arigoni F, et al. Identification of protein tyrosine phosphatases with specificity for the ligand-activated growth hormone receptor. Mol Endocrinol. 2003;17(11):2228–39.
Patrignani C, Magnone MC, Tavano P, Ardizzone M, Muzio V, Greco B, et al. Knockout mice reveal a role for protein tyrosine phosphatase H1 in cognition. Behav Brain Funct. 2008;4:36.
Patrignani C, Lafont DT, Muzio V, Greco B, Hooft van Huijsduijnen R, Zaratin PF. Characterization of protein tyrosine phosphatase H1 knockout mice in animal models of local and systemic inflammation. J Inflamm. 2010;7:16. Lond.
Pilecka I, Patrignani C, Pescini R, Curchod ML, Perrin D, Xue Y, et al. Protein-tyrosine phosphatase H1 controls growth hormone receptor signaling and systemic growth. J Biol Chem. 2007;282(48):35405–15.
Prehaud C, Wolff N, Terrien E, Lafage M, Megret F, Babault N, et al. Attenuation of rabies virulence: takeover by the cytoplasmic domain of its envelope protein. Sci Signal. 2010;3(105):ra5.
Sozio MS, Mathis MA, Young JA, Walchli S, Pitcher LA, Wrage PC, et al. PTPH1 is a predominant protein-tyrosine phosphatase capable of interacting with and dephosphorylating the T cell receptor zeta subunit. J Biol Chem. 2004;279(9):7760–9.
Wang Z, Shen D, Parsons DW, Bardelli A, Sager J, Szabo S, et al. Mutational analysis of the tyrosine phosphatome in colorectal cancers. Science. 2004;304(5674):1164–6.
Whited JL, Robichaux MB, Yang JC, Garrity PA. Ptpmeg is required for the proper establishment and maintenance of axon projections in the central brain of Drosophila. Development. 2007;134(1):43–53.
Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, et al. The genomic landscapes of human breast and colorectal cancers. Science. 2007;318(5853):1108–13.
Yang Q, Tonks NK. Isolation of a cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cytoskeletal-associated proteins band 4.1, ezrin, and talin. Proc Natl Acad Sci USA. 1991;88(14):5949–53.
Young JA, Becker AM, Medeiros JJ, Shapiro VS, Wang A, Farrar JD, et al. The protein tyrosine phosphatase PTPN4/PTP-MEG1, an enzyme capable of dephosphorylating the TCR ITAMs and regulating NF-kappaB, is dispensable for T cell development and/or T cell effector functions. Mol Immunol. 2008;45(14):3756–66.
Zhang SH, Kobayashi R, Graves PR, Piwnica-Worms H, Tonks NK. Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3beta protein. J Biol Chem. 1997;272(43):27281–7.
Zhang SH, Liu J, Kobayashi R, Tonks NK. Identification of the cell cycle regulator VCP (p97/CDC48) as a substrate of the band 4.1-related protein-tyrosine phosphatase PTPH1. J Biol Chem. 1999;274(25):17806–12.

PTPN5

► Striatal-Enriched Protein Tyrosine Phosphatase (STEP)

PTPN6

Marina Tiemi Shio[1] and Martin Olivier[2]
[1]Department of Microbiology and Immunology, The Research Institute of The McGill University Health Centre, McGill University, Montréal, Québec, Canada
[2]Departments of Medicine and of Microbiology and Immunology, The Research Institute of The McGill University Health Centre, McGill University, Montréal, Québec, Canada

Synonyms

Hematopoietic cells phosphatase (HCP); Protein tyrosine phosphatase 1 C (PTP1C); Protein tyrosine phosphatase, non-receptor type 6 (PTPN6); Src homology region 2 (SH2)-domain phosphatase or Src homology region 2 domain-containing PTP-1 (SHP-1 or SH-PTP1)

Gene Location

Mouse chromosome: 6; Location: 6 60.22 cM
Human chromosome: 12; Location: p12p13

Historical Background

The Src homology region 2 (SH) domain-containing protein tyrosine phosphatase-1 (SHP-1) is a member of the large family of protein tyrosine phosphatase (PTP). SHP-1 was identified in hematopoietic cells and organs involved in immune responses such as the spleen, thymus, lymph node, and bone marrow. SHP-1 is also known as hematopoietic cell phosphatase (HCP) because of its expression in these cells. Herein the nomenclature SHP-1 will be used. Back to the beginning of the 1990s, the study of tyrosine kinases was very well advanced; however, knowledge about their

natural counterpart, the tyrosine phosphatases, was still greatly unraveled. Early on, different research groups isolated and identified a protein that contains two SH2 domains with phosphotyrosine phosphatase activity. Soon after, the PTPN6 gene that encodes SHP-1 was mapped to chromosomes 6 and 12 of mouse and human, respectively. Chromosome 6 (and its homologue in human chromosome 12) was also correlated with the autosomal recessive motheaten ("me") phenotype that affects mice to develop a severe combined immunodeficiency and systemic autoimmunity (Tsui et al. 2006). The association of "me" mutation and SHP-1 was confirmed by Shultz et al. (1993) and Tsui et al. (1993). In line with those findings, the involvement of SHP-1 in diseases was earliest described 15 years before its discovery. The first study to report the motheaten mice was done by Green and Shultz (1975), in which they described a recessive mutation in C57Bl/6 mice in the Jackson Laboratory. Those "me" mice exhibit a motheaten phenotype, consisting of disseminated fur-free patches. In addition, these mice were found to develop skin lesion as early as 3 weeks after birth, which progress rapidly and mice did not survive over 8 weeks (Green and Shultz 1975). Subsequently, a similar mutation in the *PTPN6* loci was described, called viable motheaten ("mev") as mice can survive longer than "me" mice, up to 9–12 weeks. This mutation, different of "me" (that does not express SHP-1), provides a protein with small deletion or insertion in the PTP domain which results in around 20% of the phosphatase activity of the cells expressing wild-type SHP-1 (Tsui et al. 2006). Both "me" and "mev" mice develop an unusual pneumonia with a progressive infiltration of macrophages, neutrophils, and lymphocytes, as well as spontaneous inflammatory disorder in many organs such as the kidney and joints (Zhu et al. 2010).

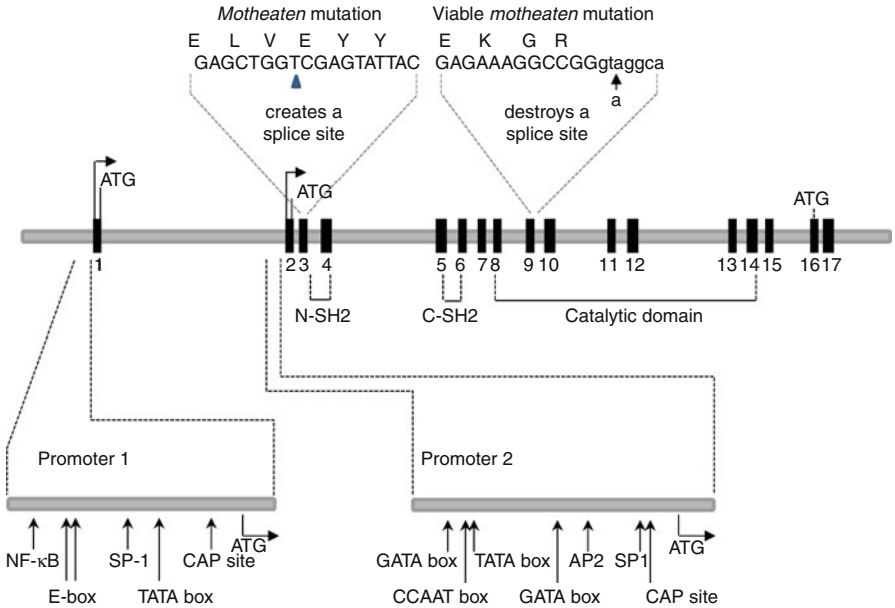
PTPN6 Gene, SHP-1 Protein Expression and Structure

Human PTPN6 gene consists of 17 exons, approximately 17 kb in length, leading to the formation of transcripts of 2.4–2.6 kb. The gene has two promoter regions (within exon 1 and 2) hence encodes two forms of proteins that do not show significant difference in their phosphatase activity (Fig. 1). However, there are differences in the initial sequence of the protein and in SHP-1 tissue expression. Promoter 1 (located 7 kb before promoter 2) encodes MLSRG amino acid sequence in the N-terminal of the form 1 of SHP-1, (I)SHP-1, and promoter 2, MVR in the form 2 of SHP-1, (II)SHP-1. The promoter 1 has two E-boxes (regulated by USF1 and/or 2), a NF-κB and SP1 binding site, as well as a TATA box. The promoter 2 has two GATA sequences, a CCAAT box, a TATA box, an AP2 and SP1 binding sites. The first promoter is active in non-hematopoietic tissue and the second, in the hematopoietic cells. Therefore, (I)SHP-1 is expressed in human epithelial cells and can be induced by PMA via ▶ NF-κB activation and (II)SHP-1, in cells related with the immune system such as macrophages, neutrophils, mast cells, lymphocytes, and so on (Wu et al. 2003; Tsui et al. 2006). In addition to immune cells, it was shown recently that SHP-1 is expressed in hepatocytes, and myocytes (Dubois et al. 2006). Concerning its cellular localization, (I)SHP-1 is located within the nuclear compartment and the (II) SHP-1 in the cytoplasm of resting cells, suggesting that the two forms of SHP-1 might have different targets (Poole and Jones 2005). The other exons of *PTPN6* are responsible for encoding different domains of the protein, hence exons 3 and 4 encode the N-terminal SH2 domain; exons 5 and 6, the C-terminal SH2 domain; and exons 8–10, the catalytic domain (Figs. 1 and 2).

Structurally the SHP-1 protein, similar to SHP-2, is constituted by two SH2 domains in the N-terminal region (N-SH2 and C-SH2), followed by a classical catalytic PTP domain and the C-terminal region containing two tyrosyl (Y536 and Y564) and one serine (S591) phosphorylation sites (Fig. 2). In addition, in the latter region, SHP-1 has a phosphatidic acid–binding active site, a functional nuclear localization signal (NLS), and a potential lipid raft-targeting motif (Poole and Jones 2005; Pao et al. 2007). Furthermore, the C-terminal region of SHP-1 can be longer in the SHP-1 L (long form of SHP-1), having 66 amino acids more than SHP-1, due to an alternative splicing (Poole and Jones 2005).

Function of SHP-1 Domains, Regulation of SHP-1 Activity and Targets

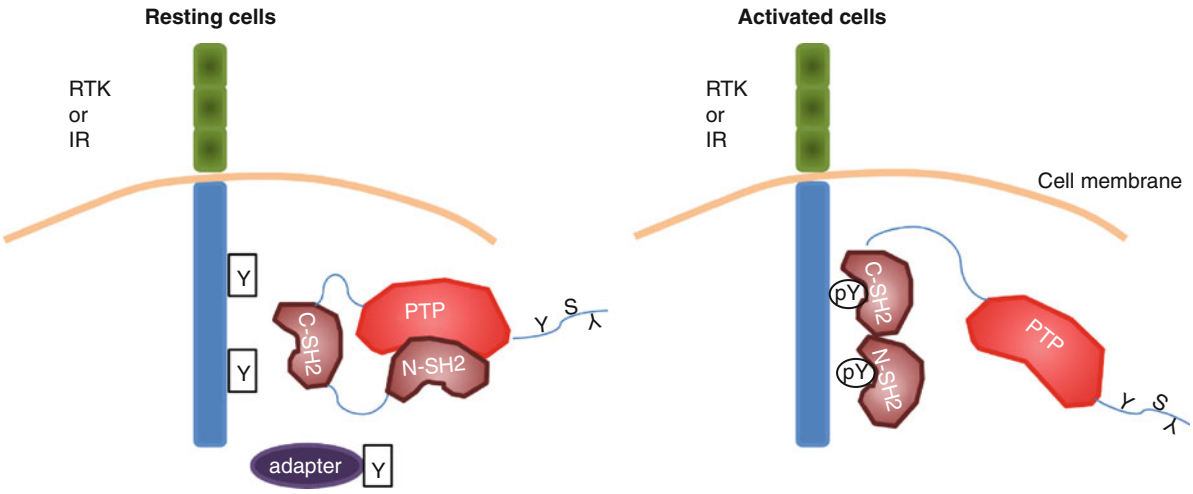
The different domains of SHP-1 protein have specific functions. Crystallographic study revealed that the N-SH2 is bound to the catalytic site in the resting



PTPN6, Fig. 1 Schematic diagram of *PTPN6* gene and mutation of murine *SHP-1*. The schematic representation of *PTPN6* gene shows introns in gray and exons in black. Exons which are responsible for encoding the N-terminus SH2 (3, 4), C-terminus SH2 (5, 6), and catalytic (8–14) domains are also shown in numbers. The bottom part represents the promoter regions 1 and 2 that encode slightly different SHP-1 proteins with comparable activity. The upper part denotes the feature of *motheaten* and viable *motheaten* mutations. The first phenotype, caused by the *motheaten* mutation, is generated by deletion of the C at position 288 that creates a cryptic 5′ splice site consensus. Once activated, the mutation leads to the deletion of 101 bp that creates a frame shift and the stop codon occurs 81 bp downstream of the deleted fragment. The second phenotype, caused by the viable *motheaten* mutation, occurs due to the substitution of T with A in the phosphatase domain. This mutation disrupts a GT dinucleotides creating two cryptic 5′ splice sites – one 15 bp before and the other 69 bp after the normal donor splice site, generating a short (less than five amino acids) and a long SHP-1 (with 23 amino acids or more), respectively. For further review see Wu et al. (2003) and Tsui et al. (2006)

state of the protein. The interaction of N-SH2 and the PTP domain is achieved via charge–charge interaction and has a bidirectional inhibitory effect. Thus N-SH2 inhibits PTP activity and the catalytic domain impedes the binding of the N-SH2 with phosphotyrosyl targets. On the other hand, C-SH2 has a minimum interaction with the PTP domain. The latter information indicates that C-SH2 can effectively serve as a sensor for the targeted phosphotyrosine residue. Once activated, the protein changes its conformation and releases the phosphopeptide-binding pocket of N-SH2 and also the catalytic site of the PTP domain (Poole and Jones 2005; Pao et al. 2007).

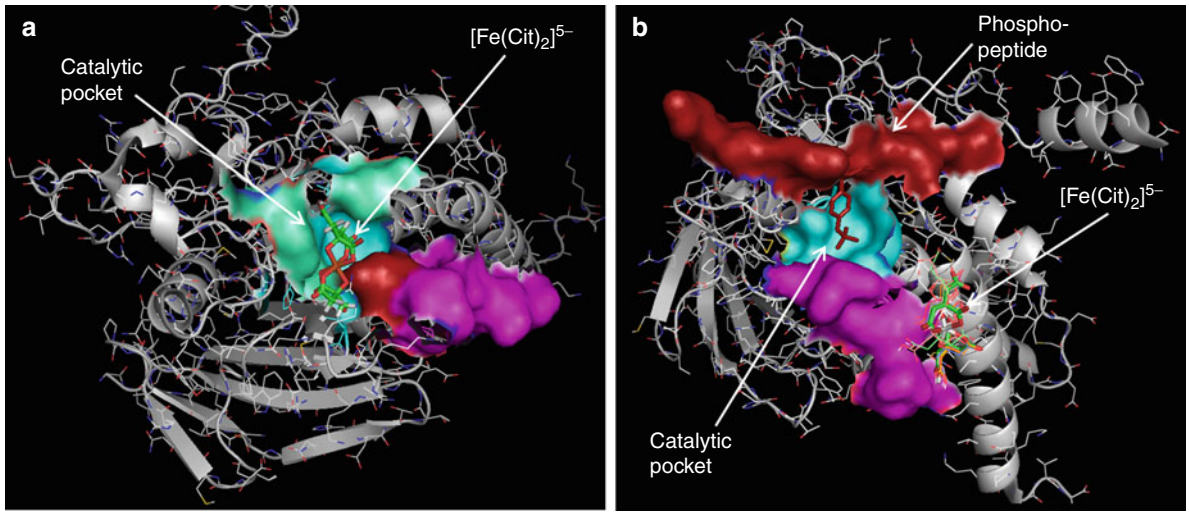
SH2 domains of SHP-1 interact with phosphotyrosyl residues present in different molecules such as scaffold proteins, receptor tyrosine kinases (RTKs), cytokine receptors, and immune inhibitory receptors. The latter are transmembrane proteins with the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) or the immunoreceptor tyrosine-based switch motif (ITSM). The first domain contains six amino acid stretches – V/L/IxpYxxL/V – and the second – TxpYxxV/I – both are phosphorylated upon receptor stimulation and recruit SHP-1. A combinatorial phosphotyrosine study revealed that SHP-1 C-SH2 domain binds selectively to (V/I/L)xpYAx(LV), whereas N-SH2 to LxpY(M/F)x(F/M) and LXpYAXL. In addition, N-SH2 binding can be increased with hydrophobic or positively charged residue at the position pY + 4 and/or pY + 5 (Pao et al. 2007; Lorenz 2009). More recently, it has been reported that several kinases of the JAK, IKK, and ▶ MAP kinase families have similar ITIM motif found mainly in the catalytic domain of the kinase allowing SHP-1 to interact with the kinase via a kinase tyrosine-based inhibitory motif (KTIM) (Abu-Dayyeh et al. 2008) and therefore conferring a better regulation over the kinase activity at resting state.



PTPN6, Fig. 2 Mechanism of SHP-1 activation. *Left* panel shows SHP-1 in its "closed" form in steady state (resting) cells. The C-SH2 domain acts as an antenna to search for phosphorylated tyrosyl residues and the N-SH2 domain interacts with the catalytic domain (PTP) preventing its activity. The C-terminus of SHP-1 consists of two tyrosine (Y) residues and one serine (S) residue, which positively or negatively regulate the SHP-1 activity. Once cells are activated (*right* panel), a receptor tyrosine kinase (RTK), an inhibitory receptor (IR), or scaffolding adapters become phosphorylated in their tyrosine residues (pY). The C-SH2 domain then binds to pY, changing the conformation of the protein and freeing the catalytic domain to act in to the substrate. For further review see Pao et al. (2007) and Poole and Jones (2005)

In addition to SH2 domains, the C-terminal of SHP-1 also controls the SHP-1 activity. As stated above, the C-terminal region contains two tyrosyl (Y536 and Y564) and one serine (S591) residues. The phosphorylation of Y536 upon insulin stimulation or apoptosis inducer reagent increases SHP-1 activation in lymphoblast, ovary, hepatoma, and monocytic cell lines (Lorenz 2009). Zhang et al. (2003) observed a 4- to 8-fold increase of Y564 phosphorylation concomitant with a 1.6-fold augmented activity of the PTP by using a protein ligation approach to install a non-hydrolyzable phosphomimetic (phosphonate) analog on Y536 and Y564 of SHP-1. On the other hand, phosphorylation of S591 negatively regulates SHP-1 activity. In human platelets SHP-1 is constitutively associated with protein kinase C (PKC) alpha and ▶ Vav1, inhibiting Vav1 function. However, when platelets are activated via a thrombin receptor, PKCα is activated and phosphorylates the S591 residue of SHP-1 inhibiting its activity; consequently Vav1 is phosphorylated. In addition to the negative regulation of SHP-1, S591 phosphorylation is also involved in determining its sub-cellular localization. It has been observed that in T cells that phosphorylated S591 SHP-1 displayed reduced translocation to lipid raft and nuclear compartment (Poole and Jones 2005). More recently, S591 phosphorylation has been implicated in the negative regulation of SHP-1 and its nuclear translocation in response to hypertonic solution (Zhang et al. 2010). To sum up, while phosphorylation of C-terminal of SHP-1 in S591 appears to negatively regulate the phosphatase, phosphorylation of Y536 and Y564 residues increase its activity.

Once activated, the catalytic domain of SHP-1 is freed and dephosphorylates its substrate by releasing phosphate. The catalytic domain of SHP-1 contains the PTP signature motif (I/V)HCxxGxxR(S/T) with an essential cysteinyl residue that can mediate hydrolysis via a thio-phosphate intermediate formation. SHP-1 can be reversibly inactivated when the cysteine residue is oxidized by reactive oxygen species (ROS). Oxidized PTP domain can be reactivated via glutathione or thioredoxin pathway (Pao et al. 2007). More recently, Gomez et al. (Gomez et al. 2010) elegantly demonstrated a new negative regulation of SHP-1 activity, as well as other PTPs, mediated by iron (Fig. 3). The iron dicitrate complex was found to compete with phosphotyrosine substrate for the catalytic pocket of the PTP, leading to inhibition of PTPs and increase of MAPK signaling pathway. In this line of thought, SHP-1 activity can be self-regulated and regulated by external oxidation or mechanism involving iron.



PTPN6, Fig. 3 Computational docking of SHP-1 and phosphotyrosyl substrate/iron citrate complexes. (**a**) Docking experiment depicts that the dicitrate iron complex C7 $[Fe(Cit)_2]^{5-}$ resides in the catalytic pocket of SHP-1 (PDB code: 1GWZ). (**b**) In vitro co-crystallization of SHPS-1 (PDB code: 1FPR) and tyrosine phospho-peptide substrate pY459) exclude the C7 complex, suggesting a possible competition between C7 complex and the phosphotyrosyl peptide for the catalytic pocket

Concerning the target of SHP-1, this phosphatase can regulate many signaling pathways triggered by different receptors such as T cell receptors (TCR), B cell receptors (BCR), and toll-like receptors (TLR). In T cells SHP-1 has been shown to directly regulate the TCR or associated co-receptor; however, the exact mechanism is unclear. It has been proposed an antagonism mechanism of SHP-1. A study done with T cells bearing TCR with different specificity revealed that stimulation of these cells with an agonist protein for one receptor and an antagonist to a second receptor lead to the binding of Lck to the agonist-activated receptor, whereas SHP-1 is recruited to the antagonist-activated TCR. In addition, in the presence of weak antigen Lck phosphorylates SHP-1 on Y564 residue, recruiting SHP-1 to associate and dephosphorylate Lck. However, in the presence of a strong ligand binding, ERK is activated and phosphorylates Lck, in this way Lck does not interact with SHP-1 and triggered signaling continues (Pao et al. 2007; Lorenz 2009). Other SHP-1 substrates such as Zap-70, SLP-76 and Vav have been proposed, those substrates and other important signaling molecules are localized constitutively or after stimulation in the lipid raft as well as SHP-1. In T cells, around 20–30% of SHP-1 is found constitutively in the lipid raft and has a negative regulation role on Lck phosphorylation and IL-2 production. Furthermore, T cells express inhibitory receptors such as the carcinoembryonic antigen–related cell adhesion molecule-1 (CEACAM-1), CD5, the cytotoxic T-lymphocyte antigen-4 (CTLA4), and the leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) that can recruit or are constitutively associated with SHP-1, resulting in its activation and consequent inhibition of T cell signaling and function (Lorenz 2009).

SHP-1 is also known to regulate BCR. In B cells it has been proposed that SHP-1 is interacting with different inhibitory receptors such as CD22, CD72, CD5, and PIR-B, as well as with various substrates like ▶ PI3K, Vav, Btk, SLP-76/65, Syk, and 3BP2. For instance, CD22 is an ITIM containing receptor member of the sialic-acid-binding immunoglobulin-like lectin (Siglec) family. CD22 associates and negatively regulates BCR response via SHP-1, as in CD22 or SHP-1 deficient B cells were found to display higher intracellular calcium mobilization and IgM secretion. However, the role of sialic acid, CD45, or IgM as ligand to CD22 is still controversial. In addition, SHP-1 activity is down-regulated in activated B cells, suggesting that SHP-1 in resting cells is active through an unknown mechanism. The production of ROS has been proposed as a mechanism of transient negative modulation of SHP-1 activity in activated B cells.

Conversely, it was shown in the same study that the production of ROS was still higher when the SHP-1 activity came back to baseline levels, suggesting that other mechanism could be regulating the phosphatase activity (Pao et al. 2007; Nitschke 2009).

Concerning TLR signaling, recently it has been demonstrated in an infectious experimental model that SHP-1 associates and rapidly inhibits the interleukin-1 receptor-associated kinase 1 (IRAK-1) and LPS-mediated macrophage functions upon TLR4 stimulation, as well as in response to other ligands targeting other TLRs utilizing IRAK-1 in their signaling cascades. SHP-1 was found to interact with an evolutionarily conserved KTIM motif on IRAK-1 (Abu-Dayyeh et al. 2008).

SHP-1 and Diseases

As discussed above, studies demonstrating implication of SHP-1 in different diseases has been based in part on phenotypes observed in "me" or "mev" mice. These mice are known to spontaneously develop inflammatory abnormalities in multiple organs such as skin, kidney, and lungs, which drive attention to certain diseases such as respiratory disorders. Of utmost interest, involvement of SHP-1 in cancer, infectious diseases, and diabetes has been reported and will be discussed in the following sections.

Respiratory Diseases

Studies done with "mev" mice revealed higher infiltration of macrophages, eosinophils, as well as neutrophils and lymphocytes in the lungs. These mice developed an allergic-type inflammatory response similar to allergic asthma, including mucus hyperproduction, metaplasia of Goblet cells, and increased airway hyperresponsiveness. Additionally, the concentration of Th2 type cytokines such as IL-4, IL-5, and IL-13 was found to be elevated, as well as chemokines (CCL2/MCP-1 and CCL11/eotaxin) that are important for recruitment of monocytes and eosinophils. As Th2 response seemed to be predominant in "mev" mice, further investigation on mast cells was done due to their capacity to produce IL-4 and IL-13, comparable to basophils and eosinophils. In vitro study using IL-13 as a growth factor showed that cells from "mev" mice grow less than their WT counterpart indicating that SHP-1 controls the number of mast cells progenitors (c-kit/FcεRI double-positive cells) and its maturation. Furthermore mast cells from these mice were found to be resistant for apoptosis, possibly due to the increased expression of the anti-apoptosis gene Bcl2. In vitro experiments with mast cells from "mev" mice showed higher production of IL-4 and IL-5 in response to superoxide, as well as to spontaneously produce those cytokines being dependent on ROS production. The role of ROS in the regulation of SHP-1 activity is well known; therefore it is not surprising that it could regulate the residual 20% of SHP-1 activity found in cells from "mev" mice. In addition to cytokines, SHP-1 also regulates mast cell degranulation as revealed by bone marrow–derived mast cells from "mev" mice that spontaneously releases β-hexosaminadase and also has greater response to IgE stimulation in comparison to WT cells. In conclusion, in experimental models SHP-1 seems to modulate lung disorders; however, correlation of human lungs disease and SHP-1 expression has still not been found. Nevertheless, low expression of SHP-1 has been correlated with human chronic myeloid leukemia and psoriasis (Zhu et al. 2010).

Cancer

SHP-1 is known to negatively regulate intracellular signaling necessary to many cellular functions including cell growth and division; therefore, it is not surprising that many cancer cells have been found not to express SHP-1. For instance, cell lines derived from T cell, B cell, and natural killer (NK) cell lymphomas (all classified as non-Hodgkin's lymphomas), as well as leukemia, were found in majority not to express or to have a reduced expression of SHP-1. However, some other cancerous cell lines such as BJAB, HUT102B, HUB102B2, AG876BL, KK124, Kem III, and WW1BL do indeed express SHP-1. Expression of SHP-1 in lymphomas has been proposed to be related with the progression, aggressivity, and the stage at which the cell is (Wu et al. 2003). Clinically, an analysis of 207 patient samples having different malignant lymphomas/leukemia revealed that 100% of NK/T cell lymphomas and 95% of samples from patients with malignant lymphomas bore complete abrogation of SHP-1 expression. One mechanism found to be responsible for the latter event is the methylation of PTPN6 promoter. DNA methyl-transferase, together with STAT3 and histone diacetylase I promote the silencing of SHP-1 in lymphoma and leukemia cells. However, methylation of PTPN6 promoter might not

be the only mechanism as treatment with demethylation drugs increase SHP-1 RNA, but not protein expression, suggesting that other posttranscriptional modification of SHP-1 could be involved (Wu et al. 2003; Tsui et al. 2006). In contrast to hematopoietic cancer, SHP-1 protein and RNA expression are increased in epithelial cancer such as ovarian and prostate cancer. In the case of prostate cancer, SHP-1 expression and activity is related with the production of somatostatin that has a paracrine/autocrine inhibitory effect on cell proliferation via SHP-1. In this way, SHP-1 expression is related with proliferation and aggressiveness. Interestingly, it has been reported in the last few years that the use of PTP inhibitors such as peroxovanadium can greatly abrogate the progression of ovarian cancer in mice (Caron et al. 2008).

Infectious Diseases

As stated above in regard to cancer, SHP-1 has an important anti-proliferative function; in this line of thought its absence is essential for the progression of the disease. However, during the course of certain infectious diseases, it has been found that the negative regulatory action of SHP-1 can be exploited by pathogens to favor their survival and development into the host cells. Two examples are the intracellular pathogens of *Leishmania* genus and *Mycobacterium tuberculosis* known to infect phagocytes of the monocyte/macrophage lineage. These cells play a critical role in innate immune response being responsible to engulf invading microorganisms and to terminate them. Macrophages display many microbicidal molecules such as ROS, nitric oxide (NO), and pro-inflammatory mediators. Even with such an impressive defense system, they can become infected by different intracellular parasites, as mentioned above. In the case of *Leishmania*, a protozoan parasite responsible for causing leishmaniasis, is known to interact with macrophages and to rapidly inactivate the IFNγ-inducible kinase JAK-2, as well as several members of the MAP kinase family, and the LPS-induced IRAK-1 kinase (critical to TLR pathway). This multiple signaling alteration results in the inhibition of several macrophage microbicidal functions. One of the mechanisms exploited by the parasite is the inactivation of the host cell by inducing macrophage SHP-1 activity upon its cleavage by *Leishmania* metalloprotease GP63 in its C-terminal portion (Gomez et al. 2010). Interestingly, as mentioned above in the text, SHP-1 was found to interact with an evolutionarily conserved kinase tyrosyl-based inhibitory motif (KTIM) being present in various kinases such as JAK-2, IRAK-1, and ERK-1/2, all being targeted by SHP-1 inhibitory action upon *Leishmania* infection (Abu-Dayyeh et al. 2008). The role of SHP-1 in leishmaniasis has been further demonstrated in an experimental model by a pharmacological intervention using the bis-peroxovanadium bpV(phen) and genetically using the "mev" mice. Treatment of mice with PTP inhibitors reduced both parasite load and skin lesion of *Leishmania* major-infected mice, and completely protected mice from the visceral *L. donovani* infection. The same phenotype was observed in "mev" mice, which developed a significantly reduced cutaneous lesion (Mansfield and Olivier 2001).

Another intra-macrophage pathogen is the bacteria *Mycobacterium tuberculosis*, which is the causative agent of tuberculosis. This bacterium enters through the respiratory airway and infects the lungs' macrophages. How the bacillus survives inside of the macrophages is not very clear. It has been proposed that *M. tuberculosis* inhibits the macrophage functions by the action of its bacterial cell wall glycolipid (LAM). LAM was reported to inhibit ERK1/2 kinase by the activation of SHP-1 (Nandan et al. 2000). LAM was also found to inhibit *M. tuberculosis*–induced apoptosis and to involve SHP-1, as LAM-induced apoptosis was shown to be absent in SHP-1 deficient "me" cells. This latter effect was shown to be related with increased LAM-induced NO production in the absence of SHP-1 (Rojas et al. 2002).

In addition to those pathogens, filarial nematodes can release excretory-secretory products (ES) influencing the immune cell functions. The ES is secreted by a majority of filarial nematodes affecting humans including *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus*, and by *Acanthocheilonema viteae* affecting rodents. ES protein has been found to inhibit the B cell functions by inducing SHP-1 recruitment to the BCR and therefore altering its signaling activity (Mansfield and Olivier 2001).

Glucose Metabolism (Diabetes)

Diabetes mellitus is described as a defect in the glucose metabolism, hence hyperglycemia, because of insufficient production of insulin or its tolerance. Recently the role of SHP-1 in the glucose homeostasis and diabetes-related pathology has been explored. A study using "mev" mice demonstrated that in the

normal condition SHP-1 collaborates to the insulin resistance profile due to its negative modulation of the insulin receptor substrate-1 (IRS-)-related signaling pathway PI3K/AKT, possibly by modulating PTEN. Furthermore, SHP-1 was also found to downregulate the transmembrane glycoprotein carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) that controls insulin clearance. Liver cells from "mev" showed a hyperphosphorylation of CEACAM-1. This adhesion molecule is also co-immunoprecipitated with SHP-1. Furthermore, SHP-1, but not PTP1B or PTP-PEST, can specifically dephosphorylate CEACAM-1. In vivo experiment showed a slower clearance of radiolabeled insulin in "mev" mice favoring the increase of glucose (Dubois et al. 2006).

One common diabetic consequence is the retinopathy and other microvascular complications due to a hyperglycemia-induced apoptosis process. The latter involves the activation of PKCα that regulates NF-κB activation (via ROS generation) and p38 MAPK. This MAPK induces SP-1 activation and consequently enhancement of SHP-1 expression. SHP-1 negatively regulates the pro-survival growth factor receptor PDGFR-β resulting in pericyte apoptosis (Geraldes et al. 2009).

Summary

Collectively, it is possible to conclude that SHP-1 – a member of the PTPs superfamily – is a critical negative regulatory phosphatase of kinases controlling several functions of immune cells. Its involvement in the development of many diseases, such as cancer, allergy, diabetes, and infection diseases, has been clearly established, and found to be related to its inability to perform its regulatory functions or to be exploited by pathogens to evade immune responses. An in-depth understanding on how it is regulated could bring about the discovery of very innovative ways to treat various pathologies.

References

Abu-Dayyeh I, Shio MT, Sato S, Akira S, Cousineau B, Olivier M. Leishmania-induced IRAK-1 inactivation is mediated by SHP-1 interacting with an evolutionarily conserved KTIM motif. PLoS Negl Trop Dis. 2008;2:e305.

Caron D, Savard PE, Doillon CJ, Olivier M, Shink E, Lussier JG, et al. Protein tyrosine phosphatase inhibition induces anti-tumor activity: evidence of Cdk2/p27 kip1 and Cdk2/SHP-1 complex formation in human ovarian cancer cells. Cancer Lett. 2008;262:265–75.

Dubois MJ, Bergeron S, Kim HJ, Dombrowski L, Perreault M, Fournes B, et al. The SHP-1 protein tyrosine phosphatase negatively modulates glucose homeostasis. Nat Med. 2006;12:549–56.

Geraldes P, Hiraoka-Yamamoto J, Matsumoto M, Clermont A, Leitges M, Marette A, et al. Activation of PKC-delta and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy. Nat Med. 2009;15:1298–306.

Gomez MA, Alisaraie L, Shio MT, Berghuis AM, Lebrun C, Gautier-Luneau I, et al. Protein tyrosine phosphatases are regulated by mononuclear iron dicitrate. J Biol Chem. 2010;285:24620–8.

Green MC, Shultz LD. Motheaten, an immunodeficient mutant of the mouse. I. Genetics and pathology. J Hered. 1975; 66:250–8.

Lorenz U. SHP-1 and SHP-2 in T cells: two phosphatases functioning at many levels. Immunol Rev. 2009;228:342–59.

Mansfield JM, Olivier M. Immune evasion by parasites. In: Kaufmann SHE, Rouse BT, Sacks DL, editors. Immunology of Infectious Disease. Washington, DC: ASM Press; 2001. p. 453–70.

Nandan D, Knutson KL, Lo R, Reiner NE. Exploitation of host cell signaling machinery: activation of macrophage phosphotyrosine phosphatases as a novel mechanism of molecular microbial pathogenesis. J Leukoc Biol. 2000; 67:464–70.

Nitschke L. CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions. Immunol Rev. 2009;230:128–43.

Pao LI, Badour K, Siminovitch KA, Neel BG. Nonreceptor protein-tyrosine phosphatases in immune cell signaling. Annu Rev Immunol. 2007;25:473–523.

Poole AW, Jones ML. A SHPing tale: perspectives on the regulation of SHP-1 and SHP-2 tyrosine phosphatases by the C-terminal tail. Cell Signal. 2005;17:1323–32.

Rojas M, Olivier M, Garcia LF. Activation of JAK2/STAT1-alpha-dependent signaling events during Mycobacterium tuberculosis-induced macrophage apoptosis. Cell Immunol. 2002;217:58–66.

Shultz LD, Schweitzer PA, Rajan TV, Yi T, Ihle JN, Matthews RJ, et al. Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell. 1993;73:1445–54.

Tsui HW, Siminovitch KA, de Souza L, Tsui FW. Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. Nat Genet. 1993;4: 124–9.

Tsui FW, Martin A, Wang J, Tsui HW. Investigations into the regulation and function of the SH2 domain-containing protein-tyrosine phosphatase, SHP-1. Immunol Res. 2006;35: 127–36.

Wu C, Sun M, Liu L, Zhou GW. The function of the protein tyrosine phosphatase SHP-1 in cancer. Gene. 2003;306:1–12.

Zhang Z, Shen K, Lu W, Cole PA. The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. J Biol Chem. 2003;278: 4668–74.

Zhang L, Oh SY, Wu X, Oh MH, Wu F, Schroeder JT, et al. SHP-1 deficient mast cells are hyperresponsive to

stimulation and critical in initiating allergic inflammation in the lung. J Immunol. 2010;184:1180–90.
Zhu Z, Oh SY, Cho YS, Zhang L, Kim YK, Zheng T. Tyrosine phosphatase SHP-1 in allergic and anaphylactic inflammation. Immunol Res. 2010;47:3–13.

PUMA (p53 Upregulated Modulator of Apoptosis), BBC3 (BCL2-Binding Component 3)

► BCL-2 Family

Purinergic Receptors

► Nucleotide Receptor P2Y

Pyk1

► ACK1

Q

Querkopf

► MOZ and MORF Lysine Acetyltransferases

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

R

R10 (Quail)

▶ TPD52 (Tumor Protein D52)

R7 Binding Protein (R7BP)

▶ R7BP/R9AP

R7BP/R9AP

Kirill A. Martemyanov[1] and Pooja Parameswaran[2]
[1]Department of Neuroscience, The Scripps Research Institute, Jupiter, Florida, USA
[2]Department of Pharmacology, University of Minnesota, Minneapolis, Minneapolis, MN, USA

Synonyms

R7 Binding Protein (R7BP); RGS9 Anchor Protein (R9AP)

Introduction and Historical Background

Regulator of G protein signaling (RGS) proteins constitute a diverse family with more than 30 members that contain the hallmark RGS domain. Most members serve as negative regulators of G protein signaling by catalyzing the GTP hydrolysis on Gα subunits leading to their inactivation (Ross and Wilkie 2000; Hollinger and Hepler 2002). Based on their structural organization and sequence homology RGS proteins are divided into 5–6 families (Ross and Wilkie 2000; Hollinger and Hepler 2002). The R7 RGS family (R7 RGS) contains multidomain proteins conserved from *C. elegans* to humans that, in mammals are represented by four members: RGS6, RGS7, RGS9, and RGS11. R7 RGS proteins play important roles in the nervous system by controlling neurotransmitter action at rhodopsin, μ-opioid, D2 dopamine, and GABA(B) receptors (Anderson et al. 2009a).

The unique feature of this group is that they form obligatory complexes with Gβ5, an atypical member of the G protein beta subunit family (Sondek and Siderovski 2001; Slepak 2009). The stability of all R7 RGS proteins crucially depends on this interaction and knockout of Gβ5 in mice leads to severe downregulation in the levels of all four R7 RGS proteins (Chen et al. 2003). Localization of R7 RGS-Gβ5 complexes in discrete membrane compartments in native cells in parallel with cytoplasmic distribution in heterologous expression systems have prompted speculations that their membrane anchoring is mediated by unidentified proteins (Hu and Wensel 2002; Lishko et al. 2002). This led to searches for additional binding partners.

For R7 RGS proteins, these studies were very productive and resulted in the identification of two homologous binding partners: RGS9 Anchor Protein (R9AP) and R7 Binding Protein (R7BP), novel proteins that now constitute a two-member family. First, proteomics search for RGS9 binding partners in the retina identified a transmembrane protein R9AP (Hu and Wensel 2002). Three years later, a homologous R7BP protein was found as a binding partner of RGS9 in the brain

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

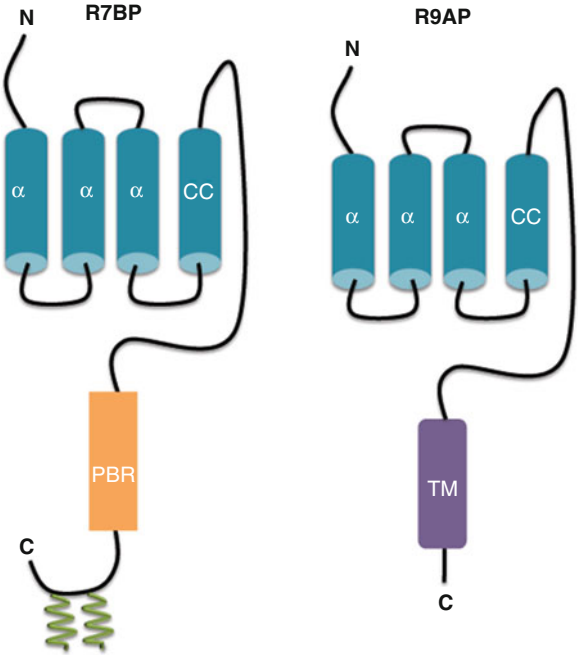
using similar approach (Martemyanov et al. 2005). R7BP was also independently discovered as a universal anchor for all R7 RGS proteins by Ken Blumer's group by in silico BLAST searches (Drenan et al. 2005).

Structural Organization

Both R7BP and R9AP are small membrane proteins that show distant homology to SNARE proteins involved in the regulation of exocytosis (Keresztes et al. 2003; Martemyanov et al. 2003). N-termini of R9AP and R7BP are predicted to contain 4 alpha helices organized in the bundle with the fourth helix containing prominent heptad repeats, a feature underlying coiled coil interactions (Fig. 1). This helical bundle region mediates binding to the R7 RGS proteins (Anderson et al. 2009a; Jayaraman et al. 2009). From the side of the RGS proteins, N-terminal DEP (Dishevelled, Egl-10, Pleckstrin) and DHEX (DEP Helical Extension) domains were found to be essential for the association (Anderson et al. 2009a; Jayaraman et al. 2009) although no high-resolution information of the determinants involved in binding have been reported. The C-termini of R7BP and R9AP contain membrane anchoring elements. In R9AP this is presented by transmembrane region, while R7BP is anchored through palmitoylation of two conserved cysteine residues which are additionally aided by a polybasic stretch with a sequence resembling membrane attachment motif of Ras proteins (Drenan et al. 2005; Song et al. 2006). In summary, while differing in amino acid composition, both R9AP and R7BP contain two domains: the N-terminal RGS binding region and C-terminal membrane localization domain.

Distribution, Subcellular Localization, and Interactions with R7 RGS Proteins

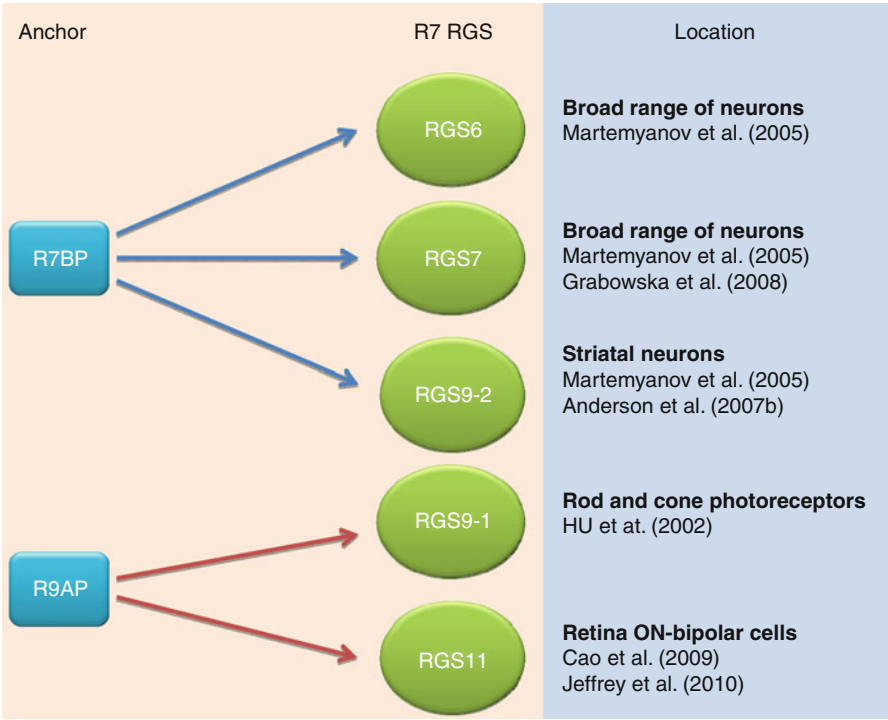
In mammals, expression of both R9AP and R7BP proteins appears to be confined to the neuronal tissues (Martemyanov et al. 2005; Grabowska et al. 2008). However, only limited set of non-neuronal tissues have been investigated and it remains possible that the proteins could be expressed more broadly, as for example, ample amounts of R9AP mRNA are found across various tissues in birds (Keresztes et al. 2003). While R7BP is expressed broadly in all regions of



R7BP/R9AP, Fig. 1 *Schematic structure of R7BP and R9AP proteins*. Membrane attachment of R7BP is mediated by palmitoylated cysteines (wavy lines) acting in conjunction with polybasic region (PBR). R9AP is anchored to the membrane by transmembrane segment (TM). Blue cylinders designate predicted alpha helical regions. CC label designates heptad repeats-containing region that is predicted to engage in coiled-coil interactions

central and peripheral nervous system, R9AP is more restricted and appears to be reliably found only in the retina, where it is found in three cell types: rod and cone photoreceptors (Hu and Wensel 2002) and ON-bipolar cells (Cao et al. 2009; Jeffrey et al. 2010).

At the subcellular level, R9AP is targeted to the disc membranes of the outer segments, a ciliated compartment of the photoreceptors and dendritic tips of the ON- bipolar neurons (Hu and Wensel 2002; Cao et al. 2009; Jeffrey et al. 2010). Likewise, R7BP was also found to be localized predominantly in the membrane compartments. Its significant fraction is found in the postsynaptic density and extrasynaptically at the plasma membrane of the spines and dendrites (Anderson et al. 2007b; Grabowska et al. 2008). Although to a lesser extent, some R7BP immunoreactivity is also present pre-synaptically in axons (Grabowska et al. 2008). Subcellular tartgeting of R7BP and R9AP to their membrane compartments requires the membrane attachment sequence at the C-terminus (Drenan et al. 2006; Song et al. 2006).



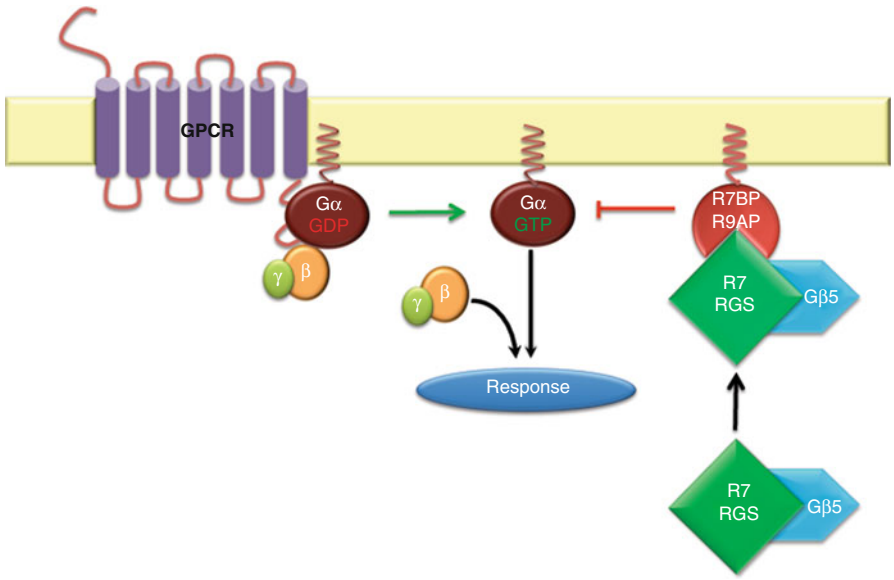
R7BP/R9AP,
Fig. 2 Physiologically relevant complexes of R7BP and R9AP in vivo

While plasma membrane compartment is the sole localization site of R9AP, R7BP has been reported to have an alternative destination – nucleus. It possesses two active nuclear localization sequences that are masked by palmitoylation (Drenan et al. 2005; Song et al. 2006). When palmitoylation is abolished, R7BP undergoes translocation to the nucleus, a phenomenon most readily demonstrated in cultured cells (Drenan et al. 2005; Song et al. 2006). However, the fraction of R7BP in the nucleus is very small and no studies have yet reported translocation in vivo under physiological conditions.

R7BP and R9AP anchors show differential selectivity in their association with individual R7 RGS members. This is determined by both their interaction specificity and co-expression patterns. At the biochemical level, R9AP can bind only to RGS9 and homologous RGS11, but not to RGS6 or RGS7 (Martemyanov et al. 2005). In contrast, R7BP associates with all R7 RGS proteins (Drenan et al. 2005; Martemyanov et al. 2005). However, the distribution of R7 RGS protein subtypes does not always overlap with that of R7BP and R9AP making some combinations physiologically irrelevant. For example, although R9AP can bind to RGS9-2, it is not expressed in the striatum, where RGS9-2 is instead found in complex with R7BP. Similarly, RGS11 can bind to R7BP, but because it is present exclusively in bipolar cells where R9AP is more abundant, most of it is found in complex with R9AP (Cao et al. 2008). See Fig. 2 for details on physiologically relevant configurations of R7BP and R9AP complexes with R7 RGS proteins.

Regulation of the RGS Protein Localization and Activity

Studies with transfected cells indicate that R7 RGS-Gβ5 complexes are predominantly cytoplasmic (Drenan et al. 2005; Song et al. 2006). In contrast, co-transfection with R7BP (or R9AP for RGS9-1) targets R7 RGS proteins to the plasma membrane (Hu and Wensel 2002; Drenan et al. 2005; Song et al. 2006). A similar situation is observed in striatal neurons in vivo for RGS9-2 that becomes mis-localized from post-synaptic densities and plasma membrane compartments upon elimination of R7BP (Anderson et al. 2007b). In striatal neurons, R7BP is also involved



R7BP/R9AP, Fig. 3 Role of membrane anchors R7BP and R9AP in G protein signaling regulation

in targeting RGS7 to the post-synaptic density (Anderson et al. 2009b). Likewise, the role of R9AP in localization of RGS9-1 to the disc membranes of the photoreceptor outer segments is also well established (Hu and Wensel 2002). Nevertheless, mechanisms governing localization of R7 RGS proteins appear to be complex and anchor-independent targeting has been observed for both RGS7 and RGS11 in the bipolar cells of the retina (Cao et al. 2008; Cao et al. 2009)

The nucleus has been repeatedly reported to be an alternative destination for relatively minor fraction of several R7 RGS proteins (Burchett 2003). Consistent with its nuclear shuttling, R7BP is capable of targeting R7 RGS to the nucleus of the transfected cells upon depalmitoylation (Drenan et al. 2005). Furthermore, knockout of R7BP abolishes nuclear localization of a significant fraction of RGS7 in the central nervous system neurons (Panicker et al. 2010). However, the functional significance of plasma membrane – nuclear shuttling of R7 RGS proteins or their functional role at this location is currently unknown.

In addition to localization, association with R9AP and R7BP influences the efficiency of R7 RGS to catalyze G protein GTPase (GAP) activity. For instance, R9AP has been shown to potentiate the ability of RGS9-1 and RGS11 to stimulate GTPase of Gαt and Gαo, respectively (Hu et al. 2003; Masuho et al. 2010). The most straightforward explanation for the stimulatory effects is facilitation of the R7 RGS complex compartmentalization with membrane bound G proteins and receptors. The restriction of the diffusion of the complex from the three-dimensional cytoplasm to the two-dimensional plane of the plasma membrane is expected to speed up Gα-GTP encounter. However, the mechanism is likely to be more complex, as at least R9AP action was shown to provide an allosteric modulation of the RGS9 and RGS11 complexes (Baker et al. 2006; Masuho et al. 2010).

Effects on the Proteolytic Stability of the R7 RGS Complexes

Perhaps the most pronounced effects of membrane anchors are on regulation of post-translational stability of R7 RGS proteins. These effects are observed only with two RGS proteins: RGS9 and RGS11. Studies with genetic knockouts indicate that elimination of R9AP severely compromises proteolytic stability of RGS9 (Keresztes et al. 2004) and RGS11 (Cao et al. 2008) in the retina. Likewise, knockout of R7BP leads to destabilization of RGS9-2 in the brain (Anderson et al. 2007a). This explains why loss-of-function mutations in R9AP produce the same phenotype as RGS9-1

mutations – slow adaptation to both light and dark conditions and difficulty in tracking moving objects (Nishiguchi et al. 2004). Similarly, knockout of R7BP causes motor co-ordination deficits characteristic of severe reduction in levels of RGS9-2 (Anderson et al. 2010). Loss of R7BP has been shown to facilitate recruitment of the destabilizing chaperone Hsc70 (Posokhova et al. 2010) to RGS9-2 and trigger its proteolysis by cellular cysteine proteases (Anderson et al. 2007b). Association of RGS9-Gβ5 with R7BP is controlled dynamically and is sensitive to changes in oxygenation and neuronal excitability (Anderson et al. 2009b). Because the abundance of R7 RGS proteins controls the extent of the G protein signaling and has direct behavioral implications, regulation of R7 RGS degradation and coupling to R7BP can be viewed as a plasticity mechanism.

Summary and Conclusions

In summary, R7BP and R9AP proteins play very important roles in controlling stability, localization and functional activity of R7 RGS proteins and as a result critically contribute to the regulation of G protein signaling (Fig. 3). There are currently a number of outstanding questions pertaining to the role of R7BP and R9AP in regulation of (1) G protein signaling selectivity, (2) RGS catalytic activity and (3) physiological processes in the nucleus. Finding answers to these questions would help gain a better understanding of G protein signaling.

References

Anderson GR, Semenov A, Song JH, Martemyanov KA. The membrane anchor R7BP controls the proteolytic stability of the striatal specific RGS protein, RGS9-2. J Biol Chem. 2007a;282:4772–81.

Anderson GR, Lujan R, Semenov A, Pravetoni M, Posokhova EN, Song JH, Uversky V, Chen CK, Wickman K, Martemyanov KA. Expression and localization of RGS9-2/Gbeta5/R7BP complex in vivo is set by dynamic control of its constitutive degradation by cellular cysteine proteases. J Neurosci. 2007b;27:14117–27.

Anderson GR, Posokhova E, Martemyanov KA. The R7 RGS protein family: multi-subunit regulators of neuronal G protein signaling. Cell Biochem Biophys. 2009a;54:33–46.

Anderson GR, Lujan R, Martemyanov KA. Changes in striatal signaling induce remodeling of RGS complexes containing Gbeta5 and R7BP subunits. Mol Cell Biol. 2009b;29:3033–44.

Anderson GR, Cao Y, Davidson S, Truong HV, Pravetoni M, Thomas MJ, Wickman K, Giesler Jr GJ, Martemyanov KA. R7BP complexes with RGS9-2 and RGS7 in the striatum differentially control motor learning and locomotor responses to cocaine. Neuropsychopharmacology. 2010;35:1040–50.

Baker SA, Martemyanov KA, Shavkunov AS, Arshavsky VY. Kinetic mechanism of RGS9-1 potentiation by R9AP. Biochemistry. 2006;45:10690–7.

Burchett SA. In through the out door: nuclear localization of the regulators of G protein signaling. J Neurochem. 2003;87:551–9.

Cao Y, Song H, Okawa H, Sampath AP, Sokolov M, Martemyanov KA. Targeting of RGS7/Gbeta5 to the dendritic tips of ON-bipolar cells is independent of its association with membrane anchor R7BP. J Neurosci. 2008;28:10443–9.

Cao Y, Masuho I, Okawa H, Xie K, Asami J, Kammermeier PJ, Maddox DM, Furukawa T, Inoue T, Sampath AP, Martemyanov KA. Retina-specific GTPase accelerator RGS11/G beta 5S/R9AP is a constitutive heterotrimer selectively targeted to mGluR6 in ON-bipolar neurons. J Neurosci. 2009;29:9301–13.

Chen CK, Eversole-Cire P, Zhang H, Mancino V, Chen YJ, He W, Wensel TG, Simon MI. Instability of GGL domain-containing RGS proteins in mice lacking the G protein beta-subunit Gbeta5. Proc Natl Acad Sci USA. 2003;100:6604–9.

Drenan RM, Doupnik CA, Boyle MP, Muglia LJ, Huettner JE, Linder ME, Blumer KJ. Palmitoylation regulates plasma membrane-nuclear shuttling of R7BP, a novel membrane anchor for the RGS7 family. J Cell Biol. 2005;169:623–33.

Drenan RM, Doupnik CA, Jayaraman M, Buchwalter AL, Kaltenbronn KM, Huettner JE, Linder ME, Blumer KJ. R7BP augments the function of RGS7/Gbeta5 complexes by a plasma membrane-targeting mechanism. J Biol Chem. 2006;281:28222–31.

Grabowska D, Jayaraman M, Kaltenbronn KM, Sandiford SL, Wang Q, Jenkins S, Slepak VZ, Smith Y, Blumer KJ. Postnatal induction and localization of R7BP, a membrane-anchoring protein for regulator of G protein signaling 7 family-Gbeta5 complexes in brain. Neuroscience. 2008;151:969–82.

Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacol Rev. 2002;54:527–59.

Hu G, Wensel TG. R9AP, a membrane anchor for the photoreceptor GTPase accelerating protein, RGS9-1. Proc Natl Acad Sci USA. 2002;99:9755–60.

Hu G, Zhang Z, Wensel TG. Activation of RGS9-1GTPase acceleration by its membrane anchor, R9AP. J Biol Chem. 2003;278:14550–4.

Jayaraman M, Zhou H, Jia L, Cain MD, Blumer KJ. R9AP and R7BP: traffic cops for the RGS7 family in phototransduction and neuronal GPCR signaling. Trends Pharmacol Sci. 2009;30:17–24.

Jeffrey BG, Morgans CW, Puthussery T, Wensel TG, Burke NS, Brown RL, Duvoisin RM. R9AP stabilizes RGS11-G beta5 and accelerates the early light response of ON-bipolar cells. Vis Neurosci. 2010;27:9–17.

Keresztes G, Mutai H, Hibino H, Hudspeth AJ, Heller S. Expression patterns of the RGS9-1 anchoring protein R9AP in the chicken and mouse suggest multiple roles in the nervous system. Mol Cell Neurosci. 2003;24:687–95.
Keresztes G, Martemyanov KA, Krispel CM, Mutai H, Yoo PJ, Maison SF, Burns ME, Arshavsky VY, Heller S. Absence of the RGS9.Gbeta5 GTPase-activating complex in photoreceptors of the R9AP knockout mouse. J Biol Chem. 2004; 279:1581–4.
Lishko PV, Martemyanov KA, Hopp JA, Arshavsky VY. Specific binding of RGS9-Gbeta 5L to protein anchor in photoreceptor membranes greatly enhances its catalytic activity. J Biol Chem. 2002;277:24376–81.
Martemyanov KA, Lishko PV, Calero N, Keresztes G, Sokolov M, Strissel KJ, Leskov IB, Hopp JA, Kolesnikov AV, Chen CK, Lem J, Heller S, Burns ME, Arshavsky VY. The DEP domain determines subcellular targeting of the GTPase activating protein RGS9 in vivo. J Neurosci. 2003;23:10175–81.
Martemyanov KA, Yoo PJ, Skiba NP, Arshavsky VY. R7BP, a novel neuronal protein interacting with RGS proteins of the R7 family. J Biol Chem. 2005;280:5133–6.
Masuho I, Celver J, Kovoor A, Martemyanov KA. Membrane anchor R9AP potentiates GTPase-accelerating protein activity of RGS11 x Gbeta5 complex and accelerates inactivation of the mGluR6-G(o) signaling. J Biol Chem. 2010; 285:4781–7.
Nishiguchi KM, Sandberg MA, Kooijman AC, Martemyanov KA, Pott JW, Hagstrom SA, Arshavsky VY, Berson EL, Dryja TP. Defects in RGS9 or its anchor protein R9AP in patients with slow photoreceptor deactivation. Nature. 2004; 427:75–8.
Panicker LM, Zhang JH, Posokhova E, Gastinger MJ, Martemyanov KA, Simonds WF. Nuclear localization of the G protein beta 5/R7-regulator of G protein signaling protein complex is dependent on R7 binding protein. J Neurochem. 2010;113:1101–12.
Posokhova E, Uversky V, Martemyanov KA. Proteomic identification of Hsc70 as a mediator of RGS9-2 degradation by in vivo interactome analysis. J Proteome Res. 2010;9:1510–21.
Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem. 2000; 69:795–827.
Slepak VZ. Chapter 6 structure, function, and localization of Gbeta5-RGS complexes. Prog Mol Biol Transl Sci. 2009;86C:157–203.
Sondek J, Siderovski DP. Ggamma-like (GGL) domains: new frontiers in G-protein signaling and beta-propeller scaffolding. Biochem Pharmacol. 2001;61:1329–37.
Song JH, Waataja JJ, Martemyanov KA. Subcellular targeting of RGS9-2 is controlled by multiple molecular determinants on its membrane anchor, R7BP. J Biol Chem. 2006;281:15361–9.

RA70 (Retinoic Acid-Induced Protein 70)

▶ SKAP-HOM

RAB18

Irene Aligianis and Mark Handley
MRC Human Genetics Unit, Western General Hospital, Edinburgh, Scotland, UK

Synonyms

AtRabC1-C2b (*Arabidopsis thaliana*); Rab18b (*Danio rerio*); Rab-RP4 (*Drosophila melanogaster*)

Historical Background

Interest in the RAB proteins stems from early work on yeast that identified essential roles for the RABs Ypt1p (Rab1) and Sec4p in pre- and post-Golgi membrane trafficking (Salminen and Novick 1987; Segev et al. 1988). These findings prompted efforts to clone other members of the RAB gene family and it was quickly established that this family had undergone significant expansion in mammals. As each new RAB was discovered, it also became clear that different RAB proteins could adopt specific subcellular localizations, associating with particular membrane compartments and regulating the functions of these organelles. RAB18 was partially cloned in 1992, and then fully cloned in 1993 (Chavrier et al. 1992; Yu et al. 1993). Initial characterization showed that it was widely expressed in different tissues and localized to endosomes in polarized epithelia (Lutcke et al. 1994). Subsequent research has suggested, however, that it adopts various different subcellular localizations and can function in a cell-type-specific manner (Dejgaard et al. 2008; Hashim et al. 2000; Hashimoto et al. 2008; Martin et al. 2005; Ozeki et al. 2005; Vazquez-Martinez et al. 2007). Currently, little is known about the protein-protein interactions that regulate and mediate RAB18 activity, but genetic evidence has linked it to the regulation of RAB3 isoforms. In a recent study, loss-of-function mutations in *RAB18* were found to cause Micro syndrome, a disorder previously associated with mutations in *RAB3GAP1* or *RAB3GAP2* (Aligianis et al. 2005, 2006; Bem et al. 2011).

RAB18 in Disease

Micro syndrome is a rare autosomal recessive disorder with both developmental and degenerative features. Patients present with micropthalmia, atonic pinpoint pupils, and congenital cataracts. Even with early cataract surgery, most have only light perception due to severe cortical visual impairment characterized by normal electroretinogram (ERG), but virtually absent visually evoked potentials (VEPs). Affected individuals have severe developmental delay and do not learn to sit independently, walk, or talk. They suffer from postnatal growth retardation, microcephaly, and hypothalamic hypogenitalism and can have cerebral anomalies including polymicrogyria and hypoplasia or agenesis of the corpus callosum. In addition to initial muscular hypotonia, they go on to develop ascending spastic paraplegia and contractures.

The pathogenesis of Micro syndrome indicates that RAB18 function is important in eye and lens development, neurotransmission and neuronal migration and homeostasis. Knockdown of rab18b in zebrafish produces a phenotype reminiscent of Micro syndrome, suggesting that RAB18 has conserved roles in these processes and that animal models might offer a valuable resource in future studies (Bem et al. 2011). Further, as it is now clear that specific physiological systems are particularly susceptible to loss of functional RAB18, characterization of this deficit at a cellular and molecular level can be better targeted. In turn, this work may inform the development of therapeutics for Micro syndrome and related disorders. However, it is important to note that the physiological roles of RAB18 may in fact be much broader than can be inferred from the effects of its absence. RAB proteins can show a varying degree of functional redundancy in different tissues, either as a result of the different expression patterns of RABs with overlapping cellular roles, or because a given RAB may mediate responses through tissue-specific effector proteins. Furthermore, RAB18 expression may be regulated in concert with other factors. Dysregulation of RAB18 expression has been observed in a number of different cancers and has been extensively studied in the context of pituitary tumors (Vázquez-Martínez and Malagón 2011). In these tumors, its downregulation has been linked to the hypersecretion of human growth hormone (hGH) that contributes to acromegaly in sufferers (Vazquez-Martinez et al. 2008). However, Micro syndrome patients who do not express any functional RAB18 do not show abnormal hGH secretion.

Rab18 Expression and Subcellular Localization

Several reports show that RAB18 mRNA is ubiquitously expressed, implying that it has a general cellular role (Lutcke et al. 1994; Schafer et al. 2000; Yu et al. 1993). However, other evidence suggests that its role is more specialized. It appears to be expressed at different levels in the different tissues examined, with it being highly expressed in the brain and the heart. Immunohistochemistry suggests that the protein is enriched in polarized epithelia (Lutcke et al. 1994). Furthermore, in a number of situations, its expression is reported to be inducible. For example, it is induced in endothelial cells stimulated with histamine (Schafer et al. 2000), in differentiating adipocytes (Pulido et al. 2011), and in the brains of alloxan-treated rats (Karthik and Ravikumar 2011). The most convincing evidence that the protein serves discrete cellular roles, though, comes from a series of divergent reports on its cellular localization and function.

RAB18 has been reported to associate with endosomes in polarized epithelia where it has been suggested to function in endocytosis (Lutcke et al. 1994). In a macrophage cell line, it localized to a specialized phagocytic compartment and was suggested to function in immune evasion (Hashim et al. 2000). Several reports have found that the protein can localize to lipid droplets in adipocyte, fibroblast, and epithelial cell lines (Martin et al. 2005; Ozeki et al. 2005; Pulido et al. 2011) and this has been linked to roles in lipogenesis (Pulido et al. 2011) and lipolysis (Martin et al. 2005; Pulido et al. 2011). However, in the same cells, it can localize to endoplasmic reticulum (ER) and Golgi under some circumstances (Dejgaard et al. 2008; Martin et al. 2005), and one report has suggested that it functions in Golgi to ER trafficking (Dejgaard et al. 2008). In endocrine cell lines and in pituitary melanotropes, it has been found associated with secretory granules, and suggested to function in modulation of the secretory response (Vazquez-Martinez et al. 2007). These reports are

difficult to reconcile. However, a common feature of several of them is that the recruitment of RAB18 to intracellular organelles can be enhanced by cellular stimulation. In adipocytes, for example, recruitment of RAB18 to lipid droplets was promoted by treatment with insulin, which stimulates lipogenesis (Pulido et al. 2011), or by the β-adrenoceptor agonist isoproterenol, which stimulates lipolysis (Martin et al. 2005; Pulido et al. 2011). Similarly, in PC12 and AtT20 cells, stimulation with KCl led to redistribution of RAB18 from the cytosol to a subpopulation of secretory granules (Vazquez-Martinez et al. 2007). It will be important to establish the extent to which different molecular interactions underlie the functions of RAB18 in different cell types, and reciprocally, that to which common molecular interactions underlie its function at diverse intracellular compartments.

Regulator and Effector Proteins

Following synthesis and posttranslational prenylation, RAB proteins are regulated by four classes of protein: GDP-dissociation inhibitors (GDIs), GDI displacement factors (GDFs), Guanine-nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs). GDIs and GDFs are thought to be general regulators of multiple RABs, while GEFs and GAPs are thought to show more specificity for particular RAB proteins. GDIs can sequester GDP-bound RABs in the cytosol by binding to their hydrophobic prenyl groups, but also coordinate with GDFs in the delivery of RABs to membranes. GEFs catalyze the exchange of bound GDP for GTP, and so can serve to concentrate RABs on a particular cellular compartment because RAB proteins are not susceptible to GDI-mediated membrane extraction when GTP-bound. GAPs stimulate a RAB's intrinsic GTP-hydrolysis activity, which converts bound GTP to GDP, thus rendering them susceptible to extraction once again.

The GEF(s) and GAP(s) that regulate RAB18 are not known. However, the identification of these proteins may be key to understanding how it can be recruited to a wide range of organelles in a cell-type-specific manner. It is possible that a single RAB18GEF might be differentially localized in different cells. However, in some cases, specific RABs are the substrates for multiple GEFs, and so differential expression of RAB18GEFs with different localizations might offer another explanation for this phenomenon. Alternatively, RAB18 localization might be regulated by its GAP(s). The presence of a RABGAP on a particular membrane compartment can effectively exclude its target RAB from this compartment by promoting GTP hydrolysis and GDI-mediated membrane extraction at this location. Thus, the translocation of the RABGAP away from a given compartment can indirectly promote RAB recruitment there.

The function of *RAB18* is genetically linked to that of *RAB3GAP1* and *RAB3GAP2*, mutations in which can also cause Micro syndrome (Aligianis et al. 2005, 2006; Bem et al. 2011). RAB3GAP1 and RAB3GAP2 form a heterodimeric RAB3GAP complex that regulates the activity of RAB3 isoforms (Fukui et al. 1997; Nagano et al. 1998). However, the activity of Rab3GAP against many other RABs, including RAB18, is unknown. Work is underway to determine whether RAB3GAP is a regulator of RAB18 function. However, it is also possible that it is a mediator of this function. RAB proteins mediate cellular responses via interacting partners generically called "effectors," and it has been proposed that RABGAPs acting on one RAB protein are frequently effectors of another (Kanno et al. 2010).

While it remains to be established whether RAB3GAP is a regulator and/or an effector of RAB18 function, several candidate RAB18-interacting proteins have previously been identified in the literature. A weak interaction between RAB18 and mammalian suppressor of Sec4 (Mss4) was found in a screen for RABs that interact with this protein (Wixler et al. 2011). Investigators used copurification of exogenously expressed proteins from HEK293 cells to identify this interaction. However, though Mss4 is proposed to be either a GEF or a RAB chaperone, they found that the protein showed no GEF activity toward RAB18 and that it interacted much more strongly with other RAB proteins. Therefore, the physiological relevance of this finding is unclear. In another screen, this time for novel RAB-binding proteins, a pull-down assay with GST-Rab18 identified a potential interaction with N-ethylmaleimide-sensitive factor (NSF), a protein involved in the disassembly of *cis*-SNARE complexes following membrane fusion (Kanno et al. 2010). This finding may implicate RAB18 in the fusion process, though it should be noted that NSF is thought to regulate fusion even in the absence of RAB proteins. In a third study, the *Arabidopsis* RAB18 orthologue

AtRabC2a was shown by yeast 2-hybrid (Y2H) and by in vitro binding assays to interact with a class V myosin, *Arabidopsis* myosin XI (Hashimoto et al. 2008). Since myosins function as molecular motors, transporting cargoes along actin filaments, this interaction may suggest a mechanism by which RAB18 could direct organelle mobility. However, because of sequence divergence of both RABs and myosins between plants and mammals and the presence of multiple RAB18 orthologues in *Arabidopsis*, it will be necessary to show that the interaction is conserved in mammals before any such conclusions can be drawn. Clearly, the validation of proposed effectors, and the identification of novel effectors, will help to better define the role of RAB18 in cells.

Structure

A RAB18 orthologue is not present in yeast, but its orthologues are found in plants, nematodes, and flies (Pereira-Leal and Seabra 2001). Despite this high degree of conservation, however, attempts to classify the protein on the basis of phylogeny and active conformation have not yielded any clues as to its function (Pereira-Leal and Seabra 2001). It has been suggested in the literature that RAB18 is one of a group of RAB proteins with a putative exocytotic role (Vázquez-Martínez and Malagón 2011; Wixler et al. 2011). However, the systematic application of phylogenetic algorithms and principal components analysis to the RAB protein family did not place the protein in this group (Collins 2005). Thus, it remains to be shown whether RAB18 is part of some larger functional subgroup of RAB proteins or possesses a distinct role.

One feature that sets RAB18 apart from most other RAB proteins is its differential posttranslational modification. Most RAB proteins have a C-terminal dicysteine motif that is subject to modification by two geranylgeranyl lipid groups. In contrast, RAB18, like RAB8, RAB13, and ▶ RAB23, has a C-terminal CAAX motif like that of RAS and RHO proteins. This motif is monoprenylated, and then sequentially cleaved and carboxymethylated by ER-resident enzymes (Leung et al. 2007). As methylation affects the susceptibility of RAB proteins to GDI-mediated membrane extraction, and is a potentially reversible modification, this may represent an additional level of RAB18 regulation.

Summary

To summarize, many questions remain to be answered about RAB18. In the context of the other members of the RAB gene family, it appears unusual in terms of the sequence determinants that aid classification, and it is subject to atypical posttranslational modification (Leung et al. 2007; Pereira-Leal and Seabra 2001). However, it is conserved in all but the simplest eukaryotes, and so future work should determine whether it is truly novel, or an unrecognized member of an established subgroup.

The function of RAB18 has been strongly liked to that of RAB3GAP1 and RAB3GAP2, as loss-of-function mutations in each of these proteins causes clinically indistinguishable Micro syndrome in humans (Aligianis et al. 2005, 2006; Bem et al. 2011). Therefore, a primary aim of future work will be to define the relationship between these proteins. Further, since this finding implicates RAB18 in a primarily neurological pathology, work in animal models and neuronal cell lines should seek to establish its role in these processes.

References

Aligianis IA, Johnson CA, Gissen P, Chen D, Hampshire D, Hoffmann K, et al. Mutations of the catalytic subunit of RAB3GAP cause Warburg Micro syndrome. Nat Genet. 2005;37:221–3.

Aligianis IA, Morgan NV, Mione M, Johnson CA, Rosser E, Hennekam RC, et al. Mutation in Rab3 GTPase-activating protein (RAB3GAP) noncatalytic subunit in a kindred with Martsolf syndrome. Am J Hum Genet. 2006;78:702–7.

Bem D, Yoshimura S, Nunes-Bastos R, Bond FC, Kurian MA, Rahman F, et al. Loss-of-function mutations in RAB18 cause Warburg micro syndrome. Am J Hum Genet. 2011; 88:499–507.

Chavrier P, Simons K, Zerial M. The complexity of the Rab and Rho GTP-binding protein subfamilies revealed by a PCR cloning approach. Gene. 1992;112:261–4.

Collins RN. Application of phylogenetic algorithms to assess Rab functional relationships. Methods Enzymol. 2005; 403:19–28.

Dejgaard SY, Murshid A, Erman A, Kizilay O, Verbich D, Lodge R, et al. Rab18 and Rab43 have key roles in ER-Golgi trafficking. J Cell Sci. 2008;121:2768–81.

Fukui K, Sasaki T, Imazumi K, Matsuura Y, Nakanishi H, Takai Y. Isolation and characterization of a GTPase activating protein specific for the Rab3 subfamily of small G proteins. J Biol Chem. 1997;272:4655–8.

Hashim S, Mukherjee K, Raje M, Basu SK, Mukhopadhyay A. Live Salmonella modulate expression of Rab proteins to persist in a specialized compartment and escape transport to lysosomes. J Biol Chem. 2000;275:16281–8.

Hashimoto K, Igarashi H, Mano S, Takenaka C, Shiina T, Yamaguchi M, et al. An isoform of Arabidopsis myosin XI interacts with small GTPases in its C-terminal tail region. J Exp Bot. 2008;59:3523–31.
Kanno E, Ishibashi K, Kobayashi H, Matsui T, Ohbayashi N, Fukuda M. Comprehensive screening for novel rab-binding proteins by GST pull-down assay using 60 different mammalian Rabs. Traffic. 2010;11:491–507.
Karthik D, Ravikumar S. Characterization of the brain proteome of rats with diabetes mellitus through two-dimensional electrophoresis and mass spectrometry. Brain Res. 2011; 1371:171–9.
Leung KF, Baron R, Ali BR, Magee AI, Seabra MC. Rab GTPases containing a CAAX motif are processed post-geranylgeranylation by proteolysis and methylation. J Biol Chem. 2007;282:1487–97.
Lutcke A, Parton RG, Murphy C, Olkkonen VM, Dupree P, Valencia A, et al. Cloning and subcellular localization of novel rab proteins reveals polarized and cell type-specific expression. J Cell Sci. 1994;107(Pt 12):3437–48.
Martin S, Driessen K, Nixon SJ, Zerial M, Parton RG. Regulated localization of Rab18 to lipid droplets: effects of lipolytic stimulation and inhibition of lipid droplet catabolism. J Biol Chem. 2005;280:42325–35.
Nagano F, Sasaki T, Fukui K, Asakura T, Imazumi K, Takai Y. Molecular cloning and characterization of the noncatalytic subunit of the Rab3 subfamily-specific GTPase-activating protein. J Biol Chem. 1998;273:24781–5.
Ozeki S, Cheng J, Tauchi-Sato K, Hatano N, Taniguchi H, Fujimoto T. Rab18 localizes to lipid droplets and induces their close apposition to the endoplasmic reticulum-derived membrane. J Cell Sci. 2005;118:2601–11.
Pereira-Leal JB, Seabra MC. Evolution of the Rab family of small GTP-binding proteins. J Mol Biol. 2001;313: 889–901.
Pulido MR, Diaz-Ruiz A, Jimenez-Gomez Y, Garcia-Navarro S, Gracia-Navarro F, Tinahones F, et al. Rab18 dynamics in adipocytes in relation to lipogenesis, lipolysis and obesity. PLoS One. 2011;6:e22931.
Salminen A, Novick PJ. A ras-like protein is required for a post-Golgi event in yeast secretion. Cell. 1987;49:527–38.
Schafer U, Seibold S, Schneider A, Neugebauer E. Isolation and characterisation of the human rab18 gene after stimulation of endothelial cells with histamine. FEBS Lett. 2000; 466:148–54.
Segev N, Mulholland J, Botstein D. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell. 1988;52:915–24.
Vazquez-Martinez R, Cruz-Garcia D, Duran-Prado M, Peinado JR, Castano JP, Malagon MM. Rab18 inhibits secretory activity in neuroendocrine cells by interacting with secretory granules. Traffic. 2007;8:867–82.
Vazquez-Martinez R, Martinez-Fuentes AJ, Pulido MR, Jimenez-Reina L, Quintero A, Leal-Cerro A, et al. Rab18 is reduced in pituitary tumors causing acromegaly and its overexpression reverts growth hormone hypersecretion. J Clin Endocrinol Metab. 2008;93:2269–76.
Vázquez-Martínez R, Malagón MM. Rab proteins and the secretory pathway: the case of Rab18 in neuroendocrine cells. Front Endocrinol 2011;2:1–9. doi:10.3389/fendo.2011.00001
Wixler V, Wixler L, Altenfeld A, Ludwig S, Goody RS, Itzen A. Identification and characterisation of novel Mss4-binding Rab GTPases. Biol Chem. 2011;392:239–48.
Yu H, Leaf DS, Moore HP. Gene cloning and characterization of a GTP-binding Rab protein from mouse pituitary AtT-20 cells. Gene. 1993;132:273–8.

Rab18b (*Danio rerio*)

► RAB18

Rab23

Marga Gual-Soler, Tomohiko Taguchi, Jennifer L. Stow and Carol Wicking
Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

Synonyms

Opb; Opb2; Open brain; RAB23, member RAS oncogene family

Historical Background

Rab23 (Ras-related protein Rab 23) belongs to the Rab family of monomeric small guanosine triphosphatases (GTPases) involved in the regulation of membrane traffic. Rab GTPases are conserved from yeast to humans and coordinate the delivery of cargo to its correct destination within eukaryotic cells. Rab proteins regulate many membrane trafficking steps, including vesicle formation, budding, motility along the cytoskeleton, docking, and membrane fusion (Zerial and McBride 2001; Stenmark 2009). More than 60 members of the Rab family have been identified in humans to date (Zerial and McBride 2001). Rab proteins function as molecular switches cycling from an active GTP (guanosine triphosphate)-bound form to an inactive GDP (guanosine diphosphate)-bound form. The GDP/GTP exchange factors (GEFs) catalyze the conversion from GDP to GTP-bound forms, whereas GTP hydrolysis to GDP is catalyzed by GTPase-activating proteins (GAPs). Once in their active state, GTP-bound Rabs can recruit specific effector

molecules to transduce signals in the transport pathway. These effectors include sorting adaptors, tethering factors, kinases, phosphatases, motor proteins, GEFs, and GAPs. Crosstalk between Rab GTPases through their effectors allows the spatiotemporal regulation of vesicle trafficking (Zerial and McBride 2001; Stenmark 2009). Post-translational modification by protein prenylation of C-terminal cysteines is generally required for membrane association and biological function of Rab proteins. (Zerial and McBride 2001; Stenmark 2009).

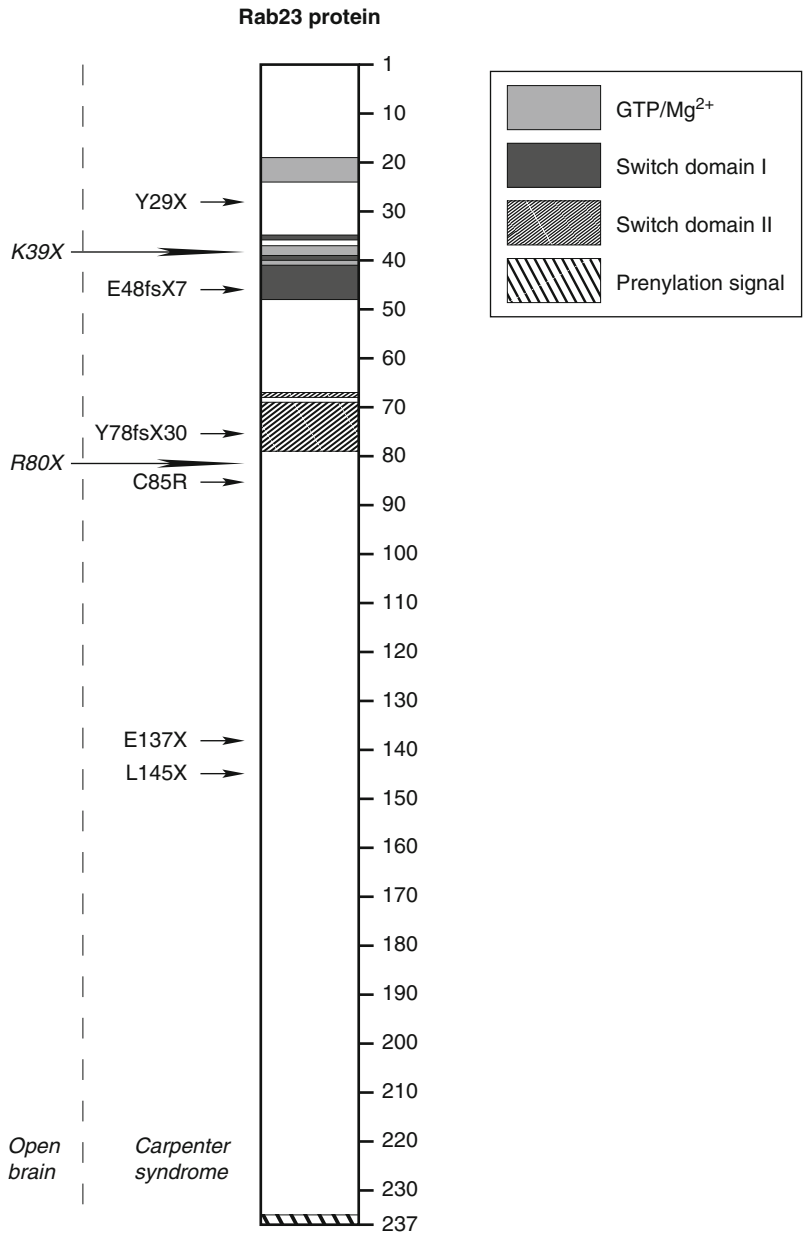
Rab23 was first identified in 1994 using a PCR-based homology cloning approach, and although it is expressed ubiquitously in many tissues, its predominant site of expression is the brain (Olkkonen et al. 1994). The human *RAB23* gene localizes to chromosome 6p11, is conserved in evolution back to *Drosophila*, and encodes a 237 amino acid protein. The Rab23 protein contains a CAAX-motif ("C" is Cysteine, "A" is an aliphatic amino acid, and "X" is variable) in its C-terminus, which acts as substrate for the post-translational prenylation modifications required for membrane anchoring (Fig. 1) (Olkkonen et al. 1994; Leung et al. 2007). As described below, several mutations in the *Rab23* gene have been described in both mouse and human (Fig. 1), providing insight into the physiological function of Rab23 (Eggenschwiler et al. 2001; Jenkins et al. 2007; Alessandri et al. 2010).

Rab23 and Hedgehog Signaling

Homozygous mutation of the *Rab23* gene is responsible for the mouse *open brain* (*opb*) phenotype (Eggenschwiler et al. 2001). There are two independent *opb* alleles, both of which encode truncated proteins (Eggenschwiler et al. 2001). The opb^1 allele is a natural mutation, while opb^2 was experimentally induced by *N*-ethyl-*N*-nitrosurea (ENU). *Opb* mice are characterized by severe defects in neural tube closure and patterning (Gunther et al. 1994), related to a failure in the correct specification of neurons along the dorsoventral axis of the neural tube. This process is highly dependent on the correct activity of ▶ Sonic hedgehog (SHH), a morphogen secreted from the notochord and floorplate (Ericson et al. 1997). The hedgehog (HH) pathway is one of the most pivotal signaling pathways directing embryonic development, and at the cellular level is regulated by trafficking events at the primary cilium (Huangfu et al. 2003). Genetic studies in mice revealed that the *opb* mutation rescues the *Shh* mutant phenotype, demonstrating that Rab23 acts as a cell autonomous negative regulator of HH signaling (Eggenschwiler et al. 2001). Since Rab proteins typically regulate vesicle trafficking, Rab23 was initially presumed to have a role in the trafficking of HH pathway components. Localization studies in a range of mammalian cell types showed distribution of exogenously expressed GFP-tagged Rab23 at the plasma membrane and on the endocytic pathway in transferrin-positive endosomes, where it co-localized with the HH receptor patched (PTCH1), but not with the co-receptor smoothened (SMO) (Evans et al. 2003). However, further genetic studies suggested a regulatory role for Rab23 downstream of both PTCH and SMO and upstream of the transcriptional regulators of the HH pathway, the Gli proteins (named after glioblastoma from where they were first isolated) (Eggenschwiler et al. 2006; Yang et al. 2008). Of the three vertebrate Gli proteins, Gli1 and Gli2 generally act as transcriptional activators, while Gli3 is primarily processed to a truncated transcriptional repressor. Studies in the mouse neural tube led to the suggestion that Rab23 negatively regulates HH signaling through trafficking of a molecule that mediates the effects of SMO on the formation of Gli2 activators and Gli3 repressors (Eggenschwiler et al. 2006). To date the identity of such a factor remains elusive, but increasing evidence suggests that the activation and processing of Gli proteins, and hence possibly Rab23 function, is intricately linked to the primary cilium.

Rab23 and Primary Cilia

The discovery that the primary cilium is an essential organelle for mammalian HH signaling (Corbit et al. 2005; Huangfu et al. 2003) is arguably one of the most significant findings in the fields of cell and developmental biology over the past decade. The primary cilium is a microtubule-based organelle that projects from the surface of virtually every vertebrate cell type. The major components of the HH pathway, including the Gli proteins, localize to the ciliary axoneme extension and shuttle in and out in a dynamic fashion (Rohatgi et al. 2007; Kim et al. 2009). The intraflagellar transport (IFT) system is primarily



Rab23, Fig. 1 *Rab23 domains and mutations.* Functional domains of the 237 amino acid protein Rab23. Human mutations responsible for Carpenter syndrome are located on the right column, and those causing the mouse *open brain* phenotype are situated on the left. All mutations produce truncated proteins except C85R, which causes a non-conservative substitution from uncharged to charged amino acid, possibly impairing normal folding of the protein. Rab23 structure was analyzed with Cn3D 4.1 software from NCBI. (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi)

responsible for trafficking of cargo between the cell body and the cilia tip, but a number of other accessory proteins also mediate ciliary trafficking. A screen for Rab GTPases involved in primary cilium formation identified Rab8 as the sole Rab localized on primary cilia. However, in the same study, biochemical analysis of Rab GTPase-activating proteins (Rab GAPs) and their attendant Rabs suggested a role for Rab23 and Rab17 in primary cilia formation (Yoshimura et al. 2007). More recently, exogenously expressed wild-type Rab23 was shown to localize to the primary cilium in Madin-Darby Canine Kidney (MDCK) epithelial cells (Boehlke et al. 2010). In these cells, shRNA-mediated depletion of Rab23 or expression of a GDP-bound form of Rab23 decreased the steady state level of SMO at the cilium, suggesting a role for Rab23 in ciliary turnover. Thus, the precise role of Rab23 at the primary cilium has yet to be determined, but the findings to date hint at a potential role in trafficking cargo to or from the cilium.

Rab23 and Planar Cell Polarity

The planar cell polarity (PCP) pathway coordinates cell polarization in a given plane across a cell layer, a process essential for the correct formation of certain highly ordered differentiated tissues during development (Fanto and McNeill 2004). A recent study in *Drosophila* provides new evidence for Rab23 as a PCP regulator (Pataki et al. 2010). Mutations in the *Drosophila Rab23* gene resulted in abnormal trichome orientation and the formation of multiple hairs on the wing, leg, and abdomen. This work also showed that Rab23 associates with the PCP protein Prickle, likely contributing to its asymmetric cellular accumulation to regulate the hexagonal packing of *Drosophila* wing cells and the orientation of cuticular hairs. Since components of the PCP pathway are important for the correct formation and positioning of cilia in vertebrate cells (Park et al. 2006), these data potentially provide a further link between Rab23 and cilia. However, cilia do not appear to be important for PCP or HH signaling in *Drosophila*, and a role for Rab23 in vertebrate PCP signaling has not yet been elucidated.

Rab23 in Human Disease

The physiological relevance of Rab23 has been highlighted by its involvement in a number of human disorders. Homozygous loss-of-function mutations in *RAB23* are responsible for Carpenter syndrome, a pleiotropic disorder with autosomal recessive inheritance, characterized by premature closure of the cranial sutures, polysyndactyly, obesity, and cardiac defects (Jenkins et al. 2007). Six independent mutations in *RAB23* have been identified in Carpenter syndrome patients (Alessandri et al. 2010; Jenkins et al. 2007). Five of the six mutations are predicted to result in premature protein truncation, and one is a missense mutation thought to interfere with protein folding (Fig. 1). They show no apparent clustering to specific domains within the RAB23 protein and all are likely to represent loss-of-function alleles. While craniosynostosis and obesity are not classic features of perturbed HH signaling, obesity in particular has been associated with disrupted cilia in humans and mice (Sheffield 2010), again reinforcing a role for Rab23 in ciliogenesis.

Rab23 appears to have functions beyond mammalian embryonic development, as overexpression of *Rab23* has been associated with human cancers. In the gastric cancer cell line Hs746T, siRNA-mediated silencing of Rab23 significantly reduced cellular migration and invasion, whereas overexpression of *Rab23* enhanced invasion in gastric epithelial (AGS) cells (Hou et al. 2008). *Rab23* expression is also upregulated in hepatocellular carcinoma (Liu et al. 2007). The finding that *Rab23* is upregulated in a number of human cancers seems contradictory given that Rab23 antagonizes HH signaling, and enhanced expression would be expected to result in pathway inhibition. In a wide range of tumor types, activation of HH signaling, rather than inhibition, is generally associated with tumorigenesis. It is possible that the role of Rab23 in cancer is unrelated to its regulation of HH signaling, or alternatively that a fine balance of Rab23 is required for correct functioning in the tumor environment. Future elucidation of the precise role of Rab23 in regulating HH signaling, ciliogenesis, and other cellular events will shed light on its involvement in cancer and other disease states.

Summary and Perspectives

Genetic and biochemical studies have implicated Rab23 in embryonic development, thus highlighting the role of vesicular trafficking in regulating embryogenesis at the cellular level. However, no Rab23 effectors or interacting partners have been identified to date in vertebrates, and as a result no molecular mechanism for Rab23 action has yet been elucidated. It may be that Rab23 acts indirectly to inhibit HH signaling. The key to Rab23 function might be found at the primary cilium, as the two signaling pathways in which Rab23 is involved (PCP and HH), converge at this organelle (Veland et al. 2009). Rab23 is not essential for *Drosophila* development (Pataki et al. 2010) but it is possible that, unlike Rab23 involvement in HH signaling, the Rab23-PCP link is evolutionarily conserved. PCP genes are required for neural tube closure, a characteristic phenotype of Rab23 and ciliogenesis mouse mutants (Doudney and Stanier 2005). Taken together, these observations suggest a key role for Rab23 at the intersection of the cilia-related HH and PCP pathways. Elucidation of such a link, along with the more precise definition of Rab23 function, is likely to come from future detailed studies at both the whole organism and cellular levels.

References

Alessandri JL, Dagoneau N, Laville JM, Baruteau J, Hebert JC, Cormier-Daire V. RAB23 mutation in a large family from Comoros Islands with Carpenter syndrome. Am J Med Genet A. 2010;152A:982–6.

Boehlke C, Bashkurov M, Buescher A, et al. Differential role of Rab proteins in ciliary trafficking: Rab23 regulates smoothened levels. J Cell Sci. 2010;123(9):1460–67.

Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate smoothened functions at the primary cilium. Nature. 2005;437:1018–21.

Doudney K, Stanier P. Epithelial cell polarity genes are required for neural tube closure. Am J Med Genet C Semin Med Genet. 2005;135C:42–7.

Eggenschwiler JT, Espinoza E, Anderson KV. Rab23 is an essential negative regulator of the mouse sonic hedgehog signalling pathway. Nature. 2001;412:194–8.

Eggenschwiler JT, Bulgakov OV, Qin J, Li T, Anderson KV. Mouse Rab23 regulates hedgehog signaling from smoothened to gli proteins. Dev Biol. 2006;290:1–12.

Ericson J, Rashbass P, Schedl A, et al. Pax6 controls progenitor cell identity and neuronal fate in response to graded shh signaling. Cell. 1997;90:169–80.

Evans TM, Ferguson C, Wainwright BJ, Parton RG, Wicking C. Rab23, a negative regulator of hedgehog signaling, localizes to the plasma membrane and the endocytic pathway. Traffic. 2003;4:869–84.

Fanto M, McNeill H. Planar polarity from flies to vertebrates. J Cell Sci. 2004;117:527–33.

Gunther T, Struwe M, Aguzzi A, Schughart K. Open brain, a new mouse mutant with severe neural tube defects, shows altered gene expression patterns in the developing spinal cord. Development. 1994;120:3119–30.

Hou Q, Wu YH, Grabsch H, et al. Integrative genomics identifies RAB23 as an invasion mediator gene in diffuse-type gastric cancer. Cancer Res. 2008;68:4623–30.

Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV. Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature. 2003;426:83–7.

Jenkins D, Seelow D, Jehee FS, et al. RAB23 mutations in Carpenter syndrome imply an unexpected role for hedgehog signaling in cranial-suture development and obesity. Am J Hum Genet. 2007;80:1162–70.

Kim J, Kato M, Beachy PA. Gli2 trafficking links hedgehog-dependent activation of smoothened in the primary cilium to transcriptional activation in the nucleus. Proc Natl Acad Sci USA. 2009;106:21666–71.

Leung KF, Baron R, Ali BR, Magee AI, Seabra MC. Rab GTPases containing a CAAX motif are processed post-geranylgeranylation by proteolysis and methylation. J Biol Chem. 2007;282:1487–97.

Liu YJ, Wang Q, Li W, et al. Rab23 is a potential biological target for treating hepatocellular carcinoma. World J Gastroenterol. 2007;13:1010–7.

Olkkonen VM, Peterson JR, Dupree P, Lutcke A, Zerial M, Simons K. Isolation of a mouse cDNA encoding Rab23, a small novel GTPase expressed predominantly in the brain. Gene. 1994;138:207–11.

Park TJ, Haigo SL, Wallingford JB. Ciliogenesis defects in embryos lacking inturned or fuzzy function are associated with failure of planar cell polarity and Hedgehog signaling. Nat Genet. 2006;38:303–11.

Pataki C, Matusek T, Kurucz E, Ando I, Jenny A, Mihaly J. Drosophila Rab23 is involved in the regulation of the number and planar polarization of the adult cuticular hairs. Genetics. 2010;184(4):1051–65.

Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. Science. 2007;317:372–6.

Sheffield VC. The blind leading the obese: the molecular pathophysiology of a human obesity syndrome. Trans Am Clin Climatol Assoc. 2010;121:172–81. Discussion 181–2.

Stenmark H. Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol. 2009;10:513–25.

Veland IR, Awan A, Pedersen LB, Yoder BK, Christensen ST. Primary cilia and signaling pathways in mammalian development, health and disease. Nephron Physiol. 2009;111:p39–53.

Yang L, Clinton JM, Blackburn ML, et al. Rab23 regulates differentiation of ATDC5 chondroprogenitor cells. J Biol Chem. 2008;283:10649–57.

Yoshimura S, Egerer J, Fuchs E, Haas AK, Barr FA. Functional dissection of Rab GTPases involved in primary cilium formation. J Cell Biol. 2007;178:363–9.

Zerial M, McBride H. Rab proteins as membrane organizers. Nat Rev Mol Cell Biol. 2001;2:107–17.

RAB23, Member RAS Oncogene Family

► Rab23

RAB7a

► Rab7a in Endocytosis and Signaling

Rab7a in Endocytosis and Signaling

Soumik BasuRay[1], Jacob O. Agola[1], Patricia A. Jim[1], Matthew N. Seaman[2] and Angela Wandinger-Ness[1]
[1]Department of Pathology and Cancer Center, MSC08-4640, University of New Mexico Health Sciences Center, Albuquerque, NM, USA
[2]Department of Clinical Biochemistry, Cambridge Institute for Medical Research, Cambridge, UK

Synonyms

BRL-Ras; CMT2B; FLJ20819; PRO2706; RAB7a; Ras-related protein Rab-7a; Ypt7p (yeast ortholog)

Rab7 Historical Background and Function

Mammalian Rab7 was first identified in a rat liver cell line as BRL-Ras [X12535; NM_023950] and subsequently named Rab7 when it was recognized to be a member of an emerging, separate branch of Ras-related GTPases now well known as the Rab family of GTPases [NP_004628.4; P51149; P09527]. Rab7a is the most widely studied form and encoded on human chromosome 3q21.3 (mouse chromosome 6) as two splice variants differing in the 3′ untranslated region. The most intensively studied mammalian forms of Rab7a (mouse, canine, rat, and human) are 99.5% identical with only a single conservative change among the 207 amino acids (D/E 196). A more recently discovered homolog, Rab7b/Rab7L1, is encoded on human and mouse chromosome 1q32 and functions in late endosome to Golgi trafficking [Q96AH8; Q8VEA8]. Human Rab7b is only 47% identical and 82% homologous to human Rab7a across its 199 aa length. Following the initial demonstration of Rab7a function in regulating membrane transport from early to late endosomes, Rab7a has been found to have critical roles in autophagy, lipid metabolism, growth factor signaling, bone resorption, and phagolysosome biogenesis (Fig. 1) (Agola et al. 2011).

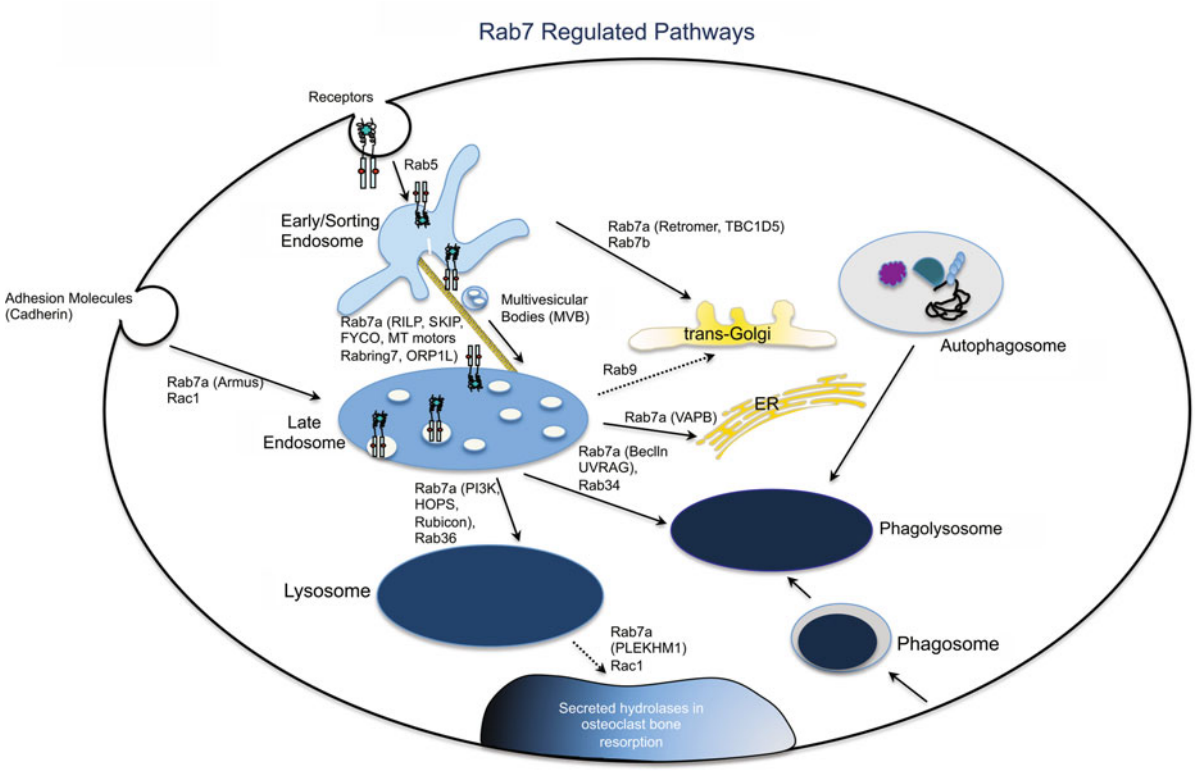
Rab7a Activation and Localization

Typical of Ras-related GTPases, Rab7a undergoes a cycle of membrane association and dissociation that is closely linked to nucleotide binding and hydrolysis (Fig. 2) (Agola et al. 2011). In the membrane-associated state, Rab7a is GTP bound and active, while upon hydrolysis the GDP-bound Rab7a is inactive and recycles to the cytoplasm. The GDP-/GTP-dependent activation cycle is regulated by two sets of proteins, the guanine nucleotide exchange factors (GEFs) that catalyze the GTP binding and conversion to the active GTP-bound state and the GTPase-activating proteins (GAPs) that stimulate the GTPase activity of the Rab protein to convert it to the inactive GDP-bound state. Early reports suggested Vps39 functioned as a Rab7a GEF. More recently, studies in yeast and *C. elegans* indicate that Ypt7p/Rab7 activation is linked to endosome conversion involving coordinate inactivation and loss of the early endosomal Rab5 and acquisition and activation of the late endosomal Rab7a through large multimeric complexes with overlapping components (Fig. 3a) (Wang et al. 2011a). SAND-1 (Mon1a-Mon1b in vertebrates) binds to the CORVET components (Vps11, VPS16A, Vps18, and Vps33) and functions to displace the Rab5 GEF (RABX-5). Subsequent recruitment of Ccz1 through Mon1 and cooperation with Vps39 is central to the endosomal recruitment and activation of Ypt7p in yeast and Rab7 *C. elegans* enabling binding to the HOPS complex (Vps11, Vps16A, Vps18, Vps33, and Vps41) (Fig. 2). Mon1 and Ccz1 are conserved in mammals (Wang et al. 2011a), though mammalian Rab7a GEF activity remains to be demonstrated.

The activation of Rab7a is dynamically regulated through differential interactions of proteins first identified to be important in autophagy called Rubicon *(RUN domain and cysteine-rich domain containing Beclin 1-interacting protein*) and UVRAG (Liang et al. 2008; Zhong et al. 2009) (Fig. 2). Rubicon, a regulatory component of the ▶ phosphatidylinositol 3-kinase complex (PI3KC3; hVps34/hVps15), can bind and sequester UVRAG and thereby block Rab7a-mediated transport (Sun et al. 2010, 2011; Lin and Zhong 2011). Conversely, membrane-bound, active Rab7a can relieve the inhibition of its activation by binding Rubicon (Sun et al. 2010).

Proteins of the Tre-Bub-CDC16 (TBC) family function as GTPase-activating proteins that stimulate nucleotide hydrolysis. Three family members (TBC1D2/Armus, TBC1D5, and TBC1D15) have all been shown to stimulate Rab7a nucleotide hydrolysis and may regulate Rab7a involvement in discrete functions in coordination with specific signaling (Seaman et al. 2009; Frasa et al. 2010; Peralta et al. 2010). For example, on endosomes the recycling of mannose 6-phosphate receptor to the Golgi via retromer is thought to be regulated by Rab7a/TBC1D5, while the disassembly of adherens junctions and degradation of E-cadherin depends on signal integration of Arf6 and a Rac1/TBC1D2/Rab7a complex. As illustrated by the specific examples given, the facilitated nucleotide binding and hydrolysis cycle brings about conformational changes in Rab7a that modulate its activity and localization.

Membrane localization is dependent on posttranslational modification with a lipid anchor (prenylation) (Fig. 2). Nascent Rab7a synthesized on cytosolic ribosomes is inactive and GDP bound. Prenylation on two C-terminal cysteine residues is mediated by the

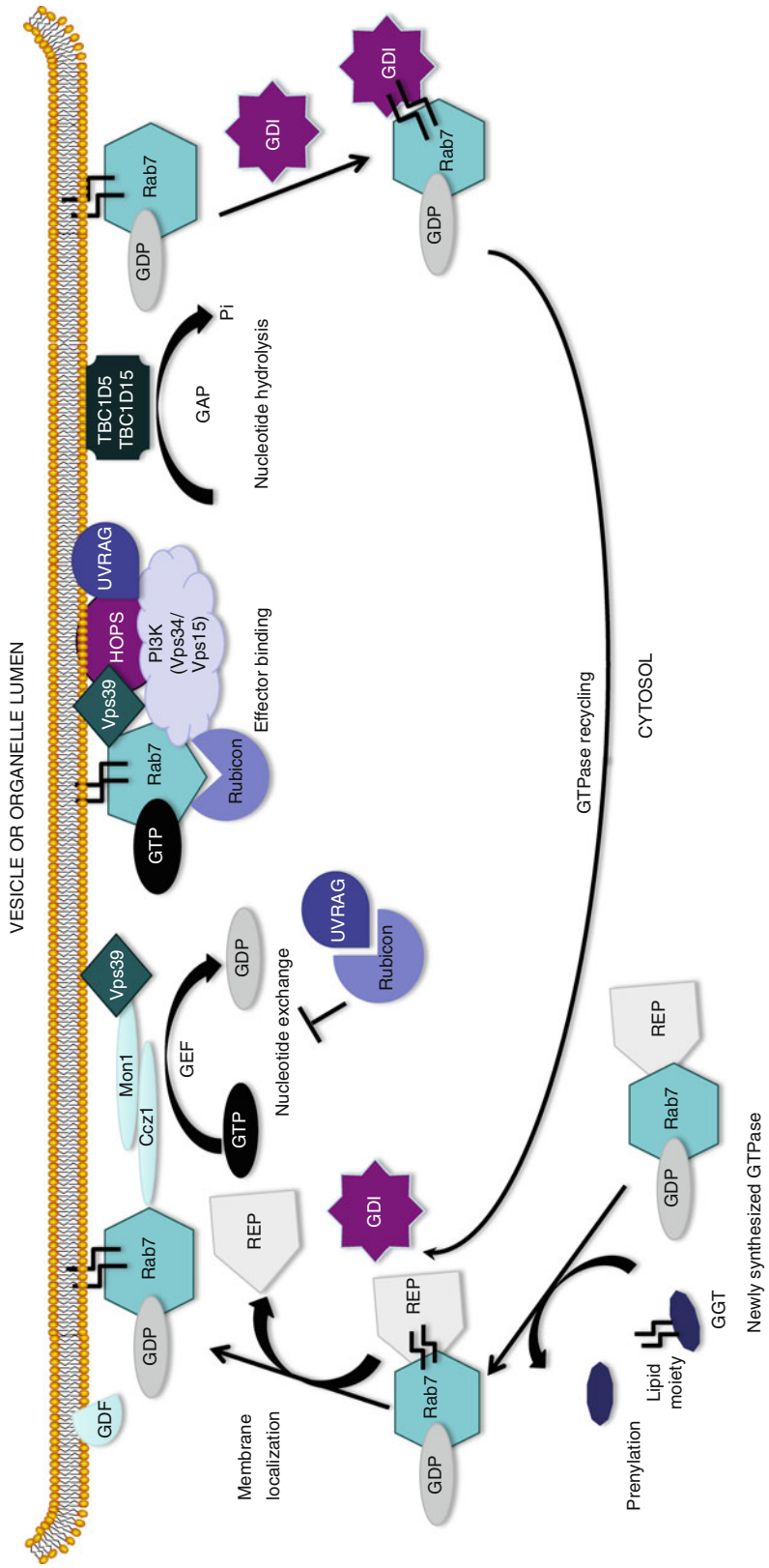


Rab7a in Endocytosis and Signaling, Fig. 1 *Rab7a-regulated pathways.* Rab7a regulates endocytic transport from early to late endosomes in a process requiring Rab5 to Rab7a conversion. Rab7a also cooperates with other Rab GTPases to facilitate late endosome-lysosome fusion and phagolysosome formation (see Table 1). Key Rab7a effectors involved on individual pathways are noted in parentheses. Rab7a cooperates with Rac1 in epithelia to promote internalization of cell adhesion molecules and in osteoclasts to promote localized hydrolase secretion for bone resorption

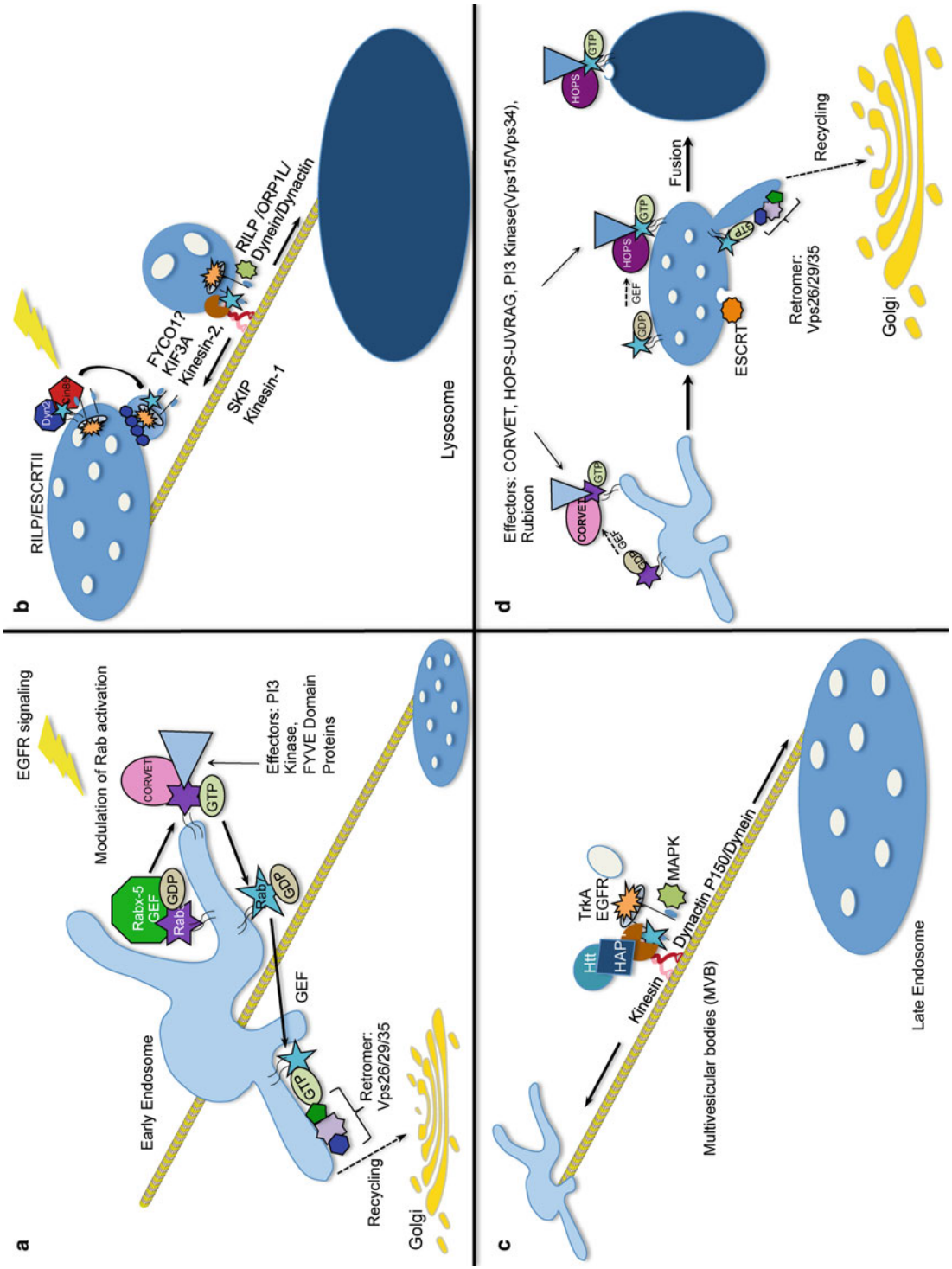
universal Rab geranylgeranyl transferase, through recognition of the last nine amino acid residues of Rab7a (Wu et al. 2009). The Rab escort protein (REP) serves as the intermediary for Rab7a presentation to the prenylating enzyme and first-time membrane association (Zhang et al. 2009; Agola et al. 2011). GDP dissociation inhibitor (GDI) functions as a universal Rab recycling factor, binding preferentially to doubly prenylated, GDP-bound Rab7a (Wu et al. 2007). GDI binding masks the isoprenyl anchor in the cytosol and renders Rab7a membrane association a reversible process that is closely linked to the nucleotide bound status, based on the fact that GDI has a 3 order of magnitude higher affinity for Rab7a-GDP than Rab7a-GTP (Wu et al. 2010). A GDI-displacement factor (GDF) has been implicated in GDI release during endosomal Rab membrane association, though GEF proteins may also perform this function in conjunction with nucleotide exchange (Wu et al. 2010). Once on the membrane and in the GTP-bound state, Rab7a interacts with diverse effectors to carry out specific functions.

Rab7a Effectors in the Control of Endocytic Trafficking

Over the years, many effector proteins have been identified to interact specifically with active GTP-bound Rab7a (Table 1). Rab7a effectors orchestrate events ranging from cargo selection to microtubule translocation to downstream membrane tethering and endosomal membrane fusion (Fig. 3). Emerging concepts are that Rab7a activation contributes to the dynamic assembly of large protein complexes in a spatially and temporally regulated manner (Wang et al. 2011a). Specific protein complexes serve discrete functions in the transport process, yet handoffs and



Rab7a in Endocytosis and Signaling, Fig. 2 *Rab7a activation cycle.* Newly synthesized Rab7a is prenylated by geranylgeranyl transferase (GGT) and delivered to endosomal membranes by rab escort protein (REP), thereafter Rab7a membrane cycling is facilitated by GDP dissociation inhibitor (GDI); pathways that are common to all Rab GTPases. A GDI displacement factor (GDF) has been implicated in membrane transfer of late endocytic Rab9 and Rab7a. Rab7a activation is closely linked to Rab5 inactivation in a conversion process that involves Mon1. A Vps39-Mon1-Ccz1 complex likely acts as a guanine nucleotide exchange factor (GEF) to promote activation, while TBC1D5 or TBC1D15 act as GTPase-activating proteins (GAPs) to promote hydrolysis and inactivation. Active, GTP-bound Rab7a acts as a scaffold for sequentially binding multiple effectors phosphoinositide 3-kinase (PI3K, Vps34/Vps15), HOPS, Rubicon, UVRAG, among others (see Table 1) to promote cargo selection, cytoskeletal translocation, and membrane fusion



Rab7a in Endocytosis and Signaling, Fig. 3 (continued)

multiple layers of regulation are common. The importance of maintaining transport fidelity is evidenced by the increasing numbers of human diseases attributable to defects in endosomal trafficking and Rab7a specifically (Charcot-Marie-Tooth disease type 2) (Cogli et al. 2009; Zhang et al. 2009; Agola et al. 2011).

On early endosomes, Rab7a functions in cargo sorting by recruiting the retromer complex (Vps26/29/35), which enables retrieval of cation-independent mannose 6-phosphate receptor, TGN38, Wntless among other cargo from early endosomes to the Golgi (Rojas et al. 2008; Seaman et al. 2009; McGough and Cullen 2011) (Fig. 3a). Interaction of retromer with Snx proteins and actin-binding proteins couples sorting with membrane tubulation (Harbour et al. 2010; McGough and Cullen 2011). Dysregulation of retromer is associated with neurologic diseases, including Alzheimer's disease, underscoring the importance of the Rab7a-retromer link.

On multivesicular bodies and late endosomes, Rab7a facilitates coordinate cargo sorting and bidirectional transport on microtubules through interactions with effectors that differentially associate with dynein or kinesin motors (Fig. 3b–c). Lysosomal sorting and perinuclear transport are mediated by the Rab7a-interacting lysosomal protein (RILP) effector (Zhang et al. 2009; Wang et al. 2011b). RILP interacts with components of the endosomal sorting complex required for transport (ESCRT-II) (Vps22 and Vps36) and based on depletion studies, RILP is shown to participate in the sorting of ubiquitinated receptors into intraluminal vesicles (Zhang et al. 2009; Wang et al. 2011b). In this manner, RILP sorts and sequesters the receptors from the cytosolic signaling machinery and targets them for lysosomal degradation. RILP is also targeted by bacterial pathogens to create a specialized intracellular niche for replication (Zhang et al. 2009). In a tripartite complex, Rab7a, RILP, and a second effector known as oxysterol-binding protein-related protein 1 L (ORP1L) serve to recruit a dynein/dynactin motor complex that in association with betaIII spectrin facilitates the perinuclear transport of endosomes on microtubules (Wang et al. 2011b) (Fig. 3b).

Dynein-/dynactin-mediated perinuclear positioning of late endosomes has also been shown to depend on the membrane-associated scaffolding protein, Huntingtin (Htt), which when mutant causes Huntington's disease, though the link between Htt and Rab7a remains unclarifed (Agola et al. 2011; Caviston et al. 2011). Htt and the Huntingtin-associated protein of 40 kDa (HAP40) are known effectors of Rab5 that facilitate transfer between microtubule-and actin-based networks (Agola et al. 2011). Parallel in vitro studies testing Rab7a did not provide evidence for a direct Rab7a-Htt or a Rab7a-HAP40 interaction, although Huntingtin-associated protein 1 (HAP1) binds dynactin p150Glued (Agola et al. 2011). Therefore, it is speculated that an Htt interaction with the Rab7a dynein/dynactin complex may occur through HAP1 (Fig. 3c). In light of the disease relevance, further study of the potential interfaces between Htt, HAP1, and Rab7a is warranted.

Anterograde movement of endosomes to the cell periphery along the microtubular network is incompletely characterized. Plus-end motility of autophagosomes is dictated by the recently identified Rab7a effector FYVE and coiled-coil domain protein 1

R

Rab7a in Endocytosis and Signaling, Fig. 3 *Rab7a trafficking and signaling complexes.* (**a**) Recycling from early endosomes to the Golgi. Cargo recycling from early endosomes to the Golgi entails sequential Rab5 (via CORVET) and Rab7a activation and Rab7a-mediated recruitment of a complex of Vps proteins (26/29/35) known as retromer. Rab5 activation can be positively modulated by EGF receptor signaling. (**b**) Transport to lysosomes. Rab7a cooperates with the Dyn2-CIN85 complex to regulate signaling and lysosomal degradation of the ligand-receptor (EGF-EGF receptor) complex. Bidirectional transport on microtubules depends on kinesin and dynein motors. (**c**) Transport from early to late endosomes. Growth factor receptor signaling is intimately coupled to endocytic transport. As illustrated, nerve growth factor receptor TrkA and EGF receptor associate with MAPK on endosomes are translocated bidirectionally on microtubules. Transport toward perinuclear late endosomes occurs through association with the Rab7a effector RILP and the p150/dynein motor complex. Association with HAP1/Htt contributes to perinuclear late endosome positioning. Transport to the cell periphery is mediated in association with kinesin motor complexes (Kif3a/Kinesin-2 via FYCO1 or Kinesin-1 via SKIP). (**d**) Distinct multi-protein complexes regulate transport to and from late endosomes. Rab5-Rab7a conversion involves coordinate inactivation of Rab5 and activation of Rab7a, transition of CORVET complex to HOPS complex, which ensures seamless cargo transport to late endosomes. Handoff to ESCRT machinery enables membrane invagination and sequestration of growth factor receptors on intraluminal vesicles of multivesicular bodies. Rab7a-retromer complex enables Golgi recycling. Rab7a HOPS complex enables late endosome-lysosome fusion

(FYCO1) and an unknown kinesin (Wang et al. 2011a). Late endosome movement is known to depend on kinesin-2 KIF3A heavy chain, while the Rab7a link and effector remain enigmatic (Loubery et al. 2008). Evidence from studies on *Salmonella* suggest that Rab9 and Rab7a associate with distinct domains on SifA and kinesin-interacting protein (SKIP), implicating kinesin-1 in anterograde motility and late endosomal sorting (Jackson et al. 2008). In sum, the function of the Rab7a-RILP complex in sorting and cytoskeletal transport is best characterized, while other Rab7a effector interactions including those with kinesins and disease relevant proteins (Htt and HAP1) await further characterization. Bacterial proteins from *Salmonella* with identified functions in interfering with Rab7a motor proteins or linkers may offer unique tools for further dissecting Rab7a motor protein interactions.

Endosomal lipids such as cholesterol and phosphoinositides are critical regulators of cargo sorting and transport on the late endosomal pathway that are integrated through Rab7a and associated motor proteins. In particular, cholesterol sensing is integrated with transport through the Rab7a effector ORP1L (Wang et al. 2011a). When cholesterol levels are low, ORP1L promotes the association of late endosomes with the endoplasmic reticulum via the dissociation of minus-end motor proteins. The ER protein VAPB contributes to motor dissociation and the peripheral movement of late endosomes. Being more peripherally localized, late endosomes are poised to receive cholesterol and other cargo internalized through early endosomes or association with the endoplasmic reticulum (ER). Conversely, when cholesterol levels are high, the conformation of ORP1L is altered and perinuclear transport is favored. In Niemann-Pick type C disease, where endosomal cholesterol levels are constitutively high, the bidirectional motility of endosomes/phagosomes and activation of Rab7a are perturbed (Chen et al. 2008; Zhang et al. 2009). The perturbations contribute to disease pathology and can be reversed by overexpression of Rab9 or Rab7a (Zhang et al. 2009).

Similar to Rab5 on early endosomes, GTP-bound Rab7a is required for class-III ▶ phosphatidylinositol 3-kinase (consisting of the hVps34 catalytic, the hVps15/p150 Rab7a-binding adaptor, and the Rubicon regulatory subunits) activation on late endosomes (Agola et al. 2011; Ho et al. 2012). The local synthesis of PI(3)P on late endosomes enables the recruitment of FYVE domain–containing proteins that promote membrane remodeling (including intraluminal vesicle formation) and eventually terminate the signal. FYVE domain–containing factors include the PI(3,5)P(2)-producing kinase PIKfyve, myotubularin lipid phosphatases, among others (Table 1). Together these downstream effectors control endolysosome morphology, membrane trafficking, acidification, among other functions. Rab7a together with the early endosomal myotubularin lipid phosphatases (MTM1) and late endosomal myotubularin-related protein 2 (MTMR2) acts as a molecular switch controlling the sequential synthesis and degradation of endosomal PI(3)P (Cao et al. 2008). Direct binding of the phosphatases to the phosphatidylinositol 3-kinase complex leads to inactivation of the myotubularins. The lipid kinase-myotubularin interaction also precludes the interaction of the activated Rab7a with the lipid kinase, illustrating the importance of protein handoffs in phosphoinositide 3-phosphate homeostasis on late endosomes. Together, the examples cited provide evidence for Rab7a function in endosomal lipid homeostasis in both metabolism and signaling, the disruption of which leads to human disease.

Two Rab7a effectors participate directly in the regulation of cargo degradation. Rabring7 (Rab7a-interacting ring finger protein) functions as an E3 ligase in conjunction with the Ubc4 and Ubc5 as E2 proteins (Zhang et al. 2009; Wang et al. 2011a). Functionally, overexpression of Rabring7 increases epidermal growth factor receptor degradation and lysosome biogenesis. The proteasome alpha-subunit XAPC7 or PSMA7 in mammals has been found to interact specifically with Rab7a and is recruited to late multivesicular endosomes (Zhang et al. 2009; Agola et al. 2011). Overexpression of XAPC7 impairs late endocytic transport of EGF receptor and hence is a negative regulator of trafficking. Together, Rabring7 and XAPC7 may coordinate the degradation of ubiquitinated growth factor receptors via a link to the proteasomal degradation machinery though further studies are required to elucidate mechanistic details.

In addition to the described Rab7a effectors whose functional activities have been detailed, there are many more putative effectors whose characterization remains to be documented (Table 1). Therefore, further complexity in Rab7a-mediated regulation of cargo

Rab7a in Endocytosis and Signaling, Table 1 Rab7 GTPase regulators and effectors, and their functions

Rab7 isoform and nucleotide-bound state	Rab7 effector/binding partner	Regulator or effector function[a]
Rab7a	ANKFY1 (ankyrin repeat and FYVE domain containing 1)/ANKHZN/ Rabankyrin-5	Possible role in vesicular trafficking. Novel interactor of Rab7. Specific role yet to be established
Rab7a	ATP6V0A1	Component of vacuolar ATPase that regulates organelle acidification required for protein sorting, receptor-mediated endocytosis, zymogen activation, and synaptic vesicle proton gradient. Novel interactor of Rab7. Specific role yet to be established
Rab7a-GDP	Ccz1 (vacuolar protein trafficking and biogenesis-associated homolog)	Recruited to endosomes by Mon1a/Mon1b and acts as Rab7 GEF in yeast. Possible human homolog C7orf28B also some similarity to HPS4 involved in biogenesis of lysosome-related organelles
Rab7a-GTP	FYCO1 (FYVE and coiled-coil domain containing 1)	Promotes microtubule plus end transport of autophagosomes presumably by functioning as a kinesin adapter
Rab7a	GNB2L1 (guanine nucleotide binding protein (G protein), beta polypeptide)	Role in intracellular signaling and activation of protein kinase C and possible interaction with Rab7 via WD40 domain. Novel interactor of Rab7. Specific role yet to be established
Ypt7p/ Rab7a-GTP	HOPS complex (Vps11,-16,-18,-33,-39, and-41)	Involved in vacuolar tethering and fusion in yeast and conserved mammalian homologs function in mammalian endolysosomal fusion. Interfaces with CORVET complex to promote rab5 to rab7 conversion in yeast. Vps39 subunit binds Mon1-Ccz1 complex that serves as a Rab7 GEF in yeast and *C. elegans*
Rab7a	hVps39	In yeast Vps39p, cooperates with Mon1-Ccz1 complex to promote Ypt7p nucleotide exchange, function of mammalian protein remains to be determined
Rab7a	IMMT (Mitofilin)	Maintains mitochondrial morphology and suggested role in protein import. Novel interactor of Rab7. Specific role yet to be established
Rab7a	KIF3A (kinesin + adapter?)	Kinesin2 heavy chain associates with late endosomes along with dynein, Rab7, and dynactin. Possible mediator of Rab7-regulated anterograde transport coordinated by Rab7-interacting adapter such as FYCO1 or other as-yet-unidentified protein
Rab7a-GDP	Mon1a-Mon1b	Mammalian homologs of *C. elegans* SAND1. Mon1a-Mon1b causes Rab5 GEF displacement and Mon1b interacts with the HOPS complex. Mon1 is an effector of Rab5, but only interacts with Rab7 when complexed to Ccz1
Rab7a-GTP	ORP1L ([oxysterol-binding protein, OSBP]-related protein 1)	Required for cholesterol sensing and regulation of dynein/ dynactin motor with Rab7 and RILP, regulates late endosome/lysosome morphogenesis and transport
Rab7a-GTP	Phosphoinositide 3-kinase complex (hVps34/hVps15)	Type III ▶ PI 3-kinase that generates phosphoinositide 3-phosphate to control endosomal trafficking and signaling. Forms complex with myotubularins for negative regulation
Rab7a-GTP	Plekhm1 (pleckstrin homology domain containing, family M [with RUN domain] member)	Regulates lysosomal secretion in osteoclasts for bone resorption by interacting with LIS1 to control microtubule transport and Rab7 and ▶ PI 3-kinase to recruit effectors for fusion
Rab7a	Prohibitin	Negative regulator of cell proliferation and a possible tumor suppressor. Novel interactor of Rab7, specific role yet to be established

(*continued*)

Table 1 (continued)

Rab7 isoform and nucleotide-bound state	Rab7 effector/binding partner	Regulator or effector function[a]
Rab7a-GTP	Rabring7	Rab7-interacting ring finger protein, functions as E3 ligase that ubiquitinates itself and controls EGF receptor degradation
Rab7a-GDP	REP1 (Rab escort protein 1)	Presents Rab7 to Rab geranylgeranyl transferase for addition of prenyl group that acts as a membrane anchor
Rab7a-GTP	Retromer (Vps26, Vps29, Vps35)	Regulates retrograde transport from late endosome to trans-Golgi network (TGN) through direct interaction with Vps26
Rab7a-GTP	RILP (Rab7-interacting lysosomal protein)	Involved in late endosomal/lysosomal maturation. Recruits dynein-dynactin motor protein complex
Rab7a-GTP	Rubicon	Regulates endosome maturation through differential interaction with UVRAG and Rab7. Rubicon binding inhibits UVRAG. Rubicon binding to active Rab7 frees UVRAG to activate the hVps34/hVps15 ▶ PI 3-kinase and HOPS, thereby simultaneously increasing the active pool of Rab7 and PI3P signaling
Rab7a-GTP	SKIP (SifA and kinesin-interacting protein)	Homolog of PLEKHM1 that binds Rab7, Rab9 and kinesin-1, and may regulate anterograde motility of late endosomes. Target of *Salmonella* SifA protein
Rab7a	Spg21	Loss of function causes autosomal recessive hereditary spastic paraplegia. Involved in vesicular transport. Novel interactor of Rab7. Specific role yet to be established
Rab7a	STOML2 (Stomatin-like 2)	Negatively modulates mitochondrial sodium calcium exchange. Novel interactor of Rab7. Specific role yet to be established
Rab7a-GTP	TBC1D2 ([tre-2/USP6, BUB2, cdc16] domain family, member 5)/Armus and Rac1	Regulates cytoskeleton organization, ruffled border formation in osteoclasts, and E-cadherin/adherens junction degradation in conjunction with Rac1, inactivates Rab7 through C-terminal GAP activity
Rab7a-GTP	TBC1D5 ([tre-2/USP6, BUB2, cdc16] domain family, member 5)	Negatively regulates retromer recruitment and causes Rab7 to dissociate from membrane and may have Rab7 GAP activity
Rab7a-GTP	TBC1D15 ([tre-2/USP6, BUB2, cdc16] domain family, member 15)	Functions as Rab7 GAP and reduces interaction with RILP, fragments lysosomes, and confers resistance to growth factor withdrawal-induced cell death
Rab7a-GTP	TrkA (neurotrophic tyrosine kinase receptor)	Interacts with Rab7 and regulates endocytic trafficking and nerve growth factor signaling as well as influencing neurite outgrowth
Rab7a-GTP	UVRAG (UV radiation resistance-associated gene)/Beclin1	UVRAG/C-Vps complex positively regulates Rab7 activity via ▶ PI 3-kinase (PI3KC) during autophagic and endocytic maturation
Rab7a-GTP	VapB ([vesicle-associated membrane protein]-associated protein B)	Involved in mediating endosome-ER interaction in response to ORP1L conformation sensing low cholesterol levels
Rab7a	Vps13c (vacuolar protein sorting 13c)	Vacuolar protein sorting and novel interactor of Rab7. Specific role yet to be established
Rab7a-GDP, GTP	XAPC7/PSMA7 (proteasome subunit, alpha type 7)	Negative regulator of late endocytic transport. Overexpression inhibits EGF receptor degradation
Rab7b	SP-A (Surfactant protein A)	Transiently enhances the expression of Rab7 and Rab7b and makes them functionally active to increase the endolysosomal trafficking in alveolar macrophages

[a]Agola, JO thesis provides reference listing for effectors

sorting, cytoskeletal transport, and membrane fusion will emerge through continued study. An important area for investigation is how Rab7a interactions with tethering factors and SNARE proteins control late endosomal fusion events which have primarily been characterized for the yeast homolog Ypt7p (Zhang et al. 2009; Wang et al. 2011a).

Rab7a in Endosomal Signaling

At the late endosome, Rab7a coordinately regulates intracellular signaling through special scaffolds, selective endosome positioning, and control of growth factor receptor trafficking. Epidermal growth factor receptor (EGF receptor), vascular endothelial growth factor receptor (VEGFR2), and nerve growth factor receptor (TrkA) all depend on Rab7a for their signaling and downregulation (Agola et al. 2011). For example, upon EGF stimulation, K-Ras is endocytosed and sorted to late endosomes where Rab7a and the p14-MP1-p18 scaffolding proteins recruit and activate ▶ MEK-Erk on late endosomes (Lu et al. 2009; Nada et al. 2009). ▶ MAP kinase signaling is further regulated by Rab7a-dependent late endosome positioning through dynactin such that peripheral mislocalization results in prolonged EGF receptor activation and downstream Erk and p38 signaling. The localization of two signaling mediators of the TGF-β superfamily to Rab7-positive late endosomes (p-Smad1 and p-Smad2) is also suggested to be critical for regulation of growth factor signaling (Rajagopal et al. 2007). At the conclusion of signaling, Rab7a may act cooperatively with dynamin 2 and CIN85 (cbl-interacting protein of 85 kDa) to promote the transfer of signaling receptors from late endosomes to lysosomes for degradation (Fig. 3b) (Schroeder et al. 2010).

In neuronal cells, the receptor tyrosine kinase, TrkA is activated by nerve growth factor (NGF). On NGF stimulation, Rab7a interacts with TrkA as it transits through early and late endosomes. Cells expressing Rab7a T22N, which is predominantly GDP bound, showed prolonged Erk1/2 signaling due to impaired trafficking of activated TrkA (Agola et al. 2011). Disease-causing Rab7a mutants that are constitutively activated have also been shown to exhibit enhanced NGF-stimulated Erk1/2 signaling (BasuRay et al. 2010). This apparently contradictory result can be explained by the duality of Rab7a in regulating transfer of cargo to lysosomes and interacting with scaffold proteins. Thus, Rab7a plays a significant role in growth factor transport by controlling both signaling scaffolds and trafficking to degradative compartments.

Phosphorylation of Rab7a in response to growth factor suggests a further layer of regulation. Large-scale proteomics analyses have identified Rab7a to be both serine and tyrosine phosphorylated. In mouse liver extracts, Rab7a was found phosphorylated on serine 72 within a highly conserved sequence near the GTP-binding pocket (Villen et al. 2007). Rab7a was phosphorylated in response to EGF stimulation on tyrosine 183 in the C-terminal region. Enhanced tyrosine 183 phosphorylation of Rab7a was also associated with mutant EGF receptor and HER2 overexpression in non–small cell lung carcinoma and mammary epithelia, respectively (Guo et al. 2008). The functional consequences of Rab7a serine and tyrosine phosphorylation with respect to membrane trafficking, GTP binding, and hydrolysis remain to be established.

Ubiquitination of activated growth factor receptors plays a crucial role in the endosomal sorting and lysosomal targeting to downregulate receptor levels. Such ubiquitination may depend on interaction with Rab7a and the Rabring7 E3 ubiquitin ligase. The sorting of ubiquitinated receptors into luminal vesicles of multivesicular bodies depends on the ESCRT0, ESCRTI, ESCRTII, and ESCRTIII complexes (Raiborg and Stenmark 2009). EGF receptor and TrkA endolysosomal degradation are both ubiquitin and proteasome dependent. The K63-linked polyubiquitin chain on activated TrkA receptors gets shuttled by the p62 scaffolding protein, possibly in association with Rab7a/XAPC7, to the proteasome for deubiquitination prior to degradation in lysosomes (Geetha and Wooten 2008). TrkA deubiquitination prior to lysosomal degradation may allow crucial recycling of ubiquitin since the ubiquitin tagging is essential for optimum interaction of activated TrkA with the transport machinery and its delivery along the long axonal route from the tip to the cell body. Only after TrkA reaches the cell body, the termination of signaling calls for deubiquitination of the cargo prior to its lysosomal degradation. As illustrated, the reversible ubiquitination is an important component of growth factor receptor downregulation.

The Rab7a-regulated, interdependent late endocytic trafficking, and signaling pathways are indispensible for translating growth factor signals into appropriate cell responses. The development of suitable in vivo models will therefore be crucial to elucidate how impaired trafficking of growth factor receptors and consequent alterations in signaling lead to neurodegenerative diseases and cancer.

Summary

Since its discovery over 20 years ago, Rab7a and its functions in late endocytic trafficking and signaling have remained under active investigation. Unflagging interest is attributed to the diverse processes that are regulated by Rab7a together with a demonstrated role in human disease. The list of Rab7a effector proteins continues to grow, though the exact functions of many recent interacting partners remains to be elucidated. Rab7a helps to coordinate signaling through the temporal and localized assembly of signaling scaffolds and a close coupling to degradative pathways. Elucidating how Rab7 nucleotide exchange and hydrolysis are regulated and how Rab7 is selectively recruited to specific macromolecular complexes to regulate individual pathways remain important areas for further investigation.

Acknowledgments We acknowledge PREP fellowship from NIGMS (R25GM075149) to PJ and research support from NSF (MCB0956027) to AWN and MRC (G0701444) to MNM.

References

Agola J, Jim P, Ward H, Basuray S, Wandinger-Ness A. Rab GTPases as regulators of endocytosis, targets of disease and therapeutic opportunities. Clin Genet. 2011;80: 305–18.

BasuRay S, Mukherjee S, Romero E, Wilson MC, Wandinger-Ness A. Rab7 mutants associated with Charcot-Marie-Tooth disease exhibit enhanced NGF-stimulated signaling. PLoS One. 2010;5:e15351.

Cao C, Backer JM, Laporte J, Bedrick EJ, Wandinger-Ness A. Sequential actions of myotubularin lipid phosphatases regulate endosomal PI(3)P and growth factor receptor trafficking. Mol Biol Cell. 2008;19:3334–46.

Caviston JP, Zajac AL, Tokito M, Holzbaur EL. Huntingtin coordinates the dynein-mediated dynamic positioning of endosomes and lysosomes. Mol Biol Cell. 2011;22:478–92.

Chen H, Yang J, Low PS, Cheng JX. Cholesterol level regulates endosome motility via Rab proteins. Biophys J. 2008;94:1508–20.

Cogli L, Piro F, Bucci C. Rab7 and the CMT2B disease. Biochem Soc Trans. 2009;37:1027–31.

Frasa MA, Maximiano FC, Smolarczyk K, Francis RE, Betson ME, Lozano E, et al. Armus is a Rac1 effector that inactivates Rab7 and regulates E-cadherin degradation. Curr Biol. 2010;20:198–208.

Geetha T, Wooten MW. TrkA receptor endolysosomal degradation is both ubiquitin and proteasome dependent. Traffic. 2008;9:1146–56.

Guo A, Villen J, Kornhauser J, Lee KA, Stokes MP, Rikova K, et al. Signaling networks assembled by oncogenic EGFR and c-Met. Proc Natl Acad Sci USA. 2008;105:692–7.

Harbour ME, Breusegem SY, Antrobus R, Freeman C, Reid E, Seaman MN. The cargo-selective retromer complex is a recruiting hub for protein complexes that regulate endosomal tubule dynamics. J Cell Sci. 2010;123:3703–17.

Ho CY, Alghamdi TA, Botelho RJ. Phosphatidylinositol-3,5-bisphosphate: no longer the poor PIP2. Traffic. 2012;13:1–8.

Jackson LK, Nawabi P, Hentea C, Roark EA, Haldar K. The Salmonella virulence protein SifA is a G protein antagonist. Proc Natl Acad Sci USA. 2008;105:14141–6.

Liang C, Lee JS, Inn KS, Gack MU, Li Q, Roberts EA, et al. Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. Nat Cell Biol. 2008;10:776–87.

Lin MG, Zhong Q. Interaction between small GTPase Rab7 and PI3KC3 links autophagy and endocytosis: a new Rab7 effector protein sheds light on membrane trafficking pathways. Small Gtpases. 2011;2:85–8.

Loubery S, Wilhelm C, Hurbain I, Neveu S, Louvard D, Coudrier E. Different microtubule motors move early and late endocytic compartments. Traffic. 2008;9:492–509.

Lu A, Tebar F, Alvarez-Moya B, Lopez-Alcala C, Calvo M, Enrich C, et al. A clathrin-dependent pathway leads to KRas signaling on late endosomes en route to lysosomes. J Cell Biol. 2009;184:863–79.

McGough IJ, Cullen PJ. Recent advances in retromer biology. Traffic. 2011;12:963–71.

Nada S, Hondo A, Kasai A, Koike M, Saito K, Uchiyama Y, et al. The novel lipid raft adaptor p18 controls endosome dynamics by anchoring the MEK-ERK pathway to late endosomes. EMBO J. 2009;28:477–89.

Peralta ER, Martin BC, Edinger AL. Differential effects of TBC1D15 and mammalian Vps39 on Rab7 activation state, lysosomal morphology, and growth factor dependence. J Biol Chem. 2010;285:16814–21.

Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature. 2009;458:445–52.

Rajagopal R, Ishii S, Beebe DC. Intracellular mediators of transforming growth factor beta superfamily signaling localize to endosomes in chicken embryo and mouse lenses in vivo. BMC Cell Biol. 2007;8:25.

Rojas R, van Vlijmen T, Mardones GA, Prabhu Y, Rojas AL, Mohammed S, et al. Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab7. J Cell Biol. 2008;183:513–26.

Schroeder B, Weller SG, Chen J, Billadeau D, McNiven MA. A Dyn2-CIN85 complex mediates degradative traffic of the EGFR by regulation of late endosomal budding. EMBO J. 2010;29:3039–53.

Seaman MN, Harbour ME, Tattersall D, Read E, Bright N. Membrane recruitment of the cargo-selective retromer subcomplex is catalyzed by the small GTPase Rab7 and inhibited by the Rab-GAP TBC1D5. J Cell Sci. 2009;122:2371–82.
Sun Q, Westphal W, Wong KN, Tan I, Zhong Q. Rubicon controls endosome maturation as a Rab7 effector. Proc Natl Acad Sci USA. 2010;107:19338–43.
Sun Q, Zhang J, Fan W, Wong KN, Ding X, Chen S, et al. The RUN domain of rubicon is important for hVps34 binding, lipid kinase inhibition, and autophagy suppression. J Biol Chem. 2011;286:185–91.
Villen J, Beausoleil SA, Gerber SA, Gygi SP. Large-scale phosphorylation analysis of mouse liver. Proc Natl Acad Sci USA. 2007;104:1488–93.
Wang T, Ming Z, Xiaochun W, Hong W. Rab7: role of its protein interaction cascades in endo-lysosomal traffic. Cell Signal. 2011a;23:516–21.
Wang T, Zhang M, Ma Z, Guo K, Tergaonkar V, Zeng Q, et al. A role of Rab7 in stabilizing EGFR-Her2 and in sustaining Akt survival signal. J Cell Physiol. 2011b;227:2788–97.
Wu YW, Tan KT, Waldmann H, Goody RS, Alexandrov K. Interaction analysis of prenylated Rab GTPase with Rab escort protein and GDP dissociation inhibitor explains the need for both regulators. Proc Natl Acad Sci USA. 2007;104:12294–9.
Wu YW, Goody RS, Abagyan R, Alexandrov K. Structure of the disordered C terminus of Rab7 GTPase induced by binding to the Rab geranylgeranyl transferase catalytic complex reveals the mechanism of Rab prenylation. J Biol Chem. 2009;284:13185–92.
Wu YW, Oesterlin LK, Tan KT, Waldmann H, Alexandrov K, Goody RS. Membrane targeting mechanism of Rab GTPases elucidated by semisynthetic protein probes. Nat Chem Biol. 2010;6:534–40.
Zhang M, Chen L, Wang S, Wang T. Rab7: roles in membrane trafficking and disease. Biosci Rep. 2009;29:193–209.
Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, et al. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. Nat Cell Biol. 2009;11:468–76.

Rab8

Heather H. Ward and Angela Wandinger-Ness
Department of Pathology and Cancer Center, MSC08-4640, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Synonyms

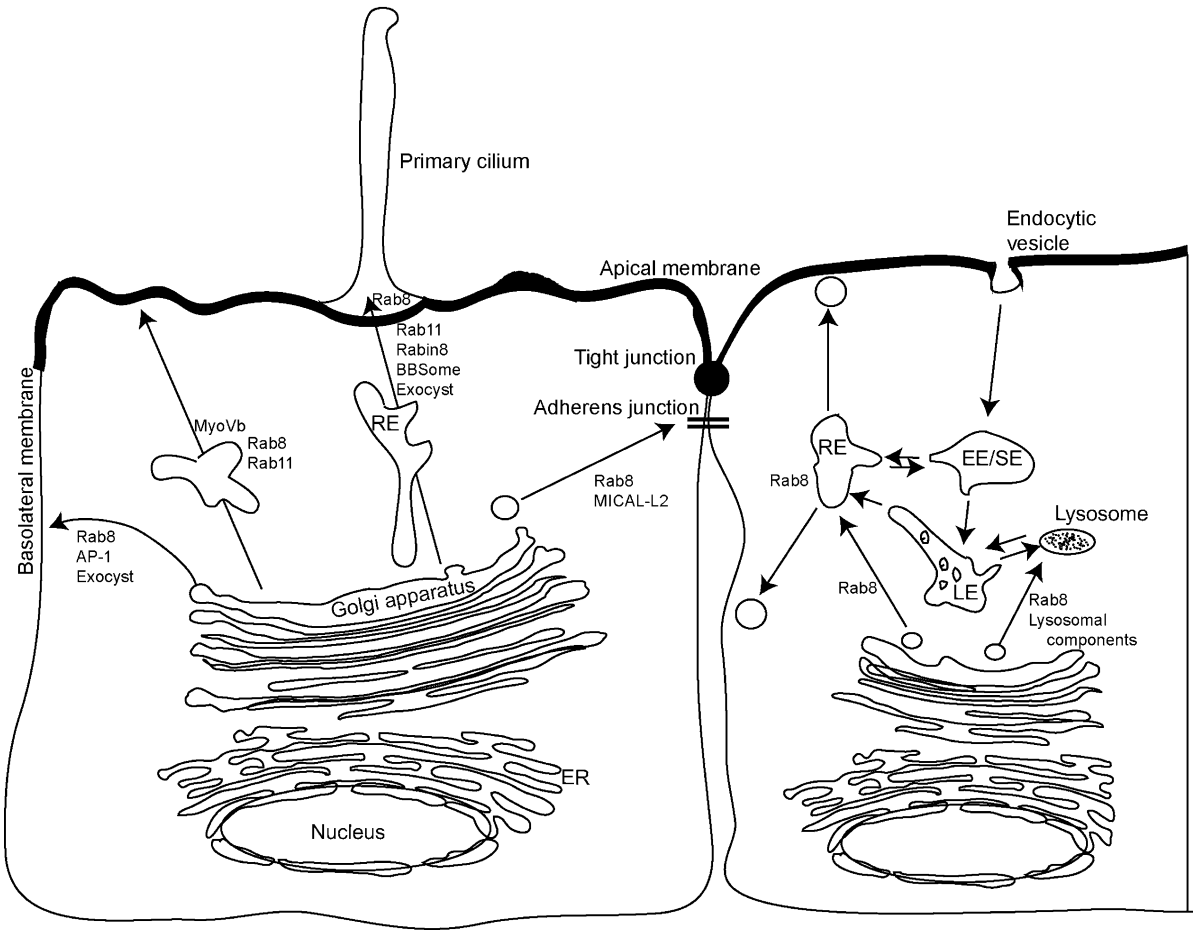
Dmel; Mel transforming oncogene (RAB8 homolog); MEL; MGC124948; Oncogene c-mel; Rab8a; Ras-related Rab8; Ras-associated protein RAB8; Ras-related protein Rab-8A

Historical Background

Ras-like Rab GTPases (guanosine triphosphatases) are regulators of membrane trafficking. Identification of two Ras-like GTPases, Sec4p and Ypt1p, which are involved in regulating secretion in yeast, rapidly led to the discovery of additional small GTPases in mammalian cells. Rab8 was among the first group of mammalian Rab GTPases to be identified and is a close functional and sequence homolog of the yeast proteins Ypt2 and Sec4p. A second isoform, Rab8b (b for basophil), was cloned from mast cells in 1996. Rab8b shares 83% sequence identity with MEL/Rab8 (now also termed Rab8a) primarily over amino acids 1–152. The two isoforms display significant overlap in tissue distribution (coexpressed in liver, skeletal muscle, and testis), though Rab8 is much more abundant in lung and kidney, while Rab8b is more prevalent in heart, brain, and spleen. This review presents a synopsis of recent developments on Rab8 function. As detailed in the following sections, Rab8 regulates transport from the trans-Golgi network to the basolateral plasma membrane of epithelia, neuronal dendrites, and the ciliary membrane. Rab8 is required in cellular polarization, cellular signaling and development. Consequently, alterations in Rab8 expression and localization affect numerous cellular events and organ systems.

Rab8 Function

Analyses of Rab8 function across diverse cell types demonstrate that Rab8 plays a pivotal role in exocytic and endocytic pathway interfaces and is particularly important in exocytic events (Fig. 1). Rab8 predominately localizes to budding vesicles at the trans-Golgi network, recycling endosomes, exocytic vesicles, and ruffling plasma membrane domains. From the trans-Golgi network, Rab8 regulates delivery of newly synthesized lysosomal enzymes to endosomes (del Toro et al. 2009), exocytosis of regulated secretory vesicles and melanosomes (Faust et al. 2008; Wandinger-Ness and Deretic 2008; Sun et al. 2010), and plasma membrane export to neuronal dendrites, as well as to the basolateral, apical and ciliary membrane domains of epithelia (Bravo-Cordero et al. 2007; Wandinger-Ness and Deretic 2008). An emerging theme is that Rab8-mediated transport of newly synthesized ciliary



Rab8, Fig. 1 *Rab8 trafficking routes*. Rab8 plays an important role in both exocytic and endocytic pathway interfaces and is particularly important in exocytic events. Through interactions with different protein complexes, Rab8 regulates delivery of newly synthesized lysosomal enzymes to endosomes, exocytosis of secretory vesicles, and cargo export basolateral, apical and ciliary membrane domains of epithelial cells. *RE* recycling endosome, *EE/SE* early endosome/sorting endosome, *LE* late endosome

membrane proteins, basolateral and apical cargo frequently occurs via recycling endosomes in coordination with Rab10 and Rab11a (Schuck et al. 2007; Cramm-Behrens et al. 2008; Wandinger-Ness and Deretic 2008; Knodler et al. 2010; Ward et al. 2011; Westlake et al. 2011). On the endosomal circuit, Rab8 may cooperate with Arf6, Rab11a or Rab13 to regulate externalization of recycling receptors (transferrin and glutamate), cell adhesion molecules (integrins and E-cadherin), and lipid regulators (ABCA1), often to specialized domains such as membrane protrusions, junctions and postsynaptic membranes (Hattula et al. 2006; Nagabhushana et al. 2010; Rahajeng et al. 2010; Roland et al. 2011).

Rab8 function in membrane trafficking is intimately connected to the cytoskeleton. Rab8 is associated with microtubules, actin and intermediate filaments, and expression of mutant Rab8 variants results in significant perturbations of both microtubule and actin networks (Omori et al. 2008; Wandinger-Ness and Deretic 2008). Rab8 has not been shown to bind microtubule-based motors, and the perturbation of microtubules induced by mutant Rab8 may be linked to Rabin8-dependent activation of Rab8 at centrosomes and the dependence of microtubule-based cilia on Rab8 membrane transport (Wandinger-Ness and Deretic 2008; Knodler et al. 2010; Westlake et al. 2011). On the other hand, Rab8-dependent actin

filament association depends on direct Rab8 interactions with myosin V(a,b,c) or myosin VI motor proteins, which in turn facilitate directed vesicle trafficking (Wandinger-Ness and Deretic 2008; Roland et al. 2009, 2011). Rab8-myosin VI interactions direct basolateral cargo to the plasma membrane (Wandinger-Ness and Deretic 2008). Rab8/Rab27/myosin Va regulate the final stages of melanosome docking to the plasma membrane via actin filaments (Wandinger-Ness and Deretic 2008). Rab8-Rab11-myosin Vb and Rab8-myosinVb interactions are involved in apical lumen formation and insulin-dependent GLUT4 exocytosis, respectively (Ishikura and Klip 2008; Roland et al. 2011). Rab8 and myosin Vc likely cooperate in the early stages of regulated secretion involving tubule-dependent transport to the cell periphery of exocrine epithelia followed by hand-off in the cell periphery to Rab27a vesicles whose cortical actin anchoring depends on myosin Vc (Jacobs et al. 2009). The three isoforms of myosin V also act as scaffolds for the binding of multiple Rab GTPases cooperating on linked pathways, including Rab8, Rab10, Rab11a, and Rab27 (Roland et al. 2009). For example, Rab8 as well as Rab10 interact via an alternatively spliced exon D in myosin Va and Vb or an exon D-like domain in myosin Vc, while Rab27a interacts via exon F in myosin Va (Roland et al. 2009). Thus, regulated interactions of distinct myosin isoforms with distinct combinations of Rab GTPases may partially explain cell-type-specific regulation of Rab8-dependent pathways (Roland et al. 2009, 2011). However, questions remain as to how Rab8 functions cooperatively with other GTPases and how specific functions are regulated, for example: Is Rab8 bound simultaneously with multiple Rab GTPases to individual myosin motors? Are there hand-offs between Rab8 and other GTPases at discrete locations and how do they occur? How are specific effectors involved in discrete transport steps enriched at Rab8-positive membranes?

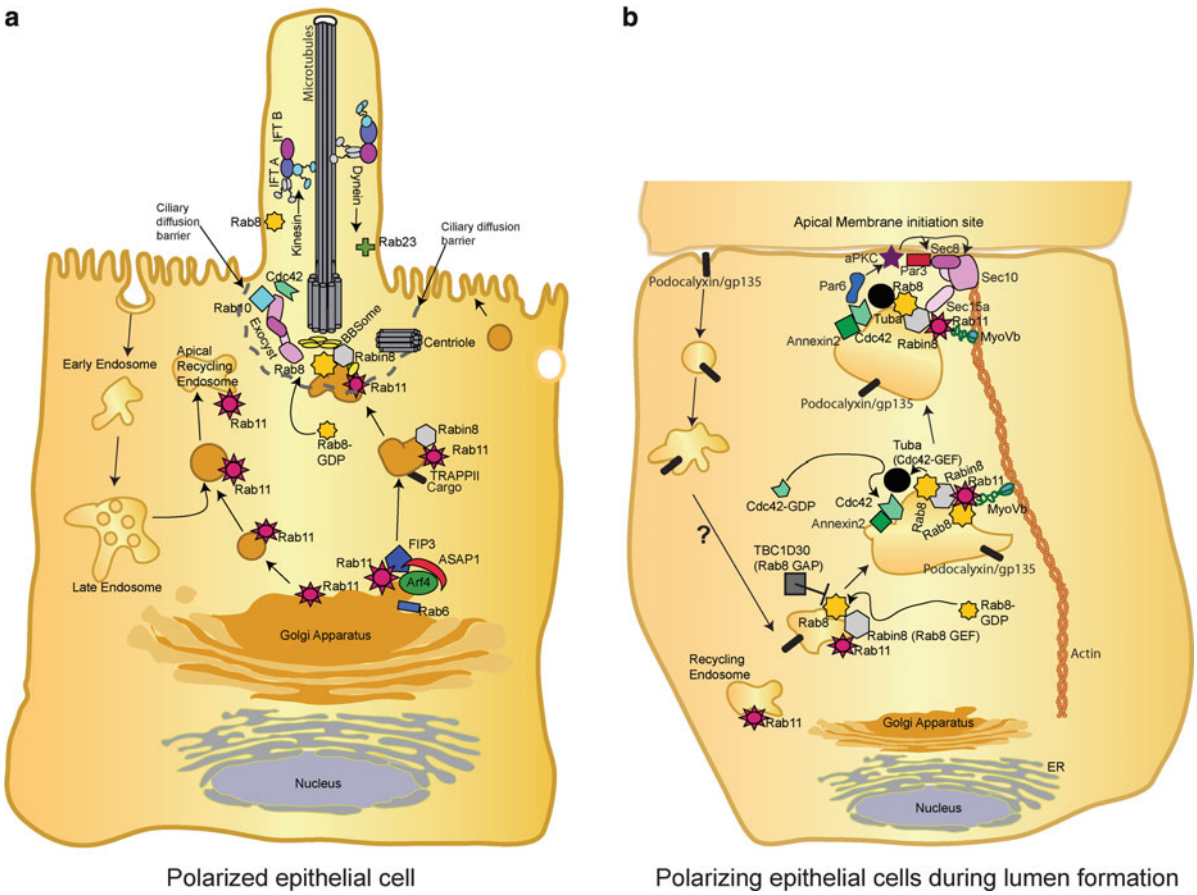
As illustrated by the given examples, Rab8 serves as a key regulator in communication between membrane transport circuits, the cytoskeleton and cellular signaling. Due to the critical role that Rab8 plays in multiple cellular pathways, loss of Rab8-protein interactions and regulation can result in numerous human diseases including open angle glaucoma, retinitis pigmentosa, microvillus inclusion disease, Huntington's disease, and cystic kidney disease.

Rab8 Regulation

As with other Rab family members, Rab8 cycles through membrane-associated, GTP-bound "on" states and cytoplasmic, GDP-bound "off" states. Membrane association depends on isoprenylation by protein geranylgeranyl transferases (primarily REP/GGTase II or GGTase I, minor pathway) [reviewed in (Wandinger-Ness and Deretic 2008)]. Activation of membrane-bound Rab8 through GTP binding may be facilitated by one of several guanine nucleotide exchange factors (GEFs) such as MSS4, Rabin8/Rabin3, Rabin 3-like GRAB, or retinitis pigmentosa GTPase regulator (RPGR). In the activated state, Rab8 is a scaffold for numerous effectors and kinases that cooperatively serve in the temporal and spatial regulation of Rab8 transport. Upon completion of the transport cycle, nucleotide hydrolysis and Rab8 inactivation can be mediated by two known GTPase activating proteins (GAPs), AS160 (Akt substrate)/Tbc1d4 (Tre-2/Bub2/Cdc16) domain containing protein or Tbc1d30/XM_037557, for which multiple isoforms may exist (Wandinger-Ness and Deretic 2008). Interestingly, AS160 also serves as a GAP for Rab10 and Rab13 suggesting that GTPases that function on interrelated pathways may also be coregulated (Ishikura and Klip 2008; Sun et al. 2010). Inactive Rab8-GDP is bound and recycled from membranes to the cytosol by GDP dissociation inhibitor-2 (GDI-2) (Shisheva et al. 1999). Given Rab8 involvement in multiple transport pathways, some of which are highly cell type specific, it remains to be clarified if and how Rab8 function is regulated in a site-specific manner.

Rab8 Effectors, Signaling Integration, and Disease

Spatial and temporal regulation of Rab8 trafficking is partially dependent on signaling receptors, as well as effector and cargo protein interactions, thereby conferring specificity to Rab8-mediated targeting in accordance with cell need and cell type (Wandinger-Ness and Deretic 2008). For example, Rab8 interactions differentially regulate α_{2B}- and β2-adrenergic receptor transport from the trans-Golgi network to the plasma membrane (Dong et al. 2010). Triggered by integrin-mediated adhesive events, Rab8 controls the polarized and regulated exocytosis of matrix metalloprotease MT1-MMP/MMP-14, which is critical for collagen degradation and tumor cell invasion (Bravo-Cordero et al. 2007). Specific cell types use conserved



Rab8, Fig. 2 *Rab8 exocytic trafficking in ciliated and polarizing epithelial cells*. (**a**) Ciliogenesis and cilial transport is regulated in part by Rab8-mediated vesicle trafficking. Vesicular transport of membranous protein cargo such as polycystin-1 begins with formation of a Golgi-exit complex consisting of Arf4 and Rab11 and recruitment of the Arf4 GAP (ASAP1) and Rab11 effector FIP3. TRAPPII components and Rab11 are required for recruitment and vesicular trafficking of Rabin8. Near the centrosome Rabin8 recruits and activates Rab8, which facilitates transit and docking with BBSome. Vesicle fusion is thought to require Rab8, Rab10, the exocyst complex and Cdc42. Kinesin and dynein motor protein isoforms transport proteins within the primary cilium, though the link between Rab GTPases and motor proteins within the cilium has not been defined. (**b**) Rab8, Rabin8, exocyst components and Cdc42 also participate in Rab11-mediated vesicle transport (recycling endosome transition to exocytic vesicle) in polarizing epithelial during the formation of tubule lumens. These vesicles interact with myosin Vb to transport to the apical surface via the actin cytoskeleton. Rab11 interacts with Rabin8, which in turn recruits and activates Rab8. Rab8 recruits Tuba, the Cdc42 GEF, which activates Cdc42. Cdc42 and Par6 recruit aPKC, which then recruits Par3-Sec8 and Sec10. Interactions with the exocyst and Rab GTPases promote association with the plasma membrane

trafficking mechanisms during different stages of polarization. For example, Rab8, Rab11, Rabin8, and the exocyst complex are used with different effectors to traffic apical protein cargo during lumen formation and ciliary protein cargo during ciliogenesis upon reaching a fully polarized, ciliated state (Fig. 2). Loss of cargo, especially signaling proteins, to the proper target membrane results in aberrant signaling and disease. Although an assortment of diverse Rab8a effectors and cargo are known, further study is needed to determine if Rab8a activity is regulated by distinct sets of regulators within specific cellular compartments.

Rab8-mediated trafficking is required to maintain cellular polarity and homeostasis, which when altered can lead to tumor formation and malignancy. Membrane type 1-matrix metalloproteinase (MT1-MMP, also known as MMP-14) plays a key role in tumor

invasiveness by participating in proteolytic degradation of surrounding tissues through activation of MMP2. MT1-MMP is exocytosed via Rab8, and trafficking to the plasma membrane is enhanced with expression of constitutively active Rab8 mutants, whereas RNAi knockdown of Rab8 prevents MT1-MMP from reaching the plasma membrane and thus prevents collagen degradation (Bravo-Cordero et al. 2007). Of note, MT1-MMP activation of MMP2 and MMP9 from pro- to active form is dependent upon interaction between MSS4 (a Rab8 GEF) and α-integrin chains (Knoblauch et al. 2007). The MSS4 binding sites for α-integrin chains and Rab8 are likely competitive. Decreased expression of MSS4 affects assembly and remodeling of the extracellular matrix. Rab8 expression can be up-regulated in breast cancer malignancies and associated lymph node metastases, and further studies on Rab8 mechanisms in tumorigenesis and malignancy may pave the way for the development of small molecule treatments with Rab8 or associated effectors as therapeutic targets.

Rab8 mediates basolateral trafficking and protein transport to adherens and tight junctions. Rab8 and Rab13 interact with effector MICAL-L2 (Molecule Interacting with CasL-Like 2) via competitive binding and formation of distinct independent Rab8a-MICAL-L2 and Rab13-MICAL-L2 protein complexes. Rab-MICAL-L2 protein interactions dictate trafficking specificity by regulating E-cadherin recycling to adherens junction (further evidenced by Rab8 knockdown causing delay of E-cadherin delivery to adherens junctions in calcium switch assays), and Rab13-MICAL-L2 interactions mediate recycling of occludin to tight junctions (Rahajeng et al. 2010). MICAL-1, MICAL-2, MICAL-3, MICAL-L1, and MICAL-L2 make up a family of large proteins with two to three common domains that mediate vesicular transport and cytoskeleton organization (Rahajeng et al. 2010). Rab8 interacts with at least three MICAL family members. Rab6-dependent recruitment of Rab8 to exocytic vesicles involves a MICAL3 intermediate in Rab8-ELKS interactions (Grigoriev et al. 2011). A related MICAL-like protein, MICAL-L1, regulates Rab8 in endocytic recycling. MICAL-L1 is proposed to serve as a Rab8 effector that serves to stably link Rab8 and EHD1 (Eps15 homology domain 1) on endocytic recycling vesicles (Rahajeng et al. 2010). EHD ATPase scaffolding proteins localize to tubular and vesicular membranes, regulate endocytic trafficking, and coordinate activity with Rab GTPases through interaction with Rab effectors. EHD also mediates GLUT4 recycling. Thus, Rab8 in conjunction with MICAL and EHD proteins form scaffolds that are crucial for integrating basolateral and endocytic pathways.

Over the last 3 years insulin-dependent signaling has been linked with Rab-mediated vesicle trafficking. Insulin-dependent glucose uptake is mediated by regulation of the surface recycling of GLUT4 transporters. AS160/TBC1D4 serves as a Rab GAP and AKT signaling target and is phosphorylated in response to insulin. Upon phosphorylation AS160 GAP activity is likely inhibited to allow activated Rab targets to function, thereby releasing the brakes on vesicle docking and fusion and allowing GLUT4 insertion at the plasma membrane of myoblasts (Randhawa et al. 2008). TBC1D1 is a second GAP that coregulates Rab activation in response to insulin stimulation. Rab8a and Rab14 are targets of the GAP TBC1D1 in skeletal myotubes and AS160, along with Rab13, in myoblasts (Ishikura and Klip 2008; Sun et al. 2010). The Rab8, Rab10, and Rab14 GTPases are required for insulin-induced GLUT4 trafficking, with GTP loading stimulated by insulin in a cell-type-specific manner (Ishikura and Klip 2008; Sun et al. 2010). Rab8 and Rab13 act as a Rab regulatory cascade that is activated sequentially to promote GLUT4 translocation in muscle cells, while Rab10 functions in adipocytes. Downregulation of GLUT4 translocation is thought to be achieved through the interaction of active Rab8a with the myosin Vb motor protein, thereby altering Rab8a localization and negatively impacting GLUT4 translocation (Ishikura and Klip 2008; Sun et al. 2010). This example is illustrative of how signaling and Rab8-regulated membrane trafficking are closely intertwined and modulated through cell-type-specific processes.

Noc2, rabphilin, Rim2 and Slp4/granuphilin are members of the ▶ synaptotagmin-like family and all bind to multiple Rab GTPases, including Rab3a, Rab8a, and Rab27a. Noc2 and rabphilin bind to active, GTP-bound Rab8a suggesting that both may serve as Rab8a effector proteins. Rim2 has been predominately studied in regulated secretion within presynaptic nerve terminals and insulin-secreting cells, where it functions in regulated exocytosis (Yasuda et al. 2010). Rabphilin is expressed in neuronal, neuroendocrine, intestinal goblet cells and kidney podocytes. Slp4 is also expressed in neuroendocrine cell dense core

vesicles and additionally localizes to the insulin-containing vesicles of pancreatic beta cells. All the members of the synaptotagmin-like family are implicated in regulated secretion and are of interest due to the fact that synaptotagmin-like proteins also bind plus-end directed myosin motors and thus, may bridge Rab8-regulated vesicle docking and fusion to cytoskeletal translocation.

Further evidence for a link between Rab8 and myosin motors is provided by studies in enterocytes. Rab8a is essential for localization of apical proteins and maintenance of the small intestine (Fig. 1) (Sato et al. 2007). Microvillus inclusion disease of the small intestine is characterized by microvillar atrophy and malabsorption. Rab8 conditional knockout in mice causes microvillus inclusion disease and one case of human disease has been linked to decreased Rab8 mRNA and protein expression in the enterocytes of the small intestine. To date, no further work has been reported on the role of Rab8 in microvillus inclusion disease. However, several reports have linked myosin Vb mutations to microvillus inclusion disease, and myosin Vb mutations cause disruption of epithelial cell polarity, as evidenced by loss of microvilli on the surface of intestinal absorptive cells and microvilli present within intracellular inclusion (Ruemmele et al. 2010). Given that both Rab8a and myosin Vb defects can result in microvillus inclusion disease, it is interesting to speculate that, as is the case in insulin-dependent signaling, the motor protein myosin Vb may interact with Rab8a and direct trafficking to the apical surface, which when perturbed, inhibits surface expression of microvilli. Further roles for Rab8 and apical protein targeting were brought to light through the study of zymogen granules in the exocrine pancreas. Rab8 localizes to zymogen granules and facilitates delivery of digestive enzymes to the apical surface, evidenced by the fact that Rab8 knockdown decreases granule numbers and causes granule proteins to accumulate in the Golgi (Wandinger-Ness and Deretic 2008). The authors speculate that Rab8-zymogen trafficking may depend on a clathrin/AP-1/dynamin association at the Golgi.

At the Golgi, Rab8a interfaces with optineurin (FIP-2) to promote cargo export, which is mediated by clathrin adaptor complex AP-1 (del Toro et al. 2009). Optineurin localizes to the cytosol, Golgi, and recycling endosomes and participates in vesicular trafficking. Optineurin binds active, but not GDP-bound, Rab8, thus suggesting that optineurin serves as a downstream effector. Optineurin interacts with myosin VI, a minus-end directed motor, to link Rab8 positive membranes to the actin cytoskeleton [reviewed in (Wandinger-Ness and Deretic 2008)]. The optineurin E50K mutation causes glaucoma and has been shown to impair endocytic trafficking (as demonstrated by impaired transferrin uptake) and slows the velocity of Rab8-GFP positive vesicles (Nagabhushana et al. 2010). Interestingly, mutant E50K optineurin completely abolishes optineurin-Rab8 interactions at the Golgi (Chi et al. 2010). In mice, the E50K mutation causes massive apoptosis and degeneration of the retina, but not broader neuronal degeneration (Chi et al. 2010). The composite data suggest there may be a conserved mechanism for zymogen and optineurin-mediated trafficking in exocrine cells and neurons.

Optineurin-Rab8 interactions also come into play in Huntington's disease. Huntington's disease results from abnormal expansion of a polyglutamine tract in the N-terminus of the huntingtin protein and has defects in lysosome function. Huntingtin regulates post-Golgi trafficking of secreted proteins and interacts with the optineurin-Rab8 complex. Huntingtin links Rab8/optineurin vesicles to microtubules via interactions with via HAP1 (a Trio-like protein with a Rac1 GEF domain), dynactin (p150glued) and dynein [reviewed in (Wandinger-Ness and Deretic 2008)]. Expression of huntingtin mutants or decreased huntingtin expression decreases Rab8 and optineurin localization at the Golgi and inhibits clathrin and optineurin/Rab8-dependent trafficking to lysosomes (del Toro et al. 2009). Loss of huntingtin causes loss of Rab11 in isolated membranes, and Rab11-GDP was shown to interact with huntingtin ~30-fold greater than Rab11-GTP. Rab8 and Rab11 both play a role in polarized outgrowth and are preferentially located in the somatodendritic domain of neurons, and Rab8 functions in neuronal maturation and polarized transport. For example, Rab8 is required for the transport and insertion of (AMPA)-type glutamatergic receptors, which mediate the fast synaptic transmission throughout the nervous system, into the postsynaptic compartment. AMPA receptors are recycled back to the membrane via Rab11 recycling endosomes, suggesting a potential Rab11/Rab8 trafficking pathway similar to those found in neuronal photoreceptor cells. However, the generalizability of the mechanism remains to be studied further.

Rab8a in Cilial Transport

The role of Rab8 in cilial transport and cilial-mediated signaling was suggested approximately 16 years ago (Wandinger-Ness and Deretic 2008), and the body of literature supporting this hypothesis has grown significantly over the last 5 years. The primary cilium is a specialized organelle that sits atop most cell types in the body and serves as an antenna to sample the extracellular space and transmit signals to the cell body. Accordingly, components of several signaling pathways such as hedgehog, WNT and JAK-STAT, which regulate cellular growth, proliferation, differentiation, and polarization, are found within the primary cilium. Cilial structures are evolutionarily conserved but can have cell-type-specific modifications to detect and respond to various stimuli such as mechanical, physical, chemical, or temperature sensation. For example, the modified cilium in photoreceptor cells responds to light, whereas the primary cilia in kidney epithelia are thought to be chemo-mechano sensors. Loss or aberrant localization of membrane-bound and cytoplasmic ciliary proteins at the site of the primary cilia is associated with a group of inherited diseases known as the ciliopathies. Ciliopathies encompass a broad range of genetic mutations and phenotypes. Some genetic mutations cause single organ, adult onset and progressive disease such as autosomal dominant polycystic kidney disease (ADPKD) and retinitis pigmentosa, whereas other genetic mutations affect multiple organs. An example of a multiorgan disorder is Bardet-Biedl Syndrome (BBS), which exhibits phenotypes that include (but are not limited to) obesity, polydactyly, retinopathy, mental impairment, and kidney abnormalities.

Rab8 function aligns strongly with the defined responsibilities of primary cilia, namely, regulation of epithelial differentiation and maintenance, development and organ function through cellular signaling. Expression of GTP-locked Rab8 induces cilial extension, whereas depletion of Rab8 inhibits cilia formation and trafficking, which often results in renal and retinal defects (Nachury et al. 2010). Numerous studies have linked Rab GTPases to the transport of membrane-bound ciliary proteins to the primary cilium; however, the distinct mechanisms are not well defined. Many questions about ciliary trafficking have been brought to the forefront of basic cell biology research, such as: How many ciliary trafficking routes exist? How are they modulated for specific cell types? What are the signaling pathways that drive ciliary transport? What are the mechanisms that switch cellular transport to direct ciliary trafficking of molecules with multiple localization patterns? How do vesicular transport pathways cooperate with other trafficking pathways such as intraflagellar transport (IFT)?

In the context of cilial function, Rab8 trafficking is best characterized in the rhodopsin transport model. Rhodopsin requires several small GTPases, including Rab8, to shuttle from the Golgi to an elaborate primary cilium, known as the rod outer segment, of photoreceptor cells; a Rab8 GDP-locked mutant (Rab8-T22N) inhibits docking and fusion of rhodopsin-containing exocytic vesicles in transgenic frogs which results in dramatic retinal degeneration (Wandinger-Ness and Deretic 2008). Rab8 in concert with the Rab6, Rab11, and Arf4 GTPases is responsible for rhodopsin transport from the Golgi to the rod outer segment, in concert with regulatory proteins (e.g., ASAP, the Arf4 GAP) and effector proteins (e.g., FIP3) (Mazelova et al. 2009a). Mutations in the extreme C-terminus of rhodopsin impair interactions between rhodopsin and the GTPase trafficking complex, which leads to autosomal dominant retinitis pigmentosa. The altered interaction results in aberrant trafficking of rhodopsin to the rod outer segment, and explains at the molecular level the cause of retinitis pigmentosa. These careful studies were the first to reveal GTPase-mediated vesicular hand-offs in ciliary transport, beginning with interaction of a cargo protein with Rab6 and Rab11 in the Golgi. The cargo recruits and binds Arf4 via a specific targeting sequence to facilitate vesicle budding and Arf4 cooperates with Rab11. ASAP1, the Arf4 GAP, recognizes membrane curvature and is recruited to bind Arf4 to promote GTP hydrolysis and removal of Arf4 from the ciliary-targeted vesicle. FIP3, the Rab/Arf effector also bridges Arf and Rab GTPases via interactions with Rab11 and ASAP1 (Fig. 2a). The described pathway of rhodopsin transport prompted further questions about mechanism conservation between different cell types, the identification of other trafficking molecules that participate in the GTPase vesicle ciliary relay, and the composition of the coat complex of the ciliary transport vesicles.

Further studies in renal epithelial cells provided clues to conservation of Rab-mediated ciliary trafficking. In normal cultured renal cells, Rab8 can be found at the perinuclear Golgi region, whereas in cultured

ADPKD cells, Rab8 is mislocalized to disperse vesicles (Wandinger-Ness and Deretic 2008). Polycystin-1, a protein that when mutant causes ADPKD, uses the same GTPase transport mechanism as rhodopsin to traffic to primary cilia of renal epithelial cells (Ward et al. 2011). Thus, the trafficking mechanism consisting of Rab GTPases (Rab6, Rab11, Rab8), Arf4 GTPase, and Arf GAP ASAP1 is conserved between retinal photoreceptor cells and renal epithelial cells (Ward et al. 2011). In contrast, fibrocystin, a protein mutant in patients with autosomal recessive PKD, also utilizes Rab8 to traffic to the primary cilium, but the fibrocystin C-terminal sequence does not bind Rab6 and Rab11 (nor Rab17, Rab23, and IFT20), suggesting that Rab8 may serve as the common GTPase between different vesicular transport mechanisms leading to ciliary delivery (Follit et al. 2010).

Until recently, the mechanism of Rab8 recruitment and activation along the cilial trafficking route was an enigma. For example, in the sequential transport of cargo that traffics with Rab6, then Rab11, and finally with Rab8 vesicles, how is Rab8 recruited to the exocytic vesicle? Identification of the interaction between Rabin8, a Rab8 GEF, and a complex of Bardet-Biedl proteins (known as the BBSome) at the centriole provided insight into the GTPase coordination and localized GTPase activation required for ciliary formation. The BBSome-Rabin8 interaction was identified at the ciliary base within pericentriolar recycling endosomes, offering the first clue for spatial regulation (Wandinger-Ness and Deretic 2008). Here BBSome components assist in recruitment of Rab8 to the pericentriolar recycling endosome for nucleotide exchange and activation, presumably by Rabin8. Further studies revealed that transport protein particle II complex (TRAPPII) components (C3, C9 and C10) and Rab11 are required for vesicular trafficking of Rabin8 to the centrosome, where Rabin8 subsequently recruits and activates Rab8 (Fig. 2a) (Knodler et al. 2010; Westlake et al. 2011). Thus, Rabin8 serves both as a Rab11 effector and as a Rab8 activator and both Rab8 and Rab11 are required for ciliogenesis. Together, the data highlight the involvement of a spatially and temporally regulated GTPase cascade in cilial formation and ciliary membrane protein targeting.

The dissection of a specific Golgi to cilia pathway is revealing key players in a ciliary targeting, but there are numerous examples of Rab8 involvement in cilial targeting that do not seem to follow the above-mentioned GTPase sequence and the unifying mechanisms remain elusive. CEP290 mutations are linked to several inherited cystic diseases including Senior-Löken syndrome, nephronophthisis, Joubert syndrome, Meckel-Grüber syndrome, and BBS. CEP290 protein satellites the base of the primary cilium, interacts with pericentriolar material 1 (PCM-1), and CEP290 knockdown significantly inhibits Rab8 localization to the primary cilium (Kim et al. 2008); however, the mechanism that mediates CEP290/PCM-1 Rab8 recruitment to the primary cilium remains undefined. An alternate Rab8-mediated ciliary trafficking mechanism has been suggested in *Caenorhabitis elegans* worms in the context of the transmembrane olfactory receptor ODR-10 (Kaplan et al. 2010). Rab8 associates with AP-1 in the clathrin-dependent delivery of ODR-10 to dendritic sensory cilia. Overexpression of GTP-locked Rab8 or mutations in the clathrin heavy chain perturbed ODR-10 trafficking and caused ODR-10 to localize to all plasma membrane compartments. Therefore, AP-1 and Rab8 likely cooperate to direct ODR-10 to the dendritic cilium. The authors suggested that a default secretory pathway directs proteins to all plasma membrane regions, and that this default pathway can be re-routed by AP-1 and Rab8 activation. The level of Rab8 activity may serve as the determining factor in protein destination. If so, then active Rab8 may serve as the switch between a general secretory and cilial-directed transport pathway. The authors' leading model predicts that AP-1 functions at the trans-Golgi network in a clathrin-dependent manner, where the cargo is packaged into budding vesicles. Upon leaving the Golgi, the vesicle uncoats, fuses with Rab8 positive vesicles, and targets to the cilium. It is interesting to speculate that Rab8 vesicles that traffic ODR-10 from neuronal soma to the dendritic cilium may resemble secretory granules in exocrine cells and contain components of the pericentriolar recycling endosomes described in epithelial cells.

At site of the cilium, Rab8 serves as an evolutionarily conserved regulator of ciliary trafficking in several cell types. In turn, the exocyst complex, first identified in *Saccharomyces cerevisiae*, also serves as a conserved trafficking regulator and as an effector of several GTPases. The exocyst complex is comprised of several subunits and regulates polarized secretion via docking of intracellular vesicles to the plasma membrane. The exocyst also localizes to the primary cilium of renal cells, and Sec6 and Sec8 exocyst components

are overexpressed or diminished in ADPKD cells. The exocyst complexes with small GTPases including RalA, Rho1, and Rab8, and regulates function via interactions with GAPs and other molecules such as aPKCs and the Arp2/3 complex (Hertzog and Chavrier 2011). Rab8a-Sec6/8 interactions are thought to control vesicle docking and fusion with the basolateral plasma membrane, thus linking the exocyst with Rab GTPase trafficking. In photoreceptor cells, Rab8 cooperates with phosphatidylinositol (4,5)-bisphosphate, moesin, Rac1 and actin to tether and fuse vesicles to the base of the modified photoreceptor cell cilium. The Sec6/8 complex likely serves as a Rab8 effector during GTPase-mediated vesicular trafficking, as Sec8 colocalizes with Rab8 at fusion sites of vesicles transporting rhodopsin, and, like Rab8, the exocyst localizes to the primary cilia of renal epithelial cells (Fig. 2a) (Nachury et al. 2010). In the case of retinal cells, the Sec6/8 complex coordinates with syntaxin 3 and SNAP-25, whose interactions are regulated by omega-3 docosahexaenoic acid, to regulate rhodopsin delivery (Mazelova et al. 2009b).

Rab8, Rab11, Rabin8 and exocyst components play dual roles in renal epithelial cells. Once renal cells are polarized, Rab8, Rab11 and Rabin proteins participate in delivery of trans-membrane protein cargo to the primary cilium. However, during polarization and lumen formation, Rab11, Rabin 8 and Rab8 mimic a yeast trafficking pathway and traffic cargo, such as podocalyxin, from the trans-Golgi to the forming pre-apical membrane (Fig. 2b). Rab11 recruits Rabin8, which recruits and activates Rab8. Rab11 (and potentially Rab8) also recruits Sec15a, an exocyst component, which binds Sec10 at the plasma membrane. Rab8 recruitment to the transport vesicle enhances active Cdc42 localization, likely driven by Rab8 activity on Tube, the Cdc42 GEF. Cdc42 and Par6 recruit aPKC, which then recruits Par3-Sec8 and Sec10. Thus, similar Rab8 trafficking mechanisms are utilized throughout the cellular polarization process, but the role that Rab8 plays in the regulation and switch between different target destinations remains to be explored.

One important question in the field of ciliary trafficking is how the intraflagellar transport (IFT) system, which transports non-membranous particles to and within the primary cilium, interfaces with the Rab8 GTPase-mediated vesicular trafficking pathway. The IFT protein Elipsa provided one of the first links to IFT-vesicular cross talk. Elipsa localizes to primary cilia and interacts with Rab8 via the Rab8 effector, Rabaptin5 (Omori et al. 2008). Elipsa also directly interacts with IFT20, which has been shown to localize to the Golgi and within primary cilia, and when knocked down, decreases the amount of polycystin-2 delivered to primary cilia (Follit et al. 2010). Combined, these data suggest that IFT plays a role in the transport of membrane-bound proteins to the primary cilium. Taken together, one can speculate that membrane-bound proteins interact in a complex associated with GTPase-mediated vesicular transport, which in turn interacts via effector proteins, such as Rabaptin5, with IFT components. Of note, IFT molecules have been linked to the formation of the immune synapse in T-lymphocytes (Nachury et al. 2010), and immune synapse formation shares similarities with cilial trafficking, namely, the use of IFT20, IFT57, IFT88, Kif3a motor protein. In contrast, IFT transport was not affected when ODR-10 transport was perturbed by Rab8 and clathrin manipulation (Kaplan et al. 2010). Further studies are necessary to dissect out the overlapping and independent roles of IFT and Rab8-mediated vesicular transport pathways to cilia.

Though advances have been made in the context of Rab8 and cilial trafficking, numerous questions remain about coat complexes used by cilia-destined vesicles in various cell types. Since Rab8, along with FIP-2, facilitates AP-1-mediated cargo export from the Golgi and may be associated with AP-1 in zymogen granule transport, could AP-1 (an Arf4 effector) also serve to generate the exocytic vesicle coat in mammalian epithelial cells? Alternatively, going back to the Rab GTPase-mediated transport in epithelial cells (Fig. 2), note that Arf4 facilitates vesicle budding. Arf1 and Arf4 regulate COPI recruitment and TRAPPII components bind COPI (Angers and Merz 2011). Thus, COPI may serve as the initial vesicle coat for some ciliary targeted vesicles. The coat-like BBSome plays a role in Rab8 recruitment and activation at the pericentrosomal region and the complex shares similarities with the COPI and clathrin coat complexes (Nachury et al. 2010). Therefore, after initial budding, does the vesicle coat morph to resemble components of the BBSome, or can the BBSome itself become the late-stage Rab8-positive vesicle coat prior to fusion with the ciliary membrane? Further questions arise about how Rab8, a potentially central ciliary component, may play a role in recruitment of the motor proteins and tethering components for ciliary transport vesicles. Recently,

helical SNARE [Soluble NSF Attachment Protein (SNAP) Receptor] tethering proteins have been incorporated into the rhodopsin trafficking model (Mazelova et al. 2009b). Syntaxin 3 and SNAP-25 regulate rhodopsin delivery and localize to the base of the cilium, where Rab8 recruitment and activation is predicted to occur. SNARE proteins can function as Rab effectors or GEFs and future studies may reveal that SNARE proteins, in addition or as an alternative to the BBSome and Rabin8, may play a role in Rab activation at the primary cilium.

Summary

Vesicular transport can be simplified into three major steps: budding from the donor membrane, translocation of the trafficking vesicle along the cytoskeleton, and docking and fusion with the acceptor membrane compartment (Wandinger-Ness and Deretic 2008). Rab GTPases facilitate this process by serving as molecular scaffolds and interacting with cargo, proteins that promote vesicle budding and coat proteins, and through recruitment of motor protein complexes and tethering molecules. Rab8 is involved in several transport pathways and interfaces with endocytic pathways, regulates exocytosis, and coordinates regulation of the cytoskeleton and trafficking of diverse cargo to multiple subcellular destinations. Rab effectors, motor proteins and specific GEFs, GAPs and kinases mediate regulation of Rab8. Defects in Rab8-mediated trafficking have profound effects on cell morphogenesis, cytoskeletal organization, and cellular polarity. Rab8 serves to coordinate vesicle recycling and delivery of newly synthesized vesicle components to target membranes and functions as a nexus between vesicular endocytic and exocytic pathways. There are a number of unanswered questions in the field of vesicle trafficking: How do cells temporally and spatially regulate the sorting decisions for GTPases? What are the molecular switch mechanisms that cause a shift of shuttling cargo from one target to another? What signals cause Rab8 to associate with different effectors and how are these signals processed? Further investigation of Rab8 in diverse cell types and polarization states is predicted to reveal the unifying and diverse mechanisms of GTPase trafficking and lead to specific signaling targets for small molecule therapies for tumor invasion, cyst formation, and neurologic disease.

Acknowledgments We acknowledge fellowships from NCRR INBRE 5P20RR016480 and NIGMS 1K12GM088021 to HW and research support from NIDDK DK50141 to AWN.

References

Angers CG, Merz AJ. New links between vesicle coats and Rab-mediated vesicle targeting. Semin Cell Dev Biol. 2011;22:18–26.

Bravo-Cordero JJ, Marrero-Diaz R, Megias D, Genis L, Garcia-Grande A, Garcia MA, Arroyo AG, Montoya MC. MT1-MMP proinvasive activity is regulated by a novel Rab8-dependent exocytic pathway. EMBO J. 2007;26:1499–510.

Chi ZL, et al. Overexpression of optineurin E50K disrupts Rab8 interaction and leads to a progressive retinal degeneration in mice. Hum Mol Genet. 2010;19:2606–15.

Cramm-Behrens CI, Dienst M, Jacob R. Apical cargo traverses endosomal compartments on the passage to the cell surface. Traffic. 2008;9:2206–20.

del Toro D, Alberch J, Lazaro-Dieguez F, Martin-Ibanez R, Xifro X, Egea G, Canals JM. Mutant huntingtin impairs post-golgi trafficking to lysosomes by delocalizing optineurin/Rab8 complex from the golgi apparatus. Mol Biol Cell. 2009;20:1478–92.

Dong C, Yang L, Zhang X, Gu H, Lam ML, Claycomb WC, Xia H, Wu G. Rab8 interacts with distinct motifs in alpha2B- and beta2-adrenergic receptors and differentially modulates their transport. J Biol Chem. 2010;285:20369–80.

Faust F, Gomez-Lazaro M, Borta H, Agricola B, Schrader M. Rab8 is involved in zymogen granule formation in pancreatic acinar AR42J cells. Traffic. 2008;9:964–79.

Follit JA, Li L, Vucica Y, Pazour GJ. The cytoplasmic tail of fibrocystin contains a ciliary targeting sequence. J Cell Biol. 2010;188:21–8.

Grigoriev I, et al. Rab6, Rab8, and MICAL3 cooperate in controlling docking and fusion of exocytotic carriers. Curr Biol. 2011;21:967–74.

Hattula K, Furuhjelm J, Tikkanen J, Tanhuanpaa K, Laakkonen P, Peranen J. Characterization of the Rab8-specific membrane traffic route linked to protrusion formation. J Cell Sci. 2006;119:4866–77.

Hertzog M, Chavrier P. Cell polarity during motile processes: keeping on track with the exocyst complex. Biochem J. 2011;433:403–9.

Ishikura S, Klip A. Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. Am J Physiol Cell Physiol. 2008;295:C1016–25.

Jacobs DT, Weigert R, Grode KD, Donaldson JG, Cheney RE. Myosin Vc is a molecular motor that functions in secretory granule trafficking. Mol Biol Cell. 2009;20:4471–88.

Kaplan OI, et al. The AP-1 clathrin adaptor facilitates cilium formation and functions with RAB-8 in *C. elegans* ciliary membrane transport. J Cell Sci. 2010;123:3966–77.

Kim J, Krishnaswami SR, Gleeson JG. CEP290 interacts with the centriolar satellite component PCM-1 and is required for Rab8 localization to the primary cilium. Hum Mol Genet. 2008;17:3796–805.

Knoblauch A, Will C, Goncharenko G, Ludwig S, Wixler V. The binding of Mss4 to alpha-integrin subunits regulates matrix metalloproteinase activation and fibronectin remodeling. FASEB J. 2007;21:497–510.
Knodler A, Feng S, Zhang J, Zhang X, Das A, Peranen J, Guo W. Coordination of Rab8 and Rab11 in primary ciliogenesis. Proc Natl Acad Sci USA. 2010;107:6346–51.
Mazelova J, Astuto-Gribble L, Inoue H, Tam BM, Schonteich E, Prekeris R, Moritz OL, Randazzo PA, Deretic D. Ciliary targeting motif VxPx directs assembly of a trafficking module through Arf4. EMBO J. 2009a;28:183–92.
Mazelova J, Ransom N, Astuto-Gribble L, Wilson MC, Deretic D. Syntaxin 3 and SNAP-25 pairing, regulated by omega-3 docosahexaenoic acid, controls the delivery of rhodopsin for the biogenesis of cilia-derived sensory organelles, the rod outer segments. J Cell Sci. 2009b;122:2003–13.
Nachury MV, Seeley ES, Jin H. Trafficking to the ciliary membrane: how to get across the periciliary diffusion barrier? Annu Rev Cell Dev Biol. 2010;26:59–87.
Nagabhushana A, Chalasani ML, Jain N, Radha V, Rangaraj N, Balasubramanian D, Swarup G. Regulation of endocytic trafficking of transferrin receptor by optineurin and its impairment by a glaucoma-associated mutant. BMC Cell Biol. 2010;11:4.
Omori Y, Zhao C, Saras A, Mukhopadhyay S, Kim W, Furukawa T, Sengupta P, Veraksa A, Malicki J. Elipsa is an early determinant of ciliogenesis that links the IFT particle to membrane-associated small GTPase Rab8. Nat Cell Biol. 2008;10:437–44.
Rahajeng J, Giridharan SS, Cai B, Naslavsky N, Caplan S. Important relationships between Rab and MICAL proteins in endocytic trafficking. World J Biol Chem. 2010;1:254–64.
Randhawa VK, Ishikura S, Talior-Volodarsky I, Cheng AW, Patel N, Hartwig JH, Klip A. GLUT4 vesicle recruitment and fusion are differentially regulated by Rac, AS160, and Rab8A in muscle cells. J Biol Chem. 2008;283:27208–19.
Roland JT, Lapierre LA, Goldenring JR. Alternative splicing in class V myosins determines association with Rab10. J Biol Chem. 2009;284:1213–23.
Roland JT, Bryant DM, Datta A, Itzen A, Mostov KE, Goldenring JR. Rab GTPase-Myo5B complexes control membrane recycling and epithelial polarization. Proc Natl Acad Sci USA. 2011;108:2789–94.
Ruemmele FM, et al. Loss-of-function of MYO5B is the main cause of microvillus inclusion disease: 15 novel mutations and a CaCo-2 RNAi cell model. Hum Mutat. 2010;31:544–51.
Sato T, et al. The Rab8 GTPase regulates apical protein localization in intestinal cells. Nature. 2007;448:366–9.
Schuck S, Gerl MJ, Ang A, Manninen A, Keller P, Mellman I, Simons K. Rab10 is involved in basolateral transport in polarized Madin-Darby canine kidney cells. Traffic. 2007;8:47–60.
Shisheva A, Chinni SR, DeMarco C. General role of GDP dissociation inhibitor 2 in membrane release of Rab proteins: modulations of its functional interactions by in vitro and in vivo structural modifications. Biochemistry. 1999;38:11711–21.
Sun Y, Bilan PJ, Liu Z, Klip A. Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells. Proc Natl Acad Sci USA. 2010;107:19909–14.
Wandinger-Ness A, Deretic D. Rab8a. UCSD Nature Molecule Pages 2008.
Ward HH, Brown-Glaberman U, Wang J, Morita Y, Alper SL, Bedrick EJ, Gattone 2nd VH, Deretic D, Wandinger-Ness A. A conserved signal and GTPase complex are required for the ciliary transport of polycystin-1. Mol Biol Cell. 2011;22:3289–33.
Westlake CJ, et al. Primary cilia membrane assembly is initiated by Rab11 and transport protein particle II (TRAPPII) complex-dependent trafficking of Rabin8 to the centrosome. Proc Natl Acad Sci USA. 2011;108:2759–64.
Yasuda T, et al. Rim2alpha determines docking and priming states in insulin granule exocytosis. Cell Metab. 2010;12:117–29.

Rab8a

▶ Rab8

Rab-RP4 (*Drosophila melanogaster*)

▶ RAB18

Rac GTPases

Diamantis Konstantinidis and Theodosia A. Kalfa
Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, OH, USA

Historical Background and Taxonomy

Rac GTPases comprise one of the eight subfamilies of the Rho (Ras homology) GTPases family, itself a subgroup of the Ras superfamily of small G proteins (Burridge and Wennerberg 2004). They were first identified as a substrate for the bacterial C3-like transferases that block Rho by ADP-ribosylation, although the C3-like transferases act on Rac rather inefficiently. More effective are the large clostridial cytotoxins (with prototypes the *Clostridium difficile* toxin A and B) which glycosylate Rac at Thr35, inhibiting its functions by preventing effector coupling (Aktories et al. 2000). Rac GTPases are preferred targets for bacteria since they act as molecular switches in a multitude of

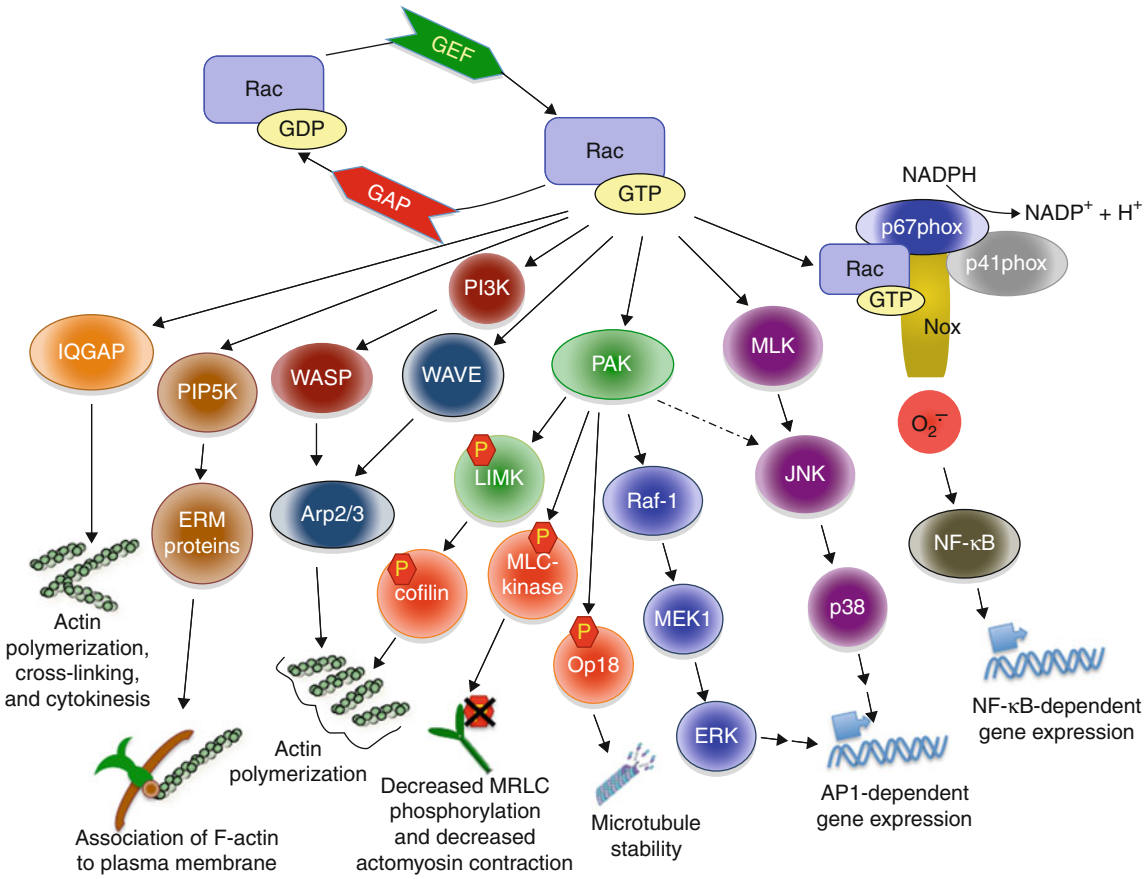
signaling processes, regulating many fundamental cellular functions, including actin cytoskeleton, cell adhesion, motility and migration, vesicular transport pathways and cytokinesis, ▶ reactive oxygen species (ROS) production via NADPH oxidase, as well as cell proliferation and survival (Hall 1998).

The Rac GTPase subfamily consists of four members; Rac1, Rac2, Rac3, and RhoG. They all have high homology, with the first three sharing over 90% amino acid sequence identity between them (Heasman and Ridley 2008). Despite this sequence similarity, studies using knockout mice indicate that many of their functions are nonredundant (Wang and Zheng 2007). Homologues of Rac GTPases have been found in all eukaryotic cells studied, but are totally absent in prokaryotes. In mammalian tissues, Rac1 is ubiquitously expressed while expression of Rac2 is restricted to hematopoietic cells. Rac3 is abundant in the brain but has also been identified in a variety of tissues including heart, placenta, and pancreas. RhoG (Ras homology growth-related) is widely expressed in a variety of organs but reaches a particularly high level in lung and placenta. Deletion of the Rac1 gene in mouse germline produces an early embryonic lethal phenotype and thus studies of Rac1 function have utilized tissue-specific conditional knockout. In contrast, Rac2, Rac3, and RhoG knockout mice are viable, fertile, and do not exhibit obvious developmental defects. Nevertheless, they do exhibit cell-type-specific functional defects and in the case of Rac3-null mice, a mild neurological phenotype (Wang and Zheng 2007; Heasman and Ridley 2008).

Activation of Rac GTPases and Downstream Signaling

Rac GTPases are classical Rho GTPases and as such act as molecular switches cycling between a GTP-bound active form and a GDP-bound inactive form. In their active form, they recognize target proteins, "turning on" signaling pathways, until they hydrolyze GTP to GDP, "switching off" (Etienne-Manneville and Hall 2002). The activation of Rac GTPases is mediated and regulated by guanine nucleotide exchange factors (GEFs), which exchange GDP for GTP on the Rac GTPase enzyme. GEFs exhibit a varied degree of specificity with some being able to activate a large number of different GTPases, while others specialize in activating only one particular isoform. GEFs are antagonized by GTPase-activating proteins (GAPs), which increase intrinsic GTPase activity resulting in GTP hydrolysis and the return of the GTPase to the inactive GDP-bound state. Another level of GTPase activity regulation is achieved through guanosine nucleotide dissociation inhibitors (GDIs), which inhibit nucleotide exchange and sequester small GTPases away from the membrane. Following the effect of specific stimuli such as soluble molecules, cell surface-bound ligands, or mechanical stress on cell surface receptors, GEFs and GAPs are mobilized accordingly to regulate the activation state of the Rac GTPases. Upon adopting an active conformation, Rac GTPases can bind to a wide variety of effectors and lead to initiation of the corresponding downstream signaling pathways (Bishop and Hall 2000; Schwartz 2004) (Fig. 1).

Rac GTPases bind and activate the p21-Activating Kinases (PAK1, PAK2, PAK3), serine/threonine kinases, which drive cytoskeletal remodeling (lamellipodia and membrane ruffling), cell adhesion and proliferation, and gene transcription. While PAK can activate c-Jun NH_2-Terminal Kinase (JNK), Rac1 activates JNK mostly independently of PAK (Westwick et al. 1997), and mainly through Mixed Lineage Kinases (MLKs). Pathways downstream PAK and MLK stimulate AP1-dependent gene expression. AP1 can upregulate the expression of genes that control cell cycle progression, such as cyclin D1 and c-myc, proteins that when overexpressed are associated with cell transformation and cancer (Bosco et al. 2009). Through PAK, Rac GTPases phosphorylate and activate ▶ LIMK, which in turn phosphorylates and inhibits cofilin, an actin filament severing protein, hence inducing actin polymerization into lamellipodia and membrane ruffling. Additionally, Rac stimulates the Wiskott–Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE) complex, which in turn activates actin-related proteins 2/3 complex (Arp2/3) that nucleates unbranched actin filaments (Heasman and Ridley 2008). Rac regulation of cell contractility includes PAK-mediated phosphorylation of myosin light chain kinase (▶ MLCK) and hence its inactivation causing decreased phosphorylation of the myosin regulatory light chain (MRLC) and reducing actomyosin assembly and contraction (Bishop and Hall 2000). PAK can also inhibit the microtubule



Rac GTPases, Fig. 1 Rac GTPases regulate their multiple functions within the cell via a variety of effectors, which initiate separate or interacting signaling cascades. Following the effect of specific stimuli GEFs and GAPs are mobilized accordingly to regulate the activation state of the Rac GTPases and direct Rac GTP to the appropriate effector

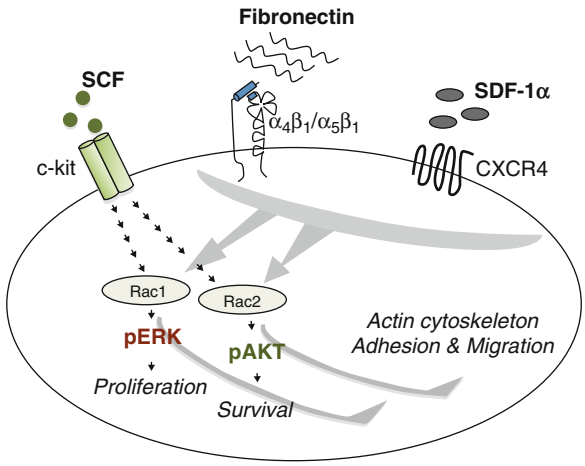
destabilizing activity of OP18/stathmin by phosphorylation. Other functions of Rac GTPases mediated by PAK are interactions with the myosin heavy chain, also leading to decreased actomyosin filaments, with filamin A to promote membrane ruffling, as well as with components of the paxillin-GIT/PKL-P1X complex to regulate cell adhesion and motility (Schwartz 2004). Independently of PAK, Rac binds to the actin-binding protein IQGAP (named GAP because of some homology with Ras GAP, but actually a Rac effector) which oligomerizes and cross-links F-actin in vitro and has been shown to arrange actin filaments into the cytokinetic contractile ring in yeast (Bishop and Hall 2000). Rac GTPases bind and activate Phosphatidylinositol-4-Phosphate 5-Kinase (PIP5K) leading to production of phosphatidylinositol (4,5)-bisphosphate (PIP_2) and activation of the ERM (ezrin, radixin, moesin) complex of proteins. ERM proteins have actin-binding domains as well as domains that bind to cytosolic domains of plasma membrane integral proteins and mediate the association of F-actin to plasma membrane. (Schwartz 2004). Phosphatidylinositol 3-Kinase (PI3K) is also a Rac effector with multiple actions. It stimulates WASP and Arp2/3, thus inducing actin polymerization and produces 3′-phosphorylated lipids that bind to and stimulate Rac GEFs, creating a positive feedback loop that maintains cell migration. In addition, it also activates AKT Ser/Thr kinase to support cell survival (Schwartz 2004; Bosco et al. 2009). Rac-GTP binds to the p67phox component of NADPH oxidase, activating the enzyme to produce superoxide. Superoxide and other reactive oxygen species (ROS) have multiple roles and effects to cells and

tissues, including signaling and stimulation of NFκB-dependent gene expression (Schwartz 2004; Hordijk 2006).

Rac GTPases in Hematopoiesis

Rac1 and Rac2 GTPases play distinct and overlapping roles in hematopoietic and mature blood cells, regulating homing, engraftment, actin cytoskeleton organization, ROS production, cell survival, and proliferation (Gu et al. 2003; Mulloy et al. 2010). Hematopoietic stem cells and progenitors (HSC/P) deficient of Rac1 demonstrate decreased proliferation when stimulated with stem cell factor (SCF), associated with nondetectable cyclin D1 levels and with decreased Extracellular Signal–Regulated Kinase (ERK) (p42/p44) phosphorylation. In contrast, loss of Rac2 activity leads to a pro-apoptotic phenotype in HSC/P as well as in mast cells, with reduced AKT activation in the presence of SCF. $Rac1^{-/-}$;$Rac2^{-/-}$ HSC/Ps display decreased adhesion to fibronectin, despite normal expression of α4β1 and α5β1 integrins on their surface and decreased migration in response to stromal-derived factor-1 (SDF-1), although they have significantly increased expression of CXCR4, the SDF-1 receptor (Gu et al. 2003) Since Rac GTPases are key components of the signaling pathways downstream of the SCF-ligand c-kit, the chemokine receptor CXCR4, and the β1 integrin-receptors for fibronectin, all significant mechanisms of interaction of HSC/Ps with the bone marrow microenvironment (Fig. 2), it is not surprising that combined Rac1 and Rac2 deficiency results in massive mobilization of progenitor colony-forming unit cells (CFU-C) into the peripheral circulation and results in increased homing of CFU-C in the spleen (Cancelas et al. 2005; Mulloy et al. 2010).

In neutrophils, Rac1 affects cell spreading and adhesion, while Rac2 regulates directed migration and superoxide production (Gu et al. 2003). Rac2-deficient mice exhibit a phagocyte immunodeficiency syndrome. Interestingly, after the description of this phenotype, the case of a patient with leukocytosis and neutrophilia but multiple, recurrent, life-threatening infections in infancy was described. A dramatic decrease of neutrophil infiltration (absence of pus) in areas of infections was noted. The neutrophils of the patient exhibited decreased chemotaxis and



Rac GTPases, Fig. 2 Rac1 and Rac2 GTPases mediate proliferation and survival, and regulate the actin cytoskeleton, adhesion, and migration of hematopoietic stem cells and myeloid and erythroid progenitors in the bone marrow microenvironment, in response to cell surface receptors triggered by cytokines (SCF), chemokines (SDF-1α), and extracellular matrix (fibronectin). Their deficiency results in massive mobilization of HSC/P and increased homing in the spleen, while it also disturbs the actin cytoskeleton of neutrophils and mature erythrocytes

superoxide generation in response to fMLP (*N*-formyl-methionyl-leucyl phenylalanine), as well as reduced rolling on the L-selectin ligand GlyCAM-1; the latter a defect that had been observed in $Rac2^{-/-}$ mouse neutrophils. After LAD (leucocyte adhesion disorder) was ruled out with normal presence of CD11b, CD11c, and CD18, the patient was found to have a p.Asp57Asn (D57N) mutation of Rac2 (Williams et al. 2000). This is a highly conserved position in Rac GTPases as well as in the Ras superfamily as a whole, since it is located in the GTP-binding pocket of the GTPase. The mutation creates a dominant negative protein that is not only dysfunctional but also antagonizes Rac1 and Rac3 for GTP.

Studies in gene-targeted mice demonstrated that Rac1 and Rac2 play an overlapping but essential role in organizing the erythrocyte cytoskeleton. Mice with combined deficiency of Rac1 and Rac2 GTPases in their hematopoietic cells develop hemolytic anemia, as evidenced by concurrent reticulocytosis. $Rac1^{-/-}$; $Rac2^{-/-}$ red blood cells exhibit a disorganized membrane cytoskeleton with increased actin-to-spectrin ratio, F-actin aggregates and meshwork gaps, irregular clamping of band 3, decreased content of the proteins adducin and dematin, and decreased cellular

deformability (Kalfa et al. 2006). These mice develop successful stress-erythropoiesis in the spleen while homeostatic erythropoiesis in the bone marrow is significantly compromised, implying different signaling pathways for homeostatic and stress erythropoiesis (Kalfa et al. 2010). Rac GTPases were also shown to play a role in enucleation by using constitutively active and dominant negative mutants of Rac1 and Rac2; both inhibited enucleation in cultured mouse fetal liver erythroblasts indicating that either inhibition or excessive activation of Rac GTPases inhibits enucleation via disruption of the contractile actin ring in enucleating erythroblasts (Ji et al. 2008).

Combined Rac1 and Rac2 deficiency has also been shown to impair T and B cell development, proliferation, survival, adhesion, and migration, while Rac1 deficiency compromises platelet aggregation, lamellipodia formation, granule secretion, and clot retraction (Mulloy et al. 2010).

Rac GTPases in Cancer

Rac GTPases have been implicated in cellular transformation, oncogenesis, cancer invasiveness, and metastasis. Rac1 can be induced by oncogenes like Ras and collaborates with p53 loss of function to promote transformation in primary fibroblasts. Although no Rac mutations have been reported in tumors, overexpression or increased activity of Rac1 have been found in breast, lung, and colon cancer (Bosco et al. 2009). Activated Rac3 was detected in the malignant precursor B-lymphoblasts in p190-BCR/ABL transgenic mice (Cho et al. 2005) while Rac1 and Rac2 gene targeting was found to significantly delay or abrogate disease development in a p210-BCR/ABL mouse model of chronic myelogenous leukemia (CML) (Thomas et al. 2007). These data suggest that targeting modulation of Rac GTPases activity may provide clinical benefit for patients with CML, Ph-positive ALL, or other cancers.

Summary

The Rac subfamily of Rho GTPases consists of four members: Rac1, Rac2, Rac3, and RhoG. Although they exhibit high-sequence similarity, a great number of their functions are nonredundant. Via proteins that activate them (guanosine exchange factors, GEFs) or deactivate them (GTPase-activating proteins, GAPs), they receive signals from the cell surface after soluble ligand-receptor binding, interaction with the extracellular matrix or mechanical stress on cell surface receptors and propagate them through the appropriate downstream signaling pathways. They regulate many fundamental cellular functions, including actin cytoskeleton, cell adhesion, motility and migration, vesicular transport pathways and cytokinesis, ROS production via NADPH oxidase, gene transcription, and cell proliferation and survival. They have been implicated in many physiological and pathological processes, including hematopoiesis and cancer, and their role continues to be investigated using gene-targeted mouse models.

References

Aktories K, Schmidt G, Just I. Rho GTPases as targets of bacterial protein toxins. Biol Chem. 2000;381:421–6.

Bishop AL, Hall A. Rho GTPases and their effector proteins. Biochem J. 2000;348(Pt 2):241–55.

Bosco EE, Mulloy JC, Zheng Y. Rac1 GTPase: a "Rac" of all trades. Cell Mol Life Sci. 2009;66:370–4.

Burridge K, Wennerberg K. Rho and Rac take center stage. Cell. 2004;116(2):167–79.

Cancelas JA, Lee AW, Prabhakar R, Stringer KF, Zheng Y, Williams DA. Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. Nat Med. 2005;11:886–91.

Cho YJ, Zhang B, Kaartinen V, Haataja L, de Curtis I, Groffen J, Heisterkamp N. Generation of rac3 null mutant mice: role of Rac3 in Bcr/Abl-caused lymphoblastic leukemia. Mol Cell Biol. 2005;25:5777–85.

Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature. 2002;420:629–35.

Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, Jasti AC, Harris CE, Lee AW, Prabhakar R, Atkinson SJ, Kwiatkowski DJ, Williams DA. Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science. 2003;302:445–9.

Hall A. Rho GTPases and the actin cytoskeleton. Science. 1998;279:509–14.

Heasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat Rev Mol Cell Biol. 2008;9:690–701.

Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. Circ Res. 2006;98:453–62.

Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. Nat Cell Biol. 2008;10:314–21.

Kalfa TA, Pushkaran S, Mohandas N, Hartwig JH, Fowler VM, Johnson JF, Joiner CH, Williams DA, Zheng Y. Rac GTPases regulate the morphology and deformability of the erythrocyte cytoskeleton. Blood. 2006;108:3637–45.

Kalfa TA, Pushkaran S, Zhang X, Johnson JF, Pan D, Daria D, Geiger H, Cancelas JA, Williams DA, Zheng Y. Rac1 and Rac2 GTPases are necessary for early erythropoietic expansion in the bone marrow but not in the spleen. Haematologica. 2010;95:27–35.
Mulloy JC, Cancelas JA, Filippi MD, Kalfa TA, Guo F, Zheng Y. Rho GTPases in hematopoiesis and hemopathies. Blood. 2010;115:936–47.
Schwartz M. Rho signalling at a glance. J Cell Sci. 2004;117:5457–8.
Thomas EK, Cancelas JA, Chae HD, Cox AD, Keller PJ, Perrotti D, Neviani P, Druker BJ, Setchell KD, Zheng Y, Harris CE, Williams DA. Rac guanosine triphosphatases represent integrating molecular therapeutic targets for BCR-ABL-induced myeloproliferative disease. Cancer Cell. 2007;12:467–78.
Wang L, Zheng Y. Cell type-specific functions of Rho GTPases revealed by gene targeting in mice. Trends Cell Biol. 2007;17:58–64.
Westwick JK, Lambert QT, Clark GJ, Symons M, Van Aelst L, Pestell RG, Der CJ. Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol Cell Biol. 1997;17:1324–35.
Williams DA, Tao W, Yang F, Kim C, Gu Y, Mansfield P, Levine JE, Petryniak B, Derrow CW, Harris C, Jia B, Zheng Y, Ambruso DR, Lowe JB, Atkinson SJ, Dinauer MC, Boxer L. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. Blood. 2000;96:1646–54.

RAC3

▶ Steroid Receptor Coactivator Family

Rad

▶ Ras-Related Associated with Diabetes

Raf1

▶ RAF-1 (C-RAF)

Raf-1

▶ RAF-1 (C-RAF)

RAF-1 (C-RAF)

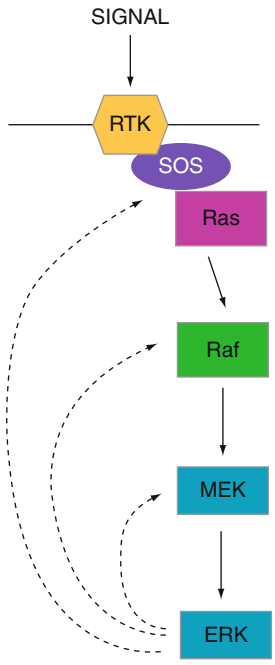
Andrea Varga and Manuela Baccarini
Department of Microbiology and Immunobiology, Center for Molecular Biology, University of Vienna, Max F. Perutz Laboratories, Vienna, Austria

Synonyms

C-Raf; c-Raf-1; Murine leukemia viral (v-raf-1) oncogene homolog 1 (3611-MSV); Murine sarcoma 3611 oncogene 1; Raf1; Raf-1; v-Raf; v-Raf-1 leukemia viral oncogene 1

Historical Background

Raf-1, also known as C-Raf-1 or C-Raf, was identified about 30 years ago as the oncogene (*v-raf*) in the murine sarcoma virus 3611 (3611-MSV) and, in parallel, in the naturally occurring avian retrovirus Mill Hill 2 (MH2). The gene was named after its enhancing effect on fibrosarcoma induction in newborn mice: *R*apidly *a*ccelerated *f*ibrosarcoma, or Raf. The sequences of the oncogenes, *v-raf* (derived from 3611-MSV) and *v-mil* (derived from MH2), were found to encode a serine/threonine protein kinase containing the catalytic, but not the N-terminal regulatory domain of the enzyme. This deletion rendered the protein constitutively active and was responsible for its transforming effect, making Raf the first oncogenic serine/threonine kinase discovered. A pseudogene (*c-raf-2*) and two paralogues of *c-raf-1*, named *a-raf* and *b-raf*, were subsequently identified (Wellbrock et al. 2004; Niault and Baccarini 2010). About 20 years ago, Raf-1 was reported to be phosphorylated in response to growth factor stimulation and was identified as the activator of the ▶ MEK/ERK pathway, the first mitogen-activated protein kinase (MAPK) module discovered that acts downstream of receptor tyrosine kinases; and finally, the finding that Raf-1 could be recruited to the membrane and stimulated by active Ras, already then recognized as a human oncogene, made the picture complete and led to the "textbook" description of the pathway as it is known today. Briefly, pathway activation involves the growth factor–induced dimerization and tyrosine



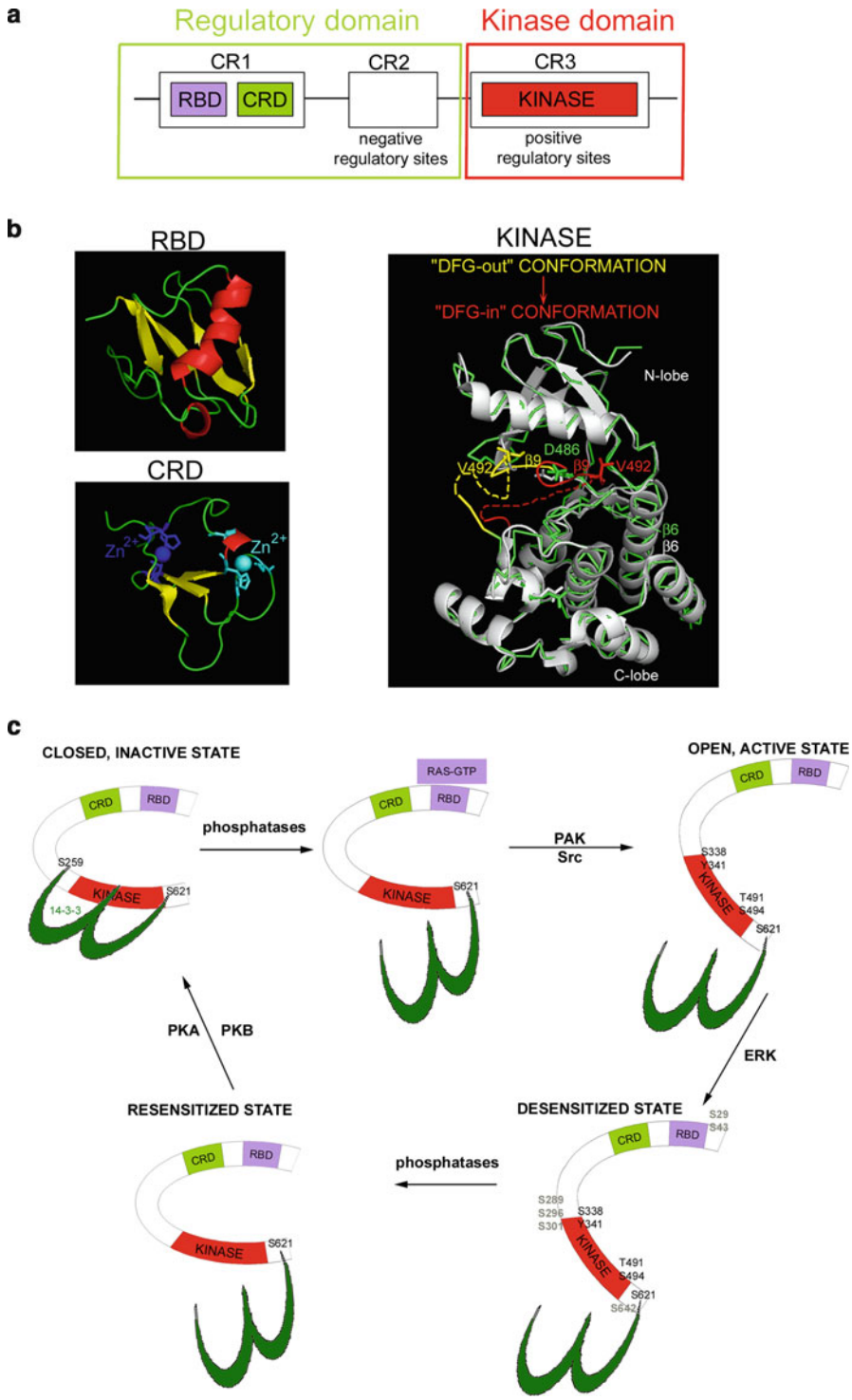
RAF-1 (C-RAF), Fig. 1 *Outline of the ERK pathway*. The Grb2-SOS complex is recruited via the binding of Grb2 to tyrosine phosphorylated residues in the cytoplasmic domain of activated Receptor Tyrosine Kinases (RTK). This brings Sos in the proximity of Ras, which is activated by the exchange of GDP for GTP. GTP-bound Ras recruits Raf to the membrane, where it is activated by phosphorylation (see Fig. 2 for more details on Raf-1 activation). From here, the signal is passed, in the form of phosphorylation, from Raf to MEK to ERK (solid arrows). ERK, in turn, quenches pathway activity at different levels by phosphorylating SOS, B-Raf, Raf-1, and MEK on negative regulatory residues (*broken arrows*)

phosphorylation of cell surface receptors, which in turn triggers the binding of a complex containing a scaffolding protein, Grb2, and a nucleotide exchange factor for Ras, SOS. The interaction with SOS catalyzes the exchange of GDP for GTP on Ras; with the help of scaffolding proteins (e.g. KSR), active, GTP-bound Ras can now induce the formation of membrane-associated Raf/MEK/ERK complexes. Each protein in the cascade can activate its downstream target via phosphorylation, i.e., Raf can phosphorylate MEK and MEK can phosphorylate ERK. Active ERK can induce the expression of several genes involved in cell proliferation, differentiation, and survival. In addition, active ERK feeds back on SOS, Raf, and MEK, providing a mechanism for signal attenuation [Fig. 1; reviewed in (Niault and Baccarini 2010)]. Adaptor proteins other than KSR have been reported to direct pathway components to distinct subcellular compartments, with potentially different signaling outcomes (Kolch 2005; McKay and Morrison 2007), and inhibitors of the pathway can regulate it at different levels (Kolch 2005).

The study of the essential functions of Raf-1 in conventional and conditional knockout mice [(Galabova-Kovacs et al. 2006) and references therein] has revealed that most of Raf-1's essential functions are not linked to the activation of the MEK/ERK pathway and to proliferation, but rather to pathways that counteract apoptosis and promote migration and differentiation. These new roles of Raf-1 are based on its interaction with three other kinases: the Rho-dependent kinase Rok-α, also known as ▶ ROCK2 (Ehrenreiter et al. 2005; Piazzolla et al. 2005; Ehrenreiter et al. 2009), involved in cytoskeletal rearrangements; the mammalian Sterile-20-like kinase-2, MST2 (O'Neill et al. 2004), homolog of *Drosophila*'s Hippo; and the apoptosis signal-regulating kinase 1, ASK1 (Yamaguchi et al. 2004), upstream regulator of the p38 and JNK pathways.

Structure of Raf and Activation of Its Kinase Function

Structure: The structure of Raf consists of three conserved regions [Fig. 2a; see also (Baccarini 2005) and references therein]. The regulatory/autoinhibitory domain of Raf is composed of two *C*onserved *R*egions, CR1 and CR2. CR1 contains the RBD – *R*as *B*inding *D*omain, which is required for membrane recruitment of the protein after activation by Ras; and the CRD – *C*ysteine-*R*ich *D*omain, which, besides being a secondary Ras binding site, is responsible for Raf-1 autoinhibition. CR2 is rich in Ser/Thr residues, whose phosphorylation can inactivate protein function (i.e., negative regulatory residues such as S259, whose dephosphorylation is prerequisite for Ras binding and Raf activation). CR3 is responsible for the catalytic activity and contains residues (S338, Y341) whose phosphorylation is involved in growth factor-induced kinase activation. The three-dimensional structure of the RBD [NMR structure, PDB 1RFA, (Emerson et al. 1995)], CRD [NMR, PDB 1FAR, (Mott et al. 1996)], and of the kinase domain [X-ray, PDB 3OMV, (Hatzivassiliou et al. 2010)] is known. The RBD domain fold resembles that of ubiquitin, while the CRD domain is an atypical C1 domain, which binds to phosphatidylserine and needs Zn^{2+} ions to preserve its folded



RAF-1 (C-RAF), Fig. 2 *Domain Structure of Raf-1 and mechanism of activation.* (**a**) Schematic representation of the domain structure of Raf-1. CR1, encompassing the RBD and the CRD, and CR2, containing some of the phosphorylation sites which restrain Raf-1 activity, comprise the regulatory domain; CR3 consists essentially of the kinase domain and contains the positive regulatory sites whose phosphorylation stimulates Raf-1 activity. (**b**) three-dimensional structure of the RBD, with its ubiquitin fold, of the Zn^{2+}-bound CRD, and of the Raf kinase domain. The inactive structure of the Raf-1 kinase domain

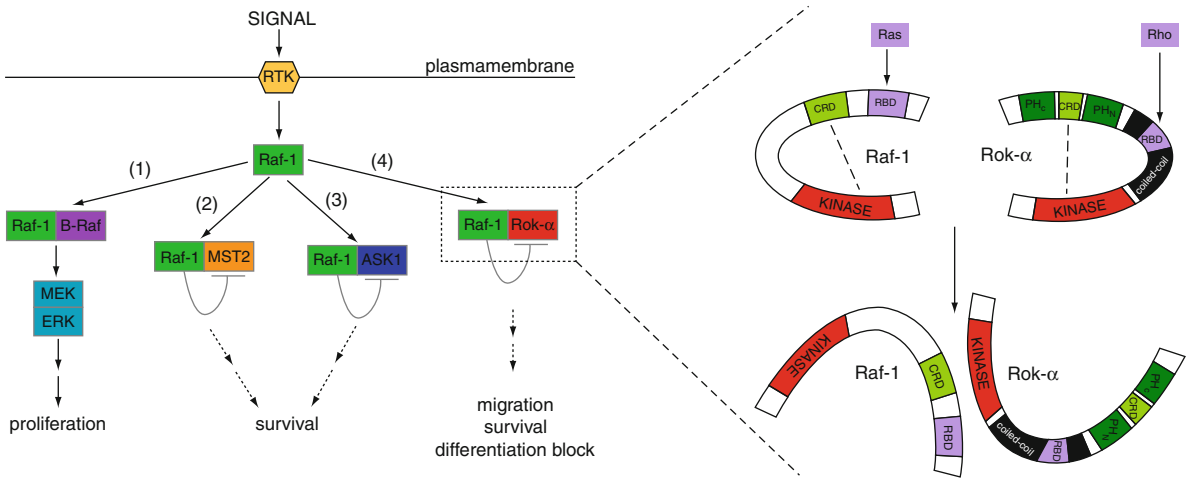
structure (Fig. 2b, left panel). The structure of the kinase domain consists of a smaller (N-terminal) and a larger (C-terminal) lobe. The latter contains the activation segment (the region between the DFG. . .APE motif, from D486 to E515), including the P-loop and β-strand 9 (β9). The P-loop is responsible for the correct positioning of both the adenosine and the gamma-phosphate of ATP for catalysis. The inactive conformation of the enzyme ("DFG-out", yellow) is stabilized by hydrophobic interactions between the P-loop and the activation segment (Fig. 2b, right panel). Kinase activation is mediated by phosphorylation of serine/threonine residues in the activation segment (T491 and S494), which results in a conformational change from the "DFG-out" to the "DFG-in" conformation (red). In the "DFG-in" conformation, the β9 strand of the N-lobe interacts with the β6 strand of the C-lobe of the kinase, closing the cleft between the two lobes and switching the enzyme to its active state. The β9 strand contains the V482 residue which corresponds to the B-Raf residue frequently mutated to E in human melanoma [V600E; activating B-Raf mutation (Wellbrock et al. 2004)]. Interestingly, corresponding mutations in Raf-1, which would have the same activating effect, have not been reported.

Activation–Inactivation: In the inactive state of Raf, the N-terminal, regulatory domain of the protein binds to its kinase domain and inhibits its activity [Fig. 2c; see also (Wellbrock et al. 2004; Niault and Baccarini 2010)]. This conformation is stabilized by the binding of 14-3-3 proteins, which recognize two phosphorylated Raf-1 residues: S259 on the N-terminal and S621 on the C-terminal part of the protein. The current model of Raf-1 activation postulates that this binding must be disrupted to enable Raf-1 activation. This process is accomplished by protein phosphatases 1 and 2A, which dephosphorylate residue S259. 14-3-3 proteins remain bound to the phosphorylated S621 and maintain a productive conformation of the kinase domain. After S259 dephosphorylation, Raf-1 can be recruited to the membrane by binding to activated Ras, primarily via the RBD. Ras binding is followed by disruption of the inhibitory interaction between the regulatory and the kinase domain of the protein. The activation process is completed by the phosphorylation of the activating residues in the CR3 region (T491, S494), which stabilizes the active, "DFG-in" conformation. Inactivation of Raf-1 occurs via the phosphorylation on its negative regulatory residues by ERK, which is followed by the dephosphorylation of activating residues by PP2A. Finally, PKA/▶ PKB rephosphorylates the residue S259, making the rebinding of 14-3-3 possible (Wellbrock et al. 2004; Niault and Baccarini 2010).

Raf-1-Containing Complexes and Their Biological Functions

Raf-1 can also be activated by (Ras-dependent) homodimerization or by heterodimerization with other Rafs, particularly B-Raf. As part of this complex, Raf-1 can stimulate the MEK/ERK pathway and therefore regulate cell proliferation and several other biological functions (Fig. 3, pathway 1). In most cells and tissues, however, Raf-1 is not essential for MEK/ERK activation and proliferation. Instead, Raf-1 is required to promote survival, either through the inhibition of proapoptotic kinases such as MST2 and ASK1 (Fig. 3, pathways 2 and 3) or by restraining the cytoskeleton-based kinase Rok-α, which regulates the trafficking of the death receptor Fas (Fig. 3, pathway 4; and right panel). Interaction with, and inhibition of Rok-α is also the molecular basis of Raf-1's role in cell migration, in keratinocyte differentiation, and in Ras-driven

RAF-1 (C-RAF), Fig. 2 (continued) (PDB: 3OMV, (Hatzivassiliou et al. 2010); ribbon representation, *in green*) is superimposed on the active structure of the B-Raf kinase domain (PDB: 2FB8, (King et al. 2006); cartoon representation, *in white*). The aminoacid numbering corresponds to the human Raf-1 protein. The inactive "DFG-out" conformation is shown in yellow, the active "DFG-in" conformation in red. Note the interaction of the β9 strand of the N-lobe (*red*) with the β6 strand of the C-lobe in the "DFG-in" conformation. The start of the DFG (D486) and the position of the V492 residue corresponding to the V600 in B-Raf frequently mutated in melanoma are indicated. (**c**) Mechanism of Raf-1 activation. In quiescent cells, intramolecular inhibition, stabilized by 14-3-3 binding to the phosphorylated S259 and S621 sites, prevents Raf-1 activation. The transition to the active state is mediated by the dephosphorylation of 259 and Ras binding, which recruits Raf-1 to the membrane, where phosphorylation of activating residues occurs. The Raf-1 signal is quenched by the phosphorylation of negative regulatory sites (*in grey*) mediated by active ERK, which results in kinase desensitization, followed by dephosphorylation of both positive and negative regulatory sites (resensitized state). Finally, rephosphorylation of S259 restores the close, inactive conformation of Raf-1. The residues phosphorylated at each step are shown at their approximate localization in the molecule. The kinases responsible for phosphorylation of the positive (PAK, Src) or negative (ERK, PKA, PKB) regulatory sites are shown



RAF-1 (C-RAF), Fig. 3 *Interactions of Raf-1 with different partners and their biological consequences. Left*, As part of a Raf dimer, Raf-1 functions as a MEK/ERK activator and stimulates many cellular functions, in particular proliferation (1). Most of the essential Raf-1 functions rely on protein/protein interaction and are independent of Raf-1 kinase activity. Binding of Raf-1 to the MST2 (2) and ASK-1 (3) kinases promotes survival by reducing the strength of the downstream proapoptotic signal. Interaction with the cytoskeleton-based kinase Rok-α (4) promotes migration and survival and restrains differentiation. *Right*, molecular basis of Rok-α inhibition by Raf-1. Raf-1 and Rok-α share similar autoinhibitory domains, which, in quiescent cells, interact with the kinase domains and restrain their activity. When the kinases are activated by the respective upstream GTPases, autoinhibition is relieved, and the regulatory domain of Raf-1 is free to bind to the kinase domain of Rok-α and modulate its kinase activity

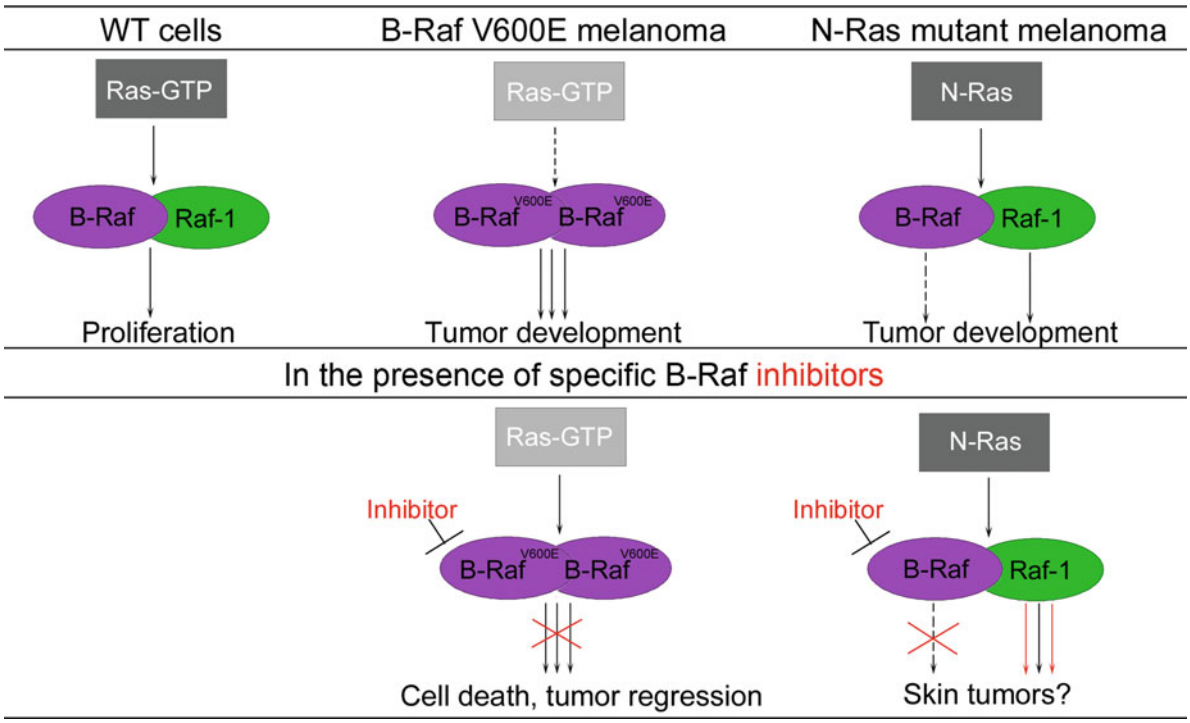
epidermal tumorigenesis (Niault and Baccarini 2010; Wimmer and Baccarini 2010; Kern et al. 2011).

The following section describes the individual Raf-1-containing complexes.

The Raf-1/B-Raf complex (Fig. 3, pathway 1): All three Raf isoforms, ▶ A-Raf, B-Raf, and Raf-1, can mediate MEK/ERK activation. Conditional mutagenesis has revealed that B-Raf is the essential MEK activator in various cells and tissues (Galabova-Kovacs et al. 2006; Niault and Baccarini 2010). A-Raf and Raf-1 can heterodimerize with B-Raf, yielding a MEK kinase more potent than the individual monomeric forms. Whether A-Raf or Raf-1 are preferred B-Raf partners in the context of the heterodimer is yet unknown; however, both A-Raf and Raf-1 must be ablated to reduce MEK/ERK phosphorylation in fibroblasts (Mercer et al. 2005), consistent with an interchangeable role of these two kinases as dimer subunits. The heterodimerization of Raf has been in the limelight since the discovery that B-Raf inhibitors currently in the process of being approved for the treatment of melanoma patients activate the Raf/MEK/ERK pathway, instead of stopping it (see below; and Fig. 4).

Raf-1 and MST2 (Fig. 3, pathway 2): In mammalian cells, MST2 is activated by stress signals and causes apoptosis acting upstream of the mammalian Hippo signaling pathway (Pan 2010). MST2 was identified as a Raf-1 interacting partner using mass spectrometry (O'Neill et al. 2004). In quiescent cells, MST2 is phosphorylated on two negative regulatory sites in its N-terminal and C-terminal region (Romano et al. 2010). This diphosphorylated form can interact with Raf-1 (region between amino acids 150–303) and this inhibits MST2 activation by preventing homodimerization. This interaction is disrupted by proapoptotic stimuli, enabling MST2 activation by the tumor suppressor RASSF1.

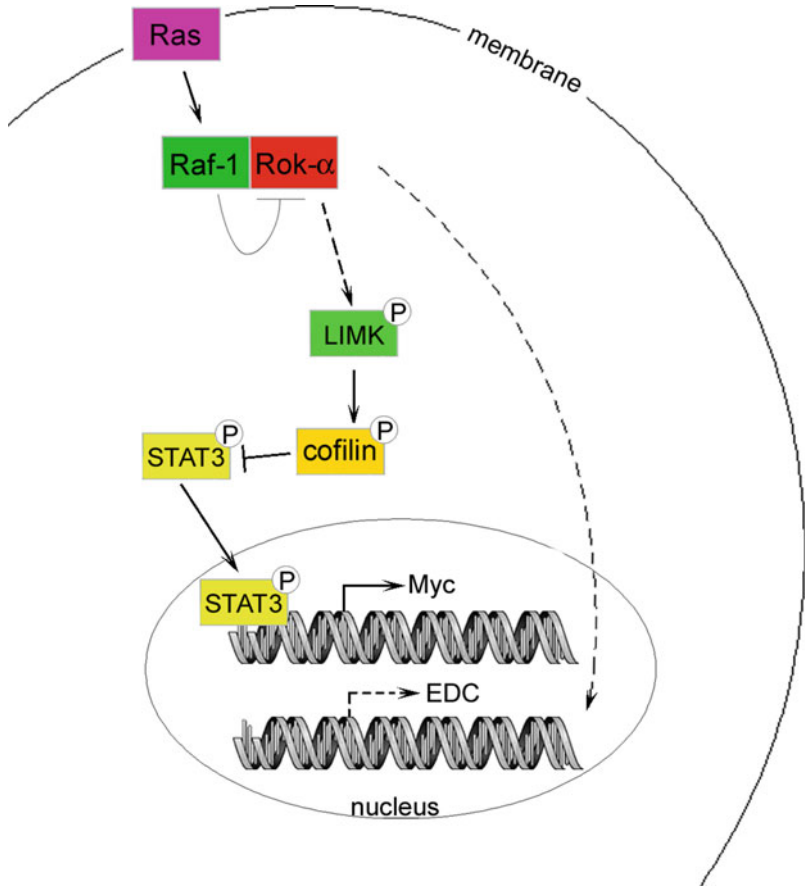
Raf-1 and ASK1 (Fig. 3, pathway 3): ASK1 is a Ser/Thr kinase which acts upstream of JNK and p38 and promotes apoptosis induced by stress or death receptors. Cardiac-specific ablation of Raf-1 induces apoptosis in the cardiac muscle in vivo and leads to a transient increase in ASK1, JNK, and p38 activity during postnatal heart development, without affecting the MEK/ERK pathway (Yamaguchi et al. 2004). Concomitant ablation of ASK1 rescues the phenotype of Raf-1 conditional knockout mice, indicating a causal role of ASK1 in the abnormalities observed in Raf-1 knockout hearts and suggesting that Raf-1 limits ASK1 activity in cardiomyocytes. How exactly Raf-1 inhibits



RAF-1 (C-RAF), Fig. 4 *Paradoxical activation of MEK/ERK pathway by B-Raf inhibitors. Upper panel*, in wild type (WT) cells, Raf dimer formation and MEK/ERK activation are stimulated by extracellular signals acting through RTKs and Ras. In melanoma cells harboring constitutively active B-RafV600E, MEK/ERK activation is Ras and Raf-1-independent; while melanoma cells harboring mutated N-Ras activate MEK/ERK by stimulating the formation of B-Raf-Raf-1 dimers. *Lower panel*, B-Raf inhibitors will block B-RafV600E kinase activity, reducing ERK activation and inducing a proliferation block and subsequent tumor regression. However, in cells harboring NRAS mutations, inhibitors can induce Raf dimerization and ERK activation. This is due to the fact that dimers containing only one functional kinase subunit are active as MEK kinases. Such dimers may arise if the inhibitors used have fast off-rates, or if low concentrations of inhibitors are used. In both situations, the conformational change induced by the inhibitors would suffice to stimulate dimerization, but not to completely inhibit the resulting dimeric kinase. This mechanism may be linked to the development of drug-related tumors observed in melanoma patients treated with B-Raf inhibitors

ASK1 has not yet been established, but it is known that this function of Raf-1 is independent of the MEK/ERK pathway, although the phosphorylation of the activating Raf-1 regulatory residues S338 and S339 is a prerequisite, at least in endothelial cells (Alavi et al. 2007). Raf-1 physically interacts with the N-terminal autoinhibitory domain of ASK1; it is therefore possible that by doing so it will promote and/or stabilize an inactive ASK1 conformation (Chen et al. 2001).

Raf-1 and Rok-α (Fig. 3, pathway 4): In mammalian cells, the Rho effector Rok-α is responsible for cytoskeletal rearrangements essential for cell adhesion and motility. Conditional gene ablation studies have revealed that Rok-α is hyperactive and mislocalized in the absence of Raf-1 (Ehrenreiter et al. 2005; Piazzolla et al. 2005). Importantly, chemical or genetic inhibition of Rok-α rescues all phenotypes of Raf-1 knockout mouse embryonic fibroblasts, defining Rok-α hyperactivity as the rate-limiting factor in this context (Piazzolla et al. 2005). In addition, both the cellular phenotypes and Rok-α hyperactivity could be rescued by complementation with Raf-1 mutants devoid of kinase activity, as well as by mutants featuring the isolated Raf-1 regulatory domain, indicating that the physical presence of at least part of Raf-1 is necessary for the inhibition of Rok-α activity (Ehrenreiter et al. 2005; Piazzolla et al. 2005). The molecular basis of Raf-1 interaction with Rok-α is understood in some detail. Both Raf-1 and Rok-α are modular kinases featuring a similar domain structure. In quiescent cells, the activity of Raf-1 and Rok-α is restrained by intramolecular inhibition. The negative



RAF-1 (C-RAF), Fig. 5 *Raf-1-mediated Rok-α inhibition is essential for the establishment and maintenance of Ras-induced epidermal tumors.* Activated Ras stimulates the interaction between Raf-1 and Rok-α. The resulting attenuation of Rok-α leads to decreased expression of the epidermal differentiation cluster genes (EDC) and reduces the phosphorylation of cofilin (via ▶ LIMK). Since phosphocofilin, in turn, inhibits the pro-proliferative STAT3/myc pathway, Raf-1-mediated Rok-α attenuation supports Ras-driven tumorigenesis

regulatory domain (cysteine-rich region, CRD) of each protein can interact with its own kinase domain and inhibit its activity, likely by preventing substrate binding. Upon mitogenic stimulation, binding to activated small G-proteins (Ras for Raf-1 and Rho for Rok-α) relieves autoinhibition and, at the same time, makes the interaction between the regulatory domain (CRD) of Raf and the kinase domain of Rok possible (Niault et al. 2009). In this situation, the autoinhibitory domain of Raf-1, much like an ill-fitting lego brick, can restrain the activity of the Rok-α kinase domain without blocking it completely (Fig. 3, right panel). This mechanism of inhibition in *trans* is the first example of kinase regulation mediated by physical interaction rather than phosphorylation on negative regulatory residues.

Raf-1 and Cancer

A wealth of reports has implicated Raf isoforms, particularly B-Raf, in different aspects of tumor development (Niault and Baccarini 2010; Maurer et al. 2011). The next chapters will focus on two recently described functions of Raf-1 in melanoma and squamous cell carcinoma, both of which relay on Raf-1's ability to form physical complexes with other kinases.

Raf-1-B-Raf interaction and melanoma: MEK/ERK signaling is particularly important in melanoma. Somatic mutations occur in B-Raf and N-Ras in ~50% and ~15% of cutaneous melanomas, respectively (www.sanger.ac.uk/genetics/CGP/cosmic/). The most frequent mutation in B-Raf is the V600E mutation, which causes constitutive activation of the kinase and thus of the MEK/ERK pathway. After this discovery, tremendous efforts were made to find inhibitors targeting the mutated form of B-Raf. Surprisingly, these inhibitors could inactivate the enzyme in vitro, but activated the RAF/MEK/ERK pathway in cells not harboring the V600E mutation. The molecular basis of the Raf-inhibitor paradox is the ability of Raf enzymes to heterodimerize and form a potent MEK kinase, even when only one dimer subunit is enzymatically active [Fig. 4; reviewed in (Cichowski and Janne 2010;

Wimmer and Baccarini 2010)]. Selective inhibitors cause a conformational change in the structure of B-Raf, which promotes the dimerization of this "inhibited" form of B-Raf with Raf-1 or A-Raf. This leads to the stabilization of an active MEK kinase and to the stimulation of the MEK/ERK pathway. Inhibitor-induced stabilization of Raf hetero- or homodimers is predicted to be particularly dangerous in cells containing mutations that stimulate dimer formation, such as melanoma cells with an N-Ras mutation, as indicated by a recent animal study (Heidorn et al. 2010), and also in any other tumor-prone cell. This mechanism might be the reason for the appearance of keratoacanthomas and squamous cell carcinomas in about 30% of patients treated with Raf inhibitors in clinical studies (Arkenau et al. 2011).

Raf-1-Rok-α interaction and Ras-driven epidermal carcinogenesis: To date, Raf-1 is the only Ras effector that has been shown to be essential for the maintenance of Ras-driven tumors. This has been achieved by conditional gene ablation experiments conducted in mice with epidermis-restricted Raf-1 ablation. Besides showing defects in wound healing, keratinocyte adhesion and migration (Ehrenreiter et al. 2005), these animals are refractory to epidermal tumors caused by Ras activation; more importantly, Raf-1 ablation causes the complete regression of established tumors. Thus, Ras-driven tumors are addicted to endogenous Raf-1, constituting a prime example of non-oncogene addiction. Mechanistically, Ras drives the formation of a complex between Raf-1 and Rok-α, ultimately resulting in Rok-α inhibition. In the absence of Raf-1, hyperactive Rok-α drives keratinocyte differentiation and tumor regression via a pathway involving phosphorylated cofilin, the inhibition of STAT3 phosphorylation, and of Myc expression (Fig. 5). Thus, inhibiting Raf-1-Rok-α complex formation, either by silencing the *Raf-1* gene or by using small molecule inhibitors which can disrupt the complex, may be a viable strategy for the (co-)therapy of Ras-driven epidermal tumors (Ehrenreiter et al. 2009).

Summary

Born as the first serine/threonine kinase oncogene and intensively studied as the link between Ras and the mitogenic MEK/ERK pathway, Raf-1 is coming of age as a versatile signal transducer with multiple partners impinging on cell motility, differentiation, and survival. The basis for this is its modular structure, featuring a kinase domain kept in check by an autoinhibitory domain. This autoinhibition is relieved by intricate regulatory mechanisms involving dephosphorylation of negative regulatory sites, Ras binding, and phosphorylation of activating sites. Once autoinhibition is relieved, Raf-1 can function as a MEK kinase, in the context of homodimers or of Raf heterodimers, but it can also exert kinase-independent functions by binding to, and directly regulating, serine/threonine kinases operating in distinct pathways. These functions in pathway cross-talk are the essential ones, as revealed by conventional and conditional gene ablation studies. One obvious unresolved question in this context is how extracellular cues direct Raf-1 (or, for that matter, other signal transducers) to the appropriate signaling complex in order to implement the correct biological response. One of the kinase-independent functions of Raf-1, the inhibition of the cytoskeleton-based kinase Rok-α, is essential for the development and maintenance of Ras-driven epidermal tumors. Will other Raf-1 interactions prove similarly essential in tumorigenesis, possibly in the context of other cell types/tissues? And if yes, will it be possible to design inhibitors for molecule-based therapy? The key to these questions lies in the further investigation of Raf-1's role in tumor models in vivo and in obtaining structural information on the complexes between Raf-1 and its interacting proteins.

Acknowledgements The authors wish to thank all the members of the Baccarini group for helpful discussions. Dr. Andrea Varga is supported by a FEBS long term fellowship. Work in the Baccarini lab is supported by funds of the Austrian National Research Fund (FWF), the Austrian Society for the Advancement of Research (FFG), the Obermann Foundation, and the European Community.

References

Alavi AS, Acevedo L, Min W, Cheresh DA. Chemoresistance of endothelial cells induced by basic fibroblast growth factor depends on Raf-1-mediated inhibition of the proapoptotic kinase, ASK1. Cancer Res. 2007;67:2766–72.

Arkenau HT, Kefford R, Long GV. Targeting BRAF for patients with melanoma. Br J Cancer. 2011;104:392–8.

Baccarini M. Second nature: biological functions of the Raf-1 "kinase". FEBS Lett. 2005;579:3271–7.

Chen J, Fujii K, Zhang L, Roberts T, Fu H. Raf-1 promotes cell survival by antagonizing apoptosis signal- regulating kinase 1 through a MEK-ERK independent mechanism. Proc Natl Acad Sci USA. 2001;98:7783–8.
Cichowski K, Janne PA. Drug discovery: inhibitors that activate. Nature. 2010;464:358–9.
Ehrenreiter K, Piazzolla D, Velamoor V, Sobczak I, Small JV, Takeda J, et al. Raf-1 regulates Rho signaling and cell migration. J Cell Biol. 2005;168:955–64.
Ehrenreiter K, Kern F, Velamoor V, Meissl K, Galabova-Kovacs G, Sibilia M, et al. Raf-1 addiction in Ras-induced skin carcinogenesis. Cancer Cell. 2009;16:149–60.
Emerson SD, Madison VS, Palermo RE, Waugh DS, Scheffler JE, Tsao KL, et al. Solution structure of the Ras-binding domain of c-Raf-1 and identification of its Ras interaction surface. Biochemistry. 1995;34:6911–18.
Galabova-Kovacs G, Kolbus A, Matzen D, Meissl K, Piazzolla D, Rubiolo C, et al. ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. Cell Cycle. 2006;5:1514–18.
Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature. 2010;464:431–5.
Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell. 2010;140:209–21.
Kern F, Niault T, Baccarini M. Ras and Raf pathways in epidermis development and carcinogenesis. Br J Cancer. 2011;104:229–34.
King AJ, Patrick DR, Batorsky RS, Ho ML, Do HT, Zhang SY, et al. Demonstration of a genetic therapeutic index for tumors expressing oncogenic BRAF by the kinase inhibitor SB-590885. Cancer Res. 2006;66:11100–5.
Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol. 2005;6:827–37.
Maurer G, Tarkowski B, Baccarini M. Raf kinases in cancer – roles and therapeutic opportunities. Oncogene. 2011; in press.
McKay MM, Morrison DK. Integrating signals from RTKs to ERK/MAPK. Oncogene. 2007;26:3113–21.
Mercer K, Giblett S, Oakden A, Brown J, Marais R, Pritchard C. A-Raf and Raf-1 work together to influence transient ERK phosphorylation and Gl/S cell cycle progression. Oncogene. 2005;24:5207–17.
Mott HR, Carpenter JW, Zhong S, Ghosh S, Bell RM, Campbell SL. The solution structure of the Raf-1 cysteine-rich domain: a novel ras and phospholipid binding site. Proc Natl Acad Sci USA. 1996;93:8312–17.
Niault TS, Baccarini M. Targets of Raf in tumorigenesis. Carcinogenesis. 2010;31:1165–74.
Niault T, Sobczak I, Meissl K, Weitsman G, Piazzolla D, Maurer G, et al. From autoinhibition to inhibition in trans: the Raf-1 regulatory domain inhibits Rok-alpha kinase activity. J Cell Biol. 2009;187:335–42.
O'Neill E, Rushworth L, Baccarini M, Kolch W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science. 2004;306:2267–70.
Pan D. The hippo signaling pathway in development and cancer. Dev Cell. 2010;19:491–505.
Piazzolla D, Meissl K, Kucerova L, Rubiolo C, Baccarini M. Raf-1 sets the threshold of Fas sensitivity by modulating Rok-{alpha} signaling. J Cell Biol. 2005;171:1013–22.
Romano D, Matallanas D, Weitsman G, Preisinger C, Ng T, Kolch W. Proapoptotic kinase MST2 coordinates signaling crosstalk between RASSF1A, Raf-1, and Akt. Cancer Res. 2010;70:1195–203.
Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. Nat Rev Mol Cell Biol. 2004;5:875–85.
Wimmer R, Baccarini M. Partner exchange: protein-protein interactions in the Raf pathway. Trends Biochem Sci. 2010;35:660–8.
Yamaguchi O, Watanabe T, Nishida K, Kashiwase K, Higuchi Y, Takeda T, et al. Cardiac-specific disruption of the *c-raf-1* gene induces cardiac dysfunction and apoptosis. J Clin Invest. 2004;114:937–43.

RAFA1

▶ A-RAF

Ramp

Debbie L. Hay[1], Patrick M. Sexton[2] and David R. Poyner[3]
[1]School of Biological Sciences, University of Auckland, Auckland, New Zealand
[2]Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, VIC, Australia
[3]School of Life and Health Sciences, Aston University, Birmingham, UK

Historical Background

The receptor activity-modifying protein (RAMP) family was first reported in 1998 during attempts to identify the cell surface receptor for a neuropeptide known as calcitonin gene-related peptide (CGRP) (McLatchie et al. 1998). Formerly, a protein known as the calcitonin receptor-like receptor (CLR) was thought to be the receptor for CGRP but no study had convincingly shown that this was the case. McLatchie and colleagues were able to show that CLR needs RAMP1 for a CGRP receptor to be formed. RAMP1 assists CLR in reaching the cell surface. Thus, RAMP1 and CLR together at the cell surface form the receptor for CGRP, which binds and activates this protein complex,

leading to downstream signaling events such as an accumulation of intracellular cAMP. In this same study, two other related proteins were found, named RAMP2 and RAMP3. Each of these proteins could also assist CLR in reaching the cell surface but remarkably, CGRP was less effective at activating these protein complexes. Instead, a peptide similar to CGRP, called adrenomedullin (AM), preferentially activated them. Thus, RAMPs can be considered as "pharmacological switches" by virtue of their ability to change the peptide hormone for which CLR has a preference.

Overview

The receptor complexes that are formed by RAMPs interacting with CLR are shown in Fig. 1. Since RAMPs were first identified, a great deal has been learned about their functions. It is now broadly understood how they change peptide recognition but also that they have other functions and interact with other receptors.

Pharmacological Importance of RAMPs

At first it was thought that the way RAMPs could change peptide preference was by changing the conformation of the receptor protein, CLR, but it is now known that RAMPs also play their own role in binding CGRP and AM and also small molecule drugs (Sexton et al. 2009). For instance, RAMP1 and CLR both participate in the binding of the drugs Olcegepant and Telcagepant. This makes the drugs extremely selective for the CGRP receptor; they have only low affinity for the two AM receptors or for the RAMP1-based AMY_1 receptor. RAMPs also change the pharmacology of another receptor, the calcitonin receptor (CTR). This is the closest protein relative to CLR and can also interact with all three RAMPs. In this case, CTR is a receptor for the peptide hormone, calcitonin, when it is expressed alone in cells. However, when CTR is expressed with RAMPs, the resulting RAMP/CTR complexes have a preference for a peptide hormone known as amylin (Fig. 1) (Poyner et al. 2002).

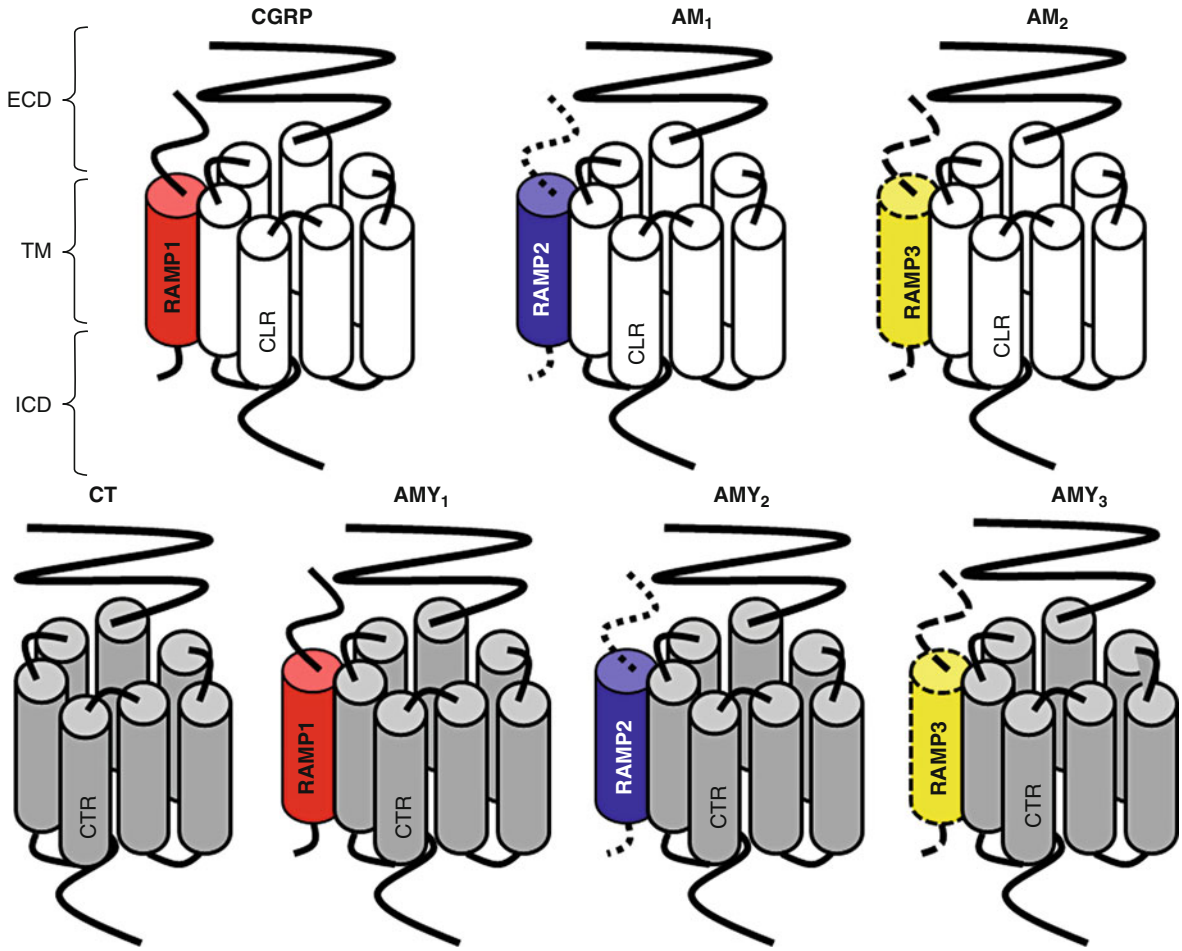
RAMPs and Cellular Signaling

CLR and CTR are proteins known as G protein-coupled receptors (GPCRs). As their name suggests, these proteins interact with G proteins (guanine nucleotide binding proteins) to initiate intracellular signaling. There are several types of G proteins that initiate different downstream signaling events and many receptors can interact with more than one G protein to regulate cellular function. For instance, G_s G proteins activate ▶ adenylate cyclase to cause increases in cAMP, while G_q G proteins activate phospholipase C to hydrolyze membrane inositol phosphates to form inositol trisphosphate (▶ IP_3 Receptors) and diacyl glycerol (DAG); these in turn mobilize intracellular calcium and activate various kinase proteins. Both CLR and CTR can couple to G_s and signal via adenylate cyclase/cAMP pathway. In the case of the RAMP/CTR complexes, that is the amylin receptors, the amount of amylin binding can be influenced by G protein type in a RAMP-dependent manner (Morfis et al. 2008), and conversely RAMP interaction with the CTR can change the receptor preference for different G proteins. For example, the RAMP1/CTR (AMY_1) and RAMP3/CTR (AMY_3) receptors have greater relative preference for G_s over G_q G proteins than the CTR when expressed alone (Morfis et al. 2008), and this may change to overall cellular response to receptor activation.

The activity of a GPCR must be carefully controlled to make sure that cells are kept responsive when they need to be. In many cases, a process called desensitization occurs after a GPCR has been activated, which reduces the amount of signal that the receptor can generate. Following this, GPCRs are often removed from the cell surface membrane; the receptors are internalized. The GPCR may then be returned to the cell surface and, thus, recycled or it can be degraded and not returned. RAMPs can also modulate these internalization and recycling processes. RAMP3 has a specific sequence of amino acids, a PDF-like domain, in its intracellular domain. This allows it to interact with different regulatory proteins to RAMP1 and RAMP2, such as N-ethylmaleimide-sensitive factor (Bomberger et al. 2005). Therefore, in model systems, RAMP3/CLR (AM_2 receptor) complexes can be recycled unlike AM_1 and CGRP receptors.

RAMP Interactions with Other Receptors

There is accumulating research which shows that RAMPs may have much broader roles and control aspects of the activity of many receptors. For example, the calcium sensing receptor which is unrelated to CLR or CTR requires RAMP1 or RAMP3 for it to reach the cell surface and therefore signal (Bouschet et al. 2005). The VPAC1 receptor can associate with RAMP2 and this leads to enhanced, agonist-mediated



Ramp, Fig. 1 Receptor complexes that are formed when CLR and RAMPs or CTR and RAMPs associate. CLR is the seven transmembrane (*TM*) protein in *white* and CTR is the seven TM protein in *gray*. RAMP1 is shown in *red*, RAMP2 in *blue*, and RAMP3 in *yellow*. RAMP1 with CLR is known as the CGRP receptor whereas RAMP2 or RAMP3 with CLR are known as AM_1 and AM_2 receptors, respectively. CTR is the receptor for calcitonin (*CT*) but with RAMP1 it is an AMY_1 (amylin subtype 1) receptor. AMY_2 and AMY_3 receptors are formed when RAMP2 and 3 associate with CTR. *ECD* extracellular domain, *ICD* intracellular domain

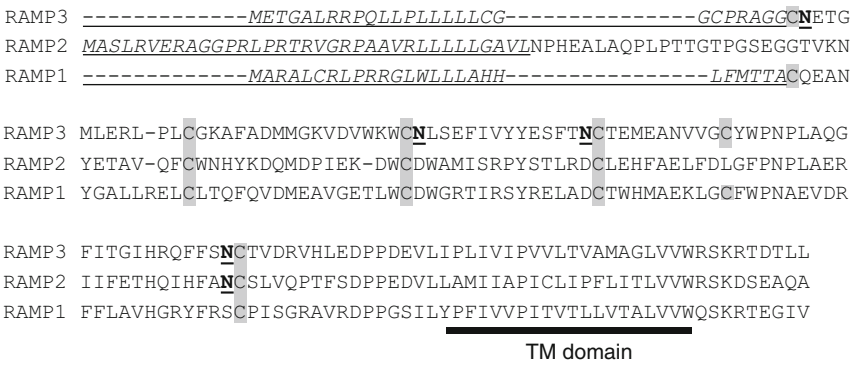
phosphoinositide breakdown but there is no effect on cAMP production (Christopoulos et al. 2003). The secretin receptor is another example of a RAMP-interacting GPCR but in this case the reason why these proteins associate has not yet been revealed.

The Structure of RAMPs

Sequence analysis shows that the RAMPs all have a single transmembrane region, with a small cytoplasmic tail of around 10 amino acids and a much larger N-terminus of around 100 amino acids (Figs. 1 and 2). All RAMPs have four cysteines that take part in formation of two disulfide bonds; RAMPs 1 and 3 have an extra cysteine pair which form a third disulfide. A crystal structure is available for the majority of RAMP1, both by itself and in combination with CLR (Kusano et al. 2008; Koth et al. 2010). The extracellular domain is a trihelical structure. Residues at the base of helix 2 and the C-terminus of helix 3, among others, appear to be important for making contact with CLR; the region connecting these two helices may be particularly important for peptide binding (Fig. 3).

RAMP Expression

RAMPs appear to be widely expressed in mammals, although there are actually very few studies where the



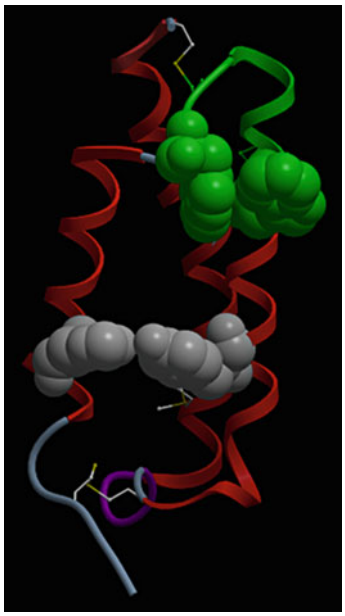
Ramp, Fig. 2 Amino acid sequences of human RAMPs. Comparison of the sequences of human RAMPs 1, 2, and 3. Signal peptides are shown in italics, potential glycosylation sites are in bold. Cysteines involved in disulfides are shaded. *TM* transmembrane

proteins themselves have been measured, rather than mRNA. This has been because there have been few reliable antibodies. This has meant that it has been difficult to properly colocalize RAMPs and receptors. A recent study has reported the co-expression of RAMP1 and CLR in neurons of the human and rodent trigeminal ganglion, which is involved in pain transmission (Eftekhari et al. 2010). More studies of this nature are needed to confirm the physiological significance of the AM and amylin receptor subtypes and the interactions of RAMPs with other receptors.

Several animal models of RAMP under or overexpression have been generated. Mice which selectively overexpress human RAMP1 in neurons are sensitized to CGRP, whereas those which overexpress RAMP2 in the smooth muscle were sensitized to the effects of AM (Tam et al. 2006; Zhang et al. 2007). These types of observation help to confirm that these are valid components of AM and CGRP receptors in vivo. Mice that genetically lack RAMP2 (RAMP2 knockout mice) have severe defects and show that RAMP2 is essential for the blood and lymphatic vascular systems to develop properly in the embryo (Fritz-Six et al. 2008). Interestingly, RAMP3 knockout mice do not have any obvious phenotype, suggesting that RAMP3, and the AM_2 receptors which it forms with CLR, has different functions to the AM_1 receptor (Dackor et al. 2007).

Evolutionary Considerations

There is some evidence that a form of CGRP first evolved in insects. In *Drosophila melanogaster*, the protein CG17415 shows homology to CLR. It is activated by the diuretic hormone DH31 and this response can be amplified when it is co-expressed with human RAMP1 or RAMP2 (Johnson et al. 2005). However,



Ramp, Fig. 3 Structure of the extracellular domain of RAMP1. Structure based on 2YX8 in the protein structure database. Helices are shown in *red*. Residues at the base of helix 2 and the C-terminus of helix 3 likely to be involved in CLR recognition are in *gray*; the region between helices 2 and 3 which may be important for ligand binding is in *green*

no ortholog of a RAMP has yet been identified in *Drosophila*. RAMPs are certainly present in bony fish, where they are found with homologues of CLR and AM. The functions of RAMPs have been best studied in the pufferfish, *Takifugu obscurus*. This expresses five forms of AM, three forms of CLR, and five RAMPs. The expanded AM/RAMP/CLR family appears to be involved in fluid homeostasis (Nag et al. 2006). The evolutionary history of RAMPs between insects and fish remains obscure.

Summary

This entry has provided a snapshot of what RAMPs are and what they are currently understood to do. Most of this research has been performed in isolated cellular systems because these have been the models that have been available. It is now important to move toward whole organism studies so it can be fully appreciated how broad the functions of these proteins may be in physiology and disease.

References

Bomberger JM, Parameswaran N, Hall CS, Aiyar N, Spielman WS. Novel function for receptor activity-modifying proteins (RAMPs) in post-endocytic receptor trafficking. J Biol Chem. 2005;280:9297–307.

Bouschet T, Martin S, Henley JM. Receptor-activity-modifying proteins are required for forward trafficking of the calcium-sensing receptor to the plasma membrane. J Cell Sci. 2005;118:4709–20.

Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, Couvineau A, Kuwasako K, Tilakaratne N, Sexton PM. Novel receptor partners and function of receptor activity-modifying proteins. J Biol Chem. 2003;278:3293–7.

Dackor R, Fritz-Six K, Smithies O, Caron K. Receptor activity-modifying proteins 2 and 3 have distinct physiological functions from embryogenesis to old age. J Biol Chem. 2007;282:18094–9.

Eftekhari S, Salvatore CA, Calamari A, Kane SA, Tajti J, Edvinsson L. Differential distribution of calcitonin gene-related peptide and calcitonin gene-related peptide receptor components (calcitonin receptor-like receptor and receptor activity-modifying protein 1) in the human trigeminal ganglion. Neuroscience. 2010;169:683–96.

Fritz-Six KL, Dunworth WP, Li M, Caron KM. Adrenomedullin signaling is necessary for murine lymphatic vascular development. J Clin Invest. 2008;118:40–50.

Johnson EC, Shafer OT, Trigg JS, Park J, Schooley DA, Dow JA, Taghert PH. A novel diuretic hormone receptor in *Drosophila*: evidence for conservation of CGRP signaling. J Exp Biol. 2005;208:1239–46.

Koth CM, Abdul-Manan N, Lepre CA, Connolly PJ, Yoo S, Mohanty AK, Lippke JA, Zwahlen J, Coll JT, Doran JD, Garcia-Guzman M, Moore JM. Refolding and characterization of a soluble ectodomain complex of the calcitonin gene-related peptide receptor. Biochemistry. 2010;49:1862–72.

Kusano S, Kukimoto-Niino M, Akasaka R, Toyama M, Terada T, Shirouzu M, Shindo T, Yokoyama S. Crystal structure of the human receptor activity-modifying protein 1 extracellular domain. Protein Sci. 2008;17:1907–14.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature. 1998;393:333–9.

Morfis M, Tilakaratne N, Furness SG, Christopoulos G, Werry TD, Christopoulos A, Sexton PM. Receptor activity modifying proteins differentially modulate the G protein-coupling efficiency of amylin receptors. Endocrinology. 2008;149:5423–31.

Nag K, Kato A, Nakada T, Hoshijima K, Mistry AC, Takei Y, Hirose S. Molecular and functional characterization of adrenomedullin receptors in pufferfish. Am J Physiol Regul Integr Comp Physiol. 2006;290:R467–78.

Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, Muff R, Fischer JA, Foord SM. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev. 2002;54:233–46.

Sexton PM, Poyner DR, Simms J, Christopoulos A, Hay DL. Modulating receptor function through RAMPs: can they represent drug targets in themselves? Drug Discov Today. 2009;14:413–9.

Tam CW, Husmann K, Clark NC, Clark JE, Lazar Z, Ittner LM, Götz J, Douglas G, Grant AD, Sugden D, Poston L, Poston R, McFadzean I, Marber MS, Fischer JA, Born W, Brain SD. Enhanced vascular responses to adrenomedullin in mice overexpressing receptor-activity-modifying protein 2. Circ Res. 2006;98:262–70.

Zhang Z, Winborn CS, Marquez de Prado B, Russo AF. Sensitization of calcitonin gene-related peptide receptors by receptor activity-modifying protein-1 in the trigeminal ganglion. J Neurosci. 2007;27:2693–703.

Ran

Carlo Petosa
Institut de Biologie Structurale Jean-Pierre Ebel, UMR 5075 (CEA/CNRS/Université Joseph Fourier), Grenoble, France

Synonyms

Gsp1; Spi1; TC4

Historical Background

Ran is an abundant member of the Ras superfamily of small GTPases that is highly conserved across eukaryotes. Originally cloned from a human teratocarcinoma cell line as one of four novel genes with sequence homology to the GTP-binding domain of Ras (Drivas et al. 1990), the gene was initially named *TC4* (*t*eratocarcinoma *c*lone 4) and found to encode a protein of 216 amino acid residues. TC4 was markedly different

from other members of the Ras superfamily in two respects: it lacked the sites required for post-translational lipid modification and it was primarily localized to the nucleus. Accordingly, it was renamed Ran for *Ra*s-related *n*uclear protein. Ran was subsequently purified as an essential cofactor for nuclear protein import (Moore and Blobel 1993) and over the following years was extensively characterized for its role in regulating nucleocytoplasmic transport. Ran was later discovered to be critical for mitotic spindle assembly (Carazo-Salas et al. 1999; Ohba et al. 1999) and for the post-mitotic assembly of the nuclear envelope (Hetzer et al. 2000; Zhang and Clarke 2000) and of nuclear pore complexes (Walther et al. 2003). Ran has more recently been implicated in diverse processes, including centrosome duplication (Budhu and Wang 2005), apoptosis (Wong et al. 2009), injury response signaling in neurons (Yudin and Fainzilber 2009), and ciliary trafficking (Dishinger et al. 2010).

Regulation of Guanine Nucleotide State and Subcellular RanGTP Distribution

Like other GTPases, Ran undergoes cycles of GTP exchange and subsequent hydrolysis to GDP. The rates of nucleotide exchange and hydrolysis by Ran are intrinsically low, and in vivo these reactions require accessory factors to proceed at physiological rates. GTP hydrolysis is stimulated by a GTPase-activating protein, RanGAP, and further enhanced by the RanGTP-binding proteins RanBP1 and RanBP2, whereas the replacement of GDP with GTP is accelerated by a guanine nucleotide exchange factor, RanGEF, which is called Regulator of Chromosome Condensation 1 (RCC1) in vertebrates (Binding partners of Ran are summarized in Table 1). These proteins act together to define an enzymatic cycle whereby Ran hydrolyzes GTP to GDP, releases the GDP, and accepts a new molecule of GTP (Fig. 1). Importantly, the accessory factors that modulate this cycle are distributed asymmetrically in the cell. RanGEF has a high affinity for chromatin and is restricted to the nucleus during interphase, whereas RanGAP, RanBP1, and RanBP2 localize to the cytosol or to the cytosolic face of the nuclear envelope. This gives rise to an asymmetric distribution of Ran, with the GTP- and GDP-bound forms prevailing in the nucleus and cytosol, respectively.

The function of Ran depends on its conformation, which in turn is determined by the state of the guanine nucleotide bound to it (Fig. 2). As with other GTPases, the conformational changes in Ran involve two regions, called switch I and II, that are sensitive to the presence of the γ-phosphate of GTP (Wittinghofer and Vetter 2011). In addition, Ran possesses a 40-residue C-terminal extension which also displays a nucleotide-dependent conformation. In the GDP-bound state, the C-terminal extension folds intimately against the core domain of Ran; in the GTP-bound state, this region detaches from the core and becomes highly solvent accessible. Thus, the GDP- and GTP-bound conformations of Ran are strikingly different, allowing Ran-interacting factors to discriminate between RanGDP and RanGTP with high selectivity. Point mutations in Ran that either block GTPase activity or prevent RanGTP generation by inhibiting RanGEF-mediated nucleotide exchange have been instrumental in elucidating the role of Ran in diverse cellular pathways. These mutations are listed in Table 1.

Role in Nucleocytoplasmic Transport

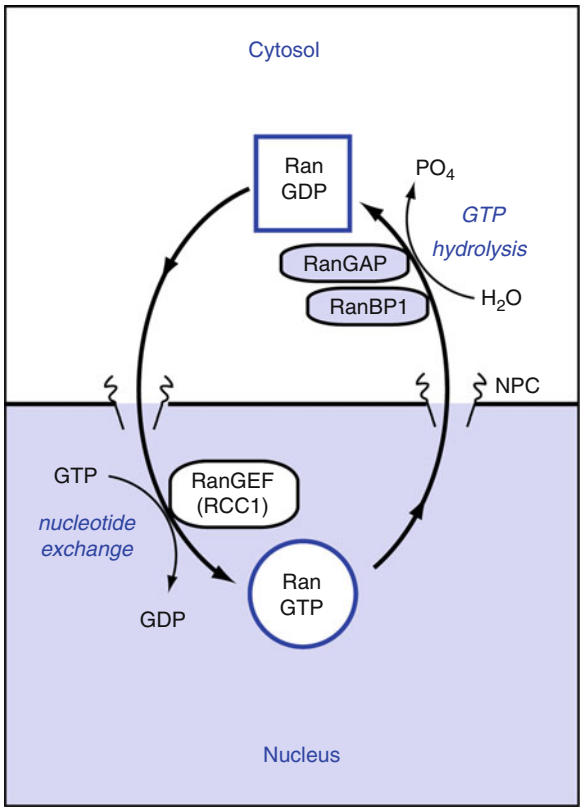
Ran regulates nuclear transport during interphase by acting as a molecular switch for the karyopherin-β family of nuclear transport receptors, also known as importins and exportins (Fried and Kutay 2003). Karyopherins are responsible for delivering various classes of macromolecular cargo through the nuclear pore complex (NPC). Representative members include Importin β, which cooperates with the adaptor protein Importin α to deliver proteins bearing a basic nuclear localization signal (NLS) from the cytosol to the nucleus; Transportin 1, which mediates the nuclear import of ribosomal proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs); CRM1/Exportin 1, which mediates the nuclear export of proteins bearing a leucine-rich nuclear export signal (NES); and Xpot, which exports tRNA. Additional family members are listed in Table 1.

Crystal structures determined for a number of transport receptors, both in isolation and in complex with binding partners, have greatly elucidated how these proteins function (Cook and Conti 2010; Stewart 2007). Karyopherins are superhelical structures made of tandem HEAT repeats that interact with the

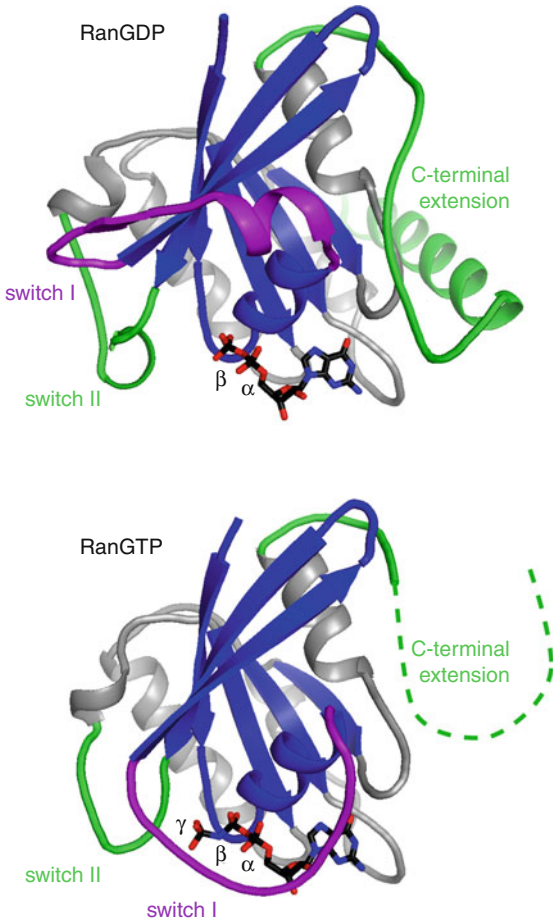
Ran, Table 1 Ran point mutants and binding partners

Protein	Function
Ran G19V, L43E, Q69L	Ran point mutants locked in the GTP-bound form due to lack of GTPase activity
Ran T24N	Ran point mutant that binds to RanGEF and inhibits its exchange activity, thereby preventing RanGTP generation
RanGAP	GTPase-activating protein for Ran. Localizes to the cytoplasm and nuclear pores during interphase and to mitotic spindle during mitosis
RanGEF/RCC1	Guanine nucleotide exchange factor for Ran. Associates with chromatin throughout the cell cycle
RanBP1	RanGTP-binding protein that localizes to the cytoplasm of nondividing cells. Acts as a cofactor for RanGAP, enhancing the rate of GTP hydrolysis on Ran
RanBP2/Nup358	Nucleoporin that localizes to the cytosolic face of the nuclear pore complex (NPC). Acts as a cofactor for RanGAP, enhancing the rate of GTP hydrolysis on Ran
RanBP3	Cofactor for CRM1-mediated nuclear export
RanBPM/RanBP9	Centrosomal protein that interacts with the GTP-bound form of Ran and is required for correct nucleation of microtubules
RanBP10	Tubulin-binding protein and cytoplasmic guanine nucleotide exchange factor for Ran
NTF2/p10	Import carrier for RanGDP
Mog1	Stimulates release of GTP from Ran. In combination with RanBP1 promotes GDP release and the selective binding of GTP to Ran
Dis3	Exoribonuclease subunit of the RNA-processing exosome complex that enhances the nucleotide-releasing activity of RCC1
Karyopherin-β family members	
Importin β/Importin β1	Associates with Importin α to mediate the nuclear import of proteins with a basic nuclear localization signal (NLS). Associates with Snurportin1 to mediate the import of UsnRNPs
Transportin 1/Importin β2	Nuclear import receptor for diverse RNA-binding proteins
	Transports the Kif17 motor protein and retinitis pigmentosa 2 protein to the primary cilium
Transportin SR	Nuclear import receptor for serine/arginine-rich (SR) proteins
Transportin SR2/ Transportin 3	Nuclear import receptor for SR proteins, stem-loop binding protein (SLBP), HIV integrase
Importin 4	Nuclear import receptor for histones, ribosomal proteins, vitamin D receptor, transition protein 2
Importin 5/Importin β3	Nuclear import receptor for histones, ribosomal proteins, recombinase protein RAG-2
Importin 7	Nuclear import receptor for ribosomal proteins, glucocorticoid receptor, HIV reverse transcriptase complex. Heterodimerizes with importin beta to import histone H1
Importin 8	Nuclear import receptor for Smad4 and signal recognition particle protein 19 (SRP19)
Importin 9	Nuclear import receptor for core histones and ribosomal proteins
Importin 11	Nuclear import receptor for ribosomal protein L12 and for the class III ubiquitin conjugating enzymes UbcM2, UbcH6, and UBE2E2
Importin 13	Nuclear import receptor for Ubc9, Rbm8, Mago-Y14, Pax6
	Export receptor for translation initiation factor eIF1A
CAS	Export receptor for Importin α
CRM1/Exportin1	Export receptor for proteins bearing a leucine-rich nuclear export signal (NES). Cooperates with Ran to recruit nucleophosmin to centrosomes
Exportin-t/Xpot	Export receptor for tRNAs
Exportin 4	Export receptor for eukaryotic translation initiation factor 5A (eIF5A) and Smad3. Import receptor for transcription factors Sox2 and SRY
Exportin 5	Export receptor for microRNA precursors and 60S ribosomal subunit
Exportin 6	Export receptor for profilin/actin complexes
Exportin 7	Export receptor for p50RhoGAP, 14-3-3σ

GTP-bound form of Ran. RanGTP binds to the N-terminal half of these proteins, while the cargo generally binds to the C-terminal half (Fig. 3a). RanGTP and cargo bind to importins in a mutually exclusive manner, whereas they bind to exportins cooperatively. This difference in binding mode, combined with the asymmetric distribution of RanGTP across the nuclear envelope, ensures the directionality of nuclear



Ran, Fig. 1 Regulation of Ran's guanine nucleotide state. The guanine nucleotide exchange factor RanGEF localizes to the nucleus, while the GTPase-activating protein RanGAP and the RanGTP-binding protein RanBP1 localize to the cytosol. This gives rise to an asymmetric subcellular distribution of RanGTP, which is at high concentration in the nucleus and at low concentration in the cytosol. *NPC* nuclear pore complex

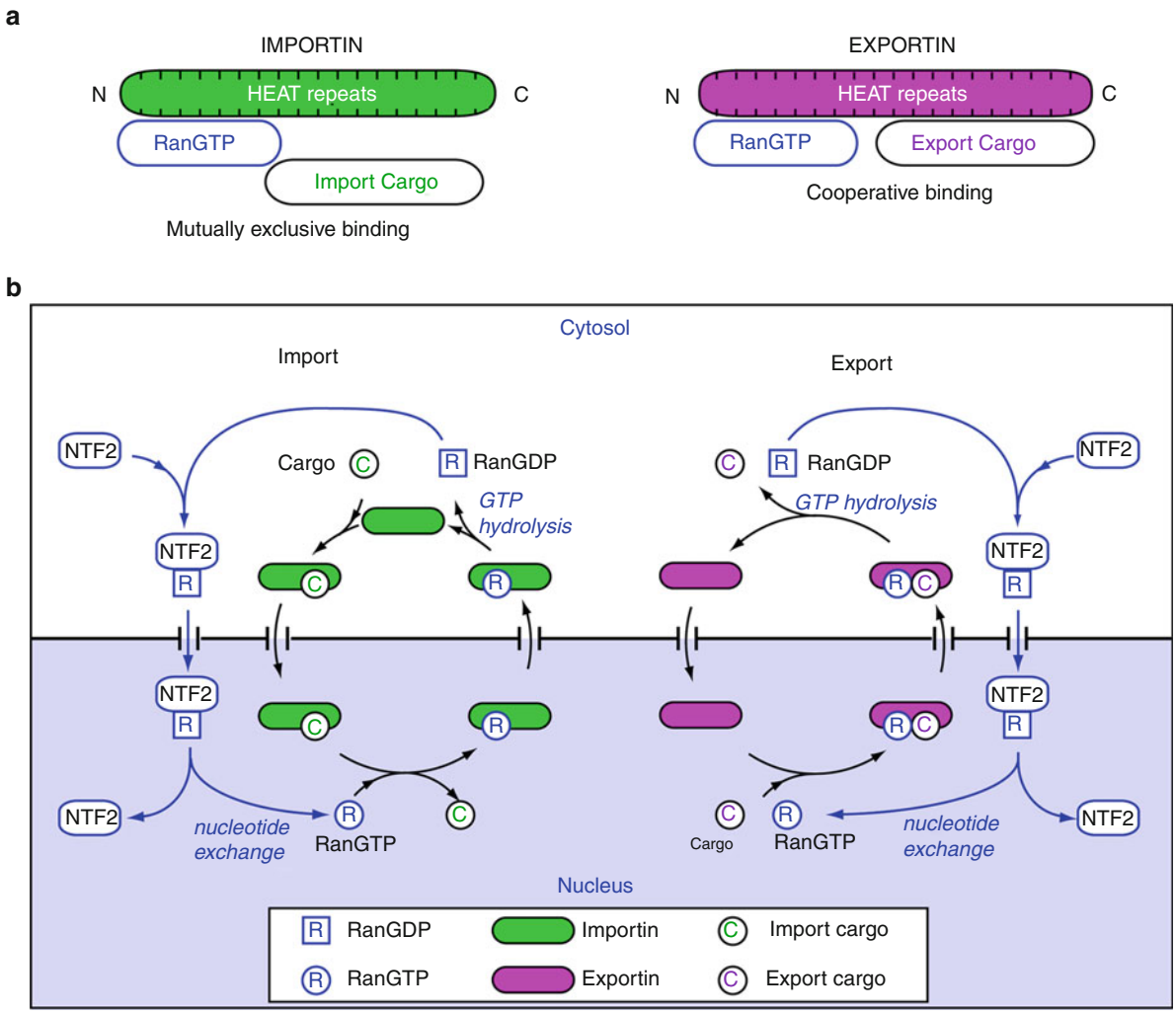


Ran, Fig. 2 Structure of Ran in the GDP- and GTP-bound states. The conformations of the switch I and II regions and of the C-terminal extension depend on the bound nucleotide. GDP and GTP are shown as stick models, with the α, β, and γ phosphate groups labeled. Structures shown are those of pdb entries 1BYU (RanGDP) and 1IBR (RanGTP)

transport. Thus, importins bind their cargo in the cytosol, translocate through the NPC, and release the cargo in the nucleus upon encountering RanGTP (Fig. 3b). In contrast, exportins associate with their cargo in the nucleus together with RanGTP, forming a ternary complex that traverses the NPC and subsequently dissociates in the cytosol. In the cytosol, the binding of RanBP1 releases RanGTP from importins and exportins, and rebinding is prevented by RanGAP-mediated hydrolysis of Ran to the GDP-bound state. Nuclear transport factor 2 (NTF2) recycles RanGDP to the nucleus, where RanGEF mediates conversion to the GTP-bound form. RanGTP, thus, acts as a positional cue that defines the nuclear compartment and directs the disassembly and assembly of import and export complexes, respectively.

Role in Mitotic Spindle Organization

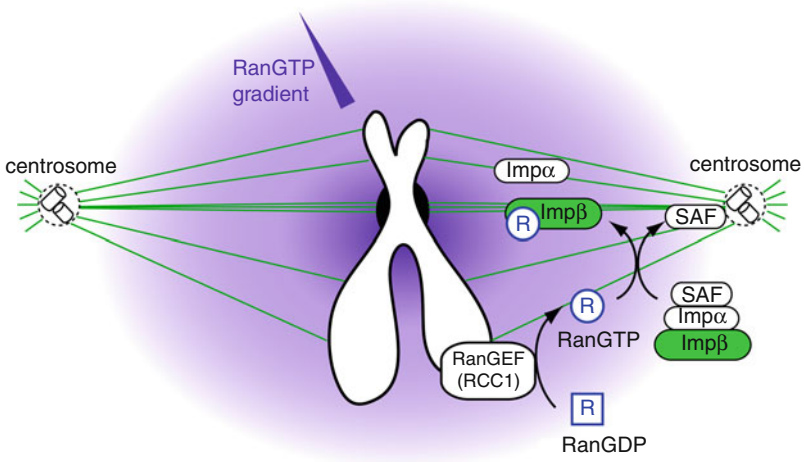
In addition to regulating nucleocytoplasmic transport during interphase, Ran plays an important role during mitosis, regulating several aspects of mitotic spindle assembly (Clarke and Zhang 2008; Gruss and Vernos 2004). These include microtubule nucleation, microtubule stability, production of antiparallel microtubule arrays, and the focusing of spindle poles. Many of these functions for Ran were discovered and characterized in studies using *Xenopus laevis* egg extracts. The importance of Ran during mitosis has been confirmed in mammalian somatic cells, where, for example, Ran regulates microtubule attachment to



Ran, Fig. 3 Role of Ran in nucleocytoplasmic transport. (**a**) Mode of interaction with importins and exportins. Whereas RanGTP and cargo associate with importins in a mutually exclusive manner, they associate with exportins cooperatively. (**b**) Generic import (*left*) and export (*right*) pathways mediated by karyopherin β members. Nucleotide exchange in the nucleus is mediated by RanGEF/RCC1, while GTP hydrolysis in the cytosol is promoted by RanGAP and RanBP1 or RanBP2

kinetochores, is required for microtubule nucleation by both centrosomes and kinetochores, and regulates centrosome cohesion during spindle pole formation (Arnaoutov and Dasso 2005; Roscioli et al. 2010).

The molecular mechanism by which RanGTP affects mitotic spindle assembly is closely related to that by which it regulates nucleocytoplasmic transport. After nuclear envelope breakdown at the onset of mitosis, RanGTP is concentrated near chromosomes by chromatin-bound RanGEF, while RanGAP and RanBP1 promote GTP hydrolysis by Ran distal to chromatin. This gives rise to a concentration gradient of RanGTP that is centered on chromosomes (Fig. 4). RanGTP influences the organization of the mitotic apparatus by activating spindle assembly factors (SAFs), which mediate microtubule stabilization and spindle assembly. One example of a RanGTP-activated SAF is TPX2 (*T*argeting *P*rotein for *X*klp2). TPX2 promotes spindle formation by targeting the kinesin-like motor protein Xklp2 to microtubule minus ends and by activating Aurora A kinase, which is involved in centrosome function. Another SAF activated by RanGTP is NuMA (*Nu*clear *M*itotic *A*pparatus protein). NuMA associates with the motor protein



Ran, Fig. 4 Role of Ran in mitotic spindle assembly. The binding of RanGTP to Importin β in the vicinity of chromosomes causes the release of spindle assembly factors such as TPX2 and NuMA. The gradient of RanGTP established by chromatin-bound RanGEF is indicated by the magenta shading. *SAF* spindle assembly factor, *Impα* importin α, *Impβ* importin β

dynein and its motility-activating complex dynactin, and translocates along microtubules to the spindle poles where it organizes and tethers microtubules to spindle poles. TPX2 and NuMA are inhibited by the Importin α/β heterodimer, which bind to the NLS motifs of these SAFs. Near chromatin, RanGTP binds and displaces Importin β, releasing and activating the SAFs. RanGTP, thus, acts as a positional marker that ensures the correct spatial regulation of spindle assembly in the vicinity of chromosomes.

Role in Nuclear Envelope Assembly

In higher eukaryotes, the nuclear envelope is reconstituted around the segregated DNA at the end of mitosis. This process occurs in three steps: first, membrane vesicles are recruited to the vicinity of chromatin; next, these vesicles fuse into a continuous nuclear membrane; and finally, nucleoporins assemble to form NPCs that insert into the nuclear envelope. Although the mechanisms underlying these events are not fully understood, Ran clearly plays an important role. RanGTP stimulates membrane fusion and nuclear pore assembly, while Importin β negatively regulates these events. More specifically, artificial beads coated with Ran and added to *X. laevis* egg extracts or other cell-free systems accumulate membrane vesicles that fuse into a continuous lipid layer, incorporate nucleoporins, and form NPCs in the absence of chromatin (Zhang and Clarke 2000). Both the generation of RanGTP by RanGEF/RCC1 and GTP hydrolysis by Ran are required for membrane fusion to occur (Hetzer et al. 2000). The generation of RanGTP is also required to release specific nucleoporins from Importin β, to target these proteins to chromatin and to allow the association of NPC subcomplexes (Walther et al. 2003).

Additional Functions of Ran

1. *Centrosome duplication*. Centrosomes, the major microtubule organizing center of mammalian cells, are duplicated once and only once during the G1/S transition of the cell cycle. Ran and the nuclear export receptor CRM1 help orchestrate this event through their effect on nucleophosmin (NPM), which has been implicated as a licensing factor that regulates centrosome synthesis (Budhu and Wang 2005). A fraction of Ran and CRM1 localizes to centrosomes, where they recruit NPM through the latter's leucine-rich NES motif to form a centrosomal CRM1/Ran/NPM complex. Mutation of the NES motif or inactivation of CRM1 leads to dissociation of NPM from centrosomes and to the premature initiation of centrosome duplication. Viral oncoproteins that cause abnormal centrosome duplication (e.g., adenovirus E1A and human papillomavirus E7 proteins) also interact physically with Ran and disrupt its centrosomal regulatory functions (Lavia et al. 2003).
2. *Apoptosis*. Apoptosis triggered by DNA damage leads to the redistribution of Ran from the nucleus to the cytosol and to an overall reduction in

RanGTP levels (Wong et al. 2009). This redistribution has been linked to the action of Mst1, a kinase localized primarily to the cytosol but which accumulates in the nucleus during apoptosis following caspase-mediated cleavage of its nuclear export signal. Nuclear Mst1 phosphorylates serine residue S14 on histone H2B. This leads to RanGEF becoming more tightly bound to chromosomes and to inhibition of its guanine nucleotide exchange activity toward Ran. The resulting dissipation of nuclear RanGTP blocks entry into the nucleus of ▶ NF-κB, a transcription factor with an important role in rescuing cells from apoptosis. Thus, dissipation of the RanGTP gradient due to histone phosphorylation prevents the initiation of an anti-apoptotic program.

3. *Ciliary trafficking*. The primary cilium is a microtubule-based organelle that projects from the cell surface and transduces environmental stimuli into intracellular signals. Entry of proteins into cilia appears to be regulated at the base of the cilia at a region known as the transition zone, where a structure analogous to the NPC has been proposed to exist. Studies of the microtubule motor Kif17 revealed a role for Ran in regulating protein entry into primary cilia which is strikingly similar to Ran's role in nucleocytoplasmic transport (Dishinger et al. 2010). Targeting of Kif17 to primary cilia depends on a short carboxy-terminal sequence that contains several basic residues and shares similarities with NLSs. This "ciliary localization signal" (CLS) mediates binding to the nuclear import receptor Transportin 1. The Kif17/Transportin 1 complex is then targeted to the intraciliary compartment, where high RanGTP concentrations release the motor protein from the importin. Ciliary import of Kif17 thus mirrors the nuclear import of an importin/cargo complex and its dissociation by nuclear RanGTP.
4. *Neuronal processes*. Ran appears to play an important role in neuron development, as RNAi knockdown of Ran revealed defects in neuron development in both drosophila and mouse neurons, while the Ran-binding protein RanBPM has been implicated in cytoplasmic signaling in neuronal processes and in the regulation of neuronal outgrowth (Yudin and Fainzilber 2009). A role for Ran has also been identified in the regulation of retrograde injury signaling in peripheral sensory neurons. In axons, RanGTP forms part of a multimeric complex that includes the nuclear export receptor CAS, Importin α, and the microtubule-associated motor protein dynein. Nerve injury causes an increase in the cytoplasmic levels of RanBP1, RanGAP, and Importin β. RanBP1 and RanGAP induce the release of RanGTP and CAS from the Importin α/dynein complex and promote the hydrolysis of RanGTP to RanGDP. This allows the newly translated Importin β to associate with Importin α/dynein, thereby creating a retrograde injury-signaling complex capable of binding (via Importin α) NLS-bearing signaling cargos. As with the ciliary trafficking example above, these findings show that Ran can act as a regulator of importin-dependent transport and signaling at sites that are distant from the nucleus.

Summary

Ran regulates several fundamental processes throughout the cell cycle, including nucleocytoplasmic transport during interphase, the organization of the mitotic apparatus after nuclear envelope breakdown, the reassembly of the nuclear envelope after mitosis, and duplication of the centrosome. Ran also plays a role in more specialized processes, such as ciliary trafficking, the apoptotic response to a variety of conditions, and neuronal development and injury signaling. In many of these processes, Ran functions as a spatial marker describing where the chromatin or nucleus is. A commonly observed feature is the role played by transport receptors of the karyopherin β family, which sequester different activators and inhibit their function until relieved by RanGTP. A challenge for the future will be to understand these Ran-regulated processes in detail, defining all the players involved and the molecular mechanisms by which these events are precisely orchestrated during the cell cycle.

References

Arnaoutov A, Dasso M. Ran-GTP regulates kinetochore attachment in somatic cells. Cell Cycle. 2005;4:1161–5.

Budhu AS, Wang XW. Loading and unloading: orchestrating centrosome duplication and spindle assembly by Ran/Crm1. Cell Cycle. 2005;4:1510–14.

Carazo-Salas RE, Guarguaglini G, Gruss OJ, Segref A, Karsenti E, Mattaj IW. Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. Nature. 1999;400:178–81.
Clarke PR, Zhang C. Spatial and temporal coordination of mitosis by Ran GTPase. Nat Rev Mol Cell Biol. 2008;9:464–77.
Cook AG, Conti E. Nuclear export complexes in the frame. Curr Opin Struct Biol. 2010;20:247–52.
Dishinger JF, Kee HL, Jenkins PM, Fan S, Hurd TW, Hammond JW, Truong YN, Margolis B, Martens JR, Verhey KJ. Ciliary entry of the kinesin-2 motor KIF17 is regulated by importin-beta2 and RanGTP. Nat Cell Biol. 2010;12:703–10.
Drivas GT, Shih A, Coutavas E, Rush MG, D'Eustachio P. Characterization of four novel ras-like genes expressed in a human teratocarcinoma cell line. Mol Cell Biol. 1990;10:1793–8.
Fried H, Kutay U. Nucleocytoplasmic transport: taking an inventory. Cell Mol Life Sci. 2003;60:1659–88.
Gruss OJ, Vernos I. The mechanism of spindle assembly: functions of Ran and its target TPX2. J Cell Biol. 2004;166:949–55.
Hetzer M, Bilbao-Cortes D, Walther TC, Gruss OJ, Mattaj IW. GTP hydrolysis by Ran is required for nuclear envelope assembly. Mol Cell. 2000;5:1013–24.
Lavia P, Mileo AM, Giordano A, Paggi MG. Emerging roles of DNA tumor viruses in cell proliferation: new insights into genomic instability. Oncogene. 2003;22:6508–16.
Moore MS, Blobel G. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature. 1993;365:661–3.
Ohba T, Nakamura M, Nishitani H, Nishimoto T. Self-organization of microtubule asters induced in *Xenopus* egg extracts by GTP-bound Ran. Science. 1999;284:1356–8.
Roscioli E, Bolognesi A, Guarguaglini G, Lavia P. Ran control of mitosis in human cells: gradients and local signals. Biochem Soc Trans. 2010;38:1709–14.
Stewart M. Molecular mechanism of the nuclear protein import cycle. Nat Rev Mol Cell Biol. 2007;8:195–208.
Walther TC, Askjaer P, Gentzel M, Habermann A, Griffiths G, Wilm M, Mattaj IW, Hetzer M. RanGTP mediates nuclear pore complex assembly. Nature. 2003;424:689–94.
Wittinghofer A, Vetter IR. Structure-function relationships of the G domain, a canonical switch motif. Annu Rev Biochem. 2011;80:943–71.
Wong CH, Chan H, Ho CY, Lai SK, Chan KS, Koh CG, Li HY. Apoptotic histone modification inhibits nuclear transport by regulating RCC1. Nat Cell Biol. 2009;11:36–45.
Yudin D, Fainzilber M. Ran on tracks–cytoplasmic roles for a nuclear regulator. J Cell Sci. 2009;122:587–93.
Zhang C, Clarke PR. Chromatin-independent nuclear envelope assembly induced by Ran GTPase in *Xenopus* egg extracts. Science. 2000;288:1429–32.

Ran/M1

► TLR4, Toll-Like Receptor 4

RANK and RANKL

Tomoki Nakashima[1,2] and Hiroshi Takayanagi[1,2,3]
[1]Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan
[2]Japan Science and Technology Agency (JST), Explorative Research for Advanced Technology (ERATO) Program, Takayanagi Osteonetwork Project, Bunkyo-ku, Tokyo, Japan
[3]Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Bunkyo-ku, Tokyo, Japan

RANK and RANKL Family Members

RANK; receptor activator of nuclear factor-κB, TNFRSF11A; tumor necrosis factor receptor superfamily, member 11a, CD265. RANKL; receptor activator of nuclear factor-κB ligand, TNFSF11; tumor necrosis factor (ligand) superfamily, member 11, OPGL; osteoprotegerin ligand, ODF; osteoclast differentiation factor, TRANCE; TNF-related activation-induced cytokine, CD254.

Historical Background

In the late 1980s, an in vitro coculture system for osteoclast formation was established. This system was shown to require cell-to-cell contact between calvarial cells and bone marrow cells for osteoclast differentiation (Suda et al. 1999). Based on this finding, it was proposed that osteoclastogenesis-supporting mesenchymal lineage cells express an osteoclast differentiation factor (ODF) in the form of a membrane-associated protein (Suda et al. 1999). In the late 1990s, the potential inhibitor of osteoclastogenesis osteoprotegerin (OPG) was cloned. OPG is a decoy receptor that associates with a transmembrane protein of the tumor necrosis factor (TNF) superfamily, OPGL, which turned out to be the long-sought ODF (Takayanagi 2007; Theill et al. 2002). Interestingly, immunologists cloned the same molecule as a stimulator of dendritic cells expressed by T cells, and named it receptor activator

of nuclear factor-κB ligand (RANKL), or TNF-related activation-induced cytokine (TRANCE) (Lorenzo et al. 2008). The receptor for RANKL is RANK, a type I transmembrane protein, which assembles into a functional trimer upon ligand binding, which is similar to other members of the TNF receptor family (Nakashima and Takayanagi 2009). The RANK and RANKL system currently provides a paradigm that enables the molecular understanding of the linkage among bone metabolism, the organization of lymphoid tissues, the regulation of body temperature, mammary gland development, and tumorigenesis.

The Role of RANKL in the Bone and the Immune Systems

Mice with a disruption of *Rank* or *Rankl* exhibit severe osteopetrosis accompanied by a defect in tooth eruption owing to a complete lack of osteoclasts. These genetic findings clearly demonstrate that RANK and RANKL are essential for osteoclastogenesis in vivo. In contrast, mice lacking *Opg* exhibit severe osteoporosis due to both an increased number and enhanced activity of osteoclasts (Takayanagi 2007; Theill et al. 2002). In humans, mutations in *RANK, RANKL*, and *OPG* have been identified in patients with bone disorders, including familial expansile osteolysis, autosomal recessive osteopetrosis, and juvenile Paget's disease of bone (Nakashima and Takayanagi 2009).

RANKL functions as a membrane-anchored molecule and is released from the cell surface as a soluble molecule following proteolytic cleavage by matrix metalloproteinases (MMPs) (Nakashima et al. 2000). Both the soluble and membrane-bound forms of RANKL function as agonistic ligands for RANK. However, previous reports have suggested that membrane-bound RANKL is more efficient than soluble RANKL (Nakashima and Takayanagi 2009). In addition, previous studies have indicated that RANKL serves as both a chemotactic and survival factor for osteoclasts, and that RANKL is mainly expressed in cells of mesenchymal lineage such as osteoblasts, bone marrow stromal cells, and synovial cells. RANKL expression can be upregulated by certain osteoclastogenic factors such as vitamin D_3, prostaglandin E_2, parathyroid hormone, interleukin (IL)-1, IL-6, IL-11, IL-17, and TNF-α (Nakashima et al. 2000; Theill et al. 2002). However, the major source of RANKL in vivo remains unclear, since RANKL is expressed by several different cell types in both the bone and bone marrow, including osteoblasts, osteocytes, bone marrow stromal cells, and lymphocytes. A recent report demonstrated that osteocytes embedded within the bone matrix both express a much higher amount of RANKL and have a much greater capacity to support osteoclastogenesis than osteoblasts or bone marrow stromal cells. Furthermore, the crucial role of RANKL expressed by osteocytes was confirmed by the severe osteopetrotic phenotype observed in mice specifically lacking RANKL in osteocytes. These results clearly indicate that the osteocytes are the major source of RANKL in bone remodeling in vivo (Nakashima et al. 2011).

Intriguingly, in addition to the defect in osteoclasts, both RANK- and RANKL-deficient mice are defective in the development and organization of secondary lymphoid tissue (Takayanagi 2007; Theill et al. 2002). However, RANKL-deficient mice also have a reduced thymus size and impaired thymocyte differentiation. Although the mRNA of RANK is present in the thymus of RANKL-deficient mice, RANK-deficient mice do not display any obvious defects in thymocytes. This phenotypic difference in the thymus is the only evident distinction between RANK- and RANKL-deficient mice (Lorenzo et al. 2008; Takayanagi 2007; Theill et al. 2002). This observation suggests that RANKL has the potential to act on another receptor during the course of thymocyte development, a subject which remains to be investigated further. Severe immunodeficiency is not observed in RANKL-deficient mice, nor are there any obvious adverse effects in the immune system due to the administration of anti-RANKL antibody in humans (McClung et al. 2006). The loss of RANKL in T cells seems to be compensated by CD40L in mice (Lorenzo et al. 2008). These observations initially suggested that the immunological function of RANKL is of lesser importance, but recent studies have revealed a crucial role for RANKL in the immune system. RANKL has been shown to play a critical role in a pathological model of inflammatory bowel disease by stimulating dendritic cells (Nakashima and Takayanagi 2009; Takayanagi 2007), suggesting that RANKL is distinctly involved in the activation of dendritic cells under certain autoimmune conditions. On the other hand, keratinocytes express RANKL in response to ultraviolet stimulation of the skin, which appears to activate Langerhans cells and trigger the

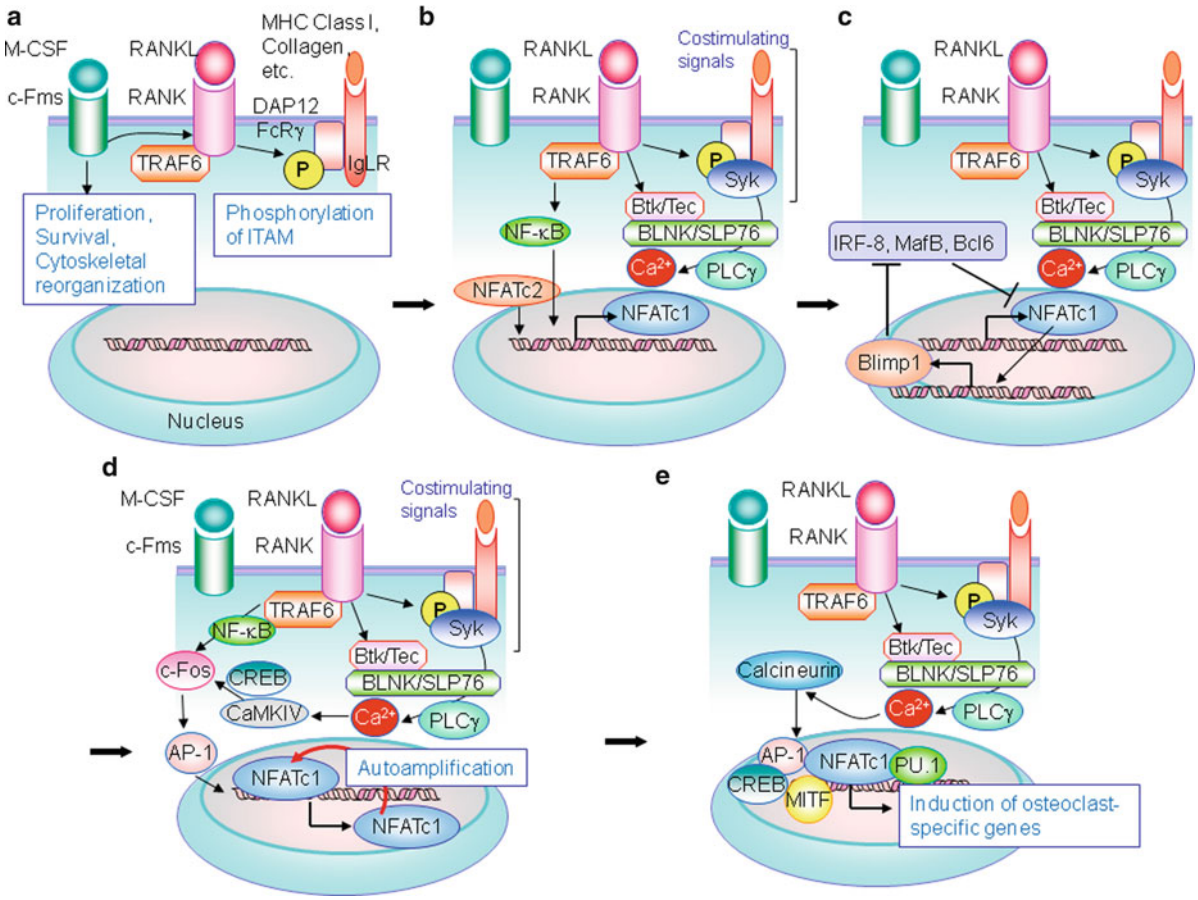
expansion of regulatory T (Treg) cells in draining lymph nodes (Nakashima and Takayanagi 2009). Vitamin D_3, which is produced in the skin in response to sun exposure, has long been known to have immunosuppressive functions and to induce RANKL on osteoclastogenesis-supporting mesenchymal cells in bone. Thus, the suggested role for RANK/RANKL might be the missing link which mediates sunlight-induced immunosuppression. In addition, recent reports suggest that RANK is a key molecule in the development of autoimmune regulator (Aire)-expressing medullary thymic epithelial cells (mTECs), and cooperation between RANK and CD40 also promotes mTEC development, thereby establishing self-tolerance (Nakashima and Takayanagi 2009). Although the functions of RANKL/RANK in the immune system need to be elucidated in greater detail, the discovery and subsequent functional analysis of RANKL has become the driving force behind advances in the understanding of the osteoimmune axis.

The Intracellular Signal Transduction of RANKL

RANK is a transmembrane molecule expressed on osteoclast precursor cells and mature osteoclasts. The ligation of RANK with RANKL results in the commitment of monocyte/macrophage precursor cells to the osteoclast lineage and the activation of mature osteoclasts. RANK lacks intrinsic enzymatic activity in its intracellular domain and transduces signals by recruiting adaptor molecules such as the TNF receptor-associated factor (TRAF) family proteins (Takayanagi 2007). Genetic approaches coupled with intensive molecular analyses have identified ▶ TRAF6 as the main adaptor molecule that links RANK to both osteoclastogenesis and lymph node development (Nakashima and Takayanagi 2009; Takayanagi 2007). By an as yet unknown mechanism, RANKL binding to RANK induces the trimerization of RANK and TRAF6, which leads to the activation of nuclear factor-κB (▶ NF-κB) and certain mitogen-activated kinases (MAPKs), including Jun N-terminal kinase (JNK) and p38. It has not yet been determined how RANK alone, among the TRAF6-binding receptors, is able to stimulate osteoclastogenesis so potently. Additional RANK-specific adaptor molecules may exist which link RANK signaling to other pathways. For example, the molecular scaffold Grb2-associated binding protein 2 (▶ Gab2) and four-and-a-half LIM domain 2 (FHL2) have been shown to be associated with RANK and to exert an important regulatory role in its signal transduction. On the other hand, recent investigation has revealed that the deubiquitinating enzyme CYLD negatively regulates RANK signaling by inhibiting TRAF6 ubiquitination and the activation of downstream signaling events (Nakashima and Takayanagi 2009). The control of the RANK signaling cascade during osteoclastogenesis is summarized in Fig. 1.

The essential role of NF-κB in osteoclastogenesis has been demonstrated genetically (Takayanagi 2007). NF-κB p50 and p52 double-deficient mice develop severe osteopetrosis because of a defect in osteoclastogenesis. The upstream kinase complex that mediates the phosphorylation and degradation of inhibitor of NF-κB (IκB) comprising the catalytic subunits IκB kinase α (IKKα), IKKβ, and the non-catalytic subunit IKKγ (also known as NEMO) are also important for RANK signaling and osteoclastogenesis. In mice, IKKβ is required for RANKL-induced osteoclastogenesis both in vitro and in vivo, whereas IKKα appears to be required only in vitro, not in vivo. Importantly, patients with X-linked osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia, and immunodeficiency (OL-EDA-ID syndrome) bear a X420W point mutation in *IKKγ* (Nakashima and Takayanagi 2009).

The activator protein 1 (AP-1) transcription factor complex is also essential for osteoclastogenesis (Wagner and Eferl 2005). RANK activates AP-1 through an induction of c-Fos. Induction of c-Fos is dependent on the activation of calcium/calmodulin-dependent protein kinase type IV (CaMKIV) and cyclic AMP-responsive element-binding protein (▶ CREB) (Sato et al. 2006), but there are several reports that suggest NF-κB is involved in the c-Fos induction (Nakashima and Takayanagi 2009). In addition, c-Fos expression is induced by treatment with macrophage colony-stimulating factor (M-CSF). Recent investigation has revealed that peroxisome proliferator-activated receptor-γ (PPAR-γ) plays an unexpected role in osteoclastogenesis by directly regulating c-Fos expression (Nakashima and Takayanagi 2009). Thus, it appears that the induction of c-Fos is not regulated by a single pathway.



RANK and RANKL, Fig. 1 *Signaling cascades during osteoclastogenesis*. Osteoclastogenesis is cooperatively induced by M-CSF, RANKL, and its costimulatory factor, immunoglobulin-like receptor. (**a**) Precursor cell stage; the binding of M-CSF to its receptor, c-Fms, activates the proliferation, survival, and cytoskeletal reorganization of osteoclast precursor cells of the monocyte/macrophage lineage and induces RANK expression. The costimulatory receptors appear to be stimulated at early stages. Proximal RANK signals; RANKL binding to RANK results in the recruitment of TRAF6 and, at the same time, the phosphorylation of the ITAM in DAP12 and FcRγ, which are adaptor proteins associating with distinct immunoglobulin-like receptors. (**b**) Initial induction of NFATc1; NFATc1, a master transcription factor for osteoclastogenesis, is initially induced by the TRAF6-activated NF-κB and NFATc2 that are present in the cell before RANKL stimulation. RANK and ITAM signals cooperate to phosphorylate PLCγ and activate calcium signaling, which is critical for the activation of NFATc1. The tyrosine kinases Btk and Tec are activated by RANK and are important for the phosphorylation of PLCγ, thus linking the two pathways. (**c**) Disinhibition of NFATc1; NFATc1 activity is negatively regulated by other transcription factors such as IRF-8, MafB, and Bcl6. The expression of such negative regulators was observed to be repressed in osteoclastogenesis. Blimp1, which is induced by RANKL through NFATc1 during osteoclastogenesis, functions as a transcriptional repressor of anti-osteoclastogenic genes. (**d**) Autoamplification of NFATc1; calcium signal-mediated persistent activation of NFATc1, as well as cooperation with AP-1, is a prerequisite for the robust induction of NFATc1. AP-1 activation is mediated by the induction and activation of c-Fos by CaMKIV-stimulated CREB and c-Fms. The NFATc1 promoter is epigenetically activated through histone acetylation and NFATc1 binds to an NFAT-binding site on its own promoter. (**e**) Induction of osteoclast-specific genes; NFATc1 works together with other transcription factors, such as AP-1, PU.1, CREB, and MITF, to induce various osteoclast-specific genes

Importantly, RANKL specifically and potently induces nuclear factor of activated T cells, cytoplasmic 1 (▶ NFATc1), the master regulator of osteoclast differentiation, and this induction is dependent on both the TRAF6-NF-κB and c-Fos pathways (Takayanagi et al. 2002). The NFAT family of transcription factors was originally discovered in T cells, but its members are involved in the regulation of a variety of biological systems (Crabtree and Olson 2002). The activation of NFAT is mediated by a specific phosphatase,

calcineurin, which is activated by calcium-calmodulin signaling. The essential and sufficient role of the *Nfatc1* gene in osteoclastogenesis has been shown both in vitro and in vivo. The *Nfatc1* promoter contains NFAT binding sites and NFATc1 specifically autoregulates its own promoter during osteoclastogenesis, thus enabling the robust induction of NFATc1 (Takayanagi 2007). AP-1 containing c-Fos, together with continuous activation of calcium signaling, is crucial for this autoamplification (Takayanagi et al. 2002). NFATc1 regulates a number of osteoclast-specific genes in cooperation with other transcription factors such as AP-1, PU.1, and MITF (Takayanagi 2007). Osteoclasts mature into multinuclear giant cells by the fusion of numerous mononuclear osteoclasts. The expression of fusion-mediating molecules such as the d2 isoform of the vacuolar ATPase Vo domain (Atp6v0d2) and the dendritic cell-specific transmembrane protein (DC-STAMP) is directly regulated by NFATc1 (Nakashima and Takayanagi 2009). A previous study indicated that CREB, activated by CaMKIV, also cooperates with NFATc1 in the activation of osteoclast-specific genes (Fig. 1). On the other hand, NFATc1 activity is negatively regulated during osteoclastogenesis by other transcription factors, such as interferon regulatory factor-8 (IRF-8), B cell lymphoma 6 (Bcl6), and v-maf musculoaponeurotic fibrosarcoma oncogene family protein B (MafB) (Miyauchi et al. 2010; Nishikawa et al. 2010; Zhao et al. 2009). The expression of such negative regulators was observed to be repressed through osteoclastogenesis (Fig. 1). This repression is consistent with the notion that high NFATc1 activity is a prerequisite for efficient osteoclastogenesis, but the mechanism by which the expression of these anti-osteoclastogenic regulators is repressed during RANKL-induced osteoclastogenesis has remained obscure. Recent data indicate that B lymphocyte-induced maturation protein-1 (Blimp1), which is induced by RANKL through NFATc1 during osteoclastogenesis, functions as a transcriptional repressor of anti-osteoclastogenic genes such as IRF-8, Bcl6, and MafB (Nishikawa et al. 2010). Therefore, NFATc1 choreographs the determination of cell fate in the osteoclast lineage by inducing the repression of negative regulators as well as through its effect on positive regulators. However, compared with the wealth of information on RANK signaling in osteoclasts, it is as yet unclear whether RANK uses the same signaling mechanisms in the immune system and other systems.

Phospholipase Cγ (PLCγ), which mediates Ca^{2+} release from intracellular stores, is crucial for the activation of the key transcription factor NFATc1 via calcineurin (Takayanagi et al. 2002). However, despite the evident importance of the calcium-NFAT pathway, it had long been unclear how RANKL activates calcium signals. RANK belongs to the TNF receptor family, which has yet to be directly connected to calcium signaling. The activation of PLCγ by RANK requires the protein tyrosine kinase Syk, along with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules, such as DNAX-activating protein (DAP12) and the Fc receptor common gamma chain (FcRγ) (Koga et al. 2004). In the osteoclast lineage, the immunoglobulin-like receptors (IgLR) associated with DAP12 include triggering receptor expressed in myeloid cells 2 (TREM-2) and signal-regulatory protein β1 (SIRPβ1) while those associated with FcRγ include osteoclast-associated receptor (OSCAR) and paired immunoglobulin-like receptor A (PIR-A). As ITAM signals are essential for osteoclastogenesis, but by themselves cannot induce osteoclastogenesis, these signals are most accurately described as costimulatory signals for RANK. The binding of M-CSF to its receptor c-Fms also generates a signaling complex comprised of phosphorylated DAP12 and the nonreceptor tyrosine kinase Syk (Nakashima and Takayanagi 2009). In addition, mutation of TREM-2 or DAP12 in humans leads to Nasu-Hakola disease, which is characterized by bone cysts (Koga et al. 2004; Takayanagi 2007). Thus, RANKL and M-CSF signals appear to converge on the ITAM signaling pathway (Fig. 1).

It is also conceivable that RANK activates an as yet unknown pathway that specifically synergizes with or upregulates ITAM signaling. Tec family tyrosine kinases such as Btk and Tec are activated by RANK and are involved in the phosphorylation of PLCγ, which leads to the release of calcium from endoplasmic reticulum (ER) through the generation of IP3 (Shinohara et al. 2008). An osteopetrotic phenotype in Tec and Btk double-deficient mice revealed these two kinases play an essential role in the regulation of osteoclastogenesis. Tec and Btk had already been reported to play a key role in proximal BCR signaling, but this study established their crucial role in linking the RANK and ITAM signaling pathways (Fig. 1).

This study also identified an osteoclastogenic signaling complex, composed of Tec kinases and scaffold proteins, which affords a new paradigm for understanding the signal transduction mechanisms involved in osteoclast differentiation.

Bone Destruction with Arthritis as a RANKL Disease

In rheumatoid arthritis (RA), a long-standing question is how abnormal T cell activation (characterized by the infiltration of $CD4^+$ T cells) mechanistically induces bone damage. The identification of osteoclast-like giant cells at the interface between synovium and bone in rheumatoid joints dates back to the early 1980s (Takayanagi 2009). These pathological findings led us to hypothesize that osteoclasts play an important role in the bone resorption that occurs in arthritis and that the osteoclasts are formed in the synovium (Takayanagi 2009). Can osteoclasts be generated from synovial cells alone? This question was answered in the affirmative by generating osteoclasts in synovial cell culture without adding any other cells, thus demonstrating that rheumatoid synovial cells contain both osteoclast precursor and osteoclastogenesis-supporting cells (Takayanagi 2009). Further studies indicated that synovial fibroblasts express membrane-bound factor(s) that stimulate osteoclastogenesis and induce the differentiation of synovial macrophages into osteoclasts, but it was not until RANKL was cloned that the membrane-bound factor in synovial cells was brought to light (Takayanagi et al. 2000a).

Importantly, inflammatory cytokines such as IL-1, IL-6, and TNF-α, which are abundant in both the synovial fluid and synovium of RA patients, have a potent capacity to induce RANKL on synovial fibroblasts/osteoblasts and to accelerate RANKL signaling, thus directly contributing to the bone destruction process. Several groups have demonstrated the high expression of RANKL in the synovium of RA patients (Takayanagi 2009). RANKL was shown to be expressed by synovial cells and T cells, both of which are found in the inflamed synovium (Takayanagi 2007, 2009). As RANKL is expressed in activated T cells, T cells may have the capacity to induce osteoclast differentiation by directly acting on osteoclast precursor cells under pathological conditions (Kong et al. 1999). However, ▶ interferon-γ (▶ IFN-γ), which is produced by T cells, potently suppresses RANKL signaling through a rapid degradation of TRAF6 (Takayanagi et al. 2000b). To fully understand the effects of T cells on osteoclastogenesis, it is absolutely necessary to elucidate the specific effects of the various cytokines which T cells produce. It has been shown that IL-17-producing Th17 cells are the exclusive osteoclastogenic T cell subset among the known Th subsets (Takayanagi 2007, 2009). Since even Th17 cells stimulate osteoclastogenesis mainly through RANKL induction on synovilal fibroblasts, it is as yet still unclear how T cell RANKL contributes to bone destruction in the face of synovial fibroblasts expressing RANKL to a higher extent.

Nevertheless, a series of reports has established that the bone damage associated with inflammation is the fundamental pathological condition caused by an abnormal expression of RANKL. Additionally, osteoclast-deficient mice and osteopetorosis patients are protected from bone erosion in arthritis (Kadono et al. 2009; Takayanagi 2009). In the absence of osteoclasts, bone destruction did not occur, despite a similar level of inflammation, indicating that RANKL and osteoclasts are indispensable for the bone loss associated with inflammation. Blocking RANKL by OPG treatment significantly prevented bone destruction in adjuvant arthritis (Kong et al. 1999). Consistent with this, anti-RANKL and anti-osteoclast therapies have been shown in clinical trials as well as in the treatment of an animal model of arthritis to be beneficial for the inhibition of bone loss without affecting the immune system (Takayanagi 2007).

RANK and RANKL in Mammary Gland Development and Tumorigenesis

During pregnancy, increased ductal side branching and the development of lobuloalveolar structures are the result of an expansion and proliferation of ductal and alveolar epithelium (Hennighausen and Robinson 2005). Previously, genetic findings demonstrated that mice with a disruption of *Rank* or *Rankl* fail to develop mammary glands during pregnancy, resulting in the death of newborns (Fata et al. 2000). These mice exhibit normal mammary development and normal ductal elongation and side-branching of the mammary epithelial tree into the mammary fat

pad during puberty. However, their mammary epithelium fails to proliferate and form lobuloalveolar structures during pregnancy (Fata et al. 2000). The mammary gland defect in female RANKL-deficient mice can be reversed by recombinant RANKL treatment. These data clearly indicate that RANKL is an essential regulator of alveolar epithelial cell proliferation. Although RANK is constitutively expressed on mammary epithelial cells, the expression of RANKL is absent in virgin glands, but gradually increases during pregnancy. RANKL expression in mammary epithelial cells is induced by pregnancy hormones such as prolactin, progesterone, and PTHrP (Fata et al. 2000). A previous report showed that kinase-dead IKKα mutant mice display a severe lactation defect due to the impaired proliferation of mammary epithelial cells (Cao et al. 2001). The phenotype can be rescued by mammary-specific overexpression of cyclin D1. These data suggest that IKKα activity in response to RANKL is required for NF-κB activation and cyclin D1 induction in mammary epithelial cells during pregnancy. However, it is reported that cyclin D1 is normally expressed in mammary epithelial cells of RANK-deficient mice, but in these animals there is a defect in nuclear translocation of the basic helix-loop-helix transcriptional regulator, inhibitor of DNA binding 2 (Id2) (Kim et al. 2006). Genetic deletion of Id2 results in a similar phenotype having a lactation defect. Id2 regulates the proliferation of mammary epithelial cells through a suppression of the cell cycle inhibitor p21 in response to RANKL. Thus, RANK/RANKL plays an essential role in mammary gland development, but further study is required to completely elucidate the signaling pathways and transcriptional regulators.

The mammary gland in the period from puberty to menopause develops through tightly choreographed stages of cell proliferation (Hennighausen and Robinson 2005). Steroid hormones such as estrogen and progesterone have a prominent role in both the healthy and diseased states of breast tissue. Reproductive history is the strongest risk factor for breast cancer, and increased risk of breast cancer is correlated with a greater number of ovarian hormone-dependent reproductive cycles (Beral et al. 2005). There is also an increased risk of breast cancer associated with pregnancy in the short term. Although a proliferative role for the steroid hormones in this gland is well accepted, it is still unclear how the mammary gland translates hormonal signals into cell proliferation. Recent studies have implied that RANK/RANKL functions in mammary stem cell (MaSC) biology. MaSC is defined as a cell that can both self-renew and propagate the full spectrum of cell types that make up the mammary gland (Shackleton et al. 2006). The MaSC activity that was increased in mice treated with steroid hormones and pregnancy led to a dramatically increased number of MaSC in mice (Asselin-Labat et al. 2010; Joshi et al. 2010). In contrast, ovariectomy or aromatase inhibitor treatment markedly reduced the MaSC number and outgrowth potential in vivo (Asselin-Labat et al. 2010). In aged mice, MaSCs also display stasis upon cessation of the reproductive cycle (Joshi et al. 2010). MaSCs carry no known receptors for estrogen or progesterone, but these stem cells are highly responsive to steroid hormone signaling. Studies have shown that neutralization of RANKL in pregnant mice reduces the capacity of the MaSC-enriched basal cell population to form colonies. These data suggest that RANKL, a known progesterone target, may act as a crucial molecule that links progesterone-responsive mammary cells to MaSCs.

Hormone replacement therapy (HRT) is associated with an increased risk of breast cancer (Beral et al. 2005). In particular, progesterones or their synthetic derivatives (progestins) such as medroxyprogesterone acetate (MPA) markedly increase the risk of an abnormal mammogram and breast cancer. Recently, it was revealed that MPA treatment triggers the induction of RANKL expression in progesterone receptor (PR)-positive luminal mammary epithelial cells, resulting in autocrine or/and paracrine stimulation of RANK signaling in the mammary epithelium (Schramek et al. 2010). Importantly, specific deletion of RANK in mammary epithelium cells prevents both the onset and progression of MPA-driven mammary cancer and impairs self-renewal of breast cancer stem cells. Tumorigenesis in an MPA-driven tumor model as well as in a spontaneous tumor model was also inhibited by a neutralizing antibody to RANKL (Gonzalez-Suarez et al. 2010). In contrast, mammary-specific overexpression of RANK results in the acceleration of preneoplasias of the mammary glands and an increase in mammary tumor formation after either multiparity or treatment with a carcinogen and progestin (Gonzalez-Suarez et al. 2010). These findings show that the RANK/RANKL system is crucial for tumorigenesis.

Bone is the most common site for the distal spread of breast and prostate cancer (Mundy 2002). Bone metastases result in serious morbidity, including skeletal-related events such as pain, fractures, and hypercalcemia, increasing the mortality risk. Therefore, the clinical priority is to prevent metastases and bone loss owing to excessive osteoclastic bone resorption. Indeed, many clinical trials have evaluated the potential activity of anti-osteoclastic agents in cancer having bone metastases. The representative anti-osteoclastic agents include bisphosphonates and a RANKL neutralizing antibody (Denosumab) (Fornier 2010). A recent clinical study showed that Denosumab is superior to bisphosphonates such as Zoledronic acid for the delay or prevention of skeletal-related events in patients with advanced breast cancer with bone metastases (Stopeck et al. 2010). Interestingly, RANK is highly expressed in several human breast cancer cell lines and primary human breast tumors (Jones et al. 2006). Functionally, it has been shown that RANKL can stimulate the directed migration of mammary epithelial cells as well as prostate cancer and melanoma cells toward a source of RANKL. Furthermore, in an in vivo metastasis model, OPG reduced the tumor burden in bones and ameliorated clinical paralysis, but did not affect the frequency of the spread of metastases into other tissues (Jones et al. 2006). A recent clinical study reported that the level of RANK expression in primary breast cancer positively correlates with the development of bone metastases and may be a predictive marker of bone metastasis risk (Santini et al. 2011). Similar to breast cancer, prostate cancer metastasizes to bone through RANK signaling. Previous reports showed that IKKα activation by RANKL inhibits the expression of Maspin, a metastasis suppressor in prostate epithelial cells. Maspin expression reduced metastatic activity, whereas Maspin ablation restored this activity (Luo et al. 2007). These findings suggest that RANK-expressing tumor cells might sense RANKL as a chemoattractant and migrate in a coordinated fashion to a source of RANKL produced in the bone.

Summary

Bone-related diseases such as osteoporosis and RA afflict a great number of patients. Women taking progesterone derivatives for contraception or HRT have been shown epidemiologically to have an increased risk of breast cancer. These diseases are presenting a tremendous burden to the health care costs. Genetic approaches have established that the RANKL/RANK system is the central regulator of osteoclastogenesis, lymph node organogenesis, mammary gland development, and thymic epithelial cell development. In addition, recent data have revealed an entirely novel and unexpected function for RANKL/RANK in female thermoregulation and the central fever response (Hanada et al. 2009). RANKL has attracted the attention of scientists and pharmaceutical companies, since it plays a pivotal role in the pathogenesis of osteoporosis, RA, tumorigenesis, and metastasis. Novel drugs specifically targeting RANK/RANKL and their signaling pathways provide a potential means to revolutionize the treatment of various diseases associated with this pathway (Kearns et al. 2008).

References

Asselin-Labat ML, Vaillant F, Sheridan JM, Pal B, Wu D, Simpson ER, et al. Control of mammary stem cell function by steroid hormone signalling. Nature. 2010;465:798–802.

Beral V, Bull D, Reeves G. Endometrial cancer and hormone-replacement therapy in the million women study. Lancet. 2005;365:1543–51.

Cao Y, Bonizzi G, Seagroves TN, Greten FR, Johnson R, Schmidt EV, et al. IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell. 2001;107:763–75.

Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. Cell. 2002;109(Suppl):S67–79.

Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. Cell. 2000;103:41–50.

Fornier MN. Denosumab: second chapter in controlling bone metastases or a new book? J Clin Oncol. 2010;28:5127–31.

Gonzalez-Suarez E, Jacob AP, Jones J, Miller R, Roudier-Meyer MP, Erwert R, et al. RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. Nature. 2010;468:103–7.

Hanada R, Leibbrandt A, Hanada T, Kitaoka S, Furuyashiki T, Fujihara H, et al. Central control of fever and female body temperature by RANKL/RANK. Nature. 2009;462:505–9.

Hennighausen L, Robinson GW. Information networks in the mammary gland. Nat Rev Mol Cell Biol. 2005;6:715–25.

Jones DH, Nakashima T, Sanchez OH, Kozieradzki I, Komarova SV, Sarosi I, et al. Regulation of cancer cell migration and bone metastasis by RANKL. Nature. 2006;440:692–6.

Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, et al. Progesterone induces adult mammary stem cell expansion. Nature. 2010;465:803–7.
Kadono Y, Tanaka S, Nishino J, Nishimura K, Nakamura I, Miyazaki T, et al. Rheumatoid arthritis associated with osteopetrosis. Mod Rheumatol. 2009;19:687–90.
Kearns AE, Khosla S, Kostenuik PJ. Receptor activator of nuclear factor κB ligand and osteoprotegerin regulation of bone remodeling in health and disease. Endocr Rev. 2008;29:155–92.
Kim NS, Kim HJ, Koo BK, Kwon MC, Kim YW, Cho Y, et al. Receptor activator of NF-κB ligand regulates the proliferation of mammary epithelial cells via Id2. Mol Cell Biol. 2006;26:1002–13.
Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, et al. Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature. 2004;428:758–63.
Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature. 1999;402:304–9.
Lorenzo J, Horowitz M, Choi Y. Osteoimmunology: interactions of the bone and immune system. Endocr Rev. 2008;29:403–40.
Luo JL, Tan W, Ricono JM, Korchynskyi O, Zhang M, Gonias SL, et al. Nuclear cytokine-activated IKKα controls prostate cancer metastasis by repressing Maspin. Nature. 2007;446:690–4.
McClung MR, Lewiecki EM, Cohen SB, Bolognese MA, Woodson GC, Moffett AH, et al. Denosumab in postmenopausal women with low bone mineral density. N Engl J Med. 2006;354:821–31.
Miyauchi Y, Ninomiya K, Miyamoto H, Sakamoto A, Iwasaki R, Hoshi H, et al. The Blimp1-Bcl6 axis is critical to regulate osteoclast differentiation and bone homeostasis. J Exp Med. 2010;207:751–62.
Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer. 2002;2:584–93.
Nakashima T, Takayanagi H. Osteoimmunology: crosstalk between the immune and bone systems. J Clin Immunol. 2009;29:555–67.
Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-κB ligand: modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Commun. 2000;275:768–75.
Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med. 2011;17:1231–4.
Nishikawa K, Nakashima T, Hayashi M, Fukunaga T, Kato S, Kodama T, et al. Blimp1-mediated repression of negative regulators is required for osteoclast differentiation. Proc Natl Acad Sci U S A. 2010;107:3117–22.
Santini D, Schiavon G, Vincenzi B, Gaeta L, Pantano F, Russo A, et al. Receptor activator of NF-κB (RANK) expression in primary tumors associates with bone metastasis occurrence in breast cancer patients. PLoS One. 2011;6:e19234.
Sato K, Suematsu A, Nakashima T, Takemoto-Kimura S, Aoki K, Morishita Y, et al. Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. Nat Med. 2006;12:1410–6.
Schramek D, Leibbrandt A, Sigl V, Kenner L, Pospisilik JA, Lee HJ, et al. Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. Nature. 2010;468:98–102.
Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature. 2006;439:84–8.
Shinohara M, Koga T, Okamoto K, Sakaguchi S, Arai K, Yasuda H, et al. Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals. Cell. 2008;132:794–806.
Stopeck AT, Lipton A, Body JJ, Steger GG, Tonkin K, de Boer RH, et al. Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study. J Clin Oncol. 2010;28:5132–9.
Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev. 1999;20:345–57.
Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol. 2007;7:292–304.
Takayanagi H. Osteoimmunology and the effects of the immune system on bone. Nat Rev Rheumatol. 2009;5:667–76.
Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, et al. Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. Arthritis Rheum. 2000a;43:259–69.
Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, et al. T cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ. Nature. 2000b;408:600–5.
Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling for terminal differentiation of osteoclasts. Dev Cell. 2002;3:889–901.
Theill LE, Boyle WJ, Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. Annu Rev Immunol. 2002;20:795–823.
Wagner EF, Eferl R. Fos/AP-1 proteins in bone and the immune system. Immunol Rev. 2005;208:126–40.
Zhao B, Takami M, Yamada A, Wang X, Koga T, Hu X, et al. Interferon regulatory factor-8 regulates bone metabolism by suppressing osteoclastogenesis. Nat Med. 2009;15:1066–71.

RANTES

► CCL5

Rap GEF Family

Hoa B. Nguyen and Lawrence A. Quilliam
Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, USA

Synonyms

Guanine nucleotide exchange factor

List of Discussed GEFs

RapGEF1: C3G
RapGEF2: PDZ-GEF1, RA-GEF, CnRasGEF, KIAA0313, nRapGEP
RapGEF3: Epac, cAMP-GEF I
RapGEF4: Epac2, cAMP-GEF II
RapGEF5: MR-GEF, Repac, GFR, KIAA0277
RapGEF6: PDZ-GEF2, RA-GEF2
RasGRP2: CalDAG-GEF I
RasGRP3: GRP3, CalDAG-GEF III, KIAA0846
NSP2: BCAR3 (human), AND34 (mouse)
NSP3: Chat, SHEP
SmgGDS: RAP1GDS1

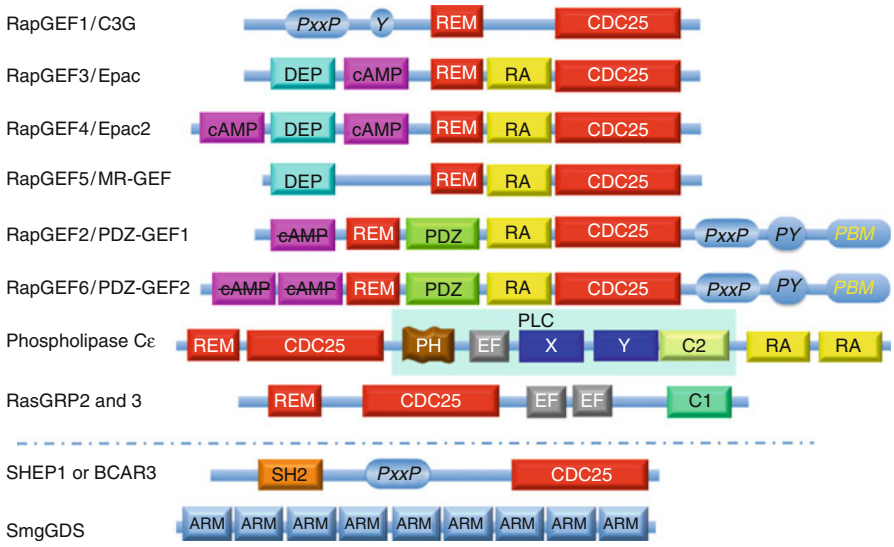
Historical Background

GTP binding proteins are extensively used in nature to regulate biological processes. Most of these proteins act as molecular switches that transition between inactive GDP-bound and active GTP-bound conformations. The largest family of GDP/GTP switches is the Ras superfamily of small-molecular-weight GTP binding proteins that constitute approximately 150 members in mammalian cells. This superfamily can be subdivided into Ras, Rho, Rab, ▶ Ran, and ARF families that regulate a myriad of cellular functions (Takai et al. 2001). True Ras proteins play major roles in coupling cell surface receptors to intracellular signaling pathways that control proliferation, differentiation, and survival. Due to their mutational activation in over 20% of human cancers, much research has focused on H-, K-, and N-Ras. In the shadow of these oncoproteins there are additionally over 30 Ras-related proteins that include the Rap proteins, Rap1A, 1B, Rap2A, 2B, and 2C. These proteins were initially discovered due to sequence similarity to Ras, their ability to revert the actions of Ras, and their abundance in leukocytes (Bokoch 1993). More recently Rap1 has been shown to play multiple roles that include inside-out signaling to control the affinity of integrins for extracellular matrix and consequently cell adhesion and migration. Rap1 also localizes to adherens junctions where it influences cell–cell adhesion and plays a number of additional roles in cell signaling, often overlapping with those of Ras (Raaijmakers and Bos 2009).

So how do Ras/Rap proteins act as molecular switches and how does the extracellular environment control their activity? Ras proteins normally exist in an inactive or resting GDP-bound state. To acquire an active GTP-bound confirmation they must exchange nucleotide. An intrinsic GTPase activity then hydrolyzes the terminal phosphate off the bound GTP enabling return to the inactive confirmation. The intrinsic rates of nucleotide exchange and GTP hydrolysis are in the order of minutes to an hour, not practical for rapid response to the extracellular environment. This means that additional factors must exist to both accelerate and tightly regulate these processes. Guanine nucleotide exchange factors (GEFs) promote the release of GDP whereas GTPase activating proteins (GAPs) have evolved to assist in the rapid hydrolysis of GTP (Quilliam et al. 2002). Whereas inhibition of GAPs and activation of GEFs both tip the balance in favor of Ras-GTP accumulation, nature most frequently uses GEFs to activate Ras proteins and enable biological responses. This article will focus on the nature and regulation of Rap family GEFs.

The baker's yeast CDC25 protein was the first Ras family GEF to be identified. Consequently the catalytic domain is referred to as a CDC25 homology domain. This region is conserved in approximately 35 mammalian GEFs, most of which also contain an REM (Ras exchange motif) that was required for a minimal CDC25 fragment to function in vitro (Quilliam et al. 2002; Raaijmakers and Bos 2009), see Fig. 1. Structural studies on the Ras GEF Sos1 and Rap GEF Epac demonstrated that the REM, which is located various distances N-terminal to the CDC25 homology region, contributes to the stabilization of the core catalytic domain.

Although there are approximately equal numbers of Ras subfamily members and GEFs, there is not a simple monogamous pairing of GEFs to Ras proteins.



Rap GEF Family, Fig. 1 *Schematic representation of Rap GEF functional domains*. The CDC25 homology domain (core catalytic unit) and associated Ras exchange motif (REM) are shown in red; protein kinase C-conserved region 1 (C1) and C2, Ca^{2+} binding motif are shades of light green; Armadillo (ARM) repeats are light blue; cAMP binding domains are pink; ~~cAMP~~ represents similar sequences not thought to bind cyclic nucleotide; DEP (Disheveled, Egl-10 and Pleckstrin) domains are turquoise; Ca^{2+}-binding EF hands are gray, PDZ (PSD-95, Dgl and ZO-1) domains are shown in green with PDZ-binding motifs (PDM), proline-rich SH3 binding motifs (PxxP), protein tyrosine kinase phosphorylation site (Y), and WW domain-binding PY site is shown in light blue. Pleckstrin homology (PH) domain is brown, Ras associated (RA) domains are shown in yellow, Src-homology (SH) 2 domain is orange, and the X and Y domains that form the catalytic domain of phospholipase C are dark blue

Interestingly more GEFs have evolved to regulate Rap than any other group. Rap GEFs contain multiple regulatory domains suggesting that Rap proteins are under the control of diverse extracellular stimuli. Regulation includes protein–protein or protein–lipid interactions, binding of second messengers, and/or posttranslational modifications. The need for so many GEFs may be to activate Rap in different tissues or different locations within a given cell, at key points in development, or in response to different hormones/growth factors, etc., that use unique signaling mechanisms. Recent knockout studies in mice have supported the notion that Rap1A and 1B play fundamental roles in mammalian development and function, and likewise, creation of mice lacking various Rap GEFs (discussed below) support the notion that individual exchange factors play equally critical functions.

RapGEF1/C3G

One of the earliest mammalian Ras family GEFs to be isolated was the *C*rk SH*3*-binding *g*uanine nucleotide exchange factor (C3G) (3, 4). In addition to the CDC25 homology and REM domains, C3G contains central proline-rich sequences that bind to the N-terminal SH3 domain of the Crk adapter protein, a more N-terminal proline-rich motif that associated with ▶ p130Cas, c-Abl, and Hck SH3s, and a central tyrosine residue (Y504) that is phosphorylated by Src family tyrosine kinases (Fig. 1). C3G is perhaps the most ubiquitously expressed Rap GEF. It is activated by numerous cell surface molecules that include integrins, T and B cell receptors, E-cadherin, and various growth factors and G-protein-coupled receptors.

C3G was initially found to activate Rap1 but has since been reported to also promote nucleotide exchange on several other small GTPases: Rap2, R-Ras, and TC10. Interestingly, TC10 is a member of the Rho family of GTPases that are typically regulated by a distinctly different family of GEFs. TC10 plays a role in insulin-stimulated glucose uptake and in 2010 a Korean study linked mutations within C3G to type II diabetes (Hong et al. 2009). Two C3G knockout mice were created and demonstrated embryonic lethality, exemplifying its important

biological role. C3G is frequently localized through its interaction with the Crk and CrkL SH2/SH3-containing adapter proteins. For example, the Abl tyrosine kinase can recruit a CrkL-C3G complex to the immune synapse.

RapGEF3 and 4/Epac1 and 2

The second messenger cyclic adenosine monophosphate (cAMP) exerts many effects on cell biology. While these were classically known to be mediated by the cAMP-dependent protein kinase/protein kinase A (PKA) and olfactory cyclic nucleotide–gated ion channels, two cAMP-activated Rap GEFs were discovered in 1998 that helped explain the PKA-independent actions of cAMP (Quilliam et al. 2002; Raaijmakers and Bos 2009; Gloerich and Bos 2010). They are called Epacs (exchange proteins activated by cAMP) 1 and 2 or cAMP-GEFs. While the former name is most popular (and used herein) their official gene names are RapGEFs 3 and 4.

In addition to the REM/CDC25 exchange region that acts on Rap1 and 2, Epac proteins have either one (Epac1) or two (Epac2) cyclic nucleotide–binding domains (CNBD), a Disheveled, Egl-10, Pleckstrin (DEP) domain, and an RA domain (Fig. 1). Structural studies on Epac2 indicate that the N-terminus folds over the C-terminus and hinders Epacs binding to its substrate Rap. This autoinhibition is relieved by the binding of cAMP to the CNBDs (Gloerich and Bos 2010). Epacs' CNBDs lack a glutamate residue found in PKA and cAMP-gated ion channels that typically interacts with the 2-OH group of the ribose of cAMP. Consequently, Epacs can bind bulky cAMP analogs such as 8-(4-chloro-phenylthio)-2'-*O*-methyladenosine-cAMP (8CPT) where the 2-OH group has been replaced with a O-Me to selectively activate Epac proteins versus other cAMP effectors. These compounds have proven to be very useful tools to implicate Epac in biological events and may have clinical potential (Gloerich and Bos 2010).

Epacs, like most other GEFs and GAPs, regulate Rap proteins in a spatial and temporal manner. For instance, localization of Epac1 and 2 is regulated by their distinct RA domains. The RA domain of Epac2 specifically binds K- and N-Ras (with weaker affinity for H-Ras), enabling Ras-GTP to translocate Epac2, but not Epac1, from the cytosol to the plasma membrane. Consequently, a pool of plasma membrane-bound Rap1 can become activated upon concurrent cAMP and Ras signaling (Li et al. 2006). Meanwhile, the RA domain of Epac1 interacts with Ran, a small G-protein best known for its role in regulating nuclear transport. Ran and its binding partner RanBP2 anchor Epac1 to the nuclear pore, allowing localized Rap1 activation at the nuclear envelope upon cAMP elevation (Liu et al. 2010). In a different scenario, Epac1 can be targeted to the plasma membrane via its DEP domain. This is necessary for Rap to regulate integrin-mediated adhesion at the membrane. However, in Rat1a fibroblasts, peripheral Rap1 activation by Epac1 is counteracted by high RapGAP activity, resulting in predominantly perinuclear Rap-GTP (Gloerich and Bos 2010). Furthermore, in both interphase and mitotic cells, Epac1 is targeted to microtubules by tubulin or the microtubule-associated protein 1 and may play a role in microtubule polymerization. Other reports show Epacs localizing to centrosomes, mitochondria, macrophage phagosomes, the apical epithelial membrane, and regulating the DNA damage–responsive kinase, DNA-PK, in the nucleus. Temporal expression also contributes to Epac action: As monocytes differentiate into macrophages, their Epac protein levels increase threefold and play a role in chemokine secretion.

Epacs regulate a variety of physiological processes that include secretion of insulin from pancreatic beta cells, permeabilization of vascular endothelium, transmigration of leukocytes, and regulation of cardiac calcium channels. Consequently, Epac activity has been associated with diabetes, vascular inflammation, and heart disease. Interestingly, PKA is also involved in these processes demonstrating the close partnership of Epac and PKA in mediating cAMP action.

Using Epac2 knockout mice, Seino's lab established that cAMP potentiates glucose-induced exocytosis via Epac2 rather than PKA. They later demonstrated that Epac2 is the direct target of the antidiabetic sulfonylurea drugs that promote insulin secretion (Zhang et al. 2009). A number of studies have implicated Epacs in inflammation both through regulation of leukocytes and vascular permeability (Borland et al. 2009). In cardiac myocytes, Epacs and another Rap GEF, phospholipase Cε both play critical synergistic roles in calcium-induced calcium release downstream of β-adrenergic receptors.

RapGEF2 and 6/PDZ-GEFI and II

Similar in structure to Epacs are PDZ-GEF-I and II, also called RA-GEF-1 and 2, or official names RapGEFs 2 and 6. PDZ-GEF-I has also been described as CNrasGEF or nRapGEP. Like Epacs, PDZ-GEFs have a REM-CDC25 GEF module, an RA domain, and a region sharing homology with CNBDs (Quilliam et al. 2002; Raaijmakers and Bos 2009) (Fig. 1). However, most reports indicate that cAMP does not bind to this latter region with high affinity. PDZ-GEFs have a PSD-95/DlgA/ZO-1 (PDZ) domain that can bind the β1 adrenergic receptor, potentially linking G-protein-coupled receptors to Ras activation. At its C-terminus, PDZ-GEFs have a proline-rich region and a PDZ-binding motif, which interact with the PDZ domains of cell junctional proteins, MAGI-1 and −2 (Sakurai et al. 2006). This links PDZ-GEF with ▶ β-catenin and contact-induced activation of Rap1 (Sakurai et al. 2006). In addition, two PY motifs at its C-terminus are responsible for binding to the WW domain of the ubiquitin protein ligase Nedd4. This interaction regulates PDZ-GEF protein turnover rate via proteasomal degradation.

PDZ-GEF1 and 2 functions have been studied through gene knockouts in both *Drosophila* and mice. In *Drosophila*, PDZ-GEF (Gef26) regulates DE-cadherin to control stem cell adhesion to its niche. Functional mutation of this GEF leads to loss of cell polarity, impaired adherens junctions, and thus reduction in stem cell number (Wang et al. 2006). Two labs found that PDZ-GEF knockout is embryonic lethal in mice. Kataoka's lab found that vascular development is impaired in PDZ-GEF1 knockout mice at around E7.5 with embryonic lethality occurring by E9.5, while Hou and colleagues found PDZ-GEF2$^{-/-}$ mouse embryos surviving to ~E11.5. Mice died of vasculature defects in the yolk sac and the allantois. Further analysis revealed dysregulation in B-Raf/ERK signaling, and a reduction in Scl/Gata transcription factor expression (Satyanarayana et al. 2010). These molecular signaling events underline the reduction of definitive hematopoietic CD41 cells. Hematopoietic progenitors isolated from these mouse embryos also lacked the potential to form erythroid or granulocyte/macrophage colonies, indicating that PDZ-GEF-2 plays a role in hematopoiesis development and progenitor functions. In addition, deletion of PDZ-GEF-2 late in embryogenesis resulted in defective fetal liver erythropoiesis. However, such deletion in the adult bone marrow, or specific deletion in B-cells, T-cells, hematopoietic stem cells, or endothelial cells had no impact on hematopoiesis (Satyanarayana et al. 2010). This reiterates the importance of temporal control and the role of various GEFs at different stages of development.

Conditional knockout of PDZ-GEF showed other crucial functions of this GEF in neural migration and in splenocyte responses. Mice with dorsal telencephalon-specific PDZ-GEF-1 knockout develop heterotopic cortical mass and commissural fiber defect. Meanwhile, PDZ-GEF-2 and Rap1 mediate TNFα-induced M-Ras activation in order to activate the integrin, lymphocyte function-associated antigen 1 (LFA-1), and subsequently, cell aggregation in response to inflammation (Yoshikawa et al. 2007).

Others also reported PDZ-GEF2 interacts through its PDZ domain with junctional adhesion molecule-A (JAM-A), which also interacts with Afadin/AF6 in human colonic epithelial cells. JAM-A and AF6 both act upstream of a signaling pathway that specifically activates Rap1A but not Rap1B in order to regulate β1 integrin and mediate cell migration (Severson et al. 2009). This is a rare report of differential signaling to Rap1A versus Rap1B.

RapGEF5/MR-GEF

MR-GEF was characterized by numerous groups, as a Rap-specific GEF (acting on Rap1 and 2) (Quilliam et al. 2002; Raaijmakers and Bos 2009). Due to sharing highest homology to Epacs it was referred to as Repac or alternatively as MR-GEF (*M*-Ras *r*egulated) due to the presence of an RA domain that selectively bound to M-Ras-GTP. M-Ras over-expression inhibited Rap1 activation but based on the experiences of us and others it is likely that M-Ras is specifically targeting the GEF to activate a plasma membrane pool of Rap1 at the expense of the activity of bulk GTPase (Li et al. 2006). An apparent splice variant that swaps the first 70 amino acid residues for an alternative 208 residue sequence (NP_036426) places a DEP domain at the extreme N-terminus that may play a role in membrane localization. A DEP domain is also found in Epacs a similar distance from the REM domain (Fig. 1). However, unlike Epacs, MR-GEF does not contain an intervening cAMP binding motif.

MR-GEF expression was induced by exposure to anthrax and expression is also turned on in developing rodent GABAergic neurons. Interestingly, MR-GEF expression is also altered in individuals with bipolar disorder. Correlation of the percentage of MR-GEF expressing neurons and 2D neuronal density between cortical layers II and IV in bipolar disorder support a growing body of evidence for its contribution to defects in cortical organization and communication in this disease (Bithell et al. 2010).

RasGRPs

In addition to RapGEFs 1–6, several other Rap1 exchange factors exist, if not implicitly acknowledged by their official gene names. The four RasGRP (Ras guanyl releasing proteins) or CalDAG-GEF gene products contain an N-terminal REM and CDC25 homology regions that are followed by two tandem Ca^{2+}-binding EF hands similar to those found in calmodulin (Quilliam et al. 2002; Raaijmakers and Bos 2009). Farther C-terminal is a C1 domain similar to the diacylglycerol (DAG)/phorbol ester binding domain found in classical and atypical protein kinases C – hence the CalDAG moniker. Both RasGRP2/CalDAG-GEF1 and RasGRP3 act as GEFs for Rap proteins. GRP2 is specific for Rap1/2 and activates it in a Ca^{2+}-dependent manner (although an N-terminally myristoylated splice variant was reported to also act on N- and K-Ras but to be inhibited by Ca^{2+} elevation). In contrast, RasGRP3 has the broadest substrate specificity of all Ras GEFs, acting on true Ras, R-Ras, and Rap subfamilies (Quilliam et al. 2002; Raaijmakers and Bos 2009). RasGRPs 1 and 4 are Ras-specific.

RasGRPs are highly abundant in brain: RasGRP2 is most highly expressed in the basal ganglia whereas RasGRP3 is found primarily in glial cells of the cerebral and cerebellar white matter. Both GRPs are also highly expressed in cells of hematopoietic origin. GRP2/Cal-DAG1 is particularly abundant in platelets (that are also replete with Rap1b) where they play a major role in coupling chemoattractant receptors to αIIbβ3 integrin activation during "inside-out" signaling as well as in thromboxane A2 release. Leukocyte adhesion deficiency (LAD) syndrome III, characterized by an inability of leukocytes to adhere and migrate during inflammatory and host defense reactions was initially attributed to mutations in RasGrp2 but recent studies suggest that kindlin-3 rather than GEF mutation is the true culprit behind this rare disease.

Phospholipase Cε

Like other phospholipases C (PLCs) PLCε contains X and Y regions that make up the phospholipase catalytic domain. This enzyme cleaves phosphatidyl inositol 4,5 bisphosphate (PIP_2) into the second messengers inositol 3,4,5 trisphosphate (IP_3) and DAG. PLCε similarly possesses, a PH domain, C2 domain, and EF hands that bind to phospholipids and Ca^{2+} (see Fig. 1). However, unlike other PLC isozymes, PLCε is also a Rap GEF. PLCε has both an N-terminal REM/CDC25 Rap GEF module and tandem RA domains located at its C-terminus (Fig. 1). The GEF function of PLCε helps maintain persistent Rap1-GTP levels following G-protein-coupled receptor stimulation (Suh et al. 2008). The C-terminal RA domain (RA2) interacts with activated Ras and Rap1 enabling PLCε recruitment to either the plasma membrane or the perinuclear area, respectively, following growth factor stimulation. Meanwhile, the other RA domain confers protein stability and possibly also autoinhibition.

Significant crosstalk between PLCε and other RapGEFs has been reported and further scenarios can readily be imagined. For example, Ca^{2+} and DAG generated by PLCε activity might promote the activation of GRP2 or 3. Additionally, upon adrenaline or prostaglandin E2 stimulation, G-protein-coupled receptors elevate cAMP levels and activate Epac. This results in Rap2B activation that in turn associates with RA2 of PLCε and induces PIP_2 hydrolysis. PLCε can also be activated by other ligands such as lysophosphatidic acid or sphingosine 1-phosphate that couple to Gα12 and Gα13. In addition, Gα12 and Gα13 can activate various RhoGEFs. GTP-loaded RhoA can bind directly to the phospholipase Y domain and stimulate PLCε activity.

PLCε knock-down or mutation is embryonic lethal in *Caenorhabditis elegans* due to its role in epidermal morphogenesis while $PLC\varepsilon^{-/-}$ mice exhibit multiple cardiac defects. These include ventricular dilation, aortic and pulmonary valve defects, and stenosis due to the thickening of valve leaflets. Additionally, cardiac myocytes from PLCε null mice have a decrease

in contractile response to acute β-adrenergic stimulation. Human kidney development also requires PLCε and truncating mutations in PLCε were found in nearly 30% of children having the nephrotic syndrome, diffuse mesangial sclerosis (Suh et al. 2008). However, loss of PLCε can be advantageous to mice, resulting in reduced susceptibility to carcinogen induced skin tumor formation (Suh et al. 2008). This is likely the result of PLCε mediating both direct agonist-dependent proliferation and an indirect inflammatory response. PLCε both mediates PDGF-, EGF-, and Rho-dependent cell growth and inhibits EGF receptor down-regulation via PIP_2-derived second messengers. Furthermore, PLCε can transduce mitogenic signals through its Rap1 GEF activity. On the other hand, PLCε-null mice have a reduction in phorbol ester-induce edema, granulocyte infiltration, and expression of the proinflammatory cytokine, interleukin-1α.

Additional Potential Rap GEFs

Dock4 is a member of the Dock180/CZH family of Rho GEFs and, in partnership with ELMO, is known to regulate the activity of Rac1. However Dock4 was reported to effectively activate Rap1 in a mouse osteosarcoma cell line (Yajnik et al. 2003). A dominant inhibitory mutant of Rap1 blocked the biological consequences of Dock4 expression and a constitutively active Rap1-63E mutant mimicked Dock4-induced adherens junction formation; however, there is no direct demonstration of RapGEF activity in vitro. Since Rap1 is intimately associated with Rac and adherens junction regulation the effect of Dock4 reported here may have been indirect.

NSP1-3 (novel SH2-containing proteins) have a C-terminal CDC25 homology domain, an N-terminal SH2 domain, and a central proline-rich region that may interact with SH3 domains (Quilliam et al. 2002) (Fig. 1). They have various alternate names such as BCAR3, AND-34, SHEP1, and Chat and have been reported to associate with Rap1. While exchange activity was attributed to NSP2/BCAR3/AND34 in one study, this was likely indirect due to its association with Crk/Src to influence Rap1 and Rac GEF activity. NSPs were identified in breast cancer (BCAR3 = breast cancer antiestrogen resistance gene 3) or associated with receptor tyrosine kinases (EGFR for NSP1, EphB2 for NSP3/SHEP1/Chat). All 3 NSPs associate with the scaffold protein p130 Cas, implicating them in adhesion. Surprisingly the CDC25 homology domain is responsible for this interaction. Thus although NSPs likely play a pivotal role in coupling adhesion receptors and tyrosine kinases to actin cytoskeletal organization their CDC25 domains act as adapter modules rather than as Rap GEFs.

SmgGDS (small-molecular-weight G-protein guanine nucleotide dissociation stimulator) is relegated to last place in this article but was the first mammalian Rap (or Ras family) GEF to be discovered in 1990, reviewed in Quilliam et al. (2002). It is comprised of ARM/armadillo repeats (similar to, e.g., catenins or importins) rather than having a CDC25 homology domain (Fig. 1) and selectivity is based on the presence of positively charged residues in C-terminal tail of Ras proteins: target substrates include Rap1, K-Ras, Rac1, RhoA, and Ral that each have multiple lysine residues in their C-terminal hypervariable regions. SmgGDS (gene name RAP1GDS1) expression is critical during development and appears to possess weak GEF activity and to promote the malignant phenotype of certain cancer cells. However, whether it acts as a true GEF in vivo has always been in question. A recent study suggests that two splice variants of smgGDS increase the activity of polybasic-region-containing GTPases such as Rap1 by facilitating their posttranslational modification by lipids and subsequent transit to the plasma membrane (Berg et al. 2010).

Summary

Multiple Rap GEFs have been characterized at both the molecular level and frequently also in mouse models of development/disease. However there is much still to be learned from animal models. The number of GEFs and variety of regulatory domains suggest that Rap plays key roles in cell signaling that need to be activated in unique spatiotemporal scenarios. While Rap1 plays many roles in cell biology and likely represents a poor drug target, targeting Rap GEFs might result in specific regulation of Rap1 in certain diseases. The 8CPT-cAMP molecule represents a specific activator of Epacs that may impact signaling events in vascular disease and diabetes, but to date no Rap GEF inhibitors have been developed.

References

Berg TJ, Gastonguay AJ, Lorimer EL, Kuhnmuench JR, Li R, Fields AP, et al. Splice variants of SmgGDS control small GTPase prenylation and membrane localization. J Biol Chem. 2010;285(46):35255–66.

Bithell A, Hsu T, Kandanearatchi A, Landau S, Everall IP, Tsuang MT, et al. Expression of the Rap1 guanine nucleotide exchange factor, MR-GEF, is altered in individuals with bipolar disorder. PLoS ONE. 2010;5(4):e10392.

Bokoch GM. Biology of the Rap proteins, members of the ras superfamily of GTP-binding proteins. Biochem J. 1993; 289(Pt 1):17–24.

Borland G, Smith BO, Yarwood SJ. EPAC proteins transduce diverse cellular actions of cAMP. Br J Pharmacol. 2009;158(1):70–86.

Gloerich M, Bos JL. Epac: defining a new mechanism for cAMP action. Annu Rev Pharmacol Toxicol. 2010;50:355–75.

Hong KW, Jin HS, Lim JE, Ryu HJ, Go MJ, Lee JY, et al. RAPGEF1 gene variants associated with type 2 diabetes in the Korean population. Diabetes Res Clin Pract. 2009;84(2): 117–22.

Li Y, Asuri S, Rebhun JF, Castro AF, Paranavitana NC, Quilliam LA. The RAP1 guanine nucleotide exchange factor Epac2 couples cyclic AMP and Ras signals at the plasma membrane. J Biol Chem. 2006;281(5):2506–14.

Liu C, Takahashi M, Li Y, Dillon TJ, Kaech S, Stork PJ. The interaction of Epac1 and Ran promotes Rap1 activation at the nuclear envelope. Mol Cell Biol. 2010;30(16): 3956–69.

Quilliam LA, Rebhun JF, Castro AF. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. Prog Nucleic Acid Res Mol Biol. 2002;71:391–444.

Raaijmakers JH, Bos JL. Specificity in Ras and Rap signaling. J Biol Chem. 2009;284(17):10995–9.

Sakurai A, Fukuhara S, Yamagishi A, Sako K, Kamioka Y, Masuda M, et al. MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. Mol Biol Cell. 2006;17(2):966–76.

Satyanarayana A, Gudmundsson KO, Chen X, Coppola V, Tessarollo L, Keller JR, et al. RapGEF2 is essential for embryonic hematopoiesis but dispensable for adult hematopoiesis. Blood. 2010;116(16):2921–31.

Severson EA, Lee WY, Capaldo CT, Nusrat A, Parkos CA. Junctional adhesion molecule A interacts with Afadin and PDZ-GEF2 to activate Rap1A, regulate beta1 integrin levels, and enhance cell migration. Mol Biol Cell. 2009; 20(7):1916–25.

Suh PG, Park JI, Manzoli L, Cocco L, Peak JC, Katan M, et al. Multiple roles of phosphoinositide-specific phospholipase C isozymes. BMB Rep. 2008;41(6):415–34.

Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev. 2001;81(1):153–208.

Wang H, Singh SR, Zheng Z, Oh SW, Chen X, Edwards K, et al. Rap-GEF signaling controls stem cell anchoring to their niche through regulating DE-cadherin-mediated cell adhesion in the Drosophila testis. Dev Cell. 2006; 10(1):117–26.

Yajnik V, Paulding C, Sordella R, McClatchey AI, Saito M, Wahrer DC, et al. DOCK4, a GTPase activator, is disrupted during tumorigenesis. Cell. 2003;112(5):673–84.

Yoshikawa Y, Satoh T, Tamura T, Wei P, Bilasy SE, Edamatsu H, et al. The M-Ras-RA-GEF-2-Rap1 pathway mediates tumor necrosis factor-alpha dependent regulation of integrin activation in splenocytes. Mol Biol Cell. 2007;18(8):2949–59.

Zhang CL, Katoh M, Shibasaki T, Minami K, Sunaga Y, Takahashi H, et al. The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. Science. 2009;325(5940): 607–10.

Rapamycin and FKBP12 Target-1 Protein (RAFT1)

► mTOR

RARα

► Retinoic Acid Receptors (RARA, RARB, and RARC)

RARβ

► Retinoic Acid Receptors (RARA, RARB, and RARC)

RARγ

► Retinoic Acid Receptors (RARA, RARB, and RARC)

RAS (H-, K-, N-RAS)

Michael S. Samuel
Centre for Cancer Biology, SA Pathology, Adelaide, SA, Australia

Synonyms

c-H-ras; c-K-ras; c-N-ras; Ha-ras; Ki-ras; Ras oncogenes; v-H-ras; v-K-ras; v-N-ras

Historical Background

Evidence that viral genes were translated in virus-transformed cells was first reported in the early 1970s (Green et al. 1971). Following on from these observations, it was discovered that certain 21 kDa proteins encoded by viral genes and possessing guanine nucleotide binding properties (Scolnick et al. 1979) were essential for the maintenance of transformation in cells infected with the Kirsten and Harvey sarcoma viruses (Shih et al. 1979). Intriguingly, sequences containing a very high degree of homology to genes encoding these transforming proteins were found in normal rat, mouse, and human genomes, suggesting a physiological role, unrelated to disease, for these proteins. Mutant versions of the oncogenes associated with the Kirsten sarcoma virus (K-ras) and the Harvey sarcoma virus (H-ras) were found in cancer cell lines of various tissue origins (Der et al. 1982). The majority of these contained point mutations that resulted in the replacement of the guanosine residue at position 12. A third Ras family member termed "N-ras," also containing activating point mutations, was subsequently identified in leukemia and neuroblastoma cell lines (Hall et al. 1983). Indeed, it was soon realized that the Ras family of proto-oncogenes are among the most frequently mutated genes in human cancers. The field of Ras research is now a large and complex enterprise owing to the discovery of this protein family's fundamental roles in cell and cancer biology, development, and disease.

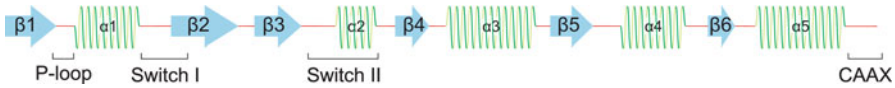
Structure and Function of Ras Proteins

The Ras proteins are 21 kDa small G proteins with intrinsic GTP binding and GTPase properties (Sweet et al. 1984). The three-dimensional structures of Ras in the GTP- and GDP-bound forms determined by crystallography were published in 1990 and revealed that Ras consists of a six stranded β-sheet and five α-helices forming a hydrophobic core and linked together by ten loops. Five of these loops mediate the high-affinity interactions between Ras and GTP and act by stabilizing the γ-phosphate of the bound GTP (Brunger et al. 1990). The p-loop, which contains the guanosine-12 often mutated in cancers, directly binds the γ-phosphate of GTP. The structures of GTP-bound and GDP-bound Ras differ in two regions called switch I and switch II (Fig. 1), which are crucial for the interaction of Ras with both its upstream regulators and downstream effector partners that mediate its function and intracellular localization.

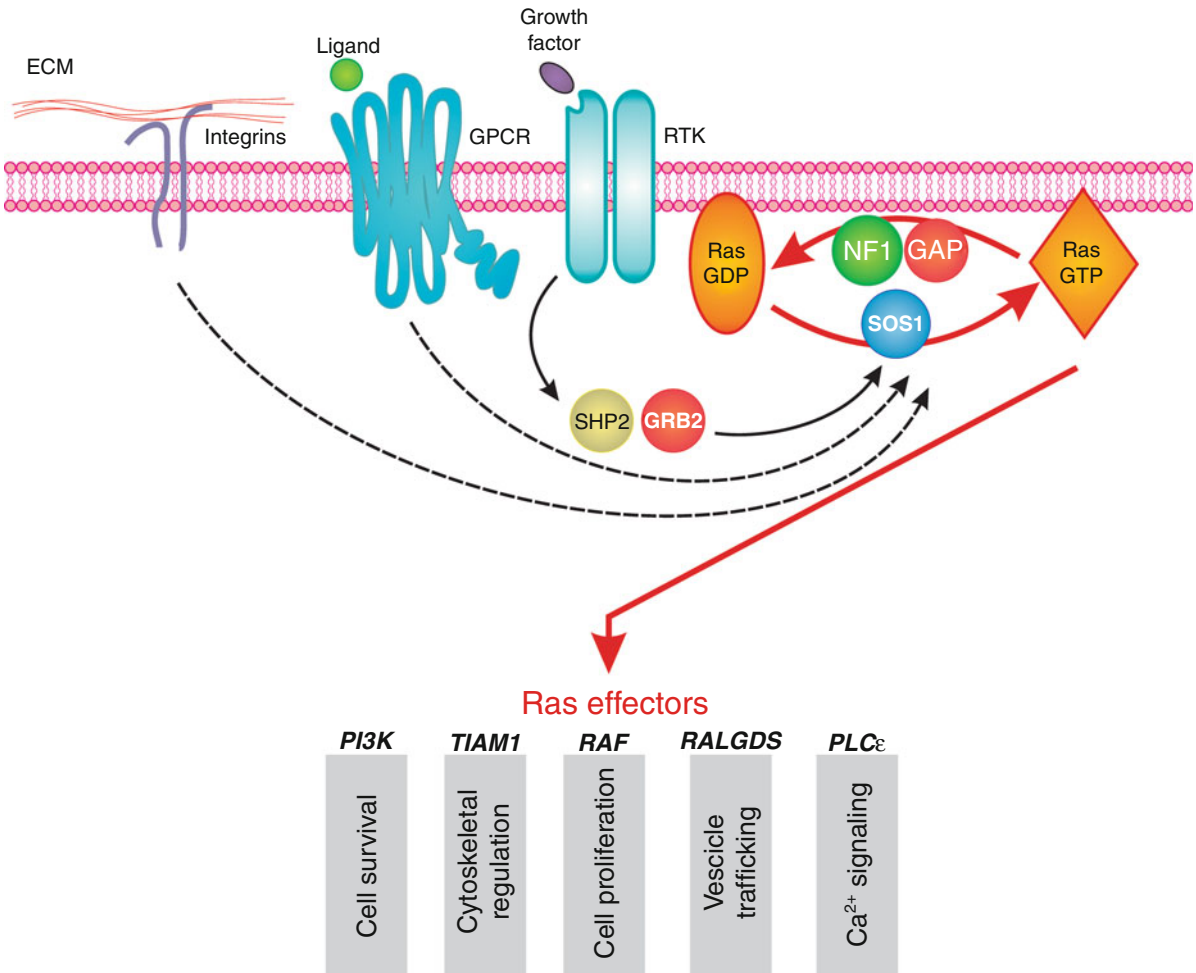
In the GTP-bound state, Ras proteins are in an active conformation and can participate in high-affinity interactions with Ras effector proteins. These proteins are usually enzymes that transduce downstream signaling cascades. However, the intrinsic GTPase activity of Ras proteins results in the hydrolysis of GTP to GDP, with which Ras proteins associate only weakly. Following hydrolysis and loss of the γ-phosphate, the GDP molecule is released and the Ras protein returns to an inactive state. This GTPase cycle is facilitated by the Ras GTPase-activating proteins (Ras GAPs) and the Ras guanine nucleotide-exchange factors (Ras GEFs) (Fig. 2). The Ras GAP proteins promote the GTPase activity of Ras and facilitate its transition from the active to the inactive state, resulting in the switching off of Ras signaling. The Ras GEF proteins, on the other hand, bind inactive Ras-GDP and promote the exchange of GDP for GTP, triggering the activation of Ras. Ras GEF proteins contain many protein-protein interaction domains that mediate their activation. One way in which Ras GEFs are activated is in response to ligand binding to receptors with which they are associated. Similarly, GAPs Ras proteins are often large and complex and contain a variety of signaling motifs that enable them to associate with the many interacting partners that regulate their activity. Mutant versions of Ras proteins often found in cancers exhibit attenuated affinity for GTP, but maintain an active conformation enabling them to interact with and activate effector proteins and signal constitutively downstream in the absence of upstream activating signals (Karnoub and Weinberg 2008).

Regulation of Ras Activity

In addition to the action of Ras GEFs and GAPs, the activity of Ras is regulated by a variety of other mechanisms. These include posttranslational modifications such as the addition of fatty acid side chains and proteolytic processing, which determine its localization. Palmitoylation of Ras at the C terminus facilitates its association with the cell membrane (Sefton et al. 1982) and this particular localization is essential for its function. A C-terminal CAAX motif within Ras is the target of a prenylation reaction by farnesyl transferase,



RAS (H-, K-, N-RAS), Fig. 1 *Linear representation of the domain structure of the Ras proteins*. The α-helices are denoted α1–α6 and the six strands making up the β-sheet are denoted β1–β6. The P-loop between β1 and α1 stabilizes the γ-phosphate of GTP and is a region often mutated in oncogenic versions of the protein. The Switch 1 and Switch 2 regions are important for protein-protein interactions and the CAAX region is a target of posttranslational modifications



RAS (H-, K-, N-RAS), Fig. 2 *Signaling through Ras*. This schematic illustration of the Ras signaling pathway highlights the modes of Ras activation and the effector pathways downstream of Ras that regulate cellular processes. Binding of growth factor to receptor tyrosine kinases (RTK) activates proteins such as GRB2 and SHP2, which activates SOS1, a Ras GEF, resulting in the accumulation of GTP-bound active Ras. GPCR and Integrin signaling can also indirectly activate Ras. NF1 and other GAPs in turn bind active Ras and catalyze its conversion to the GDP-bound inactive form, turning off Ras signaling. Downstream, Ras-GTP signals through a variety of effector pathways to regulate many cellular processes

which is followed by cleavage of the AAX sequence, leaving a C-terminal cytosine residue. This cytosine residue is then carboxy-methylated as an essential step for full Ras function. There are slight variations in the specific modifications to K-Ras, H-Ras, and N-Ras proteins, but the final outcome of these modifications is the secure tethering of the Ras protein to the cell membrane, which is essential for its association with both upstream and downstream signaling partners (Downward 2003).

Operating upstream, GEFs such as son of sevenless (SOS) that interact with and activate Ras proteins are themselves activated by various signals including those arising from receptor tyrosine kinases (RTK) such as the EGF Receptor. However, Ras may also be activated more indirectly by signals from Integrins or G protein-coupled receptors (GPCR) (Fig. 2). The function of Ras as a major signaling node is attested to by the multiple GEFs that activate it, as well as by the even larger numbers of Ras GEF binding partners that regulate their function.

Ras Signaling Pathways

MAP Kinase Signaling

Activated Ras interacts directly with the RAF1 protein, a Ser-Thr kinase, and stimulates its kinase activity. Most notable of the substrates of Raf are the mitogen-activated protein kinase kinases (MAPK, also known as MEKs) which phosphorylate the extracellular signal-regulated kinases (ERKs) to regulate the transcription of target genes through the E26-transcription factor proteins (ETS) (Fig. 2). The Ras-MAP kinase effector pathway regulates cell proliferation and is required for Ras-induced cell transformation (Khosravi-Far et al. 1995). Signaling through the MAP kinase pathway is frequently enhanced in cancers by the somatic acquisition of activating mutations in the Ras proteins or their effector Raf proteins. However, activating mutations in both Ras and Raf are only rarely seen together, illustrating the importance of abnormal signaling through the MAP kinase pathway in cancer (Karnoub and Weinberg 2008; Downward 2003).

PI3 Kinase Signaling

Upon activation, Ras proteins are capable of interacting with the catalytic subunit (p110) of the class I phosphoinositide 3-kinases (▶ PI3Ks), which results in the production of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3). This second messenger molecule binds a large number of proteins including PDK1 and the Ras homology family protein Rac, and regulates their activity. One of these pathways downstream of PtdIns(3,4,5)P_3 regulates cell survival through the serine/threonine kinase AKT/Protein Kinase B (AKT/PKB) (Fig. 2) (Downward 2003). Like the MAP kinase pathway, the PI3 kinase signaling pathway is also indispensible for Ras-mediated cell transformation (Rodriguez-Viciana et al. 1997).

Other Ras Effector Pathways

While Ras is capable of activating RalGDS, the GEF for the Ras-like (RalA/B) small GTPases, conflicting evidence exists for the involvement of the RalA/B effector pathway in transformation. This pathway was originally considered of minor importance to transformation, but new evidence suggests that signaling through RalA/B is sufficient for transformation of some human cell types (Hamad et al. 2002). Rac1 (Khosravi-Far et al. 1995; Samuel et al. 2011), tumor invasion and metastasis inducing protein (TIAM1, a Rac GEF protein), the epsilon form of phosphoinositide-specific phospholipase C (PLCε), and RASSF have all been shown to be Ras effectors, but much remains unknown of their functions downstream of Ras.

Ras in Disease

Cancers are the disease manifestations most commonly associated with aberrant signaling through Ras. Mutations in one or more of the Ras isoforms are frequently observed in cancers, with codon 12/13 mutations making up the majority of these (Loriot et al. 2009). These forms of Ras are usually able to associate with and activate effector proteins in the absence of GTP binding and indeed mutant Ras has attenuated affinity for GTP. Both the MAP kinase and PI3-kinase effector pathways are required for Ras-mediated transformation and increased signaling through both these pathways been implicated in providing cancer cells with a survival advantage.

Different isoforms of Ras are preferentially expressed in different organs, leading to isoform-specific disease manifestations resulting from aberrations in Ras activity. For instance, most pancreatic cancers exhibit K-Ras mutations, but H-Ras mutations are almost never observed in this disease. Furthermore, N-Ras mutations are common in skin cancers, but K-Ras mutations are not. That this is likely the result of differences in the expression levels of Ras isoforms, which may nevertheless function similarly in different cell types, is supported by the observation that the H-Ras coding sequence

placed under the transcriptional control of the endogenous K-Ras promoter can rescue the embryonic lethality resulting from K-Ras deficiency (Potenza et al. 2005).

Ras in Development

Aberrant signaling downstream of Ras has been identified as being the cause of several developmental disorders. Collectively termed cardio-facio-cutaneous diseases, neurofibromatosis type-1 and the Noonan and Costello syndromes are characterized by inherited lesions in effectors, GAPs or GEFs of Ras (Loriot et al. 2009) or infrequently by acquired somatic mutations in Ras genes. People suffering from these syndromes generally exhibit abnormalities in the bones of the face, insufficiency in cardiac function, stunted growth, and an increased cancer risk (Schubbert et al. 2007). The fact that activating germ-line K-Ras mutations are rarely observed in genetic syndromes is strong evidence that unregulated Ras activation is highly disruptive during development and is consistent with the embryonic lethality associated with inherited activating K-Ras mutations in mice.

Therapeutic Inhibition of Ras Signaling

Antagonizing the activity of mutant Ras has always been an attractive therapeutic modality for cancer. Many of these therapies have focused on disrupting the posttranslational modifications essential for the localization of Ras to the cell membrane, but have suffered from lack of selectivity. Agents such as farnesyl transferase inhibitors (FTI) also interfere with the processing of other proteins such as RhoB. Approaches to therapeutic targeting of Ras expression using antisense oligonucleotides are currently under trial. These approaches have run into problems associated with delivery and the lack of efficient take-up into cancer cells (Downward 2003). Small molecule inhibitors of the MAP kinase pathway protein Raf, such as Sorafenib (Sebolt-Leopold et al. 1999; Iyer et al. 2010), have been more successful, but only target a single Ras effector pathway. Sorafenib has been approved for the treatment of renal cell and hepatocellular carcinomas. Inhibitors of MEKs such as U0126 and PD98059 are effective in inhibiting activation of the MAP kinase pathway and are also currently under trial.

Summary

The Ras genes were first discovered in viruses that possess cell transforming ability before it was realized that very similar genes exist in untransformed cells. The Ras proteins have important physiological functions in transducing signals that regulate cell proliferation and survival. They constitute signaling hubs that integrate and link growth factor signals from upstream to the appropriate effector pathways downstream. Activating mutations in Ras genes or in components of Ras effector pathways are commonly observed in cancer, leading to increased cell proliferation and survival. Germ-line mutations of Ras effector pathways underlie a group of developmental disorders including the Noonan and Costello syndromes, collectively termed cardio-facial-cutaneous syndromes. Ras antagonizing therapies are beginning to be used effectively to treat cancers.

References

Brunger AT, Milburn MV, Tong L, de Vos AM, Jancarik J, Yamaizumi Z, et al. Crystal structure of an active form of RAS protein, a complex of a GTP analog and the HRAS p21 catalytic domain. Proc Natl Acad Sci U S A. 1990;87:4849–53.

Der CJ, Krontiris TG, Cooper GM. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc Natl Acad Sci U S A. 1982;79:3637–40.

Downward J, Targeting RAS. Signalling pathways in cancer therapy. Nat Rev Cancer. 2003;3:11–22.

Green M, Rokutanda H, Rokutanda M. Virus specific RNA in cells transformed by RNA tumour viruses. Nat New Biol. 1971;230:229–32.

Hall A, Marshall CJ, Spurr NK, Weiss RA. Identification of transforming gene in two human sarcoma cell lines as a new member of the ras gene family located on chromosome 1. Nature. 1983;303:396–400.

Hamad NM, Elconin JH, Karnoub AE, Bai W, Rich JN, Abraham RT, et al. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev. 2002;16:2045–57.

Iyer R, Fetterly G, Lugade A, Thanavala Y. Sorafenib: a clinical and pharmacologic review. Expert Opin Pharmacother. 2010;11:1943–55.

Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol. 2008;9:517–31.

Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol Cell Biol. 1995;15:6443–53.
Loriot Y, Mordant P, Deutsch E, Olaussen KA, Soria JC. Are RAS mutations predictive markers of resistance to standard chemotherapy? Nat Rev Clin Oncol. 2009;6:528–34.
Potenza N, Vecchione C, Notte A, De Rienzo A, Rosica A, Bauer L, et al. Replacement of K-Ras with H-Ras supports normal embryonic development despite inducing cardiovascular pathology in adult mice. EMBO Rep. 2005;6:432–7.
Rodriguez-Viciana P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, et al. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell. 1997;89:457–67.
Samuel MS, Lourenco FC, Olson MF. K-Ras mediated murine epidermal tumorigenesis is dependent upon and associated with elevated Rac1 activity. PLoS One. 2011;6:e17143.
Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer. 2007;7:295–308.
Scolnick EM, Papageorge AG, Shih TY. Guanine nucleotide-binding activity as an assay for src protein of rat-derived murine sarcoma viruses. Proc Natl Acad Sci U S A. 1979;76:5355–9.
Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat Med. 1999;5:810–6.
Sefton BM, Trowbridge IS, Cooper JA, Scolnick EM. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell. 1982;31:465–74.
Shih TY, Weeks MO, Young HA, Scolnick EM. p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J Virol. 1979;31:546–56.
Sweet RW, Yokoyama S, Kamata T, Feramisco JR, Rosenberg M, Gross M. The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature. 1984;311:273–5.

Ras Associated with Diabetes

► Ras-Related Associated with Diabetes

RAS D1/DEXRAS1 (AGS1)

► Activators of G-Protein Signaling (AGS)

Ras Guanyl Nucleotide-Releasing Protein 1

► RasGRP1

Ras Guanyl-Releasing Protein

► RasGRP1

Ras Guanyl-Releasing Protein 1

► RasGRP1

Ras Homolog Gene Family, Member C

► RhoC (RHOC)

Ras Oncogenes

► RAS (H-, K-, N-RAS)

RASA1

Philip E. Lapinski and Philip D. King
Department of Microbiology and Immunology, University of Michigan, Medical School, Ann Arbor, MI, USA

Synonyms

p120 GAP; p120 RasGAP; RasGAP

Historical Background

The small, membrane-tethered G-protein Ras plays an important role in many cellular processes, including growth, differentiation, and survival (Wennerberg et al. 2005). Ras acts as a molecular switch which is bound to GDP in its inactive state, and GTP in its active state. When Ras is active, it can directly associate with the serine/threonine kinase Raf, which can be activated by phosphorylation upon recruitment to the membrane. Raf can then activate the dual-specificity protein kinase MEK1/2, which in turn activates the

Mitogen-Activated Protein Kinase (MAPK) ERK1/2. Since Ras controls multiple cellular outcomes, its activity is tightly regulated. Inactive, GDP-bound Ras can be activated by interaction with Guanine-nucleotide Exchange Factors (GEFs), which eject GDP from the nucleotide binding site of Ras and allow GTP to bind, which is present at a much higher molar concentration than GDP in the cytoplasm. Examples of Ras GEFs include Son of Sevenless (SOS) and Ras Guanyl-Releasing Protein 1 (RasGRP1). Active, GTP-bound Ras is inactivated by association with GTPase-Activating Proteins (GAPs), which enhance the low intrinsic GTPase activity of Ras by several orders of magnitude, resulting in hydrolysis of GTP to GDP (Iwashita and Song 2008). Ras p21 Protein Activator 1 (RASA1) was the first GAP to be characterized at the molecular level. The discovery of RASA1 was initially based on the observation that Ras-GTP levels in vivo were much lower than expected based on the intrinsic GTPase activity of Ras (Trahey and McCormick 1987). Subsequently, the ubiquitously expressed RASA1 protein was purified and its cDNA cloned from human and bovine tissue (Trahey et al. 1988; Vogel et al. 1988). At least 14 Ras GAPs have since been discovered in mammals, including neurofibromin (NF1), Ca^{2+}-promoted Ras inactivator (CAPRI), and Synaptic Ras GTPase-activating protein 1 (SYNGAP1) (Bernards 2003).

Structure

GAPs are modular proteins, with numerous distinct domains in addition to the conserved, catalytic GAP domain. The RASA1 molecule is composed of six such modular domains. These include two Src-homology-2 (SH2) domains and a Src-homology-3 (SH3) domain (which recognize phospho-tyrosine residues and proline-rich sequences, respectively), a pleckstrin homology (PH), and PKC2 homology (C2) domain (both implicated in membrane phospholipid binding, the latter in a calcium-dependent manner), and a GAP domain, which confers GTPase-enhancing activity (Takai et al. 2001). The SH2-SH3-SH2 domains of RASA1 are responsible for binding to cytoplasmic proteins, which include p190 RhoGAP and Dok-1 (Iwashita and Song 2008). Dok-1 is an adapter protein that plays a role downstream of tyrosine kinase signaling, and p190 RhoGAP acts as a GAP for the Rho family of G proteins. The number of protein-binding and membrane-binding domains in RASA1 suggests that it is involved in a complex signaling network.

The GAP domain of RASA1 contains three conserved motifs that are shared with all GAP proteins. These include an arginine-finger loop, a phenylalanine-leucine-arginine region, and an α7/variable loop, with the arginine residue in the arginine-finger loop being critical for the transition state of GTP hydrolysis (Iwashita and Song 2008).

RASA1 is known to be phosphorylated by a number of protein tyrosine kinases in multiple cell types. However, the stoichiometry of phosphorylation is generally low, and no effect of phosphorylation on GAP activity or subcellular localization has been reported (Takai et al. 2001).

Function

The only known enzymatic function of RASA1 is to accelerate the hydrolysis of GTP to GDP by Ras. While the isolated GAP domain of RASA1 is sufficient to promote GTP hydrolysis of purified Ras in vitro, full activity in vivo requires the SH2-SH3-SH2 domains, suggesting that protein-protein interactions are critical for RASA1 function (Marshall et al. 1989; Gideon et al. 1992). Indeed, RASA1 interacts with active, phosphorylated PDGF Receptor and EGF Receptor via its SH2 domains, negatively regulating their activity by suppressing Ras signaling (Margolis et al. 1990; Ekman et al. 1999). PDGF Receptor and EGF Receptor are not closely related by sequence, suggesting that RASA1 associates with a broad range of growth factor receptors.

Despite being the prototypical RasGAP, RASA1 is not simply a negative regulator of Ras. In addition to controlling Ras activation, RASA1 controls certain cellular functions in a GAP-domain-independent and a Ras-independent manner. For example, RASA1 has been implicated in the control of cell motility through its interaction with p190 RhoGAP. In cell monolayer wounding assays, RASA1-deficient embryonic fibroblasts were impaired in establishing cell polarity and migration into the wound. These functions appear to require the interaction of RASA1 with p190 RhoGAP, and are independent of Ras regulation (Kulkarni et al. 2000). The Rho proteins, for which p190 RhoGAP

is a negative regulator, are known to control the formation of focal adhesions and actin fibers necessary for directed cell movement. This association between RASA1 and p190 RhoGAP implies that RASA1 can act as a positive mediator of signaling, in addition to its negative-regulator role as a RasGAP.

Another GAP-domain-independent function of RASA1 is the regulation of apoptotic cell death. In fibroblasts subjected to mild apoptotic stress, RASA1 is cleaved by activated Caspase 3. The free N-terminal fragment of RASA1 is able to directly activate AKT, a major kinase in the apoptotic pathway, via its SH2 and SH3 domains (Yang et al. 2005). Under these conditions, RASA1 is thought to provide anti-apoptotic signals that permit survival of the stressed cell. However, under more severe apoptotic stress, the N-terminal fragment of RASA1 is further cleaved by Caspase 3, resulting in two shorter N-terminal fragments. The result of this cleavage is a reduction in AKT activity, which leads to efficient apoptotic death of the cell. Therefore, the second cleavage event of RASA1 functions to abrogate the anti-apoptotic function of the longer N-terminal fragment.

RASA1-Deficiency

A null allele of mouse *rasa1* has been generated, and mice homozygous for the null allele die at embryonic day 10.5 (E10.5) (Henkemeyer et al. 1995). These embryos appear to develop normally until E9.25, at which time they display a defect in posterior elongation. No abnormal cell proliferation is observed in RASA1 deficient embryos, and in fact by E9.5 are significantly smaller than littermate controls. This is most likely due to a severe vascular developmental defect, in which blood vessel endothelial cells fail to organize into a vascular network in the yolk sac. The blood vasculature in the embryo proper is also affected, and ultimately develops local ruptures leading to leakage of blood into the body cavity. Eventually the pericardial sac becomes distended, leading to a labored heartbeat and reduced blood flow. RASA1-deficient embryos also display extensive apoptotic cell death in the brain, with large numbers of dead and dying cells as early as E9.0 in the hindbrain, optic stalk, and telencephalon.

A recently developed conditional RASA1 mouse model has been used to define a role for RASA1 in the development and survival of T cells (Lapinski et al. 2011). In this model, exon 18 of *rasa1*, which encodes the catalytic arginine-finger loop of the GAP domain, was flanked by LoxP sites (floxed). The floxed allele permits normal RASA1 expression, and thus circumvents embryonic lethality. However, excision of the floxed exon in mice by transgenic Cre recombinase results in nonsense-mediated RNA decay, and a complete loss of RASA1 expression. This system permits the study of RASA1 deficiency in adult mice with the use of transgenic tissue-specific or inducible Cre recombinase. Specific deletion of RASA1 early in thymocyte development by Cre expressed under the control of the proximal LCK promoter resulted in increased death of CD4+ CD8+ double positive thymocytes. Surprisingly, RASA1-deficient thymocytes showed increased positive selection on a Major Histocompatibility Complex (MHC) Class II background, which was associated with increased Ras/MAPK signaling. RASA1 was found to be dispensable during T cell receptor stimulation by agonist peptide/MHC complex in peripheral T cells, as measured by cytokine secretion, proliferative capacity, and activation-induced cell death. However, absence of RASA1 led to substantially reduced numbers of naive T cells in the peripheral lymphoid organs. This phenomenon was due, at least in part, to a reduced sensitivity to the pro-survival cytokine IL-7.

RASA1 in Disease

Mutations in Ras are closely linked to development of human cancer, with up to 90% of certain tumors harboring an oncogenic Ras allele. Commonly, an oncogenic Ras mutation renders it refractory to GAP activity, which leaves Ras trapped in its active, GTP-bound state (Scheffzek et al. 1997). In addition, RASA1 nonsense mutations have been associated with basal cell carcinomas in humans (Friedman et al. 1993).

A recently described human clinical disorder known as capillary malformation-arteriovenous malformation (CM-AVM) has been shown to be caused by mutations of the *RASA1* gene (Boon et al. 2005; Revencu et al. 2008). This condition is characterized by multiple randomly distributed pink lesions that result from the malformation of skin capillaries. Approximately one third of patients develop fast-flow

vascular lesions, including Parkes Weber syndrome, arteriovenous fistulas, and intracranial arteriovenous malformations. Arteriovenous fistulas are abnormal connections between arteries and veins, where the two are directly connected without branching into capillaries, and ParkesWeber Syndrome is characterized by cutaneous flush, and multiple underlying arteriovenous fistulas. It is also associated with soft tissue and skeletal hypertrophy, usually of an affected limb. Thus far, 140 individuals with RASA1 mutations have been identified, and all but 6 of these have CM-AVM. Forty-two different mutations in the RASA1 gene have been described, including insertions and deletions resulting in frame-shifts, disruption of splice sites, and nonsense, missense, or splice-site substitutions. The mutations are randomly distributed throughout the RASA1 gene, and only one germline RASA1 gene is affected in CM-AVM patients (mutation of both alleles of RASA1 would presumably result in embryonic lethality). CM-AVM is hypothesized to arise from loss of function of the intact RASA1 allele by somatic mutation, which is consistent with the focal nature of the lesions. At least two CM-AVM patients have developed chylothorax or chylous ascites, rare conditions in which lipid-laden lymph fluid, or chyle, leaks from lymphatic vessels into the thoracic or abdominal cavities, respectively. Thus, RASA1 has been found to play a role in the formation and maintenance of the blood and/or lymph vasculature in both mice and humans.

Consistent with the blood vascular phenotype of RASA1-null embryos and in RASA1-mutant humans, RASA1 has been found to play a role in the angiogenic switch (Anand et al. 2010). In this process, new blood vessels are stimulated to grow from existing ones in response to growth factor stimulation. RASA1 expression has been found to be suppressed by microRNA (mIR)-132, a genome encoded, noncoding RNA regulator of gene expression. In a human embryonic stem cell model of vasculogenesis, mIR-132 was found to be highly expressed. mIR-132 was also upregulated in the epithelium of human tumors and hemangiomas, but not in normal epithelium. RASA1, which was expressed in normal epithelium, but not tumor epithelium, was found to be a major target of mIR-132. Suppression of RASA1 expression by mIR-132 led to increased Ras activation in endothelial cells and the induction of neovascularization. This result suggests that mIR-132 acts as an angiogenic switch by downregulating RASA1 expression, and consequently increasing Ras signaling in endothelial cells. Anti-mIR-132, a specific antagonist of mIR-132, was found to restore RASA1 expression in tumor endothelium. Taken together, these results suggest that mIR-132, or RASA1 itself, might be viable targets for therapeutic anti-vasculogenesis treatments in patients.

Summary

RASA1, the prototypical Ras GAP, plays an important role in the negative regulation of Ras in growth factor receptor signaling. In addition, a number of positive regulatory roles for RASA1 have been described. Despite being the first GAP discovered, there remain many unanswered questions about its function. How RASA1 controls angiogenesis and lymphangiogenesis is unknown, but disregulated Ras signaling through one or more growth factor receptors is the most likely mechanism. The receptors VEGFR1, VEGFR2, and/or VEGFR3 are strong candidates. In addition, the precise mechanism by which RASA1 regulates naïve T cell survival remains to be elucidated. With the use of recently developed tools described above, these questions will likely be the subject of intensive study for years to come.

References

Anand S, Majeti BK, et al. MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. Nat Med. 2010;16(8):909–14.

Bernards A. GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. Biochim Biophys Acta. 2003;1603(2):47–82.

Boon LM, Mulliken JB, et al. RASA1: variable phenotype with capillary and arteriovenous malformations. Curr Opin Genet Dev. 2005;15(3):265–9.

Ekman S, Thuresson ER, et al. Increased mitogenicity of an alphabeta heterodimeric PDGF receptor complex correlates with lack of RasGAP binding. Oncogene. 1999;18(15):2481–8.

Friedman E, Gejman PV, et al. Nonsense mutations in the C-terminal SH2 region of the GTPase activating protein (GAP) gene in human tumours. Nat Genet. 1993;5(3):242–7.

Gideon P, John J, et al. Mutational and kinetic analyses of the GTPase-activating protein (GAP)-p21 interaction: the C-terminal domain of GAP is not sufficient for full activity. Mol Cell Biol. 1992;12(5):2050–6.
Henkemeyer M, Rossi DJ, et al. Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein. Nature. 1995;377(6551):695–701.
Iwashita S, Song SY. RasGAPs: a crucial regulator of extracellular stimuli for homeostasis of cellular functions. Mol Biosyst. 2008;4(3):213–22.
Kulkarni SV, Gish G, et al. Role of p120 Ras-GAP in directed cell movement. J Cell Biol. 2000;149(2):457–70.
Lapinski PE, Qiao Y, et al. A role for p120 RasGAP in thymocyte positive selection and survival of naive T cells. J Immunol. 2011;187(1):151–63.
Margolis B, Li N, et al. The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. EMBO J. 1990;9(13):4375–80.
Marshall MS, Hill WS, et al. A C-terminal domain of GAP is sufficient to stimulate ras p21 GTPase activity. EMBO J. 1989;8(4):1105–10.
Revencu N, Boon LM, et al. Parkes Weber syndrome, vein of Galen aneurysmal malformation, and other fast-flow vascular anomalies are caused by RASA1 mutations. Hum Mutat. 2008;29(7):959–65.
Scheffzek K, Ahmadian MR, et al. The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science. 1997;277(5324):333–8.
Takai Y, Sasaki T, et al. Small GTP-binding proteins. Physiol Rev. 2001;81(1):153–208.
Trahey M, McCormick F. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science. 1987;238(4826):542–5.
Trahey M, Wong G, et al. Molecular cloning of two types of GAP complementary DNA from human placenta. Science. 1988;242(4886):1697–700.
Vogel US, Dixon RA, et al. Cloning of bovine GAP and its interaction with oncogenic ras p21. Nature. 1988;335(6185):90–3.
Wennerberg K, Rossman KL, et al. The Ras superfamily at a glance. J Cell Sci. 2005;118(Pt 5):843–6.
Yang JY, Walicki J, et al. Impaired Akt activity down-modulation, caspase-3 activation, and apoptosis in cells expressing a caspase-resistant mutant of RasGAP at position 157. Mol Biol Cell. 2005;16(8):3511–20.

Ras-Associated Protein RAB8

▶ Rab8

RasGAP

▶ RASA1

RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor)

Eugenio Santos and Alberto Fernández-Medarde
Centro de Investigación del Cáncer, IBMCC (CSIC/USAL), University of Salamanca, Salamanca, Spain

Synonyms

CDC25; CDC25L; CDC25Mm; GNRP; GRF

Historical Background

The Ras guanine nucleotide releasing factor (RasGrf) proteins were isolated in an effort to find mammalian homolog(s) of the yeast CDC25 Ras activator protein. The search for mammalian Ras GEFs during the early 1990s led to the discovery and isolation of the Son of sevenless (Sos) and the RasGrf proteins. Whereas the Sos proteins had ubiquitous expression, RasGrf expression was restricted mainly to the central nervous system. Soon after the discovery of the first member of the RasGrf family (RasGrf1) in neural tissues, a second highly homologous member of this family was isolated from embryonic stem cells (RasGrf2). Later on, a number of distinct, alternatively spliced isoforms have been described for both genes in a variety of tissues or developmental stages. RasGrf1 and RasGrf2 are large, modular proteins composed by multiple functional domains accounting for protein–protein or protein–lipid interactions which are responsible for coupling to upstream and downstream signaling as well as for fine regulation of their intrinsic exchange activity (Santos and Fernandez-Medarde 2008, 2009).

Early biochemical characterization of the RasGrfs yielded identification of their specific targets in different Ras GTPase families, as well as initial characterization of their contribution to specific cellular signal transduction pathways. Whereas both RasGrf1 and RasGrf2 are able to activate canonical Ras proteins (H-Ras, N-Ras or K-Ras) and Rac1, only RasGrf1 is able to activate members of the R-Ras subfamily (R-Ras, TC21, M-Ras)

(Santos and Fernandez-Medarde 2008, 2009). Furthermore, both RasGrfs are known to be activatable in response to different signals including increases of intracellular levels of calcium or cAMP, activation of heterotrimeric G-protein-coupled receptors, or NGF receptor stimulation. More recent work using genetically modified animal models has provided significant clues to physiological roles played by the RasGrfs. Analysis of RasGrf1 and RasGrf2 knockout mice show the implication of RasGrf1 in memory and learning processes, light perception, glucose homeostasis, and substance addiction (Brambilla et al. 1997; Fernandez-Medarde et al. 2009; Font de Mora et al. 2003; Tonini et al. 2006), and the participation of RasGrf2 in memory formation and immunological responses (Li et al. 2006; Ruiz et al. 2007).

RasGrfs Protein Structure and Domain Distribution

The RasGrfs are large, highly homologous proteins (sharing ca. 80% homology and 63% identity in their sequences). RasGrf1 is slightly larger than RasGrf2 (respective molecular weights of the full-length proteins: 140 kDa and 135 kDa in mice; 145 kDa and 140 kDa in humans). These two proteins share multiple functional domains which are essential for fine regulation and control of their intrinsic catalytic activity and protein stability as well as subcellular localization and functional link to upstream/downstream signals (Fig. 1). From N- to C-terminus, these domains include:

PH1 domain- Although many PH domains are reported to mediate protein targeting to membranes, a RasGrf1 construct lacking this domain retains partial plasma membrane localization. The PH1 domain is reported to interact with G-protein β/γ subunits, and is required for normal G-protein induced ERK1/2 activation (Innocenti et al. 1999). In addition, together with the adjacent IQ and coiled coil domains, the PH1 domain is involved in interaction with scaffold proteins such as JIP2 or Spinophilin or with ribosomal proteins (Santos and Fernandez-Medarde 2009). Separate studies have also shown that this domain is necessary for complete calcium- and LPA-mediated activation of RasGrf1, and it is phosphorylated upon interaction with the TrkA NGF receptor (Innocenti et al. 1999; Robinson et al. 2005).

Coiled coil (cc) domain- All functional roles for this domain have been described in association with the flanking PH1 and IQ domains. As described above, its contribution is important for complete ERK1/2 activation upon intracellular calcium increase and for interaction with the JIP2 scaffold protein (Santos and Fernandez-Medarde 2009).

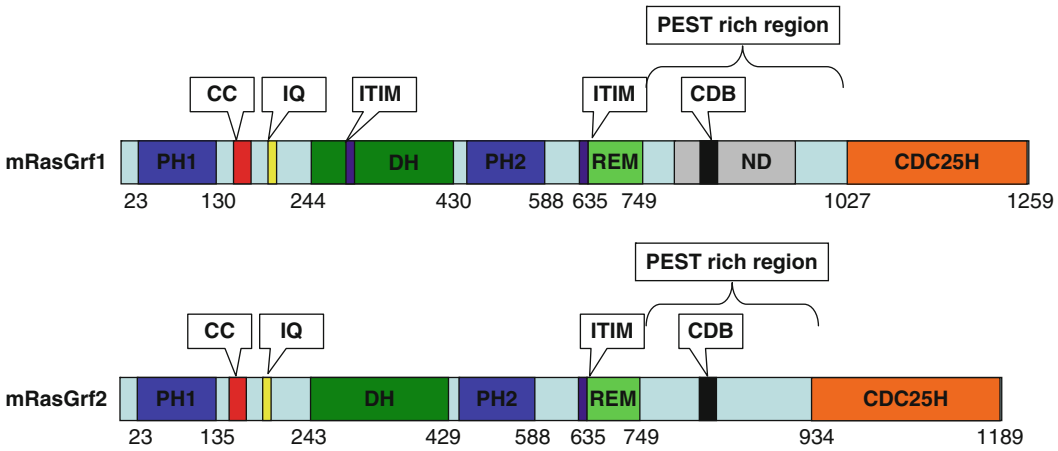
IQ domain- The main role of this domain is the interaction with Calmodulin, which is essential for activation of both RasGrfs by increases of intracellular calcium concentration. Mutations in this domain abolish ionomycin-mediated activation of RasGrf1 and stimulation of the Ras-ERK pathway. It is also involved, jointly with the PH1 and CC domains, in the interaction with the JIP2 scaffold protein (Santos and Fernandez-Medarde 2009).

DH domain- The DH-PH2 domain tandem constitutes the typical catalytically active domain facilitating GDP/GTP exchange on GTPases of the Rho subfamily. In the RasGrf proteins, this region is responsible for activation of Rac1 (Santos and Fernández-Medarde 2008, 2009). The DH domain also exerts a regulatory role on Ras activation by RasGrf1, and may also be important for Ras-independent, ionomycin-induced ERK activation (Freshney et al. 1997). This domain also mediates protein–protein interactions of RasGrf1 with β−tubulin and SCLIP and it is involved in homo- and hetero-oligomerization of the RasGrf1 and RasGrf2 proteins.

PH2 domain- A defined functional role for this domain remains unclear. In RasGrf1, some reports indicate that it is required for proper Ras and ERK activation, whereas studies in different cellular systems suggest that this domain is dispensable for Ras or ERK activation. Further work is needed to clarify these discrepancies analyzing RasGrf function in more physiological environments.

REM motif- This region is common to all Ras Guanine Nucleotide Exchange Factors and it is responsible for GEF–Ras interaction. It is likely that the REM motif has a similar role in all the Sos, ▶ RasGRP, and RasGrf1 families of GEF proteins.

CBD motif- This short domain usually targets proteins for degradation by the proteasome. It is located between the REM and CDC25H domains. Its involvement with ubiquitination and subsequent proteolytic degradation has been shown experimentally only for RasGrf2.



RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor), Fig. 1 RasGrf1 and RasGrf2 domain distribution: *PH* pleckstrin homology domain, *CC* coiled coil domain, *IQ* isoleucine (I)/glutamine (Q) motif, *DH* Dbl homology domain, *ITIM* immuno tyrosine–based inhibition motifs, *REM* Ras exchanger motif, *DB* destruction box, *ND* neurological domain, *PEST Rich Region* (P)-proline, (E)-glutamic acid, (S)-serine, and (T)-threonine rich region, *CDC25H* CDC25 homology domain

PEST motif rich region- PEST [Proline (P), Glutamic acid (E), Serine (S), Threonine (T)] regions are known targets for ▶ calpain-type protease degradation, a role that has been experimentally demonstrated in RasGrf1 (Santos and Fernandez-Medarde 2009). This area is also targeted in RasGrf1 for phosphorylation by PKA or upon muscarinic receptor–mediated activation (Mattingly 1999). It is also phosphorylated by CDK5 in both RasGrfs, inhibiting the GEF activity toward Rac1 (RasGrf2) or Ras (RasGrf1) (Kesavapany et al. 2006).

Neuronal domain- Only found in RasGrf1, it is responsible for RasGrf1 binding to the NR2B subunit of the NMDA receptors and subsequent activation of downstream signaling pathways (Krapivinsky et al. 2003).

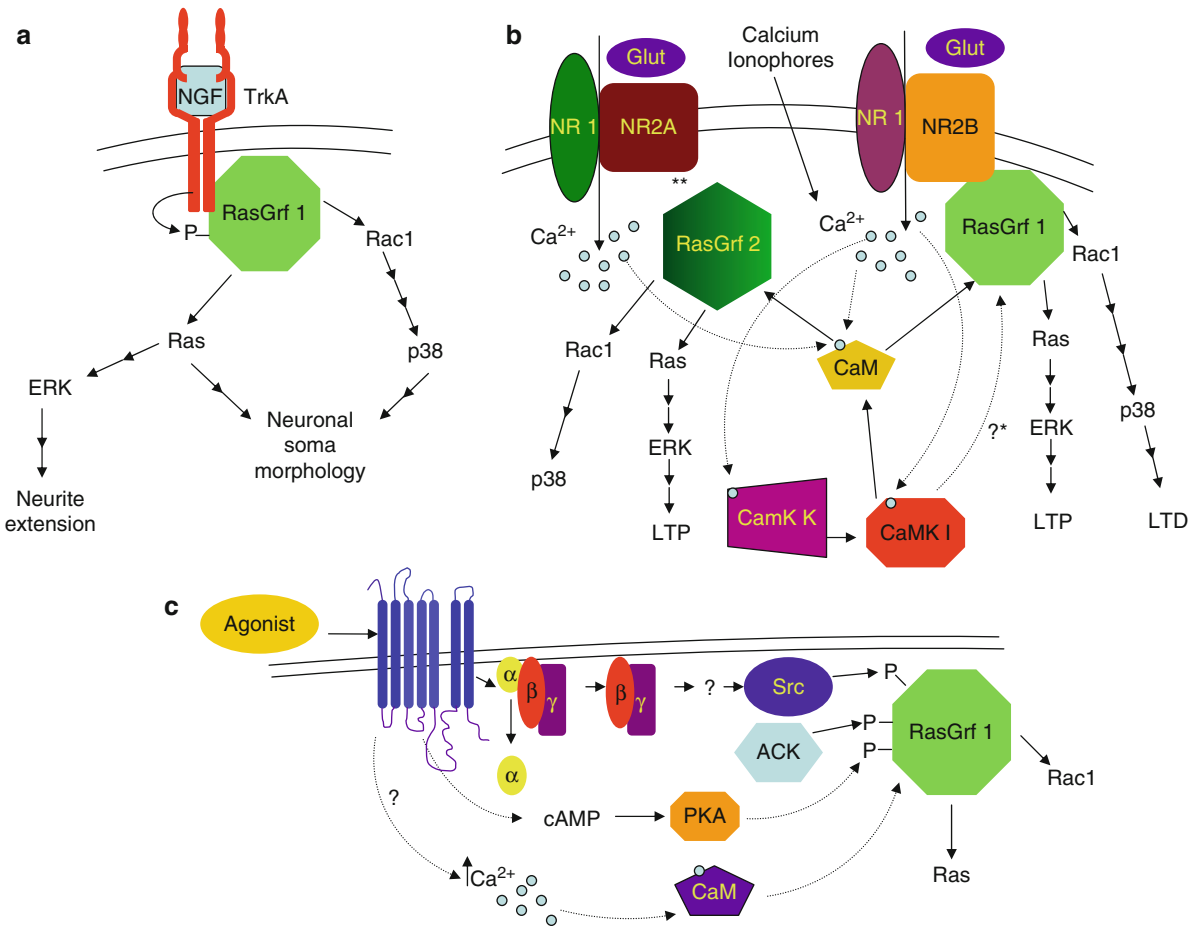
CDC25H domain- This C-terminal domain contains the catalytic region responsible for GDP/GTP exchange (GEF activity) on Ras family members. It is shared by all GEFs acting on canonical Ras proteins and shows a markedly high degree of conservation through evolution. This domain is necessary and sufficient for "in vitro" Ras activation by RasGrf1, and it is also responsible for Ras activation by RasGrf2. "In vivo" modulation of the GEF activity of the CDC25H domain in the context of full-length RasGrf proteins can be exerted through various intramolecular interactions or biochemical modifications (Santos and Fernandez-Medarde 2009, 2008).

Signaling Through the RasGrfs

Activation of RasGrfs in Response to Increase in Intracellular Calcium Concentration

Cellular calcium influx can modulate the activation of the RasGrfs through a mechanism involving Calmodulin binding to their IQ domains (Fig. 2). Mutations in this domain abolish ionomycin-mediated activation of RasGrf1 and stimulation of the Ras-ERK pathway. In addition, the N-terminal region of RasGrf1 cooperates synergistically with the IQ domain to potentiate RasGrf1-mediated activation of ERK1/2 upon stimulation by LPA or calcium (Santos and Fernandez-Medarde 2009).

RasGrf2 can also be activated by increased intracellular calcium concentration. In 293T cells, calcium influx causes translocation of RasGrf2 to the cell periphery, localizing it in close proximity to membrane GTPases, through a mechanism not yet fully understood. Although RasGrf2 interacts with calmodulin through its IQ domain, a full, functionally productive interaction appears to need the additional cooperation of other N-terminal domains (de Hoog et al. 2000). Interestingly, a RasGrf2 construct lacking the IQ domain is still able to activate Ras, suggesting that interaction with calmodulin is not necessary for RasGrf2 GEF activity, although both the IQ domain and Calmodulin interaction with RasGrf2 are indispensable for ERK activation.



RasGrf (RAS Protein-Specific Guanine Nucleotide-Releasing Factor), Fig. 2 The main upstream factors activating RasGrf proteins include the NGF receptor (**a**), NMDA receptors, intracellular increases of calcium (**b**), non-receptor protein kinases, PKA or G-protein coupled receptors (**c**). *CaMK1 activation of RasGrf1 has been proposed, but not demonstrated. **No physical interaction has been demonstrated between NR2A and RasGrf2

These observations suggest the existence of Ras-independent mechanisms of ERK1/2 activation, or the need of other interacting partners for full coupling of Ras and ERK1/2 activation (Santos and Fernández-Medarde 2008).

Activation of RasGrfs in Response to G-Protein Coupled Receptors (GPCR)

The intrinsic activity of intracellular RasGrf1 can be enhanced by stimulation with LPA or serum, but not with PDGF. This activation is inhibited by pretreatment with pertussis toxin, but not with genistein, suggesting that GPCRs, but not receptor tyrosine kinases (RTK) play a role in serum activation of RasGrf1 (Zippel et al. 1996). LPA treatment of NIH3T3 cells overexpressing RasGrf1 induces phosphorylation of RasGrf1 in serine residues, and calcium is also needed for full GEF activation (Fig. 2). Besides LPA, other GPCRs can also activate RasGrf1. Overexpression of subtype 1 human muscarinic receptor induces RasGrf phosphorylation and its GEF activity upon carbachol stimulation. This activation is prevented by phosphatases and G-protein α-subunit overexpression, and is constitutively established when G-protein βγ subunits are overexpressed. The 5-HT4 serotonin receptor is another GPCR able to activate RasGrf1. Its overexpression induces RasGrf1 phosphorylation by PKA (at Serine 916) and

IQ-dependent activation upon serotonin stimulation, suggesting that the complete activation of RasGrf1 by these receptors involves both cAMP and calcium/calmodulin-dependent signaling (Fig. 2) (Norum et al. 2007).

Overexpression of G-protein βγ subunits and LPA treatment are also known to induce RasGrf1 GEF activity toward Rac1, leading to JNK and c-fos promoter activation, but to fully activate Rac1, RasGrf1 needs to be phosphorylated in tyrosine by ▶ Src (Kiyono et al. 2000) (Fig. 2).

Activation of RasGrfs in Response to Receptor and Non-receptor Tyrosine Kinases

In PC12 cells, the TrkA nerve growth factor (NGF) receptor has been reported to interact with, and induce phosphorylation of, RasGrf1 in its PH1 domain (Robinson et al. 2005) (Fig. 2). In addition, RasGrf1 potentiates NGF-induced differentiation of PC12 cells in a process dependent on H-Ras and ERK1/2, but independent of Rac1 or ▶ PI3K pathways. On the other hand, RasGrf1 coordinates H-Ras and Rac1 pathways in a PI3K/AKT-dependent manner, to induce soma expanded morphology in PC12 cells (Santos and Fernandez-Medarde 2009).

The Src tyrosine kinase can also mediate transduction of G-protein-dependent signals from RasGrf1 to Rac1 proteins, but not to Ras canonical proteins (Kiyono et al. 2000). Other tyrosine kinases, such as ▶ ACK1 and Lck, are also able to phosphorylate RasGrf1, resulting in enhanced Ras GEF activity (Santos and Fernandez-Medarde 2009). In particular, ACK1 is known to be activated by Cdc42, which in its inactive GDP-Cdc42 conformation is also able to inhibit RasGrf1 activation of Ras (Arozarena et al. 2001). A similar mechanism may be applicable to RasGrf2, as the expression of dominant negative Cdc42 (Cdc42N17) abolishes RasGrf2 recruitment to the plasma membrane, Ras activation and ERK phosphorylation (Santos and Fernández-Medarde 2008). RasGrf2 and RasGrf1 are activated upon T-cell receptor stimulation, a process requiring the contribution of tyrosine kinase(s) of the Src family. Activation of RasGrf2 induces Ras-dependent and PLC-γ1-mediated signaling pathways, producing the activation of ▶ NF-AT, a transcriptional factor crucial for T-cell activation and differentiation (Ruiz et al. 2007).

Control of RasGrf Expression and Cellular Protein Levels

Transcriptional Control

RasGrf1 is an imprinted gene expressed only after birth. In mice, the paternal allele of the *RasGrf1* locus is methylated on a differentially methylated domain (DMD) located 30 kbp 5′ of the promoter. In mouse neonatal brain, RasGrf1 expression occurs exclusively from the paternal allele and accounts for ca. 90% of the total RasGrf1 expressed. A repeat sequence located immediately downstream of the DMD controls its methylation and is therefore required to establish RasGrf1 methylation in the male germ line. CTCF (a CCCTC-binding factor) binds to the DMD in a methylation-sensitive manner, acting as an "enhancer blocker." In the unmethylated maternal allele, CTCF is bound to DMD thus silencing expression, whereas CTCF cannot bind to the methylated paternal allele, thus allowing expression. The repeats and the DMD thus constitute a dual switch regulating RasGrf1 imprinting and timing of expression (Yoon et al. 2005).

Although *RasGrf2* appears not to be an imprinted gene, genomic methylation may still play a significant role in control of expression of this locus. The shortage of specific studies on RasGrf2 expression determines that the mechanisms controlling expression in the postnatal brain remain largely unknown. Interestingly, in colon, pancreatic and lung (NSCLC) tumors and cell lines, hypermethylation of *RasGrf2* locus and reduced protein expression are frequently associated (Santos and Fernández-Medarde 2008).

Some external factors are also known to modulate postnatal RasGrf1 expression. Cocaine induces overexpression in dorsal and ventral striatum, whereas the Alzheimer-related amyloid precursor protein with the Swedish mutation (APPSw) or oncogenic ErbB2/Neu and luteinizing hormone repress its expression in hippocampus and mammary gland, respectively (Santos and Fernandez-Medarde 2009).

Control of Proteolytic Degradation

The intracellular concentration of the RasGrf proteins is also posttranslationally regulated by cellular proteases. Both RasGrf1 and RasGrf2 contain a type A cyclin destruction box (CDB), located between the REM and CDC25 domains (Fig. 1). These domains have been reported to trigger ubiquitination and degradation of RasGrf2 by the proteasome upon Ras

binding. There is no direct experimental evidence showing a similar role of this domain in RasGrf1, although the interaction between RasGrf1 and the deubiquitinating enzyme mUBPy results in increased RasGrf1 half-life, and in M2 melanoma cells, the actin-binding protein Filamin A induces destabilization and ubiquitination of RasGrf1 with a subsequent reduction of MMP9 expression (Santos and Fernandez-Medarde 2009).

Calpain can also cleave RasGrf1, but the functional significance of such cleavage is unclear. Some reports described that this cleavage increases RasGrf1 GEF activity toward Ras proteins by releasing the C-terminus from inhibition by the N-terminal portion, whereas other studies show that phosphorylation by p35/CDK5 targets RasGrf1 for proteolysis by ▶ m-calpain, resulting in reduced Ras activation and AKT phosphorylation (Santos and Fernandez-Medarde 2009; Kesavapany et al. 2006). Phosphorylation of RasGrf2 by p35/CDK5 is also reported to result in accumulation and increased local concentration of RasGrf2 protein in the body of neurons (Santos and Fernández-Medarde 2008).

Genetically Modified Animal Models of the RasGrfs

RasGrf1

Analysis of the phenotypes of a number of independent RasGrf1 KO strains generated in different laboratories has contributed to a better understanding of the functional "in vivo" roles of RasGrf1. Two main types of phenotypes have been reported: those related to defects in memory consolidation and learning, and those related to growth retardation and glucose homeostasis responses.

An initial report on *RasGrf1*-KO mice (Brambilla et al. 1997) described LTP defects and associated impairment of amygdala-dependent learning, whereas later studies on a separate KO strain reflected LTD defects and impairment of hippocampus-dependent learning (Li et al. 2006). The discrepancy may be due to the use of different gene targeting strategies or different mouse genetic backgrounds. In any event, a role of RasGrf1 in memory and learning is supported by separate studies of the hippocampus of RasGrf1 KO mice that showed specific transcriptional alterations involving genes related to those neural processes. RasGrf1 also shows a role in cannabinoid tolerance, as the KO mice show lower tolerance to Δ9-tetrahydrocannabinoid, probably through alterations in cannabinoid receptor- and cAMP-mediated signaling (Tonini et al. 2006). Finally, RasGrf1 is also important to maintain normal photoreception, as the RasGrf1 KO mice develops light perception problems worsening with age progression (Fernandez-Medarde et al. 2009).

Analysis of the knockout mice has also revealed the role of RasGrf1 in the control of postnatal growth. Adult RasGrf1 null mice are 15–25% smaller than wild-type controls. The reduced size is probably directly associated to the lower levels of growth hormone and circulating plasma insulin observed in *RasGrf1*-deficient mice pituitary. The association between hypoinsulinemia and reduced pancreatic beta-cell mass observed in the KO mice is indicative of a role of RasGrf1 in control of beta cell proliferation and neogenesis (Font de Mora et al. 2003).

RasGrf2

RasGrf2 is dispensable for mouse development, postnatal growth, fertility, and normal aging, as the RasGrf2 KO mice are morphologically indistinguishable from their wild-type controls. A more subtle, brain phenotype is suggested by studies of a different RasGrf2 KO strain, pointing to lower levels of ERK activation upon NMDA-induction, and linking the absence of RasGrf2 to defective LTP in the CA1 region of the hippocampus (Li et al. 2006). Additional insights into the role of RasGrf2 "in vivo" have been obtained through the analysis of mice harboring combinations of a disrupted *RasGrf2* locus with null mutations for other GEFs. Thus, elimination of both RasGrf2 and RasGrf1 results in higher sensitivity to the neurotoxic effects of ischemia in the mouse brain (Santos and Fernandez-Medarde 2009). Furthermore, analysis of RasGrf2/▶ Vav3 and RasGrf2/▶ Vav1 null mice suggest a role for RasGrf2 in T-receptor signaling responses in lymphocytes (Ruiz et al. 2007).

Summary

The RasGrfs are the main GEF activators of mammalian Ras GTPases in the adult central nervous system. The experimental in vivo evidence indicates that both RasGrfs are able to activate the canonical Ras proteins (H-, N- and K-Ras) and Rac1. RasGrf1 and RasGrf2 are

large, highly homologous proteins sharing a modular structure composed of multiple distinct, functional domains which are instrumental for regulation of their intrinsic GEF activity and for modulation of their participation in signal transduction connecting a variety of upstream signals to their respective, specific downstream targets and elicited cellular responses. The GEF activity of the RasGrfs becomes activated in response to a variety of cellular signals including LPA, increased cytosolic concentration of calcium or cAMP, and activation of cell surface receptors for various signaling molecules such as ▶ NMDA, AMPA, serotonin, muscarinic agonists (G-protein coupled receptors), or NGF (trkA) (Fig. 2). Known downstream effects of the participation of the RasGrfs in cellular signaling pathways include control of cellular shape and nuclear organization, neurite extension, neuronal synaptic plasticity, induction of LTP or LTD, and neuroprotection against ischemia. Analysis of genetically modified animal models has uncovered the specific functional roles of RasGrf1 in memory and learning, postnatal growth, pancreatic beta cell proliferation, retinal photoreception, and neuroprotection against ischemia. Likewise, RasGrf2 has also been implicated in memory formation, neuroprotection, and immunological responses in lymphocytes. The participation of the RasGrfs in processes leading to human disease is also suspected. Increased RasGrf2 gene methylation is frequently observed in various human tumors and cancer cell lines. Other observations suggest the implication of RasGrf1 in visual defects, drug addiction, and Alzheimer-like neurodegenerative diseases.

In spite of the vast amount of published information on the RasGrf proteins, a number of key questions still remain unanswered. Because of their prevalent expression in the CNS, most functional studies on the RasGrfs have been restricted to neural tissues and cell lineages. However, as both RasGrfs are also expressed outside the CNS, their functional roles at those external locations remain less defined and require further, extensive studies. Another poorly understood area is the functional significance of the great variety of small RasGrf transcripts and peptides detected for both RasGrf1 and RasGrf2 in many tissues and/or states of development. An interesting hypothesis would be that such an assortment of RasGrf isoforms may contribute to the fine-tuned regulation of the activation of their cellular Ras/Rho targets at the spatial and temporal level. A better understanding of the mechanisms linking functional observations made for the RasGrfs at the cellular level with those made at the organism level would also be desirable. This pertains questions such as: Is the participation of RasGrf1 in cytoskeleton remodeling necessary for neuritogenesis? Is there a connection between the role of RasGrf1 in neuritogenesis and its contribution to memory formation processes? Finally, future work efforts should be strongly aimed at getting definitive answers to the current hints linking the RasGrf proteins to the development of different human illnesses and pathological processes.

References

Arozarena I, Matallanas D, Crespo P. Maintenance of CDC42 GDP-bound state by Rho-GDI inhibits MAP kinase activation by the exchange factor Ras-GRF. evidence for Ras-GRF function being inhibited by Cdc42-GDP but unaffected by CDC42-GTP. J Biol Chem. 2001;276(24):21878–84.

Brambilla R, Gnesutta N, Minichiello L, White G, Roylance AJ, Herron CE, et al. A role for the Ras signaling pathway in synaptic transmission and long-term memory. Nature. 1997;390:281–6.

de Hoog CL, Fan W-T, Goldstein MD, Moran MF, Koch CA. Calmodulin-independent coordination of Ras and extracellular signal-regulated kinase activation by Ras-GRF2. Mol Cell Biol. 2000;20:2727–33.

Fernandez-Medarde A, Barhoum R, Riquelme R, Porteros A, Nunez A, de Luis A, et al. RasGRF1 disruption causes retinal photoreception defects and associated transcriptomic alterations. J Neurochem. 2009;110(2):641–52.

Font de Mora J, Esteban LM, Burks DJ, Nunez A, Garces C, Garcia-Barrado MJ, et al. Ras-GRF1 signaling is required for normal beta-cell development and glucose homeostasis. EMBO J. 2003;22(12):3039–49.

Freshney NW, Goonesekera SD, Feig LA. Activation of the exchange factor Ras-GRF by calcium requires an intact Dbl homology domain. FEBS Lett. 1997;407:111–5.

Innocenti M, Zippel R, Brambilla R, Sturani E. CDC25(Mm)/Ras-GRF1 regulates both Ras and Rac signaling pathways. FEBS Lett. 1999;460(2):357–62.

Kesavapany S, Pareek TK, Zheng YL, Amin N, Gutkind JS, Ma W, et al. Neuronal nuclear organization is controlled by cyclin-dependent kinase 5 phosphorylation of Ras guanine nucleotide releasing factor-1. Neurosignals. 2006;15(4):157–73.

Kiyono M, Kaziro Y, Satoh T. Induction of rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm) following phosphorylation by the nonreceptor tyrosine kinase Src. J Biol Chem. 2000;275(8):5441–6.

Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, et al. The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron. 2003;40(4):775–84.

Li S, Tian X, Hartley DM, Feig LA. Distinct roles for Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) and Ras-GRF2 in the induction of long-term potentiation and long-term depression. J Neurosci. 2006;26(6):1721–9.

Mattingly RR. Phosphorylation of serine 916 of Ras-GRF1 contributes to the activation of exchange factor activity by muscarinic receptors. J Biol Chem. 1999;274:37379–84.
Norum JH, Dawood H, Mattingly RR, Sandnes D, Levy FO. Epac- and Rap- independent ERK1/2 phosphorylation induced by Gs-coupled receptor stimulation in HEK293 cells. FEBS Lett. 2007;581(1):15–20.
Robinson KN, Manto K, Buchsbaum RJ, MacDonald JI, Meakin SO. Neurotrophin-dependent tyrosine phosphorylation of Ras guanine-releasing factor 1 and associated neurite outgrowth is dependent on the HIKE domain of TrkA. J Biol Chem. 2005;280(1):225–35.
Ruiz S, Santos E, Bustelo XR. RasGRF2, a guanosine nucleotide exchange factor for Ras GTPases, participates in T-cell signaling responses. Mol Cell Biol. 2007;27(23):8127–42.
Santos E, Fernandez-Medarde A. RasGrf1. UCSD-nature molecule pages. 2009. doi:10.1038/mp.a002032.01.
Santos E, Fernández-Medarde A. RasGrf2. UCSD-nature molecule pages. 2008. doi:10.1038/mp.a002441.01.
Tonini R, Ciardo S, Cerovic M, Rubino T, Parolaro D, Mazzanti M, et al. ERK-dependent modulation of cerebellar synaptic plasticity after chronic Delta9-tetrahydrocannabinol exposure. J Neurosci. 2006;26(21):5810–8.
Yoon B, Herman H, Hu B, Park YJ, Lindroth A, Bell A, et al. Rasgrf1 imprinting is regulated by a CTCF-dependent methylation-sensitive enhancer blocker. Mol Cell Biol. 2005;25(24):11184–90.
Zippel R, Orecchia S, Sturani E, Martegani E. The brain specific Ras exchange factor CDC25 Mm: modulation of its activity through Gi-protein-mediated signals. Oncogene. 1996;12(12):2697–703.

RasGRP

▶ RasGRP1

RasGRP1

John J. Priatel[1], Kevin Tsai[1] and Kenneth W. Harder[2]
[1]Department of Pathology and Laboratory Medicine, Child and Family Research Institute, University of British Columbia, Vancouver, BC, Canada
[2]Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada

Synonyms

Calcium and DAG-regulated guanine nucleotide exchange factor II; CalDAG-GEFII; Guanine nucleotide exchange factor, calcium- and DAG-regulated; MGC129998; MGC129999; Ras guanyl nucleotide-releasing protein 1; Ras guanyl-releasing protein; Ras guanyl-releasing protein 1; RasGRP

Historical Background

Antigen receptor stimulation of T cells and B cells results in the rapid conversion of the small GTPase Ras from its "inactive" GDP-bound form to its "active" GTP-bound form (Alberola-Ila and Hernández-Hoyos 2003). However, the mechanisms of Ras regulation in lymphocytes, particularly by phorbol esters, had long remained a mystery as known signaling pathways could not account for its activation. The discovery of the Ras guanyl-nucleotide exchange factor RasGRP1, cloned by two groups searching for novel Ras activators in rat brain and murine T cells, was a major advance leading to a better understanding of Ras regulation in lymphocytes (Stone 2011). Ectopic expression of RasGRP1 in rodent fibroblasts was found to be capable of activating Ras and inducing cellular transformation. Subsequently, investigations of human T cells and RasGRP1-deficient mice have corroborated key roles for RasGRP1 in both T-cell receptor- and phorbol ester-induced Ras activation (Stone 2011). Originally coined RasGRP (Ras guanyl nucleotide-releasing protein), RasGRP1 is the prototypic member of the RasGRP family of proteins that share a number of conserved elements that have been implicated in its function and regulation (Stone 2011), including REM (Ras exchange motif) domain, ▶ CDC25 (cell division cycle 25)-related domain, calcium-binding "EF-hand" motif, and diacylglycerol-binding "C1" domain (Fig. 1). Besides RasGRP1, the RasGRP family also comprises of RasGRP2, RasGRP3, and RasGRP4.

RasGRP1 Functions in Developing T Cells

The small GTPase Ras acts as a molecular switch, cycling between GDP-bound "off" and a GTP-bound "on" conformations, and serves to link signals from cell surface receptors to intracellular effector pathways. Ras activation can be modulated by its own intrinsic GTPase activity, converting GTP to GDP, and guanine nucleotide exchange. However, the Ras' rates of GTP hydrolysis and nucleotide exchange are

RasGRP1, Fig. 1 Conserved domains of RasGRP1. Domain structure of human RASGRP1 protein (NCBI accession: AAH67298; 765 aa), annotated by NCBI Blast Search, is schematically shown. REM (Ras Exchange Motif; E-value: 1.44e-15) and ▶ CDC25 (also known as RasGEF; E-value: 1.44e-15) domains participate in the catalytic activity of the exchange factor. The Protein kinase C conserved region 1 (C1; E-value: 1.54e-12) domain binds diacylglycerol and phorbol esters. EF-hand domains (E-value: 8.42e-04) are suspected to act as calcium sensors and calcium signal modulators

very low unless paired with catalytic proteins. Guanine nucleotide exchange factors (Ras GEFs) control the activation of Ras by catalyzing GDP release from Ras and facilitating its association with more prevalent cellular GTP. Conversely, Ras GTPase-activating proteins (Ras GAPs) accelerate GTP hydrolysis reaction converting Ras-GTP to its inactive GDP-bound form. Tight control of Ras activity is essential for regulating cell activation, proliferation, differentiation, and apoptotic programs in multiple cell types. By associating with various effector proteins, activated Ras initiates signaling through multiple downstream pathways such as the mitogen-activated protein kinase (MAPK) cascade.

T-cell receptor (TCR) stimulation results in the rapid activation of the small GTPase Ras whose signals are essential for the development of T cells in the thymus (Alberola-Ila and Hernández-Hoyos 2003). Taking cues from Ras studies on non-lymphocytes, the Ras GEF SOS (Son of Sevenless) has been postulated to regulate Ras upon TCR activation. SOS proteins are ubiquitously expressed and their functions are modulated through their association with the adaptor GRB2 (growth factor receptor-bound protein 2). Moreover, the SH2 domain of GRB2 targets SOS to phosphorylated tyrosine residues of surface receptors and adaptor proteins.

According to the SOS-based model, the following sequence of events leads to Ras activation in T cells: TCR ligation activating the ▶ Src protein tyrosine kinases (PTKs) LCK (lymphoid cell kinase) and Fyn leading to the phosphorylation of CD3s ITAMs (immunoreceptor tyrosine-based activation motifs), these phosphorylated ITAMs mobilizing the Syk PTK ▶ ZAP-70 (zeta-associated protein – 70 kDa) by way of ▶ ZAP-70s SH2 domain and becoming activated through the action of ▶ Src PTKs, activated ▶ ZAP-70 phosphorylating multiple tyrosine residues on the docking adaptor transmembrane protein ▶ LAT (Linker for Activated T cells) and phosphorylated ▶ LAT recruiting the GRB2/SOS complex in close proximity of plasma membrane–bound Ras and facilitating its displacement of GDP. However, a SOS based model of Ras activation cannot explain at least two T-cell phenomena: (1) Why phorbol esters or their analogs activate Ras? and (2) Why PKC inhibitors dampen Ras activation?

The cloning of RasGRP1 identified the first of a novel class of Ras guanyl nucleotide-releasing proteins that possessed calcium- and diacylglycerol (DAG)-responsive elements and elucidated a critical mechanism by which TCR signal transduction and phorbol ester stimulation is linked to the activation of the Ras-MAPK cascade in T cells (Stone 2011). TCR signaling incorporates RasGRP1 function through the mobilization and enzymatic activities of PLCγ1. Similarly to GRB2/SOS, PLCγ1 is recruited to phosphorylated ▶ LAT through its SH2 domain and becomes phosphorylated by Tec family protein kinases. Subsequently, the action of activated PLCγ1 converts PIP2 (phosphatidylinositol 4, 5 bisphosphate) into IP3 (inositol 3, 4, 5 triphosphate) and DAG, a second messenger previously thought to solely activate PKC through its DAG-binding C1 domain. However, DAG also causes RasGRP1 to become membrane localized through its own DAG-binding C1 domain. Furthermore, DAG indirectly impacts RasGRP1 as activated PKC regulates RasGRP1 activity through its phosphorylation (Roose et al. 2005). As a consequence, the integration of RasGRP1 into T-cell signaling provides an explanation for the well-documented activation of Ras by DAG analogs and phorbol esters such as PMA (phorbol myristate acetate). In addition, the integration of RasGRP1 also provides a mechanism for why PKC inhibitors block Ras activation.

Ras-MAPK signaling downstream of the pre-TCR and TCR is critical for two developmental checkpoints as thymocytes undergo an ordered series of maturation steps within the thymic microarchitecture (Alberola-Ila and Hernández-Hoyos 2003). Thymocyte development is most often tracked through the variable expression of the cell surface markers CD4 and CD8. After productive rearrangement of the TCRβ chain and pairing with the pre-TCRα, pre-TCR expression by the most immature $CD4^-$ $CD8^-$ double-negative (DN) thymocytes drives ligand-independent Ras-MAPK

signaling at the first developmental checkpoint and differentiation into $CD4^+$ $CD8^+$ double-positive (DP) thymocytes. Accompanying rearrangement and expression of the TCRα chain, TCR-dependent Ras-MAPK signaling at the second developmental checkpoint (called "thymocyte selection") becomes contingent upon the recognition of self-antigens (i.e., self-peptides presented in the context of self-MHC molecules). The intensity of TCR interaction with self-antigens on thymic cortical epithelial cells and bone marrow–derived cells is presumed to determine the strength of signal and the fate of the developing DP thymocyte. According to the strength of signal hypothesis, cells that do not recognize self-antigens fail to receive TCR signaling resulting in death by neglect; cells that recognize self-antigens robustly receive strong TCR signaling dying via active apoptosis (negative selection); and cells that recognize self-antigens weakly receive moderate TCR signaling differentiating into mature $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single-positive (SP) T cells (positive selection).

DP thymocytes discriminate graded TCR-dependent Ras-MAPK signals and translate them into a cell fate decision (Alberola-Ila and Hernández-Hoyos 2003). TCR-induced Ras signaling results in the activation of three distinct families of MAPKs: ERK (extracellular signal-regulated kinases), JNK (c-Jun N-terminal kinases), and p38. The activation of the MAPKs plays qualitatively and quantitatively distinct roles in thymocyte selection: ERK has been most often paired with positive selection whereas JNK and p38 are associated with negative selection. How might different MAPKs selectively pair and become activated upon TCR-induced Ras signaling was not clear. To elucidate the role of RasGRP1 in T-cell development, analyses of *RasGRP1*$^{-/-}$ mice revealed a near-normal number of DN and DP thymocytes but a severe deficiency in mature thymocytes, suggesting a block in thymocyte selection (Stone 2011). Furthermore, *RasGRP1*$^{-/-}$ thymocytes failed to activate both Ras and ERK upon phorbol ester stimulation. In addition, one-month old *RasGRP1*$^{-/-}$ mice had very few splenic T cells. As a consequence of the T-cell phenotype present in *RasGRP1*$^{-/-}$ mice along with the governing role that TCR signaling plays in T-cell development, RasGRP1 was hypothesized to link Ras activation with TCR signaling.

To examine the role of RasGRP1 in thymocyte selection, two lines of *RasGRP1*$^{-/-}$ TCR transgenic mice were generated to determine the effect of RasGRP1 under conditions of defined TCR signaling strength (Priatel et al. 2002). Results from these experiments indicated that positive selection, particularly a weakly selecting TCR, and TCR-induced ERK activation are critically dependent on RasGRP1. By contrast, RasGRP1-deficiency had no effect on negative selection or JNK and p38 MAPK activation. These conclusions were consistent with complementary findings from another study investigating *Grb2*$^{+/-}$ mice (Gong et al. 2001). Halving of the amount of GRB2/SOS led to decreased Ras, JNK, and p38 activation and impaired negative selection. However, ERK activation and positive selection were unaffected by *Grb2* haploinsufficiency, suggesting that the RasGRP1-ERK pathway may have a lower threshold of activation than GRB2/SOS. The rationale for TCR signaling to employ two different Ras GEFs may be to subject Ras to differential regulation or to pair it with a unique subset of effectors. Based on the findings from the above studies, a hypothesis was formulated that the Ras GEFs RasGRP1 and GRB2/SOS may serve to selectively pair Ras activation with differential MAPKs pathways. According to this model, DP thymocytes expressing a positively selecting TCR will activate Ras and solely the MAPK ERK via RasGRP1 whereas DP thymocytes expressing a negatively selecting TCR will activate Ras and the full range of MAPKs (ERK, JNK, and p38) via the use of both GRB2/SOS and RasGRP1 pathways.

RasGRP1 Functions in Other Blood Cells

RasGRP1 was originally envisioned to have very restricted expression, transcripts being detected solely in T cells and some neuronal cell lineages (Stone 2011). More recent studies have found that RasGRP1 has a much broader tissue distribution than previously thought. RasGRP1 is also expressed in B cells and is presumed to couple the B-cell antigen receptor (BCR) to Ras-ERK signaling in an analogous fashion to the way it functions in TCR signal transduction (Coughlin et al. 2005). However, RasGRP1 function in BCR signaling appears to be partially masked through the coexpression of RasGRP3 and the sharing of some redundant functions with this RasGRP family member. Studies using the immature B cell line WEHI-231 have linked

a RasGRP1-pathway that is ERK-independent to BCR-induced apoptosis (Guilbault and Kay 2004). Analyses of human NK (natural killer) cells using RNA interference have demonstrated that RasGRP1 regulates ITAM-dependent cytokine production and NK cell cytotoxicity (Lee et al. 2009). In addition, RasGRP1 knockdown in NK cells was found to result in dampened Ras, ERK, and JNK activation.

RasGRP1 is also expressed by mast cells and signals downstream of the high affinity IgE receptor FcεR1 (Liu et al. 2007). Moreover, FcεR1 degranulation and cytokine production were greatly reduced in *RasGRP1*$^{-/-}$ mast cells relative to wild type and *RasGRP1*$^{-/-}$ mice failed to elicit anaphylactic allergic reactions. Interestingly, RasGRP1 in mast cells was found to link FcεR1-mediated Ras signaling to ▶ PI3K (phosphatidyl inositol 3-kinase) pathway rather than to ERK activation. By contrast, a concentrated effort to establish a connection between RasGRP1 signaling and ▶ PI3K activation in lymphocytes has been unsuccessful (Stone 2011). In addition, RasGRP1 expression and function has been recently described outside of the hematopoietic system, although those studies are described elsewhere (Stone 2011).

RasGRP1 Activity and Subcellular Localization

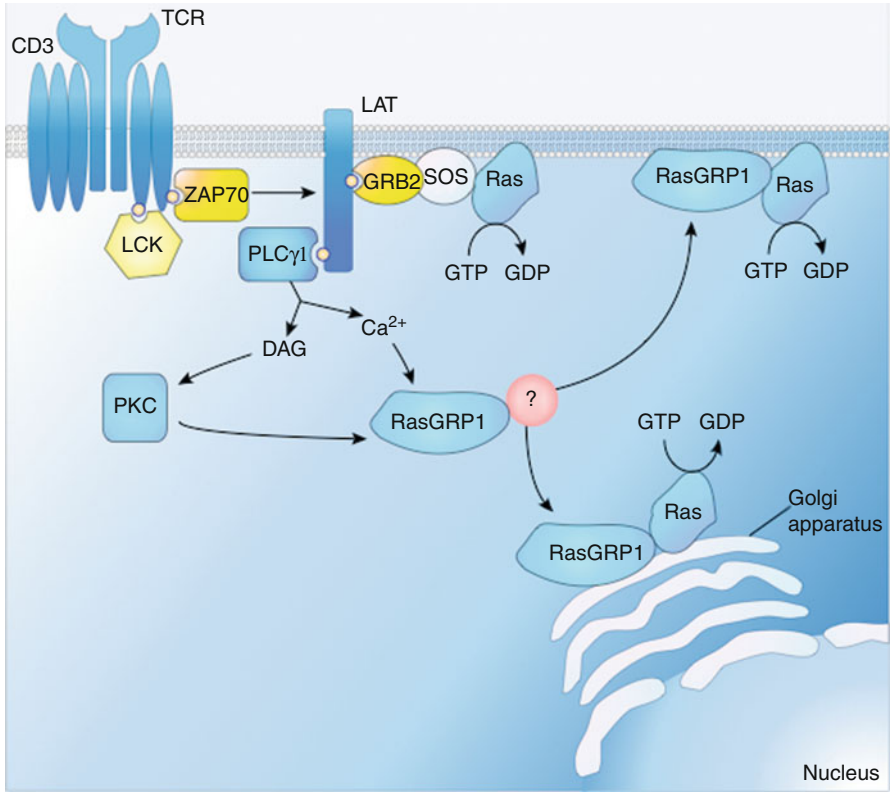
The most controversial issue regarding RasGRP1 is its subcellular locale when active. Long-held dogma asserts that Ras operates at the plasma membrane, however, a number of reports have challenged this belief by arguing that TCR-induced Ras activation occurs at the Golgi in a PLCγ1- and RasGRP1-dependent fashion (Mor and Philips 2006). As Ras proteins transit through endomembranes on their way to the plasma membrane, there is the potential for subcellular compartmentalization of Ras activation to add another dimension of complexity to Ras signaling and possible cellular responses (Fig. 2). Nevertheless, other studies have found evidence solely for plasma membrane-associated-Ras and -RasGRP1 upon TCR and BCR ligation (Stone 2011), raising the question of whether observations of active RasGRP1 and activated Ras at internal membranes are an artifact of protein overexpression or the tracking of fluorescently tagged rather than native endogenous molecules.

An exquisite investigation monitoring endogenous signaling molecules has suggested that subcellular compartmentalization of the Ras/RasGRP1/ERK pathway plays a key role in developing thymocytes undergoing selection (Daniels et al. 2006). Using OT-1 TCR transgenic preselection $CD4^+CD8^+$ thymocytes and specific peptides that span the boundary of positive and negative selection, it was revealed that negatively selecting peptides targeted Ras, ▶ Raf-1, and RasGRP1 to the plasma membrane, whereas these molecules colocalized to endomembranes when thymocytes were stimulated with peptides mediating positive selection. Notwithstanding, as this study determined the localization of total Ras rather than active Ras (i.e., Ras-GTP), it is conceivable that sufficient signaling necessary to mediate positive selection is initiated via trace amounts of RasGRP1 and Ras that is situated at the plasma membrane.

A recent study utilizing novel high affinity probes for Ras-GTP imaged in live Jurkat T cells was capable of discerning the accumulation of endogenous Ras-GTP solely at the plasma membrane (Rubio et al. 2010). In addition, the failure of a palmitoylation-defective mutant of N-Ras that is restricted to endomembranes to become activated upon TCR stimulation further asserts that plasma membrane localization is required for Ras activation. Future studies will require sophisticated tools like the one discussed above and high-resolution microscopic imaging to settle the debate over the localization of active RasGRP1 and Ras-GTP.

RasGRP1 and Autoimmunity

T cells are a vital component of the body's defense system and their capacity to differentiate self- from foreign-antigens is crucial to protect against both pathogenic challenge and autoimmune-mediated self-destruction. The dependence of T-cell development, T-cell function, and T-cell tolerance on TCR signaling suggests that mutations affecting TCR signal transduction may cause a multitude of deleterious health-related effects. Immunodeficiency may arise from alterations to the TCR repertoire and T-cell function. In addition, aberrant TCR signaling may promote autoimmunity by influencing central- (deletion of autoreactive T cells in the thymus) and peripheral-T-cell tolerance (T-cell anergy, activation-induced



RasGRP1, Fig. 2 The activation of Ras by T-cell receptor signal transduction. T-cell receptor stimulation induces the activation of the protein tyrosine kinases LCK (lymphoid cell kinase) and ▶ ZAP-70 (zeta-associated protein – 70 kDa) and subsequently, phosphorylation of ▶ LAT (Linker for Activated T cells). Phosphorylated ▶ LAT results in the GRB2 (Growth factor Receptor-Bound protein 2)/SOS (Son of sevenless) complex being targeted to the plasma membrane through Grb2's SH2 (Src homology 2) domain. Simultaneously, phosphorylated LAT also recruits PLCγ1 (phospholipase Cγ1), resulting in its activation. Activated PLC-γ1 leads to the production of DAG (diacylglycerol) and increases in cytosolic calcium. DAG and perhaps rises in cytosolic calcium cause the cytoplasmic protein RasGRP1 to become translocated, localizing at the plasma membrane or endomembranes such as the Golgi apparatus. The concentrations of these two second messengers along with phosphorylation of RasGRP1 by PKC (protein kinase C) are speculated to determine the cellular site of RasGRP1's mobilization. The subcellular compartmentalization of Ras signaling may serve to subject Ras to differential regulation or to pair it with a unique subset of effectors

cell death [AICD] and suppression by regulatory T cells). Importantly, abnormal Ras-ERK signaling in T cells has been described in a number of autoimmune diseases in humans and animal models.

One reported consequence of decreased activation of Ras-ERK pathway in T cells is reduced DNA methyltransferase I (DNMT1) expression causing the derepression of autoimmune genes (Gorelik et al. 2007). Recently, two microRNAs, miRNA-21 and miRNA-148a, overexpressed in T cells from both patients with systemic lupus erythematosus (SLE) and lupus-prone MRL/lpr mice have been found to downmodulate DNMT1 directly and indirectly by turning down Ras-ERK signaling and targeting RasGRP1 transcripts (Pan et al. 2010). By contrast, defective RasGRP1 expression in a subset of SLE patients has been proposed to result from aberrant RNA splicing (Stone 2011). Additionally, dysregulated RasGRP1 expression has been implicated in another autoimmune disease through genome-wide association studies linking RasGRP1 variants to type 1 diabetes (Stone 2011).

The severely impaired T-cell maturation in the thymus of young *RasGRP1*-deficient mice is correlated with a small but activated population of peripheral T cells, particularly of the CD4 lineage (Layer et al. 2003; Priatel et al. 2007). However, with age, *RasGRP1*-deficient mice (on a mixed

C57BL/6:129SvJ genetic background), derived through a classical gene targeting approach (*RasGRP1*$^{-/-}$) and a spontaneous mouse mutant of RasGRP1 (*RasGRP1*^{lag}; *lag* is an acronym representing *l*ymphoproliferation-*a*utoimmunity-*g*lomerulonephritis), were found to exhibit massive lymphoproliferation and autoimmunity with similarity to SLE (Layer et al. 2003). At 5 months of age, *RasGRP1*^{lag} and *RasGRP1*$^{-/-}$ mice displayed splenomegaly, lymphadenopathy, glomerulonephritis, lymphocytic infiltrates within many organs, elevated antinuclear antibodies (ANAs), anorexia, and lethargy. However, the penetrance of a severe autoimmune phenotype within one animal colony of *RasGRP1*$^{-/-}$ mice disappeared after successive backcrossing of the targeted mutation onto the C57BL/6 background. C57BL/6 *RasGRP1*$^{-/-}$ mice remained lymphopenic and free of severe autoimmune disease up to 1 year of age despite high-serum ANA levels (Priatel et al. 2007). It is possible that genetic modifiers from the 129/SvJ genetic background or environmental factors, such as distinct microfloral, may synergize with *RasGRP1*-deficiency to promote fulminant disease.

The lack of RasGRP1 in developing thymocytes may push the balance toward autoimmunity. It has been proposed that DP thymocytes capable of maturing into mature SP thymocytes need to express more strongly self-reactive TCRs if they lack RasGRP1 to overcome their signaling deficits (Priatel et al. 2002; Layer et al. 2003). As TCR transgenic studies have argued that RasGRP1 is not necessary for central tolerance (Priatel et al. 2002), the affinity/avidity of TCRs expressed by *RasGRP1*$^{-/-}$ mature SP thymocytes perhaps straggle the boundary between positive and negative selection. In addition, RasGRP1 has been shown to play a critical role in the formation of natural Foxp3-expressing regulatory T cells, suggesting that impaired development or function of this lineage may contribute to disease in RasGRP1 mutant mice (Stone 2011).

There are also a number of peripheral mechanisms by which RasGRP1-deficiency may collude with defective thymocyte development to cause disease. Firstly, the lymphopenic compartment within *RasGRP1*$^{-/-}$ mice that results from decreased thymic output may favor oligoclonal T-cell outgrowth and generation of T-cell effectors through abundance of cytokines like IL-7 and increased availability of self-peptides/self-MHC molecules. Notably, *RasGRP1*$^{-/-}$ T cells have a distinct TCR repertoire relative to wild type animals resulting from altered T-cell development or peripheral T-cell homeostasis (Priatel et al. 2007). Secondly, aberrant TCR signaling or TCR repertoire in mature T cells may lead to weakened immune responses, chronic infections, and proinflammatory conditions. Viral challenge experiments demonstrated that *RasGRP1*$^{-/-}$ mice generate drastically fewer antigen-specific T cells and delayed pathogen clearance as compared to wild type mice (Priatel et al. 2007). Thirdly, the resistance to AICD exhibited by RasGRP1-deficient T cells in vitro has been postulated to enhance their pathogenicity in vivo by escaping apoptosis (Layer et al. 2003). Fourthly, the function or maintenance of regulatory T cells may be impacted by diminished IL-2 production observed for *RasGRP1*$^{-/-}$ T-cell effectors (Layer et al. 2003; Priatel et al. 2010). Collectively, these findings suggest multiple means by which aberrant RasGRP1 signaling may enhance susceptibility to immunologic disease.

RasGRP1 and Cancer

Ras signaling regulates proliferation, differentiation and survival and activating Ras mutations are present in approximately 30% of all human cancers. As the original descriptions of RasGRP1 documented its capacity to transform rodent fibroblasts in vitro (Stone 2011), it raises the question as to whether altered RasGRP1 expression or activity can lead to tumorigenesis. To date, findings from several studies have supported this hypothesis.

The observation that the RasGRP1 is a frequent site of proviral insertion in retrovirus-induced murine T-cell lymphomas suggests that RasGRP1 can act as an oncogene (Stone 2011). Corroborating these findings, overexpression of RasGRP1 in the thymus was able to initiate thymic lymphomas in a pre-TCR/TCR-independent manner (Klinger et al. 2005). An investigation into genes able to induce acute myeloid leukemia (AML) found that RasGRP1 can act alone as a leukemic initiator/driver or act in concert with other leukemic causing genes to cause disease (Vassiliou et al. 2011). In addition, therapeutically targeting the Ras-ERK pathway using MEK inhibitors in a mouse model of AML revealed that increased RasGRP1 expression correlated with tumor resistance to the drug, although the precise mechanism of action

remained unclear (Lauchle et al. 2009). Besides its association with blood cancers, RasGRP1 overexpression in murine epidermal keratinocytes led to spontaneous development of squamous cell papillomas in the absence of chemical tumor initiators (Diez et al. 2009). Consequently, RasGRP1 is relevant to tumorigenesis in hematopoietic and non-hematopoietic cells.

Summary

RasGRP1 is the prototypical member of the RasGRP family of guanyl nucleotide exchange factors whose function is to couple surface receptor signaling to the activation of the small GTPase Ras and downstream MAPK pathways. The activity of RasGRP1 is regulated through its mobilization to membranes and pairing with Ras by the second messenger DAG (diacylglycerol). RasGRP family members are composed of a REM (Ras exchange motif) and ▶ CDC25 (cell division cycle 25)-related domains, functioning in Ras recognition and catalysis of GDP exchange, and "EF-hand" and DAG-binding "C1" motifs, serving to modulate its recruitment to membranes. Initial investigations revealed that RasGRP1 links TCR signaling to the activation of Ras-ERK pathway, playing crucial roles in both T-cell development and mature T-cell function. More recent studies have found that RasGRP1 mediates critical regulation of surface receptor signaling and effector functions in B cells, NK cells, and mast cells. Perturbations in RasGRP1 function are suspected to underlie immunodeficiency, autoimmunity, and blood cell malignancies.

References

Alberola-Ila J, Hernández-Hoyos G. The Ras/MAPK cascade and the control of positive selection. Immunol Rev. 2003;191:79–96.

Coughlin JJ, Stang SL, Dower NA, Stone JC. RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. J Immunol. 2005;175:7179–84.

Daniels MA, Teixeiro E, Gill J, Hausmann B, Roubaty D, Holmberg K, Werlen G, Holländer GA, Gascoigne NRJ, Palmer E. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. Nature. 2006;444:724–9.

Diez FR, Garrido AA, Sharma A, Luke CT, Stone JC, Dower NA, Cline JM, Lorenzo PS. RasGRP1 transgenic mice develop cutaneous squamous cell carcinomas in response to skin wounding: potential role of granulocyte colony-stimulating factor. Am J Pathol. 2009;175:392–9.

Gong Q, Cheng AM, Akk AM, Alberola-Ila J, Gong G, Pawson T, Chan AC. Disruption of T cell signaling networks and development by Grb2 haploid insufficiency. Nat Immunol. 2001;2:29–36.

Gorelik G, Fang JY, Wu A, Sawalha AH, Richardson B. Impaired T cell protein kinase C delta activation decreases ERK pathway signaling in idiopathic and hydralazine-induced lupus. J Immunol. 2007;179:5553–63.

Guilbault B, Kay R. RasGRP1 sensitizes an immature B cell line to antigen receptor-induced apoptosis. J Biol Chem. 2004;279:19523–30.

Klinger MB, Guilbault B, Goulding RE, Kay RJ. Deregulated expression of RasGRP1 initiates thymic lymphomagenesis independently of T-cell receptors. Oncogene. 2005;24:2695–704.

Lauchle JO, Kim D, Le DT, Akagi K, Crone M, Krisman K, Warner K, Bonifas JM, Li Q, Coakley KM, et al. Response and resistance to MEK inhibition in leukaemias initiated by hyperactive Ras. Nature. 2009;461:411–4.

Layer K, Lin G, Nencioni A, Hu W, Schmucker A, Antov AN, Li X, Takamatsu S, Chevassut T, Dower NA, et al. Autoimmunity as the consequence of a spontaneous mutation in Rasgrp1. Immunity. 2003;19:243–55.

Lee SH, Yun S, Lee J, Kim MJ, Piao Z-H, Jeong M, Chung JW, Kim T-D, Yoon SR, Greenberg PD, et al. RasGRP1 is required for human NK cell function. J Immunol. 2009;183:7931–8.

Liu Y, Zhu M, Nishida K, Hirano T, Zhang W. An essential role for RasGRP1 in mast cell function and IgE-mediated allergic response. J Exp Med. 2007;204:93–103.

Mor A, Philips MR. Compartmentalized Ras/MAPK signaling. Annu Rev Immunol. 2006;24:771–800.

Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, Li J, Zhou H, Tang Y, Shen N. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. J Immunol. 2010;184:6773–81.

Priatel JJ, Teh S-J, Dower NA, Stone JC, Teh H-S. RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. Immunity. 2002;17:617–27.

Priatel JJ, Chen X, Zenewicz LA, Shen H, Harder KW, Horwitz MS, Teh H-S. Chronic immunodeficiency in mice lacking RasGRP1 results in CD4 T cell immune activation and exhaustion. J Immunol. 2007;179:2143–52.

Priatel JJ, Chen X, Huang Y-H, Chow MT, Zenewicz LA, Coughlin JJ, Shen H, Stone JC, Tan R, Teh HS. RasGRP1 regulates antigen-induced developmental programming by naive CD8 T cells. J Immunol. 2010;184:666–76.

Roose JP, Mollenauer M, Gupta V, Stone J, Weiss A. A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. Mol Cell Biol. 2005;25(11):4426–41.

Rubio I, Grund S, Song S-P, Biskup C, Bandemer S, Fricke M, Förster M, Graziani A, Wittig U, Kliche S. TCR-induced activation of Ras proceeds at the plasma membrane and requires palmitoylation of N-Ras. J Immunol. 2010;185:3536–43.

Stone J. Regulation and function of the RasGRP family of Ras activators in blood cells. Genes Cancer. 2011;2(3):320–34.
Vassiliou GS, Cooper JL, Rad R, Li J, Rice S, Uren A, Rad L, Ellis P, Andrews R, Banerjee R, et al. Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. Nat Genet. 2011;43:470–5.

Rasl2-8

► TLR4, Toll-Like Receptor 4

Ras-Related Associated with Diabetes

Jose-Luis González de Aguilar
Laboratory of Molecular Signaling and Neurodegeneration, INSERM, Faculty of Life Sciences, University of Strasbourg, Strasbourg, France

Synonyms

Rad; Ras associated with diabetes; RRAD

Historical Background

Ras associated with diabetes (Rad) was identified in the beginning of the 1990s as a clone differentially expressed in two subtraction cDNA libraries prepared from skeletal muscle of normal individuals and patients with Type II (non-insulin-dependent) diabetes mellitus (Reynet and Kahn 1993). Analysis of the newly identified clone revealed about 50% identity at the nucleotide level with members of the Ras superfamily, which consists of more than a hundred low-molecular-weight guanine nucleotide–binding proteins, also referred to as small GTPases. The major feature of this class of molecules is their ability to cycle between a GDP-bound inactive and a GTP-bound active conformation. Small GTPases are divided into six subfamilies: Ras, Rho, Arf, Rab, ► Ran, and RGK, in which Rad is included. They participate in important cellular processes such as growth and differentiation, cytoskeletal dynamics, membrane trafficking, vesicle transport, and signal transduction. In their seminal report, Reynet and Kahn observed that the expression of Rad is typically highest in skeletal muscle, cardiac muscle, and lung of normal individuals. They also found that Rad mRNA levels appeared increased in Type II diabetes muscle as compared to Type I diabetes or nondiabetic muscle (Reynet and Kahn 1993). These findings provided the framework for follow-up studies on the comprehension of the biology of Rad in relation to muscle (patho) physiology and cancer.

Structure and Regulation of the Activity of Rad

The human Rad gene is localized in chromosome 16q22, spans 3.75 kb, and is composed of five exons and four introns. Translation presumably starts from an in-frame ATG codon in the second exon, which gives rise to a protein of 269 amino acids with a predicted molecular weight of 29,266 kDa (Caldwell et al. 1996). Rad is the prototype member of the small GTPases in the RGK family, which also includes Rem, Rem2, and Gem/Kir. From a structural point of view, Rad possesses the five highly conserved GTPase domains G1 to G5, characteristic of the Ras-related proteins, but it also displays particular features distinct from that commonly observed in other small GTPases. First, Rad exhibits several nonconserved amino acids in G1, G2, and G3 domains that may affect its GTPase function. Second, Rad shows longer NH_2 and COOH termini of 88 and 31 amino acids, respectively. Third, The COOH terminus of Rad does not present the typical CAAX isoprenylation motif that usually facilitates the attachment to cell membranes (Reynet and Kahn 1993).

Rad has been shown to interact with a variety of proteins that can thereby determine its function and/or subcellular distribution. For example, overexpressing Rad in a neuroblastoma cell line triggers cellular flattening and neurite extension, and it has been proposed that these effects are the consequence of binding of Rad to the Rho-associated protein kinase ROCK, which is known to regulate the shape and movement of cells by acting on the cytoskeleton. Such an interaction impedes ROCK activity which, in turn, results in the inhibition of contraction and retraction (Ward et al. 2002). The subcellular distribution of Rad greatly depends on the interactions with the Ca^{2+}-binding protein calmodulin (CaM) and the multifunctional regulatory protein 14-3-3. It has been reported that

the lack of binding to CaM induces the accumulation of Rad in the nucleus, whereas the association with 14-3-3 maintains Rad within the cytoplasm (Mahalakshmi et al. 2007). Interestingly, Rad presents several consensus phosphorylation sites in the extended COOH terminus, which are close to the CaM-binding domain. Rad can be phosphorylated on serine residues by Ca^{2+}-/CaM-dependent protein kinase II (CaMKII), PKA, ▶ casein kinase II and PKC. These post-translational modifications do not seem to affect the ability of Rad to hydrolyze GTP but alter its affinity to bind CaM and 14-3-3, thus interfering with the subcellular localization of the protein (Moyers et al. 1998; Mahalakshmi et al. 2007).

Little is known about the mechanisms controlling the expression of Rad at the transcriptional level. Early studies indicated the localization of Rad to thin filaments in skeletal muscle, as well as its increased expression during myoblast fusion (Paulik et al. 1997). In agreement with these observations, subsequent studies showed that several transcription factors involved in myogenesis, including MEF2, MyoD, and Myf5, stimulate the transcriptional activity of the Rad promoter (Hawke et al. 2006). Increased Rad expression has also been observed in experimental conditions characterized by acute accumulation of toxic reactive oxygen species in skeletal muscle, such as hind limb ischemia–reperfusion and sciatic nerve axotomy–induced muscle denervation (Halter et al. 2010).

Role of Rad in Tissue Remodeling

In vascular proliferative diseases, such as atherosclerosis, the formation of vascular lesions is mainly provoked by aberrant migration, attachment, and proliferation of vascular smooth muscle cells. Multiple factors may contribute to produce this pathological remodeling. Using adenovirus-mediated gene delivery, it has been reported that the expression of Rad is able to inhibit the migration and attachment of vascular smooth muscle cells, and that this effect is dependent on GTP loading. Experimental evidence supports that Rad reduces the formation of stress fibers and focal contacts necessary to the remodeling process, by interfering with the Rho/ROCK signaling pathway (Fu et al. 2005).

Pathological remodeling is also observed in heart in response to injury and stress, and it leads to myocardial hypertrophy and fibrosis, and subsequent heart failure. The expression of Rad is normally high in the myocardium but decreases in diseased heart. Transgenic mice lacking the Rad gene are more prone to develop cardiac hypertrophy, and this is associated with an increase in the phosphorylation of CaMKII. Through its interaction with CaMKII, it has been proposed that Rad decreases the phosphorylation and activity of this kinase, and hence reduces cardiac hypertrophy (Chang et al. 2007). Rad-deficient mice also show severe myocardial fibrosis. By binding the transcription factor C/EBP-δ, Rad impedes the expression of connective tissue growth factor, which is a key stimulator of the production of extracellular matrix leading to fibrosis. Rad therefore acts as a negative regulator of fibrosis in the heart (Zhang et al. 2011).

Role of Rad in Cancer

First reports indicated that Rad may be an oncogenic protein, since it increases the rate of growth of breast cancer cells in vitro. In addition, the ability of these cells to trigger the formation of tumors in nude mice is also exacerbated in the presence of Rad. The oncogenic potential of Rad resides within the NH_2 and COOH termini and seems not to be dependent on its GTPase activity. However, the metastasis suppressor gene, nm23, which stimulates GTP hydrolysis by Rad, is able to diminish its tumor-promoting effect (Tseng et al. 2001).

Other studies postulated that Rad is a tumor suppressor factor, because its expression appears frequently inhibited in lung and breast cancers (Suzuki et al. 2007). In support of this notion, it has been shown that Rad is a direct transcriptional target of the tumor suppressor p53 and, thus, can reduce migration and invasiveness of cancer cells by acting on cytoskeleton reorganization (Hsiao et al. 2011). Moreover, Rad triggers the apoptotic death of cardiomyocytes in vitro by stimulating the phosphorylation of p38 MAPK and by decreasing the levels of the pro-survival molecule Bcl-x_L (Sun et al. 2011).

Role of Rad in Ca^{2+} Channel Activity

Rad is a potent inhibitor of Ca^{2+} currents through L-type voltage-dependent channels. This occurs via the interaction of Rad with auxiliary $Ca_V\beta$ subunits,

which are involved in the trafficking toward the cell surface of the principal subunits forming the channels. The interaction between Rad and the $Ca_V\beta$ subunit prevents channel expression on the cell surface, by sequestering $Ca_V\beta$ subunits to the nucleus. The interaction depends on critical amino acid residues in the COOH terminus, and is facilitated by the absence of 14-3-3 and CaM binding, both of which allow relocalization of Rad within the cytoplasm (Finlin et al. 2003; Béguin et al. 2006). On the other hand, heart-specific overexpression of a dominant negative form of Rad, that binds GDP but not GTP, leads to an increase in the number of L-type voltage-dependent Ca^{2+} channels on the cell surface and, subsequently, cardiac arrhythmogenesis (Yada et al. 2007).

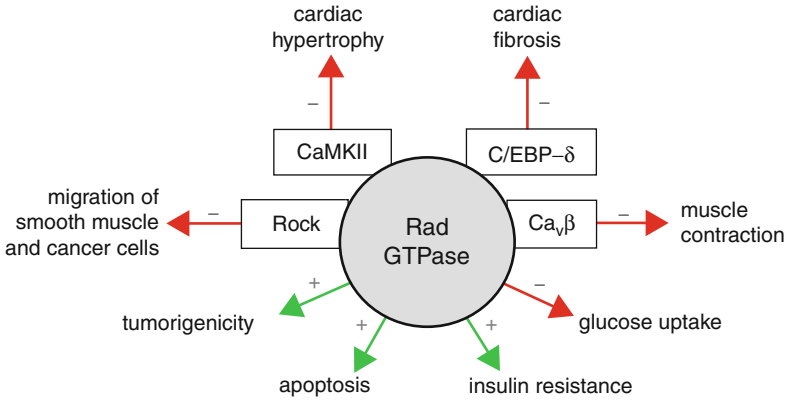
Role of Rad in Skeletal Muscle Metabolism, Development, and Disease

Rad was initially identified as being upregulated in diabetic patients. Follow-up studies did not confirm this finding, since Rad expression appeared normal in particular populations of diabetic patients and in the Zucker rat model of diabetes and obesity (Paulik et al. 1997). Of note, however, additional in vitro and in vivo experimental evidence strongly suggests the implication of Rad in glucose metabolism. First, overexpressing Rad in C2C12 and L6 myocyte cell lines reduces insulin-stimulated glucose uptake (Moyers et al. 1996). Second, using mice that overexpress Rad in muscle, it was shown that the increase in the expression of Rad acts synergistically with a high-fat diet to induce insulin resistance as observed in Type II diabetes (Ilany et al. 2006).

Rad is expressed during normal rat muscle development and in regenerating muscle in response to injury. Such an expression is located in the myogenic progenitor cell population, as well as in the newly regenerated myofibers as occurs in the *mdx* mouse model of Duchenne muscular dystrophy (Hawke et al. 2006). Extending these observations, it was also found that Rad is upregulated in skeletal muscles affected by the chronic neuromuscular degenerative condition, amyotrophic lateral sclerosis. In this disease, however, the upregulation of Rad is intimately associated with muscle atrophy, since it takes place within the myofibers that suffer from the degenerative process (Halter et al. 2010).

Summary

Rad is a multifunctional GTPase involved in many different molecular and cellular processes, mainly in muscle tissue, during development, and under normal adult and disease conditions. A major feature of Rad is the ability to interact with a variety of proteins, which determine not only the activity but also the specific role of this GTPase in a given physiological or pathological context (see Fig. 1). Rad can exert different inhibitory and stimulatory actions and, intriguingly, it can exhibit opposite roles for a particular situation. Thus, studies on the contribution of Rad to cancer showed both pro- and antitumoral activity. Not all the functions of Rad have been completely deciphered, such as its implication in muscle metabolism. In addition, the molecular mechanisms underlying Rad functions remain unclear and sometimes controversial. Finally, further efforts are needed to elucidate the regulatory factors, either



Ras-Related Associated with Diabetes, Fig. 1 Biological Functions of Rad. Rad interacts with many different proteins and participates in a variety of cellular processes. Interacting proteins are shown in boxes. Arrows indicate inhibitory (−) or stimulatory (+) actions. See text for further details

extra- or intracellular, that control Rad actions. From a pathological point of view, a better knowledge of these aspects of the biology of Rad would provide the basis for future therapeutic interventions.

References

Béguin P, Mahalakshmi RN, Nagashima K, Cher DH, Ikeda H, Yamada Y, et al. Nuclear sequestration of beta-subunits by Rad and Rem is controlled by 14-3-3 and calmodulin and reveals a novel mechanism for Ca2+ channel regulation. J Mol Biol. 2006;355:34–46.

Caldwell JS, Moyers JS, Doria A, Reynet C, Kahn RC. Molecular cloning of the human rad gene: gene structure and complete nucleotide sequence. Biochim Biophys Acta. 1996;1316:145–8.

Chang L, Zhang J, Tseng YH, Xie CQ, Ilany J, Brüning JC, et al. Rad GTPase deficiency leads to cardiac hypertrophy. Circulation. 2007;116:2976–83.

Finlin BS, Crump SM, Satin J, Andres DA. Regulation of voltage-gated calcium channel activity by the Rem and Rad GTPases. Proc Natl Acad Sci USA. 2003;100:14469–74.

Fu M, Zhang J, Tseng YH, Cui T, Zhu X, Xiao Y, et al. Rad GTPase attenuates vascular lesion formation by inhibition of vascular smooth muscle cell migration. Circulation. 2005;111:1071–7.

Halter B, Gonzalez de Aguilar JL, Rene F, Petri S, Fricker B, Echaniz-Laguna A, et al. Oxidative stress in skeletal muscle stimulates early expression of Rad in a mouse model of amyotrophic lateral sclerosis. Free Radic Biol Med. 2010;48:915–23.

Hawke TJ, Kanatous SB, Martin CM, Goetsch SC, Garry DJ. Rad is temporally regulated within myogenic progenitor cells during skeletal muscle regeneration. Am J Physiol Cell Physiol. 2006;290:C379–87.

Hsiao BY, Chen CC, Hsieh PC, Chang TK, Yeh YC, Wu YC, et al. Rad is a p53 direct transcriptional target that inhibits cell migration and is frequently silenced in lung carcinoma cells. J Mol Med (Berl).. 2011;89:481–92.

Ilany J, Bilan PJ, Kapur S, Caldwell JS, Patti ME, Marette A, et al. Overexpression of Rad in muscle worsens diet-induced insulin resistance and glucose intolerance and lowers plasma triglyceride level. Proc Natl Acad Sci USA. 2006;103:4481–6.

Mahalakshmi RN, Ng MY, Guo K, Qi Z, Hunziker W, Béguin P. Nuclear localization of endogenous RGK proteins and modulation of cell shape remodeling by regulated nuclear transport. Traffic. 2007;8:1164–78.

Moyers JS, Bilan PJ, Reynet C, Kahn CR. Overexpression of Rad inhibits glucose uptake in cultured muscle and fat cells. J Biol Chem. 1996;271:23111–16.

Moyers JS, Zhu J, Kahn CR. Effects of phosphorylation on function of the Rad GTPase. Biochem J. 1998;333:609–14.

Paulik MA, Hamacher LL, Yarnall DP, Simmons CJ, Maianu L, Pratley RE, et al. Identification of Rad's effector-binding domain, intracellular localization, and analysis of expression in Pima Indians. J Cell Biochem. 1997;65:527–41.

Reynet C, Kahn CR. Rad: a member of the Ras family overexpressed in muscle of type II diabetic humans. Science. 1993;262:1441–4.

Sun Z, Zhang J, Zhang J, Chen C, Du Q, Chang L, et al. Rad GTPase induces cardiomyocyte apoptosis through the activation of p38 mitogen-activated protein kinase. Biochem Biophys Res Commun. 2011;409:52–7.

Suzuki M, Shigematsu H, Shames DS, Sunaga N, Takahashi T, Shivapurkar N, et al. Methylation and gene silencing of the Ras-related GTPase gene in lung and breast cancers. Ann Surg Oncol. 2007;14:1397–404.

Tseng YH, Vicent D, Zhu J, Niu Y, Adeyinka A, Moyers JS, et al. Regulation of growth and tumorigenicity of breast cancer cells by the low molecular weight GTPase Rad and nm23. Cancer Res. 2001;61:2071–9.

Ward Y, Yap SF, Ravichandran V, Matsumura F, Ito M, Spinelli B, et al. The GTP binding proteins Gem and Rad are negative regulators of the Rho-Rho kinase pathway. J Cell Biol. 2002;157:291–302.

Yada H, Murata M, Shimoda K, Yuasa S, Kawaguchi H, Ieda M, et al. Dominant negative suppression of Rad leads to QT prolongation and causes ventricular arrhythmias via modulation of L-type Ca2+ channels in the heart. Circ Res. 2007;101:69–77.

Zhang J, Chang L, Chen C, Zhang M, Luo Y, Hamblin M, et al. Rad GTPase inhibits cardiac fibrosis through connective tissue growth factor. Cardiovasc Res. 2011;91:90–8.

Ras-Related Protein Rab-7a

► Rab7a in Endocytosis and Signaling

Ras-Related Protein Rab-8A

► Rab8

Ras-Related Rab8

► Rab8

RCN-1

► Regulator of Calcineurin 1 (RCAN1)

Receptor Related to FPR (RFP)

► FPR2/ALX

Recoverin

Pavel P. Philippov and Evgeni Yu Zernii
Department of Cell Signalling, A.N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, Moscow, Russia

Synonyms

A-protein; Cancer-associated retinopathy antigen; CAR-antigen; 23 kDa photoreceptor cell-specific protein; p26; p^{26}; S-modulin

Historical Background

In 1989, P. Philipopov's group from M.V. Lomonosov Moscow State University invented a method for purification of the visual G-protein transducin (G_t) and some other G-proteins. The idea of the method was based on the ability of visual rhodopsin to bind and to release transducin in the absence and in the presence of GTP, respectively. For this aim, a column with delipidated visual rhodopsin immobilized on Concanavalin A Sepharose was used. Chromatography of a crude extract of bovine rod outer segments on the column allowed one to obtain a set of transducin subunits with a slight contamination of cGMP-phosphodiesterase. Also, an admixture of an unknown protein with an apparent molecular weight of 26 K could be seen on the electrophoregram. The unknown protein attracted the attention of the group since the capability of binding to rhodopsin had been a characteristic feature of several key photoreceptor proteins, such as transducin, ▶ rhodopsin kinase, and arrestin. That is why the group decided to study this protein in more detail. The protein named "p26" was purified to a homogeneous state and used to arise specific antibodies. Screening of the retina and a number of other tissues for the presence of p26 with the use of the antibodies detected this protein only in the retina, in particular in the photoreceptor layer. It was also demonstrated that the amino acid sequence of p26 exhibited several calcium binding sites of the EF-hand type and the ability of p26 to bind Ca^{2+} was confirmed by experiments with calcium-45. In addition, p26 was suggested to be a Ca^{2+}-specific regulator of photoreceptor ▶ guanylate cyclase, a key enzyme of photoreceptors recovery, and due to this ability it was rechristened as "▶ recoverin." Afterward, a 26 K protein named "S-modulin" was purified from frog rod outer segments and shown to have a primary structure similar to bovine recoverin. Later it became clear that the binding of recoverin to rhodopsin is not quite specific as recoverin, due to its Ca^{2+}-myristoyl switch, is capable of binding to hydrophobic substances, e.g., to Phenyl-Sepharose, in a Ca^{2+}-dependent manner. That recoverin is capable of activating guanylate cyclase was, however, disproved in subsequent works. The mistake in the initial assignment of the recoverin function might apparently be explained by the presence of endogenous guanylate cyclase activator(s), GCAP1 and/or GCAP2, in the recoverin preparations used in the preceding works. Nevertheless, recoverin continues to be considered as a participant of the photoreceptor recovery but now as a Ca^{2+}-sensor of rhodopsin kinase, the enzyme catalyzing phosphorylation and thus desensitization of the visual receptor rhodopsin (for reviews, see Senin et al. 2002; Philippov et al. 2006). After the discovery of recoverin, a large number of other EF-hand-containing Ca^{2+}-binding proteins were described, which form a family of the neuronal calcium sensor (NCS) proteins. The expression of the NCS proteins is restricted within neurons and neuroendocrine cells, in which these proteins provide a Ca^{2+}-sensitivity to a number of protein targets (for a review, see Burgoyne and Weiss 2001; Philippov et al. 2006).

Another line of the recoverin research was started in 1987, when an antigen with an apparent molecular weight of 23 K was found in sera of patients with cancer-associated retinopathy (CAR). This antigen named "CAR-antigen" was then purified from bovine rod outer segments and shown to be identical to recoverin. These works have sprung an intensive recoverin study as a paraneoplastic antigen in cancer (for reviews, see Senin et al. 2002; Adamus 2006; Philippov et al. 2006). More recently, recoverin has become the first member of a new group of cancer-specific antigens designated as "cancer-retina antigens." In addition to recoverin, this group includes several key retinal proteins, such as rhodopsin, transducin, rhodopsin kinase, and some others. In health, these proteins are highly specific for the retina, but in cancer they can be expressed in malignant tumors localized outside the retina (Bazhin et al. 2007).

Tissue and Cellular Distribution of Recoverin

Immunochemical analysis demonstrated the presence of recoverin in the adult retina of all investigated species. Among these are: man, bull, monkey, mouse, rat, rabbit, frog, chameleon, and newt. In the case of the chicken retina, contradictory data were obtained: recoverin-positive immunoreaction was described in one case, but it was not found in another work. In addition to the retina, recoverin immunoreactivity was observed in the ocular ciliary epithelium, pinealocytes of the pineal organ, and rat olfactory epithelium. Within the retina, recoverin-positive reaction was found in photoreceptor cells as well as in higher order neurons (bipolar and ganglion cells) of a number of species and in amacrine cells of lamprey *Lampetra fluviatilis*. As already noted, recoverin is suggested to function in photoreceptors as a Ca^{2+}-sensor of rhodopsin kinase, but its role in neurons different from photoreceptors and in tissues different from the retina remains unknown (for reviews, see Senin et al. 2002; Philippov et al. 2006).

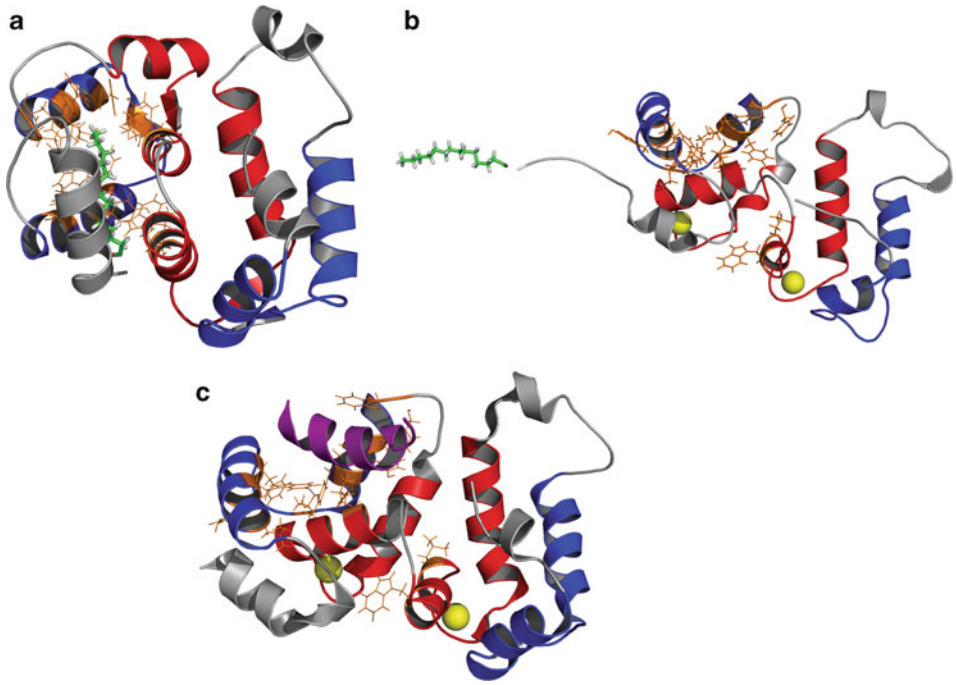
Within photoreceptors, recoverin is detected in the outer and inner segments, cell bodies, and synaptic pedicles. Most of the protein is localized in rod inner segments, with approximately 12% present in the outer segments in the dark and less than 2% remaining in that compartment in the light (Strissel et al. 2005). Thus, light causes a reduction of recoverin in rod outer segments, accompanied by its redistribution toward rod synaptic terminals.

Recoverin Structure

Recoverin is a compact (23.4 K, 201 amino acids) protein consisting of two globular N- and C-terminal domains, separated by a short linker. N-terminal glycine of recoverin is acylated predominantly with the myristic acid residue (C14:0) or, to a lesser extent, with one of the following fatty acid residues: C14:1 (5-*cis*), C14:2 (5-*cis*, 8-*cis*), or C12:0. Each of recoverin domains contains a pair of potential Ca^{2+}-binding sites of the EF-hand type: in total, the recoverin molecule contains four potential Ca^{2+}-binding sites that are disposed evenly along the amino acid chain of the protein. Of these, only two – EF-hand 2 and EF-hand 3 – are capable of binding calcium ions. Whereas EF-hand 1 and EF-hand 4 are inactive in this respect due to the following structural "defects": (1) in the sequences of EF-hand 1 and EF-hand 4, residues of negatively charged amino acids critical for the coordination of Ca^{2+} are missing in the 1st and 3rd positions of the 12-mer Ca^{2+}-binding loops, (2) EF-hand 1 cannot accept the conformation needed for the binding of calcium as P40 is present in the fourth position of the EF-hand 1 loop, (3) EF-hand 4 contains a salt bridge between the side chains of the K161 and G171 in the 2nd and 12th positions of the Ca^{2+}-binding loop, and (4) the highly conserved glycine at position 6 of the loop is replaced by the aspartic acid residue (D165) (for reviews, see Senin et al. 2002; Philippov et al. 2006).

The comparison of the structural data for apo- and Ca^{2+}-containing forms of myristoylated recoverin obtained by X-ray diffraction and NMR-spectroscopy revealed structural changes in the protein molecule, accompanying the binding of calcium (Fig. 1). In the apo-form, the myristoyl moiety of recoverin is buried into a deep hydrophobic cavity or hydrophobic "pocket," consisting of a cluster of aromatic and other nonpolar amino acid residues (L28, W31, Y32, F35, I44, F49, I52, Y53, F56, F57, Y86, L90, W104, and L108) of the protein molecule. Binding of calcium by recoverin leads to a 45° rotation of the N- and C-terminal domains around G96 and to significant conformational changes in the N-terminal domain. As a result (1) initially antiparallel α-helices of EF-hand 2 become perpendicular one to another and (2) α-helices of EF-hand 1 turn around G42, allowing myristoyl group to move outward from the hydrophobic environment. The consequence of these changes is the exposure of the hydrophobic amino acid cluster of the pocket and the myristoyl group of recoverin in solution – the so-called "Ca^{2+}-myristoyl switch" mechanism. The exposed myristoyl group allows recoverin to associate to membranes, while the amino acid cluster of the pocket participates in the interaction with the target enzyme, rhodopsin kinase (G-protein-coupled receptor kinase 1, GRK-1) (for reviews, see Senin et al. 2002; Philippov et al. 2006).

It should be added that the C-terminus of recoverin, in addition to ten α-helices "A-J" normally present in other NCS proteins, contains a variable C-terminal segment with an extra α-helix "K." Recent studies have demonstrated that C-terminal segment in recoverin is involved in regulating its Ca^{2+}-binding properties, as well as in recognizing and regulating of rhodopsin kinase (Weiergräber et al. 2006; Zernii et al. 2011).



Recoverin, Fig. 1 *Three-dimensional structures of recoverin.* Ribbon diagrams represent different recoverin forms: (**a**) Ca^{2+}-free recoverin. Image of 1iku.pdb (Tanaka et al. 1995) created with PyMol v.0.99 (DeLano Scientific LLC); (**b**) Ca^{2+}-bound recoverin. Image of 1jsa.pdb (Ames et al. 1997) created with PyMol v.0.99 (DeLano Scientific LLC); (**c**) recoverin in a complex with peptide 1–25 of rhodopsin kinase. Image of 2i94.pdb (Ames et al. 2006) created with PyMol v.0.99 (DeLano Scientific LLC). Structural elements are drawn in different colors: EF-hand 1 and EF-hand 4 (*blue*), EF-hand 2 and EF-hand 3 (*red*), N-terminal myristoyl group (*green*), calcium ions (*yellow*), nonpolar amino acid residues of the hydrophobic pocket (*orange*), and peptide 1–25 of rhodopsin kinase (*magenta*)

Molecular Properties of Recoverin

Recoverin molecule is characterized by a set of key properties required for the signaling activity of the protein. Among them the most important are calcium binding, N-terminal myristoylation and the ability to bind to phospholipid membranes. In recombinant non-myristoylated recoverin, the binding of calcium to EF-hands 2 and 3 occurs independently with different affinities: $K_d = 6.9$ and 0.11 μM, respectively. In contrast, the binding of calcium to recombinant myristoylated recoverin is a cooperative sequential process (Hill coefficient = 1.75), wherein EF-hand 3 is occupied first, facilitating the subsequent filling of EF-hand 2 (an apparent K_d of the complex formed is equal to 17 μM). Thus, N-terminal myristoylation confers onto recoverin the cooperativity in calcium binding to EF-hands 2 and 3. Also, the myristoyl residue significantly stabilizes the conformation of the Ca^{2+}-free protein during the stepwise transition toward the fully Ca^{2+}-occupied state (for reviews, see Senin et al. 2002; Philippov et al. 2006).

Myristoylated recoverin is capable of binding to hydrophobic surfaces, such as the photoreceptor and artificial lipid membranes. Depending on calcium concentration, compartmentalization of recoverin reversibly changes from a soluble Ca^{2+}-free form to a membrane-bound Ca^{2+}-containing form. This process is due to the mechanism of the Ca^{2+}-myristoyl switch that operates in recoverin: after EF-hand 3 is filled by calcium, EF-hand 2 is subsequently filled, which triggers the exposition of the myristoyl group that attaches recoverin to the membrane. Solid-state nuclear magnetic resonance studies revealed that the Ca^{2+}-bound protein is positioned on the membrane surface so that its long molecular axis is oriented 45° with respect to the normal membrane. The myristoyl group is buried inside the membrane, whereas the N-terminal region of recoverin points toward the

membrane surface, with close contacts formed by basic residues K5, K11, K22, K37, R43, and K84. This orientation of the membrane-bound protein allows an exposed hydrophobic crevice, near the membrane surface, to serve as a binding site for the target protein, rhodopsin kinase (Valentine et al. 2003). The half-maximal binding of recoverin to photoreceptor membranes in vitro occurs at 2.5 μM of a free calcium concentration ($[Ca^{2+}]_f$), which is slightly out of the physiological range of cytoplasmic $[Ca^{2+}]_f$. However, extrapolation to in vivo conditions in rod outer segments, which bear stacks of densely packed membranes, reveals that the apparent affinity of recoverin to calcium is in the submicromolar (i.e., physiological) range of $[Ca^{2+}]_f$ (for reviews, see Senin et al. 2002; Philippov et al. 2006). The binding of recoverin to membranes depends on their lipid composition: it is enhanced with the elevation of the content of phosphatidylserine (Senin et al. 2007), polyunsaturated phospholipids (Calvez et al. 2011), and most notably cholesterol. High cholesterol content in photoreceptor disk membranes found at the base of rod outer segments might favor the affinity of recoverin to the membranes and shift its binding to the physiological range of $[Ca^{2+}]_f$ (for a review, see Philippov et al. 2006). The Ca^{2+}-dependence of the recoverin binding to photoreceptor or artificial lipid membranes is also regulated by the C-terminal segment of recoverin, which serves as an internal modulator of its Ca^{2+}-sensitivity and functional activity (Weiergräber et al. 2006; Senin et al. 2007; Zernii et al. 2011).

Along with Ca^{2+}-binding, which is a key molecular property of recoverin, the protein can bind Zn^{2+} with stoichiometry of 1:1 and apparent K_d of 30 and 7.1 μM for apo- and Ca^{2+}-loaded protein forms, respectively (Permyakov et al. 2003). Also, recoverin molecules are able to form a disulfide dimer and thiol oxidized monomer under mild oxidizing conditions, using unique C39 highly conserved within NCS family (Permyakov et al. 2007). It is unclear yet, whether the above properties have the physiological significance.

Targets and Functions of Recoverin

A major intracellular target of recoverin in rod outer segments is suggested to be rhodopsin kinase (GRK-1). The filling of EF-hand 2 with calcium (in myristoylated recoverin the filling of EF-hand 2 occurs only after EF-hand 3 is already filled) results in the exposition of a cluster of the hydrophobic amino acids that provides recoverin with an ability to interact with rhodopsin kinase and thus inhibits the activity of the enzyme. According to the surface plasmon resonance studies, the half-maximal binding of rhodopsin kinase to immobilized recoverin occurs at approximately 0.51 μM of rhodopsin kinase. Myristoylation has a little effect on the binding of recoverin to the kinase, but it shifts the half-maximal effect of calcium on the binding from 150 nM for non-acylated recoverin to 400 nM for myristoylated recoverin (for reviews, see Senin et al. 2002; Philippov et al. 2006). Recoverin binds to a region of residues 1–15 at the N-terminus of rhodopsin kinase (Higgins et al. 2006). Nuclear magnetic resonance studies of the complex between Ca^{2+}-bound recoverin and a N-terminal fragment of rhodopsin kinase, residues 1–25 ($RK^{1\text{-}25}$) revealed that the hydrophobic face of the $RK^{1–25}$ helix (L6, V9, V10, A11, A14, and F15) interacts with an exposed hydrophobic groove on the surface of recoverin, lined by side chains of the residues W31, F35, F49, I52, Y53, F56, F57, Y86, and L90. In that structure, the first eight residues of recoverin at the N-terminus are solvent-exposed, enabling the N-terminal myristoyl group to interact with target membranes (Ames et al. 2006). The half-maximal inhibition of rhodopsin kinase by recoverin is observed at 2–3 μM and 1.5–1.7 μM of calcium in the case of non-acylated and myristoylated recoverin, respectively. At saturating calcium concentrations, the half-maximal inhibition of rhodopsin kinase occurs at 6.5–8 μM of non-myristoylated recoverin and at 0.8–3 μM of myristoylated recoverin, suggesting that photoreceptor membranes enhance inhibitory effect of recoverin upon rhodopsin kinase. The inhibition of rhodopsin kinase by recoverin is facilitated when the cholesterol content of membranes is increased. As the cholesterol content in photoreceptor disk membranes changes along the axis of rod outer segment from 5% at the tip to 30% at the base, the above-mentioned effect of cholesterol might be of physiological importance (for reviews, see Senin et al. 2002; Philippov et al. 2006). The activity of recoverin as a Ca^{2+}-sensor of rhodopsin kinase is also regulated by the C-terminal segment of recoverin (Weiergräber et al. 2006; Zernii et al. 2011).

Therefore, a number of the in vitro data suggest that recoverin functions as a Ca^{2+}-sensor of rhodopsin

kinase in photoreceptor cells. According to these data, at high calcium, corresponding to a dark state of photoreceptor cells, rhodopsin kinase forms a complex with recoverin and becomes inactive; at low calcium, corresponding to the bleached state of photoreceptor cells, the complex dissociates allowing activation of the enzyme (for reviews, see Senin et al. 2002; Philippov et al. 2006). However, the in vivo data on recoverin function are contradictive. On the one hand, recoverin is suggested to be implicated in the light adaptation of photoreceptor cells by Ca^{2+}-dependent prolongation of the photoresponse due to (1) regulating the lifetime of photoactivated rhodopsin through feedback on rhodopsin kinase and (2) regulating the speed of the light-induced change of $[Ca^{2+}]_f$ through a Ca^{2+}-buffering mechanism (for a review, see Philippov et al. 2006). On the other hand, there are data suggesting that the effect of recoverin on the photoresponse could not be explained by its effect on phototransduction as such. Instead, the prolonged signal transmission that enhances visual sensitivity is the effect of recoverin downstream of phototransduction in rods (Sampath et al. 2005). Recent data have advanced a new argument in favor of recoverin as a Ca^{2+}-sensor of rhodopsin kinase in vivo (Chen et al. 2010). According to these data, (1) background light accelerates inactivation of photoexcited rhodopsin and (2) recoverin is required for the light-dependent modulation of the photoexcited rhodopsin lifetime, probably due to its capability to regulate rhodopsin kinase in a Ca^{2+}-dependent manner.

The function(s) of recoverin in the structures different from photoreceptor outer segments still remains unknown, but it is possible that the following data could help to provide a clue to this issue. In the ribbon synapse of photoreceptors, recoverin is co-localized with membrane palmitoylated protein-4 (MPP4), a retina-specific scaffolding protein, which has been implicated in organizing presynaptic protein complexes. Western blot analysis of bovine retinal anti-recoverin precipitates detects co-precipitating MPP4, supporting an association between the MPP4-containing protein complex and recoverin in vivo. However, immunoprecipitation experiments do not show a direct interaction between recoverin and MPP4 in 293-BNA cells co-transfected with both proteins (Förster et al. 2009). More recently, pull-down assay and surface plasmon resonance study have revealed a neuron-specific Ca^{2+}-binding protein caldendrin as a potential target for recoverin in retinal bipolar cells and pineal gland. In particular, both proteins are co-localized in these structures and an increase of intracellular calcium facilitates the translocation of caldendrin to intracellular membranes, which is under control of the complex formation with recoverin (Fries et al. 2010).

Recoverin in Cancer

In health, the expression of recoverin is mainly restricted within the retina. In cancer, recoverin can also be a paraneoplastic (or onconeural) antigen which expressed in tumors localized outside the nervous system. The aberrant expression of recoverin in malignant cells causes an autoimmune response in some cancer patients what is followed by the development of paraneoplastic retina degeneration or cancer-associated retinopathy, CAR. Autoantibodies against recoverin (AAR) are detected in patients with different kinds of cancer (for reviews, see Adamus 2006; Bazhin et al. 2007).

A model of antibody-induced apoptosis of photoreceptor cells, underlying the CAR syndrome, has been proposed (for a review, see Adamus 2006). Serum AAR should be in sufficiently high titers to enter the eye and cause retinopathy. Circulating AAR cross the blood-retinal barrier and penetrate into retinal layers, where AAR attack photoreceptors which express recoverin. AAR then penetrate into retinal cells by an active process of endocytosis. Once in the cell, AAR block the recoverin function in phototransduction, which results in the enhancement of rhodopsin phosphorylation and an increase in the concentration of intracellular calcium ions. The high intracellular calcium activates the mitochondria-dependent and caspase-9-dependent activation of caspase 3, leading to DNA fragmentation and cell death. Massive death of photoreceptor cells leads to retinal dysfunction and degeneration.

The CAR syndrome, similar to other paraneoplastic neurological syndromes, is a very rare event: its occurrence is of the order of 1%. However, underlying AAR might occur much more frequently (Bazhin et al. 2004). An important feature of the CAR syndrome, as well as other paraneoplastic syndromes, is that it can be manifested long before the clinical diagnosis of the underlying tumor (for a review, see Adamus 2006).

Such a feature of the CAR syndrome and underlying AAR could be useful to clinicians to predict the future development of a particular cancer.

Summary

Recoverin, initially named "p26," is a Ca^{2+}-binding protein with a predominantly retinal localization, which belongs to the neuronal calcium sensor (NCS) protein family. The recoverin molecule consists of 201 amino acid residues and contains four potential EF-hand Ca^{2+}-binding sites, of which only two – EF-hands 2 and 3 – are capable of binding calcium. The N-terminus of recoverin is acylated, mainly myristoylated. Due to the mechanism of the Ca^{2+}-myristoyl switch, compartmentalization of recoverin is changed from a soluble Ca^{2+}-free form to a membrane-bound Ca^{2+}-containing form, and vice versa, depending on an external calcium concentration. In the Ca^{2+}-free form, the N-terminal myristoyl moiety of recoverin is buried into the hydrophobic pocket of the protein; on calcium binding, the myristoylated N-terminus is exposed, providing membrane association of recoverin. Recoverin is suggested to operate as a Ca^{2+}-sensor of rhodopsin kinase (G-protein-coupled receptor kinase 1, GRK-1), which catalyzes phosphorylation and thus desensitization of the visual receptor rhodopsin. In cancer, recoverin can also be a paraneoplastic (or onconeural) antigen, the aberrant expression of which in malignant tumors of some patients causes an autoimmune response and the development of paraneoplastic retina degeneration or cancer-associated retinopathy. An important feature of the CAR syndrome and underlying autoantibodies against recoverin is that they can be detected long before the clinical diagnosis of the corresponding tumor. Such a feature of the autoantibodies could be useful to clinicians to predict the future development of a particular cancer.

References

Adamus G. The role of recoverin in autoimmunity. In: Philippov PP, Koch KW, editors. Neuronal calcium sensor proteins. New York: Nova; 2006. p. 181–99.

Ames JB, Ishima R, Tanaka T, Gordon JI, Stryer L, Ikura M. Molecular mechanics of calcium-myristoyl switches. Nature. 1997;389:198–202.

Ames JB, Levay K, Wingard JN, Lusin JD, Slepak VZ. Structural basis for calcium-induced inhibition of rhodopsin kinase by recoverin. J Biol Chem. 2006;281:37237–45.

Bazhin AV, Savchenko MS, Shifrina ON, Demoura SA, Chikina SY, Jaques G, Kogan EA, Chuchalin AG, Philippov PP. Recoverin as a paraneoplastic antigen in lung cancer: the occurrence of anti-recoverin autoantibodies in sera and recoverin in tumors. Lung Cancer. 2004;44:193–8.

Bazhin AV, Schadendorf D, Willner N, De Smet C, Heinzelmann A, Tikhomirova NK, Umansky V, Philippov PP, Eichmüller SB. Photoreceptor proteins as cancer-retina antigens. Int J Cancer. 2007;120:1268–76.

Burgoyne RD, Weiss JL. The neuronal calcium sensor family of Ca^{2+}-binding proteins. Biochem J. 2001;353:1–12.

Calvez P, Demers E, Boisselier E, Salesse C. Analysis of the contribution of saturated and polyunsaturated phospholipid monolayers to the binding of proteins. Langmuir. 2011;27:1373–9.

Chen CK, Woodruff ML, Chen FS, Chen D, Fain GL. Background light produces a recoverin-dependent modulation of activated-rhodopsin lifetime in mouse rods. J Neurosci. 2010;30:1213–20.

Förster JR, Lochnit G, Stöhr H. Proteomic analysis of the membrane palmitoylated protein-4 (MPP4)-associated protein complex in the retina. Exp Eye Res. 2009;88:39–48.

Fries R, Reddy PP, Mikhaylova M, Haverkamp S, Wei T, Müller M, Kreutz MR, Koch K-W. Dynamic cellular translocation of caldendrin is facilitated by the Ca^{2+}-myristoyl switch of recoverin. J Neurochem. 2010;113:1150–62.

Higgins MK, Oprian DD, Schertler GF. Recoverin binds exclusively to an amphipathic peptide at the N terminus of rhodopsin kinase, inhibiting rhodopsin phosphorylation without affecting catalytic activity of the kinase. J Biol Chem. 2006;281:19426–32.

Permyakov SE, Cherskaya AM, Wasserman LA, Khokhlova TI, Senin II, Zargarov AA, Zinchenko DV, Zernii EY, Lipkin VM, Philippov PP, Uversky VN, Permyakov EA. Recoverin is a zinc-binding protein. J Proteome Res. 2003;2:51–7.

Permyakov SE, Nazipova AA, Denesyuk AI, Bakunts AG, Zinchenko DV, Lipkin VM, Uversky VN, Permyakov EA. Recoverin as a redox-sensitive protein. J Proteome Res. 2007;6:1855–63.

Philippov PP, Senin II, Koch K-W. Recoverin: a calcium-dependent regulator of the visual transduction. In: Philippov PP, Koch KW, editors. Neuronal calcium sensor proteins. New York: Nova; 2006. p. 139–51.

Sampath AP, Strissel KJ, Elias R, Arshavsky VY, McGinnis JF, Chen J, Kawamura S, Rieke F, Hurley JB. Recoverin improves rod-mediated vision by enhancing signal transmission in the mouse retina. Neuron. 2005;46:413–20.

Senin II, Koch KW, Akhtar M, Philippov PP. Ca^{2+}-dependent control of rhodopsin phosphorylation: recoverin and rhodopsin kinase. Adv Exp Med Biol. 2002;514:69–99.

Senin II, Churumova VA, Philippov PP, Koch K-W. Membrane binding of the neuronal calcium sensor recoverin – modulatory role of the charged carboxy-terminus. BMC Biochem. 2007;8:24.

Strissel KJ, Lishko PV, Trieu LH, Kennedy MJ, Hurley JB, Arshavsky VY. Recoverin undergoes light-dependent intracellular translocation in rod photoreceptors. J Biol Chem. 2005;280:29250–5.

Tanaka T, Ames JB, Harvey TS, Stryer L, Ikura M. Sequestration of the membrane-targeting myristoyl group of recoverin in the calcium-free state. Nature. 1995;376:444–7.
Valentine KG, Mesleh MF, Opella SJ, Ikura M, Ames JB. Structure, topology, and dynamics of myristoylated recoverin bound to phospholipid bilayers. Biochemistry. 2003;42:6333–40.
Weiergräber OH, Senin II, Zernii EY, Churumova VA, Kovaleva NA, Nazipova AA, Permyakov SE, Permyakov EA, Philippov PP, Granzin J, Koch K-W. Tuning of a neuronal calcium sensor. J Biol Chem. 2006;281:37594–602.
Zernii EY, Komolov KE, Permyakov SE, Kolpakova T, Dell'orco D, Poetzsch A, Knyazeva EL, Grigoriev II, Permyakov EA, Senin II, Philippov PP, Koch KW. Involvement of the recoverin C-terminal segment in recognition of the target enzyme rhodopsin kinase. Biochem J. 2011;435:441–50.

Reggie-1 (Reg-1)

▶ Flotillin-2 (FLOT2)

Reggie-2 (Reg-2)

▶ Flotillin-1 (flot1)

Regulator of Calcineurin 1 (RCAN1)

Masakazu Fujiwara and Mohammad Ghazizadeh
Department of Molecular Pathology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School, Kawasaki, Kanagawa, Japan

Synonyms

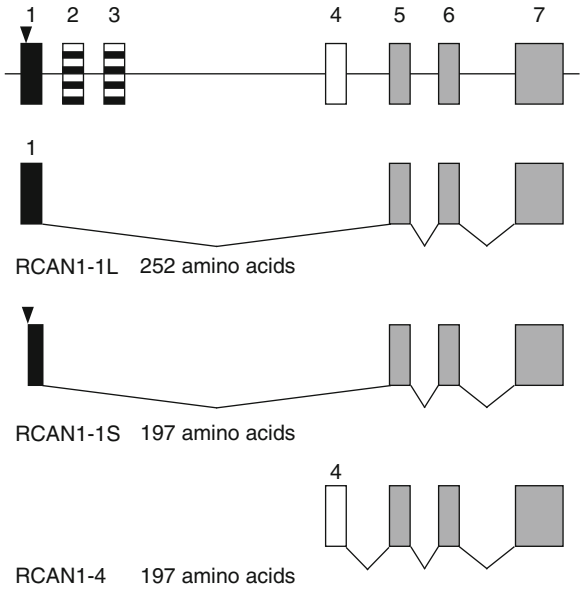
ADAPT78; Calcipressin1; CBP1; Down syndrome candidate region 1 (DSCR1); Down syndrome critical region 1 (DSCR1); MCIP1; Nebula; RCN-1; Sarah

Historical Background

Regulator of calcineurin 1 (RCAN1) was first isolated by Fuentes et al. in 1995 during a search for genes associated with clinical features of Down syndrome (e.g., mental retardation and congenital heart disease) (Fuentes et al. 1995). Coding sequences of RCAN1 were identified from the 21q22.1–q22.2 region of human chromosome 21 by an Alu-splice PCR method. It was initially thought that RCAN1 was significantly associated with the Down syndrome phenotype (Fuentes et al. 1997b), thus RCAN1 was first designated Down Syndrome Critical Region 1 (DSCR1). Future studies showed that the DSCR1 gene product is a calcineurin regulator, and the new name "regulator of calcineurin (RCAN)" was adopted to describe the gene function. Although RCAN1 is still referred to as DSCR1, ADAPT78, MCIP1, Calcipressin1, RCN-1, Nebular, Sarah, or CBP1, both the HUGO Gene Nomenclature Committee (HGNC) and the Mouse Genomic Nomenclature Committee (MGNC) (Davies et al. 2007) have adopted the RCAN1 nomenclature.

RCAN1 is an endogenous inhibitor of the serine phosphatase calcineurin (Fuentes et al. 2000; Gorlach et al. 2000; Kingsbury and Cunningham 2000). Calcineurin is a heterodimer that is composed of the catalytic subunit calcineurin A (CnA) and regulatory subunit calcineurin B (CnB). RCAN1 directly binds to CnA and inhibits the catalytic activity of calcineurin. Calcineurin controls the phosphorylation/dephosphorylation of several transcription factors, such as the nuclear factor of activated T cells (NFAT) (Crabtree and Olson 2002), CREB (Liu and Graybiel 1996), and the MEF2 transcription factor families (Mao and Wiedmann 1999). Dephosphorylation of NFAT by activated calcineurin promotes translocation of NFAT to the nucleus, where the transcription factor binds to DNA and activates gene transcription (Crabtree and Olson 2002). A wide variety of physiological processes, such as lymphocyte activation (Fruman et al. 1995), neurite outgrowth (Chang et al. 1995), aging (Mair et al. 2011), heart development (Yang et al. 2000), skeletal muscle fiber type differentiation (Olson and Williams 2000), and cardiac function (Ryeom et al. 2003) are regulated by calcineurin-activated transcription factors. Thus, RCAN1, which controls calcineurin activity, regulates various physiological functions.

RCAN1 is highly conserved from unicellular eukaryotes to multicellular animals, and orthologs have been identified in *Saccharomyces cerevisiae* (Kingsbury and Cunningham 2000), *Cryptococcus neoformans* (Gorlach et al. 2000), *Caenorhabditis elegans*



Regulator of Calcineurin 1 (RCAN1), Fig. 1 The human *RCAN1* mRNA isoforms. The 252-amino acid RCAN1-1 L, 197-amino acid RCAN1-1 S, and 197-amino acid RCAN1-4 each have different ATG start codons

(Lee et al. 2003), and *Drosophila melanogaster* (Chang et al. 2003). The human *RCAN1* gene contains seven exons, where exons 1–4 can be alternatively transcribed or spliced to produce different mRNA isoforms (Fuentes et al. 1997a). Among the four potential transcripts, the major transcripts are *RCAN1-1* and *RCAN1-4*, which have exon 1 and exon 4 for the first exon, respectively (Fig. 1). Additionally, there are two start codons in exon 1; the long form of RCAN1-1, which encodes 252 amino acids, is referred to as RCAN1-1 L (Genesca et al. 2003), and the short form of RCAN1-1, which encodes 197 amino acids, is referred to as RCAN1-1 S (Fuentes et al. 1997a).

RCAN1 Expression

RCAN1 is highly expressed in the human fetal brain and adult heart. Lower expression levels have been detected in the adult brain, lung, liver, skeletal muscle, kidney, and pancreas. The fetal lung, liver, kidney, and placenta also have low RCAN1 expression (Fuentes et al. 1995). RCAN1 expression in rat and mice is similar to the associated human tissues, and there is high expression in the rodent brain and heart (Fuentes et al. 1995, 1997a). In the rat brain, the in situ hybridization signal for *RCAN1* expression was high in the olfactory bulb, the piriform cortex, the dentate granule cell layer, the pyramidal cell layer of the hippocampus, the striatum and the cerebellar cortex. No signal for *RCAN1* expression was detected in the white matter (Fuentes et al. 1995). The brains of 2–7-day-old neonatal rats had higher *RCAN1* expression in the neocortex and the hypothalamus compared to adults rat older than 16 days (Fuentes et al. 1995).

RCAN1 expression is diverse throughout many different cell and tissue types and is regulated by various stimuli. Vascular endothelial growth factor (VEGF) (Minami et al. 2004; Yao and Duh 2004), thrombin (Minami et al. 2004), oxidative stress (Crawford et al. 1997), Ca^{2+}-mediated stress (Cano et al. 2005), ß-amyloid fragments (Ermak et al. 2001), TNFα (Minami et al. 2004; Yao and Duh 2004), and endoplasmic reticulum stress (Zhao et al. 2008) have been reported to induce RCAN1 expression. Oxidative stress in hamster HA-1 cells induced RCAN1 expression as early as 90 min after peroxide exposure, indicating that the transcriptional response of RCAN1 is rapid and robust. A previous study indicated that the maximal increase in RCAN1 expression was 7.8-fold after 5 h of initial exposure (Crawford et al. 1997). Similarly, rapid and robust RCAN1 expression occurs with other types of stimuli, such as VEGF and thrombin. After 1 h of treatment, VEGF and thrombin stimulation increased RCAN1 expression in human endothelial cells by 22.3-fold and 17.7-fold, respectively (Minami et al. 2004).

RCAN1 transcription is regulated via a negative feedback loop with RCAN1-4, which is an isoform that is abundant in the fetal kidney, adult heart, placenta, and skeletal muscle (Ermak et al. 2002; Fuentes et al. 1997a, 2000). RCAN1 expression is induced by the transcription factor NFAT, and NFAT activation and nuclear translocation are regulated by calcineurin phosphatase activity. Therefore, an excessive amount of RCAN1 inhibits calcineurin and attenuates the calcineurin–NFAT signaling pathway, thereby establishing a negative feedback loop that suppresses its own expression. Calcineurin/NFAT signal-dependent RCAN1-4 transcription is controlled by a promoter region that is located upstream of exon 4 (between nucleotides −350 and −166); this promoter region also contains putative NFAT and AP-1 binding

sites (Cano et al. 2005; Yang et al. 2000; Zhao et al. 2008). In contrast, RCAN1-1 L, which is the isoform that is predominantly expressed in the fetal and adult brains (Ermak et al. 2002; Fuentes et al. 1997a, 2000) is controlled by a conserved muscle-specific CAT (M-CAT) site located 1,426 bp upstream of exon 1 (Liu et al. 2008). Transcription enhancer factor 3 directly interacts with the M-CAT site in the promoter and is required for RCAN1-1 L expression (Liu et al. 2008).

Inhibitory and Stimulatory Effects of RCAN1

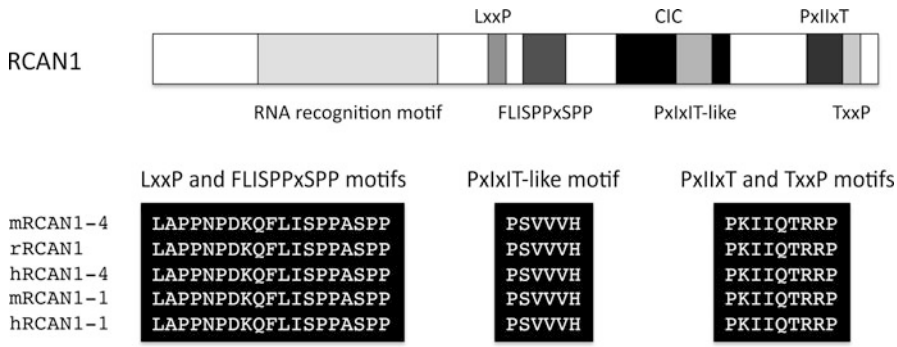
RCAN1 is an endogenous inhibitor of calcineurin; RCAN1 overexpression inhibits NFAT-mediated calcineurin signaling by directly binding to the catalytic subunit of calcineurin (Fuentes et al. 2000). However, RCAN1 gene disruption also inhibits calcineurin–► NFAT signaling instead of stimulating the signaling. In yeast, disruption of the *RCAN1* orthologus gene (*RCN1*) results in significantly decreased calcineurin–NFAT signaling (Kingsbury and Cunningham 2000). In $RCAN1^{-/-}$ mice, calcineurin activity is decreased in response to cardiac hypertrophy induced by pressure overload (Vega et al. 2003). These conflicting results suggest that reciprocal effect of RCAN1 to the calcineurin activities, as high RCAN1 expression is associated with calcineurin inhibition, while physiological levels of RCAN1 expression are associated with calcineurin stimulation.

A number of reports have indicated that phosphorylation of RCAN1 is important for RCAN1-mediated biphasic effects on calcineurin. Using both human and yeast RCAN1, Hilioti et al. showed that RCAN1-mediated calcineurin stimulation requires phosphorylation at the conserved FLISPPxSPP motif when RCAN1 is expressed at low concentrations (Hilioti et al. 2004). Moreover, Liu et al. also showed that phosphorylation of low concentrations of RCAN1 activates calcineurin–NFAT signaling (Liu et al. 2009). Because the phosphorylated FLISPPxSPP RCAN1 motif is rapidly degraded by the SCF^{Cdc4} ubiquitin ligase complex (Genesca et al. 2003; Kishi et al. 2007), it has been hypothesized that phosphorylated RCAN1 rapidly degrades and releases calcineurin from the inactive complex (Kishi et al. 2007). RCAN1-mediated stimulation of calcineurin activity is observed only at physiological RCAN1 expression levels because the net balance of phosphorylated RCAN1 is much higher. On the other hand, when RCAN1 is overexpressed, a majority of the protein is not phosphorylated; this stable RCAN1 binds to calcineurin, which covers the catalytic domain and inhibits phosphatase activity. Overexpressed RCAN1 also increases calcineurin proteolysis (Lee et al. 2009), which also contributes to RCAN1-mediated inhibition. Collectively, RCAN1 inhibits calcineurin signaling by binding to calcineurin and interfering with substrate binding; phosphorylated RCAN1 has a shorter half-life and appears to be stimulatory when expressed at low levels.

Domains and Motifs of RCAN1

Structural and functional analysis of RCAN1 indicates that specific domains or motifs are required to modulate calcineurin activity (Fig. 2). Both the N- and C-terminals of RCAN1 contain motifs that can bind to calcineurin and inhibit calcineurin catalytic activity (Fuentes et al. 2000; Vega et al. 2002). In the C-terminal, the PxIIxT motif at exon 7 is required for calcineurin binding and inhibition (Chan et al. 2005). The CIC motif (consensus sequence in mammals is LGPGEKYELHA(G/A)T(D/E)(S/T)TPSVVVHVC (E/D)S) at the C-terminal is also necessary for inhibition of calcineurin–NFAT signaling (Mulero et al. 2009). The PxIxIT-like motif within the CIC motif efficiently blocks calcineurin activity by binding to the calcineurin surface with the help of the LxxP motif when RCAN1 is overexpressed. At the N-terminal, a domain that resembles three-dimensional structures of RNA recognition motifs binds and inhibits calcineurin activity (Mehta et al. 2009).

The PxIxIT-like and LxxP motifs appear to be important for the stimulation of calcineurin signals at low RCAN1 expression level in addition to their roles in inhibition at high expression level. The TxxP motif, which is adjacent to the PxIIxT motif at exon 7, is only required for the stimulatory effects (Mehta et al. 2009). Together with the FLISPPxSPP motif phosphorylation site (see section "Inhibitory and Stimulatory Effects of RCAN1"), these motifs are important for RCAN1-mediated stimulation of calcineurin signaling (Fig. 2).



Regulator of Calcineurin 1 (RCAN1), Fig. 2 The RCAN1 motifs and aligned amino acid sequences of the LxxP, FLISPPxSPP, PxIxIT-like, PxIIxT, and TxxP motifs in mouse RCAN1-4 (mRCAN1-4), rat RCAN1 (rRCAN1), human RCAN1-4 (hRCAN1-4), mouse RCAN1-1 (mRCAN1-1), and human RCAN1-1 (hRCAN1-1)

Role of RCAN1 in Angiogenesis

Patients with Down syndrome have an extremely low incidence of solid tumors, which indicates that RCAN1 plays an important role in both angiogenesis and tumor development (Hasle 2001). RCAN1 gain-of-function studies show that constitutive expression of RCAN1 in endothelial cells impairs NFAT nuclear localization, proliferation, and tube formation. RCAN1 also reduces vascular density in Matrigel plugs and melanoma tumor growth in mice (Minami et al. 2004). Another study with transgenic mice with three copies of RCAN1 showed that these mice have significantly suppressed growth of Lewis lung carcinoma and B16F10 melanoma cells in vivo. Moreover, a study by Baek et al. showed that a modest increase in RCAN1 expression (2.4-fold increase in mRNA relative to littermate controls) is sufficient for tumor growth suppression (Baek et al. 2009). In Xenopus laevis, overexpression of RCAN1 decreased the number of branching points that sprouted from intersomitic vessels and decreased the vascular density of the microvessels (Fujiwara et al. 2011).

Consistent with the previous loss-of-function studies, studies in *RCAN1*$^{-/-}$ mice have shown that RCAN1 inhibits angiogenesis. *RCAN1* deletion suppressed subcutaneous and metastatic tumor growth, and endothelial cells isolated from these knockout mice showed decreased VEGF-induced proliferation (Ryeom et al. 2008). Specific knock down of *RCAN1* expression by antisense oligonucleotides also inhibited VEGF-stimulated migration of endothelial cells (Iizuka et al. 2004). Researchers have hypothesized that the appropriate RCAN1 expression level, as well as the phosphorylation state influences RCAN1 regulation of calcineurin activity. High RCAN1 expression may reduce calcineurin activity and block proliferation of endothelial cells, whereas low RCAN1 expression may hyperactivate calcineurin activity and trigger apoptosis (Ryeom et al. 2008).

RCAN1 and Down Syndrome

Because RCAN1 is highly expressed in the brains of Down syndrome patients, it is thought to be associated with the Down syndrome phenotype (Fuentes et al. 1995). Overexpression of the *Drosophila* ortholog of *RCAN1* (*nebula*) causes neuronal defects that are similar to Down syndrome, such as impaired synaptic development, synaptic terminal structure, vesicle recycling, and locomotor activity (Chang and Min 2009). Additionally, reduced or overexpression of nebula impairs mitochondrial enzyme activity, the number and size of mitochondria, and accumulation of toxic reactive oxygen species (ROS) in the fly brains, which are all characteristic of pathologies associated with Down syndrome (Chang and Min 2005). These results strongly suggest that altered expression of RCAN1 contributes to the neurological defects in Down syndrome. Furthermore, cardiac defects, which are another common feature associated with Down syndrome, have been reported in *RCAN1*/*DYRK1A* double transgenic mice. A craniofacial defect was also reported in Nfatc2$^{-/-}$/Nfatc4$^{-/-}$ double-knockout mice, which have impaired RCAN1-calcineurin–NFAT signaling (Arron et al. 2006). These results indicate that *RCAN1* is an important gene that is associated with the Down syndrome phenotype.

RCAN1 and Alzheimer's Disease

Overexpression of RCAN1 has been observed in Alzheimer's disease patient brains, where RCAN1 expression was twofold higher in the cerebral cortex and threefold higher in the hippocampus (Ermak et al. 2001). Of the RCAN isoforms that are expressed in the brain, RCAN1-1 L is upregulated in the neurons of Alzheimer's disease patients (Harris et al. 2007). Overexpressed RCAN1 colocalizes with the neurodegenerative disease-associated proteins huntingtin (Q148) and ataxia-3 (Q84) in cultured primary neurons (Ma et al. 2004). Additionally, RCAN1 expression is directly stimulated by the aggregated amyloid Aß peptide, which is a peptide that plays a role in neuronal degeneration in Alzheimer's disease and human neuroblastoma cell lines (Ermak et al. 2001).

Functional analyses of RCAN1 further indicate that it is closely associated with Alzheimer's disease. Hypomorphic fly mutants with RCAN1 overexpression have decreased long-term memory compared to control *D. melanogaster*, which display 40% memory retention 24 h after training. Correspondingly, calcineurin activity is 40% higher in these RCAN1 mutants (Chang et al. 2003). Inside the cell, RCAN1 regulates the number of vesicles undergoing exocytosis and the speed of vesicle fusion, which opens and closes the pore (Keating et al. 2008). These data indicate that RCAN1 is highly associated with neuronal memory and learning and is thus a strong candidate for Alzheimer's disease neuropathology.

Other Unique Functions of RCAN1

Interestingly, female-specific RCAN1 functions have been reported in non-vertebrates, such as *C. elegans* and *D. melanogaster*. In *C. elegans*, the *RCAN1* ortholog (*RCN-1*) is expressed in the vulva epithelial and muscle cells, and overexpression of RCN-1 results in egg retention (Lee et al. 2003). Calcineurin null mutants, which carry a large deletion in the calcineurin B-regulatory subunit gene, have similar defects in fertility and egg-laying. In *D. melanogaster*, the *RCAN1* ortholog (*sarah*) is expressed in the oocytes and nurse cells of normal flies and is critical for ovulation and female courtship behavior. Inhibition of sarah expression decreases the number of eggs laid, and a majority of the eggs arrest at metaphase I of meiosis (Ejima et al. 2004). Moreover, misexpression affects female courtship behavior, and *D. melanogaster* virgin mutant females frequently display extrusion behavior (Ejima et al. 2004). These studies indicate that RCAN1 may exert female-specific effects via regulation of calcineurin signaling.

Summary

RCAN1 plays an important role in the cell by regulating the multifunctional phosphatase calcineurin. RCAN1 function remains an area of ongoing investigation, although a number of reports have identified several RCAN1 molecular mechanisms. However, there is little information regarding the functional difference(s) between the RCAN1 isoforms, which are differentially expressed by various human tissues. Previous reports have indicated that RCAN1-1 L overexpression activates the transcription factor NFAT and promotes pathologic angiogenesis in human endothelial cells, while RCAN1-4 inhibits angiogenesis (Qin et al. 2006). The mechanism for these opposing effects of the two isoforms remains unknown because this data does not agree with the current model of dual RCAN1 function. Moreover, additional analyses of the nonconserved N-terminal are needed. The N-terminus of RCAN1 contains an aggregation-prone domain, and overexpression of RCAN1 results in the formation of aggresome-like aggregates in cultured primary neurons, and the number of synapses is reduced in these neurons (Ma et al. 2004). Therefore, studies of the RCAN1 N-terminus may be informative for neurodegenerative diseases, such as Down syndrome or Alzheimer's disease. In conclusion, RCAN1, which is an endogenous inhibitor of calcineurin, has wide variety of physiological functions; future studies are necessary to identify the molecular mechanisms of RCAN1.

References

Arron JR, Winslow MM, Polleri A, Chang CP, Wu H, Gao X, Neilson JR, Chen L, Heit JJ, Kim SK, et al. NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. Nature. 2006;441:595–600.

Baek KH, Zaslavsky A, Lynch RC, Britt C, Okada Y, Siarey RJ, Lensch MW, Park IH, Yoon SS, Minami T, et al. Down's syndrome suppression of tumour growth and the role of the calcineurin inhibitor DSCR1. Nature. 2009;459:1126–30.

Cano E, Canellada A, Minami T, Iglesias T, Redondo JM. Depolarization of neural cells induces transcription of the Down syndrome critical region 1 isoform 4 via a calcineurin/nuclear factor of activated T cells-dependent pathway. J Biol Chem. 2005;280:29435–43.

Chan B, Greenan G, McKeon F, Ellenberger T. Identification of a peptide fragment of DSCR1 that competitively inhibits calcineurin activity in vitro and in vivo. Proc Natl Acad Sci USA. 2005;102:13075–80.

Chang KT, Min KT. Drosophila melanogaster homolog of Down syndrome critical region 1 is critical for mitochondrial function. Nat Neurosci. 2005;8:1577–85.

Chang KT, Min KT. Upregulation of three Drosophila homologs of human chromosome 21 genes alters synaptic function: implications for Down syndrome. Proc Natl Acad Sci USA. 2009;106:17117–22.

Chang HY, Takei K, Sydor AM, Born T, Rusnak F, Jay DG. Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin. Nature. 1995;376:686–90.

Chang KT, Shi YJ, Min KT. The Drosophila homolog of Down's syndrome critical region 1 gene regulates learning: implications for mental retardation. Proc Natl Acad Sci USA. 2003;100:15794–9.

Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. Cell. 2002;109(Suppl):S67–79.

Crawford DR, Leahy KP, Abramova N, Lan L, Wang Y, Davies KJ. Hamster adapt78 mRNA is a Down syndrome critical region homologue that is inducible by oxidative stress. Arch Biochem Biophys. 1997;342:6–12.

Davies KJ, Ermak G, Rothermel BA, Pritchard M, Heitman J, Ahnn J, Henrique-Silva F, Crawford D, Canaider S, Strippoli P, et al. Renaming the DSCR1/Adapt78 gene family as RCAN: regulators of calcineurin. FASEB J. 2007;21:3023–8.

Ejima A, Tsuda M, Takeo S, Ishii K, Matsuo T, Aigaki T. Expression level of sarah, a homolog of DSCR1, is critical for ovulation and female courtship behavior in *Drosophila melanogaster*. Genetics. 2004;168:2077–87.

Ermak G, Morgan TE, Davies KJ. Chronic overexpression of the calcineurin inhibitory gene DSCR1 (Adapt78) is associated with Alzheimer's disease. J Biol Chem. 2001;276:38787–94.

Ermak G, Harris CD, Davies KJ. The DSCR1 (Adapt78) isoform 1 protein calcipressin 1 inhibits calcineurin and protects against acute calcium-mediated stress damage, including transient oxidative stress. FASEB J. 2002;16:814–24.

Fruman DA, Bierer BE, Benes JE, Burakoff SJ, Austen KF, Katz HR. The complex of FK506-binding protein 12 and FK506 inhibits calcineurin phosphatase activity and IgE activation-induced cytokine transcripts, but not exocytosis, in mouse mast cells. J Immunol. 1995;154:1846–51.

Fuentes JJ, Pritchard MA, Planas AM, Bosch A, Ferrer I, Estivill X. A new human gene from the Down syndrome critical region encodes a proline-rich protein highly expressed in fetal brain and heart. Hum Mol Genet. 1995;4:1935–44.

Fuentes JJ, Pritchard MA, Estivill X. Genomic organization, alternative splicing, and expression patterns of the DSCR1 (Down syndrome candidate region 1) gene. Genomics. 1997a;44:358–61.

Fuentes JJ, Pucharcos C, Pritchard M, Estivill X. Alu-splice PCR: a simple method to isolate exon-containing fragments from cloned human genomic DNA. Hum Genet. 1997b;101:346–50.

Fuentes JJ, Genesca L, Kingsbury TJ, Cunningham KW, Perez-Riba M, Estivill X, de la Luna S. DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum Mol Genet. 2000;9:1681–90.

Fujiwara M, Hasebe T, Kajita M, Ishizuya-Oka A, Ghazizadeh M, Kawanami O. RCAN1 regulates vascular branching during Xenopus laevis angiogenesis. J Vasc Res. 2011;48:104–18.

Genesca L, Aubareda A, Fuentes JJ, Estivill X, De La Luna S, Perez-Riba M. Phosphorylation of calcipressin 1 increases its ability to inhibit calcineurin and decreases calcipressin half-life. Biochem J. 2003;374:567–75.

Gorlach J, Fox DS, Cutler NS, Cox GM, Perfect JR, Heitman J. Identification and characterization of a highly conserved calcineurin binding protein, CBP1/calcipressin, in Cryptococcus neoformans. EMBO J. 2000;19:3618–29.

Harris CD, Ermak G, Davies KJ. RCAN1-1 L is overexpressed in neurons of Alzheimer's disease patients. FEBS J. 2007;274:1715–24.

Hasle H. Pattern of malignant disorders in individuals with Down's syndrome. Lancet Oncol. 2001;2:429–36.

Hilioti Z, Gallagher DA, Low-Nam ST, Ramaswamy P, Gajer P, Kingsbury TJ, Birchwood CJ, Levchenko A, Cunningham KW. GSK-3 kinases enhance calcineurin signaling by phosphorylation of RCNs. Genes Dev. 2004;18:35–47.

Iizuka M, Abe M, Shiiba K, Sasaki I, Sato Y. Down syndrome candidate region 1, a downstream target of VEGF, participates in endothelial cell migration and angiogenesis. J Vasc Res. 2004;41:334–44.

Keating DJ, Dubach D, Zanin MP, Yu Y, Martin K, Zhao YF, Chen C, Porta S, Arbones ML, Mittaz L, et al. DSCR1/RCAN1 regulates vesicle exocytosis and fusion pore kinetics: implications for Down syndrome and Alzheimer's disease. Hum Mol Genet. 2008;17:1020–30.

Kingsbury TJ, Cunningham KW. A conserved family of calcineurin regulators. Genes Dev. 2000;14:1595–604.

Kishi T, Ikeda A, Nagao R, Koyama N. The SCFCdc4 ubiquitin ligase regulates calcineurin signaling through degradation of phosphorylated Rcn1, an inhibitor of calcineurin. Proc Natl Acad Sci USA. 2007;104:17418–23.

Lee JI, Dhakal BK, Lee J, Bandyopadhyay J, Jeong SY, Eom SH, Kim DH, Ahnn J. The Caenorhabditis elegans homologue of Down syndrome critical region 1, RCN-1, inhibits multiple functions of the phosphatase calcineurin. J Mol Biol. 2003;328:147–56.

Lee JE, Jang H, Cho EJ, Youn HD. Down syndrome critical region 1 enhances the proteolytic cleavage of calcineurin. Exp Mol Med. 2009;41:471–7.

Liu FC, Graybiel AM. Spatiotemporal dynamics of CREB phosphorylation: transient versus sustained phosphorylation in the developing striatum. Neuron. 1996;17:1133–44.

Liu X, Zhao D, Qin L, Li J, Zeng H. Transcription enhancer factor 3 (TEF3) mediates the expression of Down syndrome candidate region 1 isoform 1 (DSCR1-1 L) in endothelial cells. J Biol Chem. 2008;283:34159–67.

Liu Q, Busby JC, Molkentin JD. Interaction between TAK1-TAB1-TAB2 and RCAN1-calcineurin defines a signalling nodal control point. Nat Cell Biol. 2009;11:154–61.

Ma H, Xiong H, Liu T, Zhang L, Godzik A, Zhang Z. Aggregate formation and synaptic abnormality induced by DSCR1. J Neurochem. 2004;88:1485–96.
Mair W, Morantte I, Rodrigues AP, Manning G, Montminy M, Shaw RJ, Dillin A. Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB. Nature. 2011;470:404–8.
Mao Z, Wiedmann M. Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. J Biol Chem. 1999;274:31102–7.
Mehta S, Li H, Hogan PG, Cunningham KW. Domain architecture of the regulators of calcineurin (RCANs) and identification of a divergent RCAN in yeast. Mol Cell Biol. 2009;29:2777–93.
Minami T, Horiuchi K, Miura M, Abid MR, Takabe W, Noguchi N, Kohro T, Ge X, Aburatani H, Hamakubo T, et al. Vascular endothelial growth factor- and thrombin-induced termination factor, Down syndrome critical region-1, attenuates endothelial cell proliferation and angiogenesis. J Biol Chem. 2004;279:50537–54.
Mulero MC, Aubareda A, Orzaez M, Messeguer J, Serrano-Candelas E, Martinez-Hoyer S, Messeguer A, Perez-Paya E, Perez-Riba M. Inhibiting the calcineurin-NFAT (nuclear factor of activated T cells) signaling pathway with a regulator of calcineurin-derived peptide without affecting general calcineurin phosphatase activity. J Biol Chem. 2009;284:9394–401.
Olson EN, Williams RS. Calcineurin signaling and muscle remodeling. Cell. 2000;101:689–92.
Qin L, Zhao D, Liu X, Nagy JA, Hoang MV, Brown LF, Dvorak HF, Zeng H. Down syndrome candidate region 1 isoform 1 mediates angiogenesis through the calcineurin-NFAT pathway. Mol Cancer Res. 2006;4:811–20.
Ryeom S, Greenwald RJ, Sharpe AH, McKeon F. The threshold pattern of calcineurin-dependent gene expression is altered by loss of the endogenous inhibitor calcipressin. Nat Immunol. 2003;4:874–81.
Ryeom S, Baek KH, Rioth MJ, Lynch RC, Zaslavsky A, Birsner A, Yoon SS, McKeon F. Targeted deletion of the calcineurin inhibitor DSCR1 suppresses tumor growth. Cancer Cell. 2008;13:420–31.
Vega RB, Yang J, Rothermel BA, Bassel-Duby R, Williams RS. Multiple domains of MCIP1 contribute to inhibition of calcineurin activity. J Biol Chem. 2002;277:30401–7.
Vega RB, Rothermel BA, Weinheimer CJ, Kovacs A, Naseem RH, Bassel-Duby R, Williams RS, Olson EN. Dual roles of modulatory calcineurin-interacting protein 1 in cardiac hypertrophy. Proc Natl Acad Sci USA. 2003;100:669–74.
Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN, Bassel-Duby R, Williams RS. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ Res. 2000;87:E61–8.
Yao YG, Duh EJ. VEGF selectively induces Down syndrome critical region 1 gene expression in endothelial cells: a mechanism for feedback regulation of angiogenesis? Biochem Biophys Res Commun. 2004;321:648–56.
Zhao P, Xiao X, Kim AS, Leite MF, Xu J, Zhu X, Ren J, Li J. c-Jun inhibits thapsigargin-induced ER stress through up-regulation of DSCR1/Adapt78. Exp Biol Med (Maywood). 2008;233:1289–300.

Regulator of G-Protein Signaling 13

► RGS13

Regulators of G-Protein Signaling

► RGS Protein Family

Relaxin Family Peptide Receptors (RXFP) 1 and 2

Roger J. Summers[1], Michelle L. Halls[2] and Emma T. van der Westhuizen[3]
[1]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia
[2]Department of Pharmacology, University of Cambridge, Cambridge, UK
[3]Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montréal, QC, Canada

Synonyms

GREAT; RXFP1: LGR7; RXFP2: LGR8

Historical Background: Relaxin Family Peptides and Their Receptors

Relaxin was one of the first reproductive hormones to be identified, following the observation that a factor in the serum of pregnant guinea pigs induced relaxation of the birth canal (Hisaw 1926). Until recently, relaxin was considered purely a hormone of pregnancy and little was known of its potential roles in males and nonpregnant females; the purification of relaxin from animal sources led to the determination of its peptide structure, biological actions, and development of reliable bioassays (Schwabe and McDonald 1977; James et al. 1977; John et al. 1981), and this knowledge precipitated the use of recombinant DNA techniques to clone the rat (Hudson et al. 1981) and pig (Haley et al. 1982) relaxin

genes, followed soon after by human gene-1 (RLN1) (Hudson et al. 1983) and gene-2 relaxin (RLN2) (Hudson et al. 1984). The identification of additional relaxin peptides (including the neuropeptide relaxin-3), in addition to the recent de-orphanization of multiple G protein-coupled receptors (GPCRs) for relaxin, more than 75 years after the identification of the peptide itself, stimulated a resurgence of interest in this pleiotropic hormone.

Relaxin is a two-chain peptide with a high level of structural homology to insulin. This established the structural determinants of the insulin/relaxin peptide family, and precipitated a search for related peptides containing similar structural motifs. Additional mammalian peptides were revealed: insulin-like growth factor (IGF)-I and IGF-II, and the insulin/relaxin-like peptides (INSL) INSL3, INSL4, INSL5 and INSL6.

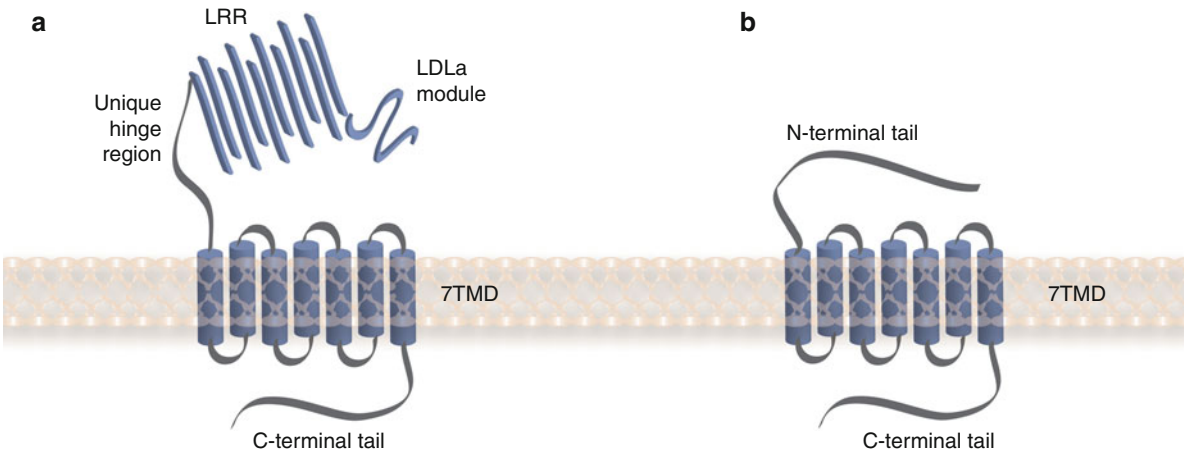
Since the publication of the human genome, many orphan GPCRs including the relaxin family peptide receptors were de-orphanized following sequence-based prediction of a seven transmembrane-spanning domain structure. A search of the human genome for paralogs of the orphan GPCR, leucine-rich repeat-containing GPCR (LGR) 7 (RXFP1), identified an additional orphan GPCR, LGR8 (RXFP2) (Hsu et al. 2002). Based upon the phenotypic similarities of mice lacking either INSL3 or the mouse LGR8 (GREAT) gene, the insulin/relaxin peptides were tested for activity at the two orphan receptors; both LGR7 and LGR8 responded to relaxin with a dose-dependent increase in cAMP production, and the expression patterns of LGR7 were consistent with known relaxin binding sites (Hsu et al. 2000). The de-orphanization of LGR7 led the way for ligand identification for the highly similar orphan GPCR, LGR8; thus INSL3 was identified as the specific peptide ligand that bound LGR8 and stimulated cAMP production (Hsu et al. 2002; Kumagai et al. 2002). In addition, receptor mRNA expression was found in tissues that express INSL3, and humans with a deletion in the LGR8 gene displayed cryptorchidism phenotypically identical to that seen in INSL3-knockout mice. These receptors, and two additional orphan GPCRs, were later renamed ▶ relaxin family peptide receptors (RXFP) 1–4, where RXFP1 is the relaxin receptor, RXFP2 is the receptor for INSL3, RXFP3 is the receptor for relaxin-3, and RXFP4 is the INSL5 receptor (see RXFP3/4 page for further information; reviewed in [Bathgate et al. 2006; Halls et al. 2007; Ivell and Anand-Ivell 2009]).

Molecular Biology of RXFP1 and RXFP2

Human RXFP1 is located on chromosome 4q32.1, and RXFP2 is located on 13q13.1. The receptors share 60% amino acid sequence identity and 80% homology. Both RXFP1 and RXFP2 have multiple alternatively spliced isoforms (reviewed in [Halls et al. 2007]). Thus far, 29 splice variants of RXFP1 and RXFP2 have been identified; four of these variants have been studied in greater detail, and show a wide range of tissue expression, but cannot bind either relaxin or INSL3 or increase cAMP accumulation. Although one isoform was highly expressed (RXFP2.1; deletion of exon 11, corresponding to leucine-rich repeat [LRR] 7), others were expressed either at very low levels (RXFP1.10; deletion of exon 3, flanking the low-density lipoprotein class a [LDLa] module), retained within the cell (RXFP1.2; deletion of exon 12 and 13, corresponding to LRRs 8 and 9), or in the case of one variant (RXFP1.1; stop codon in exon 6, resulting in only the LDLa module and two LRRs), secreted, raising interesting questions regarding their role in endogenous regulation of full-length RXFP1 (reviewed in [Halls et al. 2007]). Another even smaller secreted variant, RXFP1-truncate, has been identified in mouse, rat, and pig (reviewed in [Halls et al. 2007]). RXFP1-truncate consists of the receptor signal peptide, the LDLa module, 33 residues of the LRR flanking sequence, and a nonhomologous sequence of seven residues. When a construct encoding the truncate peptide was transiently expressed in HEK293T cells, RXFP1-truncate was secreted and inhibited relaxin-stimulated cAMP signaling mediated by the full-length receptor, acting as a functional "antagonist" of relaxin. Expression of RXFP1-truncate is increased during pregnancy in both mouse and rat, suggesting a functional role of localized antagonism of relaxin.

Structural Features and Functional Domains of RXFP1 and RXFP2

Although all receptors of the RXFP family are classified as family A GPCRs, similar to the rhodopsin receptor, they can be further subdivided into two distinct subgroups: the LGRs (Fig. 1a), and the small peptide GPCRs (Fig. 1b). Generally, LGRs comprise a LRR domain, a hinge region, seven transmembrane-spanning domains, and a C-terminal tail. The type



Relaxin Family Peptide Receptors (RXFP) 1 and 2, Fig. 1 *Schematic diagram of the structure of relaxin family peptide receptors (RXFP)*. (**a**) The relaxin and INSL3 receptors, (RXFP1 and RXFP2, respectively) are G protein-coupled receptors composed of the seven transmembrane spanning domains (*7TMD*), with a large N-terminal ectodomain consisting of a unique hinge-like region, 10 leucine-rich repeats (*LRR*), and a low-density lipoprotein class A (*LDLa*) module. (**b**) The relaxin-3 and INSL5 receptors (RXFP3 and RXFP4, respectively) are also G protein-coupled receptors that contain the 7TMD but lack the LRR structure found in RXFP1 and RXFP2

C LGRs, RXFP1 and RXFP2, are differentiated from the type A and type B LGRs by the presence of a low-density lipoprotein class A (LDLa) module at the extreme N-terminus, leading into 10 LRRs and a unique hinge region.

While relaxin binds to both RXFP1 and RXFP2, INSL3 is unable to bind RXFP1. Furthermore, rat relaxin is unable to bind RXFP2 suggesting that the relaxin interaction with RXFP2 is a species-specific event (Halls et al. 2005). RXFP1 and RXFP2 form constitutive homodimers (and may form heterodimers); dimerization is dependent upon the transmembrane region, with stabilization provided by the ectodomain (Svendsen et al. 2008a, b). Both receptor homodimers exhibit negative cooperativity in ligand binding (INSL3 at RXFP2; relaxin at RXFP1) (Svendsen et al. 2008a, b). RXFP1 is also glycosylated, and this plays a role in receptor cell surface expression and activation of cAMP (reviewed in [Bathgate et al. 2006; Halls et al. 2007]).

Both RXFP1 and RXFP2 contain two ligand-binding sites: a high-affinity site within the ectodomain, and a lower-affinity site within the transmembrane region (Halls et al. 2005; Sudo et al. 2003). Molecular modeling of the LRR region of RXFP1 has revealed a likely binding cassette for relaxin, which occurs at an angle of 45° across five of the parallel LRRs (Bullesbach and Schwabe 2005). Deletion of any of these residues within the receptor abolished relaxin binding (Bullesbach and Schwabe 2005).

Despite the RXFP2 sequence containing all of the residues required for the binding of relaxin to RXFP1, a number of recent studies have suggested that these amino acids are not essential for INSL3 binding (Bullesbach and Schwabe 2006; Rosengren et al. 2006; Scott et al. 2007). Thus, at RXFP2, relaxin and INSL3 use subtly different B-chain residues and thus bind to different, but overlapping sites of the receptor. Although five of the identified RXFP2 residues that interact with INSL3 are conserved in RXFP1, the only two non-conserved residues interact with the residues within INSL3 (Arg^{B20} and Trp^{B27}) that are critical for peptide binding and activity (Scott et al. 2007), thus providing an explanation for the lack of INSL3 binding at RXFP1.

Mutagenesis studies of the LDLa region highlighted the essential role of this module in receptor signaling. Mutation of conserved residues, those that compromise correct folding, or entire deletion of the LDLa module from either RXFP1 or RXFP2, results in receptors that retain binding of their cognate peptide but are unable to signal by increasing cAMP (Hopkins et al. 2007; Kern et al. 2007). The LDLa module also plays a role in receptor maturation and delivery to the cell surface (Kern et al. 2007).

Signal Transduction Pathways of RXFP1 and RXFP2

Constitutively active mutants of both RXFP1 and RXFP2 (transmembrane helix 6: D637Y) increase cAMP accumulation in a ligand-independent manner (Hsu et al. 2000; Hsu et al. 2002). Consequently, much research has focused on the cAMP accumulation modulated by these two receptors (reviewed by [Bathgate et al. 2006; Halls et al. 2007; van der Westhuizen et al. 2008; Du et al. 2010]) (Figs. 2 and 3).

Both RXFP1 and RXFP2 couple to $G\alpha_s$ to increase cAMP, which is negatively modulated by coupling to $G\alpha_{oB}$. Only RXFP1 can also couple to $G\alpha_{i3}$ to activate further cAMP accumulation via a $G\beta\gamma$-phosphatidylinositol 3-kinase (PI3K)-protein kinase C (PKC) ζ pathway to stimulate adenylyl cyclase 5. Activation of the $G\alpha_{i3}$ pathway is dependent upon the final 10 amino acids of the RXFP1 C-terminal tail (requiring Arg^{752}) and localization within lipid-rich membrane domains.

For RXFP1, there is also evidence for activation of other signaling pathways in response to relaxin (reviewed in [Bathgate et al. 2006; Halls et al. 2007; van der Westhuizen et al. 2008; Du et al. 2010]). In THP-1 and human endometrial stromal cells, both of which endogenously express RXFP1, there is evidence for tyrosine kinase or mitogen-activated protein kinase (MAPK)-dependent cAMP accumulation, which may involve relaxin-mediated inhibition of a phosphodiesterase. A number of cell types that express RXFP1 rapidly activate extracellular regulated kinase (ERK)1/2 (< 5 min) upon relaxin stimulation, including human endometrial stromal cells, THP-1 cells, and primary cultures of human coronary artery cells, pulmonary artery smooth muscle cells, and renal myofibroblasts. There is also evidence for relaxin-mediated increases in nitric oxide both acutely and chronically, and in renal myofibroblasts, activation of a nitric oxide pathway mediates the effects of relaxin/RXFP1 on differentiation and collagen production. Relaxin has also been reported to interact with the glucocorticoid receptor.

In cell systems that endogenously express RXFP2, the receptor appears to mediate a variety of responses (reviewed in [Bathgate et al. 2006; Halls et al. 2007; van der Westhuizen et al. 2008; Ivell and Anand-Ivell 2009]). In gubernacular cells, stimulation with INSL3 causes an increase in cAMP. However, and conversely, in both male and female germ cells, stimulation of RXFP2 causes inhibition of cAMP accumulation mediated by pertussis toxin (PTX)-sensitive G-proteins. Thus, it appears that the signaling outcomes mediated by these receptors may vary greatly depending upon the cell type, and perhaps reflecting differences in the population of G proteins expressed.

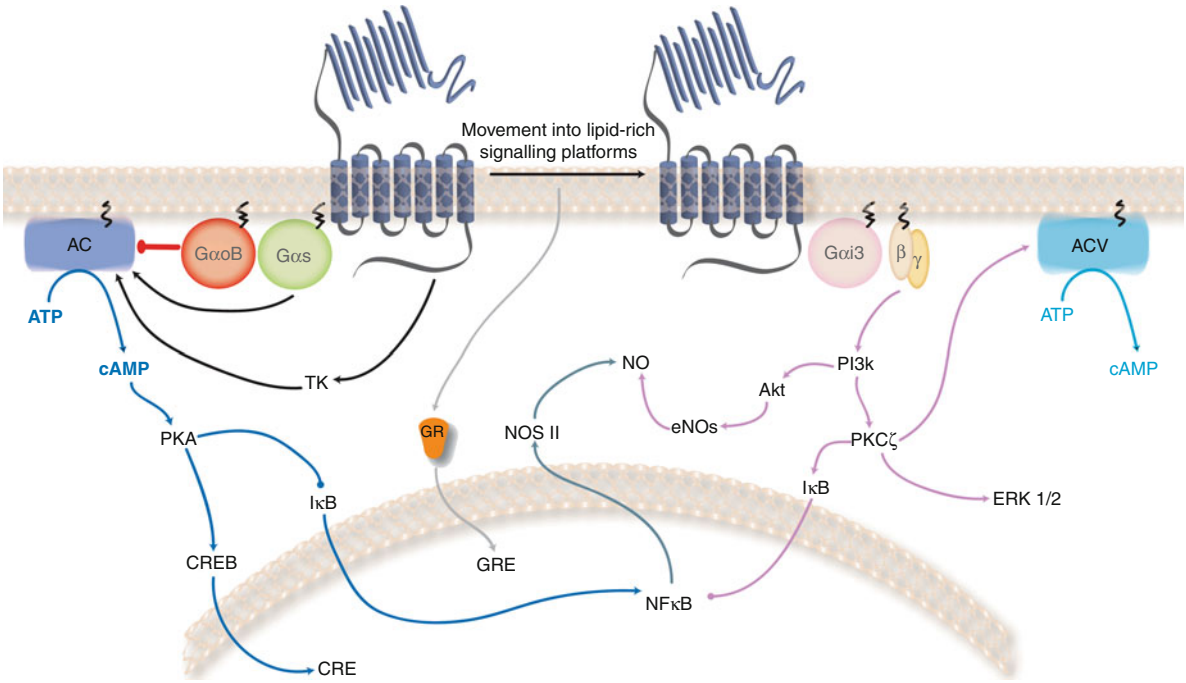
Localization of RXFP1 and RXFP2 Receptors

RXFP1 is present in a variety of tissues (reviewed in [Bathgate et al. 2006; Halls et al. 2007; van der Westhuizen et al. 2008]). Northern blots of human tissue identified relaxin receptor mRNA in ovary, uterus, placenta, testis, prostate, brain, kidney, heart, lung, liver, adrenal gland, thyroid gland, salivary glands, muscle, peripheral blood cells, bone marrow, and skin (Table 1). An additional shorter length receptor mRNA was also identified in oviduct, uterus, colon, and brain. Human relaxin receptor protein expression has been identified by immunohistochemical analysis in uterus, cervix, vagina, nipple, and breast (Table 1). Studies using Northern blots of rat tissue have additionally identified receptor mRNA in small intestine and oviduct, and LacZ reporter expression was also identified in oviduct in a relaxin receptor knockout mouse model.

INSL3 receptor mRNA expression in human (reviewed in [Bathgate et al. 2006; Halls et al. 2007; van der Westhuizen et al. 2008; Ivell and Anand-Ivell 2009]) occurs in the uterus, testis, brain, pituitary, kidney, thyroid, muscle, peripheral blood cells, and bone marrow as determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Additional expression was identified in the ovary and gubernaculum in the mouse as determined by RT-PCR and specifically in the mesenchymal and cremaster muscle of the gubernaculum using immunohistochemistry, in the rat gubernaculum using RT-PCR and Northern blot analysis, and in the rat ovary using RT-PCR, Northern blot analysis, and in situ hybridization (Table 1).

Physiological Roles of Relaxin/RXFP1 and INSL3/RXFP2

Initially, the principal role of relaxin was thought to involve preparation of the birth canal for parturition, as

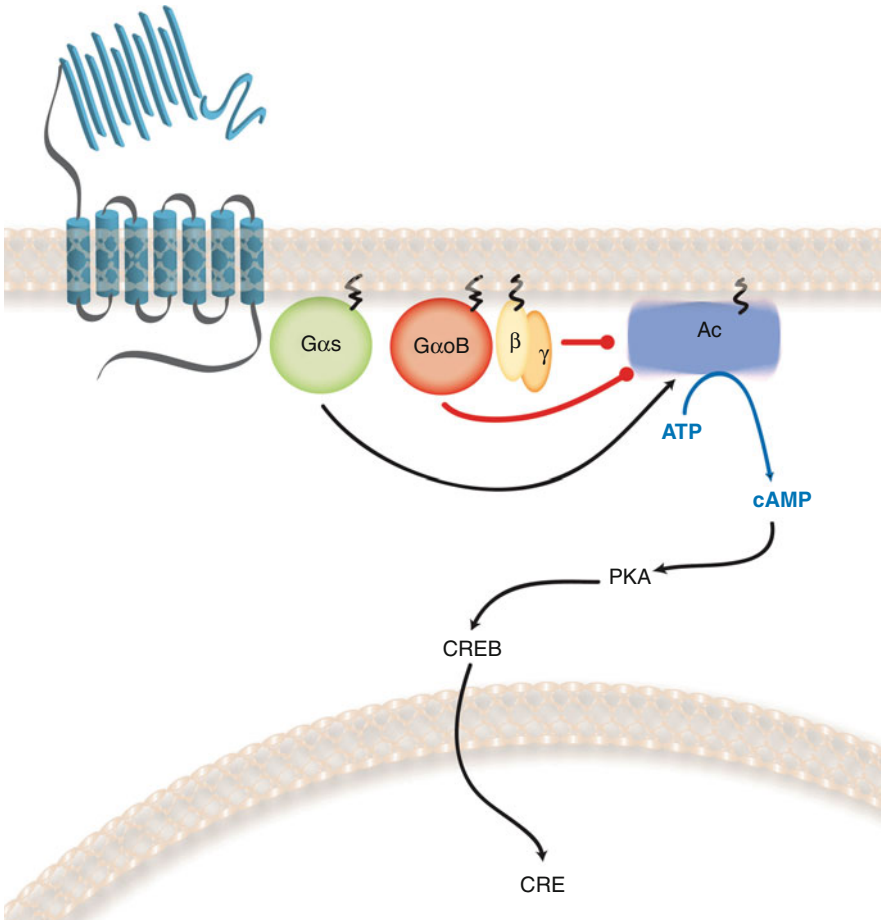


Relaxin Family Peptide Receptors (RXFP) 1 and 2, Fig. 2 *Signaling pathways activated by RXFP1*. Activation of RXFP1 by relaxin promotes receptor coupling to $G\alpha_s$ (to stimulate) and $G\alpha_{oB}$ (to inhibit) cAMP accumulation by modulating adenylyl cyclase (*AC*) activity. The receptor may also increase cAMP accumulation by a tyrosine kinase (*TK*) dependent mechanism. The cAMP downstream of $G\alpha_s$ and $G\alpha_{oB}$ activates protein kinase A (*PKA*) and cAMP response element binding protein (*CREB*) to increase the transcriptional activity of the cAMP response element (*CRE*) transcription factor. PKA may also inhibit inhibitor κB (*IκB*), allowing increased activity of the nuclear factor κB (*NFκB*), and production of nitric oxide (*NO*) via nitric oxide synthase II (*NOSII*). RXFP1 also couples to $G\alpha_{i3}$ (dependent upon lipid-rich microdomains) allowing activation of a Gβγ, phosphoinositide 3-kinase (*PI3K*), protein kinase C (*PKC*)ζ pathway, to increase cAMP via activation of AC5. As a result of $G\alpha_{i3}$ coupling, PI3K activation may also activate an Akt, eNOS pathway to increase NO, and PKCζ may activate extracellular regulated kinase (*ERK*) 1/2, or activate IκB to inhibit the activity of NκB

first described in pregnant guinea pigs (Hisaw 1926). Although this special endocrine function is found in species such as rodents and some mammals, it is now apparent that it is of reduced importance in higher species. Currently it is established that in women, maximum circulating levels of relaxin occur during the first trimester of pregnancy, and thus the role of relaxin in pregnancy is likely to be associated with first trimester physiological events such as embryo implantation. The peptide also causes a number of additional physiological changes associated with pregnancy, including uterine growth and development, myometrial contractility, central control of plasma osmolality, and cardiovascular adaptations (reviewed in [Sherwood 2004]).

Away from the reproductive system, relaxin is postulated to exert a local influence upon the circulation by increasing vasodilation and passive compliance (reviewed in [Du et al. 2010]). Recent observations suggest that relaxin exerts these local effects upon vasodilation through the synthesis and production of nitric oxide, particularly in situations of natural or induced myocardial infarction. Furthermore, recent phase II clinical trials have shown efficacy for relaxin as a vasodilator in acute heart failure.

Another physiological effect of relaxin with therapeutic potential is its effect upon connective tissue regulation and fibrosis. Importantly from a therapeutic viewpoint, relaxin is able to decrease excess collagen deposition in fibrotic lesions, with a conservation of endogenous connective tissue structure. Relaxin also exerts anti-inflammatory effects (reviewed in [Bathgate et al. 2006; Halls et al. 2007; van der Westhuizen et al. 2008]), can inhibit the activation of human neutrophils by pro-inflammatory agents and prevents histamine and granule release by activated basophils and mast cells. These studies



Relaxin Family Peptide Receptors (RXFP) 1 and 2, Fig. 3 *Signaling pathways activated by RXFP2*. Activation of RXFP2 by INSL3 or relaxin causes increased cAMP accumulation via $G\alpha_s$-mediated adenylyl cyclase (*AC*) activation. The receptor also couples to $G\alpha_{oB}$, and both $G\alpha$ and $G\beta\gamma$ subunits negatively modulate AC activity. The cAMP pool (downstream of $G\alpha_s$ and $G\alpha_{oB}$) activates protein kinase A (*PKA*) and cAMP-response element binding protein (*CREB*) to modulate the transcriptional activity of the cAMP response element (*CRE*)

indicate a protective effect of relaxin during allergic inflammatory responses. In rodent models of angiogenesis and wound healing, relaxin treatment resulted in increased vascularization and neoangiogenesis of ischemic wound sites. There was no effect of relaxin upon cytokine expression in cells taken from non-wound sites, indicating promising specificity for therapeutic applications.

In a manner that mirrors its effects in fibrosis, evidence suggests that relaxin is recruited as an endogenous factor for tissue remodeling in cancer cells (reviewed in [Klonisch et al. 2007]). Increased expression of relaxin (but not RXFP1) has been detected in both tissue from prostate carcinoma and prostate cancer cell lines. Interestingly, downregulation of either relaxin or RXFP1 caused significant inhibition of growth and invasiveness, in addition to increased apoptosis. A human prostate cancer cell line engineered to express relaxin exhibited increased tumor volume and vascularization compared to controls. Relaxin is associated with increased invasiveness of endometrial, breast, and thyroid carcinomas. Serum relaxin concentrations are also elevated in patients with bone metastasis, and relaxin is a potent stimulator of osteoclastogenesis (Ferlin et al. 2010).

The major physiological effects of INSL3 are observed within the reproductive system (reviewed in [Bathgate et al. 2006; Halls et al. 2007; van der Westhuizen et al. 2008; Ivell and Anand-Ivell 2009]). Lack of INSL3 (or alternatively, lack of RXFP2) in mice results in cryptorchidism, or failure of the testes to descend during development. Female INSL3-knockout mice exhibit only a mild phenotype of disturbed cycle length and increased ovarian apoptosis, whereas overexpression of the peptide leads to ovarian descent and bilateral inguinal hernia. The influence of the peptide extends beyond early development, as INSL3 serum levels increase with the onset of puberty in males, and

Relaxin Family Peptide Receptors (RXFP) 1 and 2, Table 1 Tissue localization of RXFP1 and RXFP2 receptors

	RXFP1			RXFP2		
Tissue/species	Rat	Mouse	Human	Rat	Mouse	Human
Ovary	mRNA[a]	Protein[f]	mRNA[b]	mRNA[a,e]		
Oviduct	mRNA[a]	mRNA[d]	Protein[f]			
Uterus	mRNA[a] Protein[c,f]	mRNA[d,b,a]	mRNA[b] Protein[c]			mRNA[b]
Uterine smooth muscle	Protein[f]	mRNA[d] Protein[c]	Protein[c]			
Endometrium	mRNA[b]	Protein[c]	mRNA[a] Protein[f,c]			
Cervix,vagina	Protein[c,f]	mRNA[d,a,b] Protein[b]	Protein[c]			
Placenta		mRNA[a,b]	*mRNA*[b]			
Nipple	Protein[c]	mRNA[d,a,b]	Protein[c]			
Breast	Protein[c]	Protein[c]	Protein[c]			
Testis	mRNA[a,b]	mRNA[d,b,a]	mRNA[b]	mRNA[a,e]	mRNA[b]	mRNA[b]
Prostate		mRNA[b]	mRNA[b]			
Gubernaculum				mRNA[b,a]	mRNA[b]	
Brain	mRNA[a,b] Protein[f]	mRNA[d,a,b]	mRNA[b]	mRNA[e]	mRNA[b]	mRNA[b]
Brain regions	mRNA[e] Protein[f]	mRNA[e] Protein[f]		mRNA[e] Protein[f]	mRNA[e] Protein[f]	
Pituitary		mRNA[d]				
Kidney	mRNA[a]		mRNA[b]	mRNA[e]		mRNA[b]
Heart	mRNA[a,b] Protein[f]	mRNA[d,a,b]	mRNA[b]			
Lung		mRNA[a,b]	mRNA[b]			
Liver			mRNA[b]			
Intestine	mRNA[a]	mRNA[a,b]				
Colon	mRNA[a]					
Adrenal	mRNA[a]		mRNA[b]			
Thyroid			mRNA[b]			mRNA[b]
Thymus	mRNA[a]					
Salivary glands			mRNA[b]			
Muscle			mRNA[b]			mRNA[b]
Blood cells			mRNA[b]			mRNA[b]
THP-1 monocytes			mRNA[b] Protein[g]			mRNA[b]
Bone marrow			mRNA[b]			mRNA[b]
Skin		mRNA[a,b]	mRNA[b]			

Italics = low levels. For detailed references see Bathgate et al. (2006)
[a]Northern blot
[b]RT-PCR
[c]Immunohistochemistry
[d]lacZ reporter expression
[e]In situ hybridization
[f]Receptor autoradiography
[g]Receptor binding

are dependent upon the level of luteinizing hormone stimulation of Leydig cells. Luteinizing hormone also stimulates expression of INSL3 in ovarian theca and testicular Leydig cells, which through activation of the INSL3 receptor, causes meiotic progression of arrested oocytes in preovulatory follicles, and suppresses male germ cell apoptosis. Interestingly, in a manner similar to relaxin, the INSL3/RXFP2 system has also been

identified in human prostate carcinoma cell lines and human thyroid carcinomas, and treatment of the tumor cell lines with INSL3 resulted in increased tumor cell motility. INSL3 and RXFP2 may also have a role in osteoporosis.

Summary

RXFP1 is coupled to cAMP and many other signaling pathways in different cell types. Linking these effectors of RXFP1 activation to specific physiological end points should allow the design and development of targeted therapies. RXFP1 has therapeutic potential for treatment of fibrosis, cancer metastasis, and is currently in Phase III clinical trials for heart failure. Although not as extensively studied, RXFP2 appears to have a simpler physiological role, and fewer downstream effectors compared to RXFP1. RXFP2 has possible therapeutic potential for the treatment of some types of cryptorchidism and could be used to control fertility; however, more extensive research is required to assess its true therapeutic potential.

References

Bathgate RA, Ivell R, Sanborn BM, Sherwood OD, Summers RJ. International union of pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. Pharmacol Rev. 2006;58(1):7–31.

Bullesbach EE, Schwabe C. The trap-like relaxin-binding site of LGR7. J Biol Chem. 2005;280:14051–6.

Bullesbach EE, Schwabe C. The mode of interaction of the relaxin-like factor (RLF) with the leucine-rich repeat G protein-activated receptor 8. J Biol Chem. 2006;281:26136–43.

Du XJ, Bathgate RA, Samuel CS, Dart AM, Summers RJ. Cardiovascular effects of relaxin: from basic science to clinical therapy. Nat Rev Cardiol. 2010;7(1):48–58.

Ferlin A, Pepe A, Facciolli A, Gianesello L, Foresta C. Relaxin stimulates osteoclast differentiation and activation. Bone. 2010;46(2):504–13.

Haley J, Hudson P, Scanlon D, John M, Cronk M, Shine J, et al. Porcine relaxin: molecular cloning and cDNA structure. DNA. 1982;1(2):155–62.

Halls ML, Bond CP, Sudo S, Kumagai J, Ferraro T, Layfield S, et al. Multiple binding sites revealed by interaction of relaxin family peptides with native and chimeric relaxin family peptide receptors 1 and 2 (LGR7 and LGR8). J Pharmacol Exp Ther. 2005;313(2):677–87.

Halls ML, van der Westhuizen ET, Bathgate RA, Summers RJ. Relaxin family peptide receptors–former orphans reunite with their parent ligands to activate multiple signalling pathways. Br J Pharmacol. 2007;150(6):677–91.

Hisaw FL. Experimental relaxation of the pubic ligament of the guinea pig. Proc Soc Exp Biol Med. 1926;23:661–3.

Hopkins EJ, Layfield S, Ferraro T, Bathgate RA, Gooley PR. The NMR solution structure of the relaxin (RXFP1) receptor lipoprotein receptor class A module and identification of key residues in the N-terminal region of the module that mediate receptor activation. J Biol Chem. 2007;282(6):4172–84.

Hsu SY, Kudo M, Chen T, Nakabayashi K, Bhalla A, van der Spek PJ, et al. The three subfamilies of leucine-rich repeat-containing G protein-coupled receptors (LGR): identification of LGR6 and LGR7 and the signaling mechanism for LGR7. Mol Endocrinol. 2000;14(8):1257–71.

Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, et al. Activation of orphan receptors by the hormone relaxin. Science. 2002;25(295):637–8.

Hudson P, Haley J, Cronk M, Shine J, Niall H. Molecular cloning and characterization of cDNA sequences coding for rat relaxin. Nature. 1981;291(5811):127–31.

Hudson P, Haley J, John M, Cronk M, Crawford R, Haralambidis J, et al. Structure of a genomic clone encoding biologically active human relaxin. Nature. 1983;301(5901):628–31.

Hudson P, John M, Crawford R, Haralambidis J, Scanlon D, Gorman J, et al. Relaxin gene expression in human ovaries and the predicted structure of a human preprorelaxin by analysis of cDNA clones. EMBO J. 1984;3(10):2333–9.

Ivell R, Anand-Ivell R. Biology of insulin-like factor 3 in human reproduction. Hum Reprod Update. 2009;15(4):463–76.

James R, Niall H, Kwok S, Bryand-Greenwood G. Primary structure of porcine relaxin: homology with insulin and related growth factors. Nature. 1977;267(5611):544–6.

John MJ, Borjesson BW, Walsh JR, Niall HD. Limited sequence homology between porcine and rat relaxins: implications for physiological studies. Endocrinology. 1981;108(2):726–9.

Kern A, Agoulnik AI, Bryant-Greenwood GD. The low-density lipoprotein class A module of the relaxin receptor (leucine-rich repeat containing G-protein coupled receptor 7): its role in signaling and trafficking to the cell membrane. Endocrinology. 2007;148(3):1181–94.

Klonisch T, Bialek J, Radestock Y, Hoang-Vu C, Hombach-Klonisch S. Relaxin-like ligand-receptor systems are autocrine/paracrine effectors in tumor cells and modulate cancer progression and tissue invasiveness. Adv Exp Med Biol. 2007;612:104–18.

Kumagai J, Hsu SY, Matsumi H, Roh JS, Fu P, Wade JD, et al. INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. J Biol Chem. 2002;277(35):31283–6.

Rosengren KJ, Lin F, Bathgate RA, Tregear GW, Daly NL, Wade JD, et al. Solution structure and novel insights into the determinants of the receptor specificity of human relaxin-3. J Biol Chem. 2006;281(9):5845–51.

Schwabe C, McDonald JK. Primary structure of the B-chain of porcine relaxin. Biochem Biophys Res Commun. 1977;75(2):503–10.

Scott DJ, Wilkinson TN, Zhang S, Ferraro T, Wade JD, Tregear GW, et al. Defining the LGR8 residues involved in binding insulin-like peptide 3. Mol Endocrinol. 2007;21(7):1699–712.

Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocr Rev. 2004;25(2):205–34.
Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RA, et al. H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. J Biol Chem. 2003;278(10):7855–62.
Svendsen AM, Zalesko A, Konig J, Vrecl M, Heding A, Kristensen JB, et al. Negative cooperativity in H2 relaxin binding to a dimeric relaxin family peptide receptor 1. Mol Cell Endocrinol. 2008a;296(1–2):10–7.
Svendsen AM, Vrecl M, Ellis TM, Heding A, Kristensen JB, Wade JD, et al. Cooperative binding of insulin-like peptide 3 to a dimeric relaxin family peptide receptor 2. Endocrinology. 2008b;149(3):1113–20.
van der Westhuizen ET, Halls ML, Samuel CS, Bathgate RA, Unemori EN, Sutton SW, et al. Relaxin family peptide receptors–from orphans to therapeutic targets. Drug Discov Today. 2008;13(15–16):640–51.

Relaxin Family Peptide Receptors (RXFP) 3 and 4

Emma T. van der Westhuizen[1], Michelle L. Halls[2] and Roger J. Summers[3]
[1]Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montréal, QC, Canada
[2]Department of Pharmacology, University of Cambridge, Cambridge, UK
[3]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia

Synonyms

GPR100; RXFP3: GPCR135, SALPR; RXFP4: GPCR142

Historical Background: Relaxin Family Peptides and Their Receptors

Relaxin family peptides including the relaxins 1–3, insulin-like peptides (INSL) 3–6, and insulin-like growth factors I and II have a similar architecture to insulin. These peptides are generally involved in the regulation of cell growth and metabolism. Relaxin was originally identified as a hormone important during pregnancy but is also now known to have roles in collagen remodeling, wound healing, cardiovascular responses, and as a brain neuropeptide. In the human, three independent genes produce three relaxin peptides, named relaxin-1, relaxin, and the recently discovered relaxin-3 (Bathgate et al. 2002). Relaxin-3 is primarily expressed in the brain as a neuropeptide that mediates stress and feeding responses in rats (Tanaka et al. 2005; McGowan et al. 2005, 2007). Relaxin-3 peptide sequences from different species are well conserved (Bathgate et al. 2002; Wilkinson et al. 2005) and the degree of sequence identity between the relaxin-3 peptides suggests that it is the ancestral relaxin peptide from which the other relaxin and insulin-like peptides evolved (Wilkinson et al. 2005).

The receptors for the relaxin family peptides are G protein-coupled receptors (GPCRs), named relaxin family peptide receptors (RXFP) 1–4, where RXFP1 is the relaxin receptor, RXFP2 is the INSL3 receptor, RXFP3 is the relaxin-3 receptor, and RXFP4 is the INSL5 receptor. RXFP3 was discovered by probing a human cortical cDNA library (Matsumoto et al. 2000). Although it has high amino acid sequence similarity to the somatostatin and the angiotensin II receptors, it was not activated by these ligands, and therefore it was initially named somatostatin and angiotensin-like peptide receptor (SALPR). The ligand for RXFP3 was later discovered by using the receptor as bait to fish for peptides in extracts derived from various rat tissues (Liu et al. 2003b). Only the brain extracts increased GTPγS binding (Liu et al. 2003b), and purification led to the identification of relaxin-3 (Liu et al. 2003b). RXFP4 was subsequently identified by searching the human genome database (Genbank™) with the RXFP3 sequence (Liu et al. 2003a). Since RXFP4 had 43% amino acid sequence identity with RXFP3 it was hypothesized that these receptors may share or have similar ligands (Liu et al. 2003a). Relaxin-3 was also found to activate RXFP4 (Liu et al. 2003a); however, RXFP4 was later identified as the receptor for INSL5 (Liu et al. 2005).

Molecular Biology of RXFP3 and RXFP4

Human RXFP3 is located on chromosome 5p15.1-5p14 (Matsumoto et al. 2000) and human RXFP4 is located on chromosome 1q22, and both receptors are coded by a single exon sequence. In the

rat RXFP3 gene, there are two potential start codons (ATG) that are not seen in the human or the mouse genes (Wilkinson et al. 2005). Transcription initiating from the first potential start codon produces rat RXFP3-long, with seven additional residues at the amino terminus. Transcription initiation from the second potential start codon produces rat RXFP3-short, which is equivalent to the human and mouse RXFP3 sequences. The mouse RXFP4 sequence has 74% homology with the human RXFP4 sequence, however, the gene equivalent to RXFP4 in the rat is a pseudogene that does not code a functional protein (Chen et al. 2005), suggesting the INSL5-RXFP4 system is redundant in rats.

Structural Features and Functional Domains of RXFP3 and RXFP4 Receptors

The relaxin family peptide receptors (RXFPs) belong to two distinct subclasses of family A GPCRs, the leucine-rich repeat-containing GPCRs (LGRs) and the small peptide receptor-like GPCRs (RXFP3 and RXFP4) (Fig. 1). RXFP3 and RXFP4 consist of seven transmembrane spanning domains (7TMDs), an extracellular amino-terminal tail, and an intracellular carboxy-terminal tail.

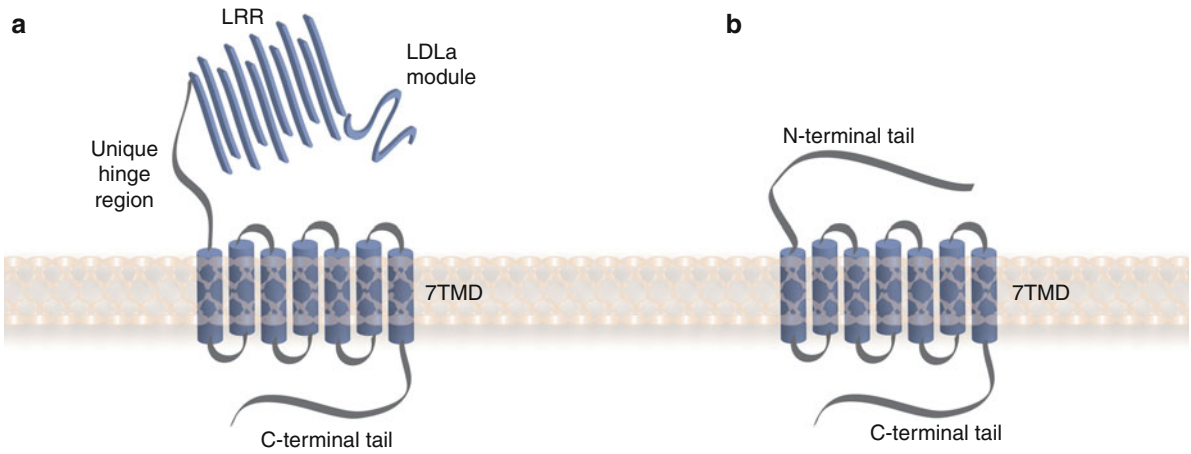
Receptor chimeras between RXFP3 and RXFP4 identified the amino-terminal tail and extracellular loop 2 as important regions of the receptors for binding to relaxin-3 or INSL5, respectively (Zhu et al. 2008). These regions are also important for the activation of RXFP3 by relaxin-3 (Zhu et al. 2008); however, transmembrane helicies 2, 3, 5 and extracellular loop 2 are important for activation of RXFP4 by INSL5 (Zhu et al. 2008).

Human relaxin-3 has a similar affinity for mouse, rat, and human RXFP3 receptors, likewise, mouse/rat relaxin-3 have similar affinities for human, mouse, and rat RXFP3 (Chen et al. 2005). The potency of human, mouse, and rat relaxin-3 at human, mouse, and rat RXFP3 is similar in cAMP, GTPγS binding, and calcium assays (Chen et al. 2005), suggesting the minor variations in the ligand and receptor amino acid sequences do not affect the function of the receptor. The dog and rat INSL5 DNA sequences contain a frameshift mutation, resulting in multiple stop codons within the exonic sequence; therefore, INSL5 is considered to be a nonfunctional pseudogene in both the dog and the rat (Wilkinson et al. 2005). The INSL5 gene is functional in mice; however, mouse INSL5 is not expressed in the brain. Since the expression pattern of RXFP4 and INSL5 overlap and are pseudogenes in the dog and rat this suggests they are a ligand–receptor pair (Liu et al. 2005); however, the roles of both INSL5 and RXFP4 remain to be determined.

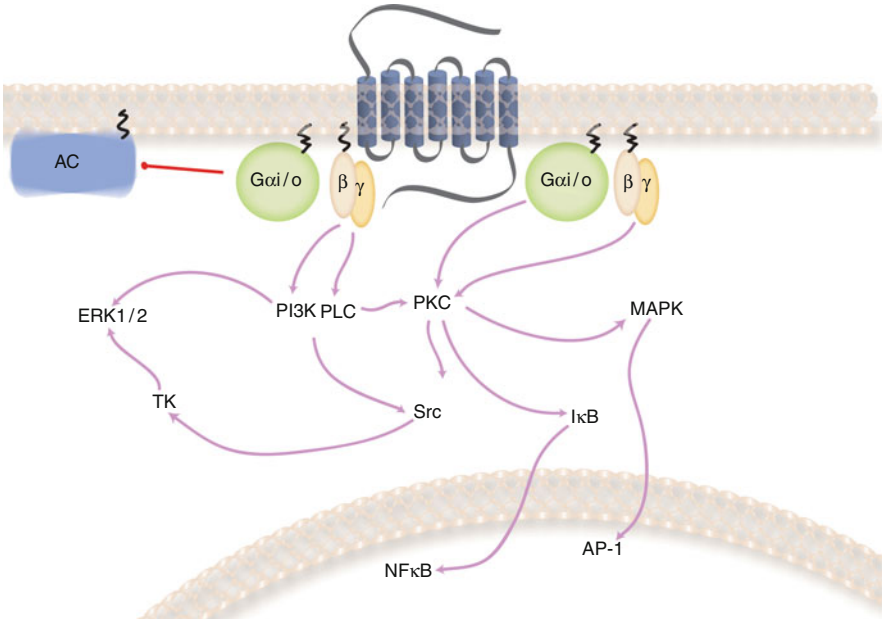
Signal Transduction Pathways of RXFP3 and RXFP4

Relaxin-3 interacts with both RXFP3 and RXFP4 to increase GTPγS binding, suggesting that it initiates G protein-mediated signaling events in cells that express the receptors (Liu et al. 2003a,b). Unlike RXFP1 and RXFP2 that increase cAMP in cells when treated with relaxin or INSL3, respectively, none of the relaxin family peptides tested increased cAMP levels in cells expressing RXFP3 or RXFP4 (Liu et al. 2003a, b, 2005; van der Westhuizen et al. 2010). In fact, relaxin-3 inhibited forskolin-stimulated cAMP accumulation in RXFP3-expressing cell lines in a concentration-dependent manner (Liu et al. 2003b; van der Westhuizen et al. 2010) (Fig. 2). Weaker inhibition of forskolin-stimulated cAMP accumulation occurs in CHO-K1, HEK293, or SN56 cells expressing RXFP3 receptors when treated with the B-chain of relaxin-3 (Liu et al. 2003b), or with human relaxin or porcine relaxin (van der Westhuizen et al. 2010). INSL5 also inhibits forskolin-stimulated cAMP accumulation in cells expressing RXFP4 but not in cells expressing RXFP3 (Liu et al. 2005).

In addition to inhibition of the cAMP signaling pathway, RXFP3 also activates ERK1/2 when stimulated with relaxin-3, the B-chain of relaxin-3 or relaxin. ERK1/2 activation involves Gαi/o proteins and either a PI 3-kinase or PKC-dependent pathway (van der Westhuizen et al. 2007; 2010) (Fig. 2). Increased transcription of AP-1 reporter genes is also observed in RXFP3-expressing cells following stimulation with relaxin-3, human relaxin, or porcine relaxin in a cell-type and peptide-dependent manner, potentially through activation of ERK1/2, ERK5, p38 MAPK, or JNK (van der Westhuizen et al. 2010) (Fig. 2). Increased transcription of NF-κB reporter genes was also observed in RXFP3-expressing cells following stimulation with relaxin-3 but not with



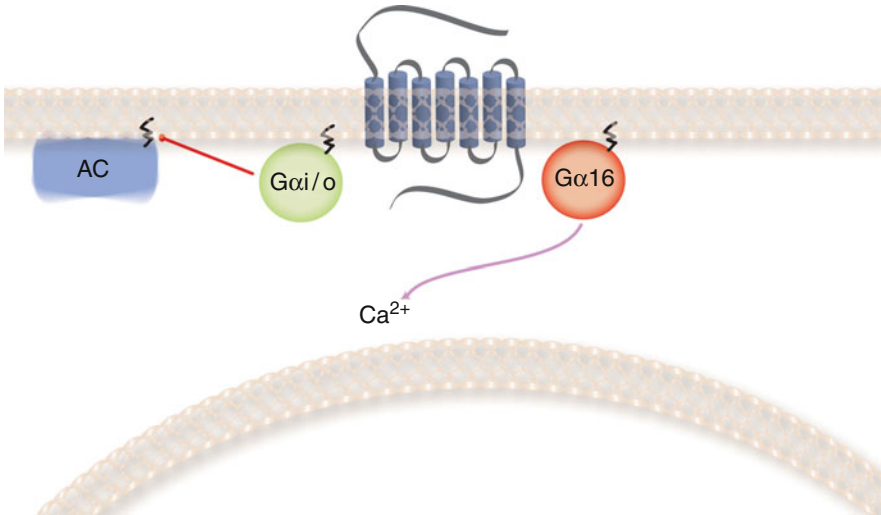
Relaxin Family Peptide Receptors (RXFP) 3 and 4, Fig. 1 Schematic diagram of the structure of relaxin family peptide receptors (RXFPs). (**a**) The relaxin and INSL3 receptors, (RXFP1 and RXFP2, respectively) are G protein-coupled receptors composed of the seven transmembrane spanning domains (7TMDs), with a large N-terminal ectodomain consisting of a unique hinge-like region, 10 leucine-rich repeats (LRRs) and a low-density lipoprotein class A (LDLa) module. (**b**) The relaxin-3 and INSL5 receptors (RXFP3 and RXFP4, respectively) are also G protein-coupled receptors that contain the 7TMDs but lack the LRR structure found in RXFP1 and RXFP2



Relaxin Family Peptide Receptors (RXFP) 3 and 4, Fig. 2 Signaling pathways activated downstream of RXFP3. RXFP3 inhibits cAMP production via inhibitory G proteins (Gαi/o) and activates extracellular signal-regulated kinase (ERK) 1/2 via Gαi/o proteins and either a phosphatidylinositol 3-kinase (PI3K) or protein kinase C (PKC)-dependent pathway. RXFP3 is coupled to increased gene transcription from nuclear factor (NF)-κB and activator protein (AP)-1 transcription factors. MAPK indicates either ERK, p38 MAPK, or JNK, and TK indicates receptor tyrosine kinase

human relaxin, porcine relaxin, or human INSL3 (van der Westhuizen et al. 2010). Thus, RXFP3 couples to at least four different signaling pathways to elicit responses in cells; ligand-biased signaling is also observed at this receptor, with some pathways activated exclusively by relaxin-3, while others are activated by relaxin-3, human relaxin, porcine relaxin, and human INSL3 (van der Westhuizen et al. 2010).

RXFP4 is potentially coupled to the calcium signaling pathway as INSL5 or relaxin-3 stimulation of CHO-K1 cells co-transfected with RXFP4 and



Relaxin Family Peptide Receptors (RXFP) 3 and 4, Fig. 3 Signaling pathways activated downstream of RXFP4. RXFP4 inhibits cAMP production via inhibitory G proteins (Gαi/o) and releases calcium via Gα16 proteins

Gα16 increased intracellular calcium, suggesting that RXFP4 couples to Gα16 signaling pathways in cell lines that express this G protein (Liu et al. 2003a, 2005) (Fig. 3). RXFP3 on the other hand, does not activate calcium signaling (Liu et al. 2003b).

Localization of RXFP3 and RXFP4

RT-PCR and in situ hybridization studies showed that RXFP3 is principally expressed in the rat brain (Table 1), with strongest expression in the hypothalamus, paraventricular nucleus, cortex, septal nucleus, preoptic area, supraoptic nucleus, periaqueductal gray, nucleus insertus, and central gray regions in the brainstem (Sutton et al. 2004; Ma et al. 2007). The mouse ortholog of RXFP3 is also expressed in the brain, as shown by Northern blots (Boels et al. 2004; Sutton et al. 2005; Lein et al. 2007). In the periphery, however, RXFP3 mRNA was only detected in the testis (Liu et al. 2003b). Direct radioligand binding studies have also mapped RXFP3 binding sites in the rat brain (Table 1) (Sutton et al. 2004; Ma et al. 2007) (RXFP4 is a pseudogene in the rat, therefore not expressed). This study used an INSL5 A-chain/relaxin-3 B-chain chimeric peptide (that does not bind to RXFP1 or RXFP2) to identify RXFP3 binding sites in many regions involved in sensory perception. The INSL5/relaxin-3 chimera binding sites may have terminating projections from rat relaxin-3-containing neurons, suggesting that relaxin-3 produced locally by the brain, acts at regions where RXFP3 is expressed (Sutton et al. 2004; Ma et al. 2007).

RT-PCR demonstrated RXFP4 expression in human colon, thyroid, salivary gland, prostate, placenta, thymus, testis, kidney, uterus, and brain (Table 1) (Conklin et al. 1999; Liu et al. 2003a; Boels and Schaller 2003). In situ hybridization did not show INSL5 or RXFP4 mRNA expression in the mouse brain and mouse INSL5 did not displace [^{125}I]-INSL5/relaxin-3 binding, suggesting that there were very few if any RXFP4 sites in the mouse brain (Sutton et al. 2005). RXFP4 mRNA is expressed in the human colon, thyroid, salivary gland, prostate, placenta, thymus, testis, kidney, and brain (Table 1) (Liu et al. 2003a; Boels and Schaller 2003).

Physiological Roles of Relaxin-3

The strongest expression of relaxin-3 mRNA occurs in the nucleus incertus (Sutton et al. 2004). In neurons, relaxin-3 is found in vesicles, suggesting that it is a neurotransmitter released from activated neurons (Tanaka et al. 2005). Based on the location of the projections of relaxin-3-containing neurons, the peptide is potentially important in motivational and emotional behaviors, and may have a role in motor function, sensory perception, processing sensory

Relaxin Family Peptide Receptors (RXFP) 3 and 4, Table 1 Tissue distribution of RXFP3 and RXFP4

	RXFP3			*RXFP4*
Tissue/ species	Rat	Mouse	Human	Human
Ovary				mRNA[c]
Uterus				mRNA[c]
Placenta				mRNA[b,c]
Testis			mRNA[a]	mRNA[b,c]
Prostate				mRNA[b,c]
Brain	Protein[e]		mRNA[a,d]	mRNA[b,c]
Brain regions	mRNA[f,g] Protein[f,g]	mRNA[h,i,j] Protein[i]	mRNA[a,d]	mRNA[c]
Pituitary			mRNA[d]	
Kidney				mRNA[b]
Heart				mRNA[c]
Intestine				mRNA[c]
Colon				mRNA[b,c]
Pancreas			mRNA[a,d]	
Adrenal			mRNA[a,d]	mRNA[c]
Thyroid				mRNA[b,c]
Thymus			mRNA[a]	mRNA[b,c]
Salivary glands	mRNA[a,d]			mRNA[b,c]
Muscle				mRNA[c]
Peripheral blood cells				mRNA[c]
Bone marrow				mRNA[c]

[a]mRNA by RT-PCR (Liu et al. 2003b)
[b]mRNA by RT-PCR (Liu et al. 2003a)
[c]mRNA by multi-tissue expression array or Northern blot analysis (Boels and Schaller 2003)
[d]mRNA by RT-PCR (Matsumoto et al. 2000)
[e]Receptor autoradiography (Liu et al. 2005)
[f]In situ hybridization, receptor autoradiography (Sutton et al. 2004)
[g]In situ hybridization, receptor autoradiography (Ma et al. 2007)
[h]In situ hybridization (Boels et al. 2004)
[i]In situ hybridization, receptor autoradiography (Sutton et al. 2005)
[j]In situ hybridization (Lein et al. 2007)

information and in learning and memory (Sutton et al. 2004; Tanaka et al. 2005). However, whether these physiological roles are dependent upon the RXFP3 receptors around the terminal regions of the relaxin-3-containing neurons remains to be determined. Injection of relaxin-3 into rat brain cerebral ventricles, the paraventricular nucleus, supraoptic nucleus, arcuate nucleus, or the anterior preoptic area increased food intake in rats (McGowan et al. 2005, 2007), possibly by acting at RXFP3 receptors in these areas.

Summary

RXFP3 activates many signaling pathways in various cell lines and exhibits different but overlapping signaling profiles dependent upon the activating ligand. On stimulation with relaxin family peptides, RXFP3 inhibits cAMP production and activates MAP kinase signaling pathways. RXFP3 also increases gene transcription from AP-1 and NF-κB promoters, which may be important in regulating feeding and stress responses mediated by relaxin-3 in rats. Linking these signaling pathways to pathophysiology should encourage the design and development of antianxiety and antiobesity therapies that specifically target particular RXFP3 signaling pathways. Extensive study of RXFP4 is required to determine its physiological role, its importance in pathophysiology, and to assess its potential as a drug target.

References

Bathgate RA, Samuel CS, Burazin TC, Layfield S, Claasz AA, Reytomas IG, et al. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. J Biol Chem. 2002;277(2):1148–57.

Boels K, Schaller HC. Identification and characterisation of GPR100 as a novel human G-protein-coupled bradykinin receptor. Br J Pharmacol. 2003;140:932–8.

Boels K, Hermans-Borgmeyer I, Schaller HC. Identification of a mouse ortholog of the G-protein-coupled receptor SALPR and its expression in adult mouse brain and during development. Brain Res Dev Brain Res. 2004;152:265–8.

Chen J, Kuei C, Sutton SW, Bonaventure P, Nepomuceno D, Eriste E, et al. Pharmacological characterization of relaxin-3/INSL7 receptors GPCR135 and GPCR142 from different mammalian species. J Pharmacol Exp Ther. 2005;312(1):83–95.

Conklin D, Lofton-Day CE, Haldeman BA, Ching A, Whitmore TE, Lok S, et al. Identification of INSL5, a new member of the insulin superfamily. Genomics. 1999;60(1):50–6.

Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, et al. Genome-wide atlas of gene expression in the adult mouse brain. Nature. 2007;445:168–76.

Liu C, Chen J, Sutton S, Roland B, Kuei C, Farmer N, et al. Identification of relaxin-3/INSL7 as a ligand for GPCR142. J Biol Chem. 2003a;278(50):50765–70.

Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, et al. Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. J Biol Chem. 2003b;278(50):50754–64.

Liu C, Chen J, Kuei C, Sutton S, Nepomucendo D, Bonaventure P, et al. Relaxin-3/insulin-like peptide 5 chimeric peptide, a selective ligand for G protein-coupled receptor (GPCR)135

and GPCR142 over leucine-rich repeat-containing G protein-coupled receptor 7. Mol Pharmacol. 2005;67:231–40.
Ma S, Bonaventure P, Ferraro T, Shen PJ, Burazin TC, Bathgate RA, et al. Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G-protein-coupled receptor-135 in the rat. Neuroscience. 2007;144:165–90.
Matsumoto M, Kamohara M, Sugimoto T, Hidaka K, Takasaki J, Saito T, et al. The novel G-protein coupled receptor SALPR shares sequence similarity with somatostatin and angiotensin receptors. Gene. 2000;248(1–2):183–9.
McGowan BM, Stanley SA, Smith KL, White NE, Connolly MM, Thompson EL, et al. Central relaxin-3 administration causes hyperphagia in male Wistar rats. Endocrinology. 2005;146(8):3295–300.
McGowan BM, Stanley SA, White NE, Spangeus A, Patterson M, Thompson EL, et al. Hypothalamic mapping of orexigenic action and Fos-like immunoreactivity following relaxin-3 administration in male Wistar rats. Am J Physiol. 2007;292(3):E913–9.
Sutton SW, Bonaventure P, Kuei C, Roland B, Chen J, Nepomuceno D, et al. Distribution of G-protein-coupled receptor (GPCR)135 binding sites and receptor mRNA in the rat brain suggests a role for relaxin-3 in neuroendocrine and sensory processing. Neuroendocrinology. 2004;80(5):298–307.
Sutton SW, Bonaventure P, Kuei C, Nepomuceno D, Wu J, Zhu J, et al. G-protein-coupled receptor (GPCR)-142 does not contribute to relaxin-3 binding in the mouse brain: further support that relaxin-3 is the physiological ligand for GPCR135. Neuroendocrinology. 2005;82(3–4):139–50.
Tanaka M, Iijima N, Miyamoto Y, Fukusumi S, Itoh Y, Ozawa H, et al. Neurons expressing relaxin 3/INSL 7 in the nucleus incertus respond to stress. Eur J Neurosci. 2005;21:1659–70.
van der Westhuizen ET, Werry TD, Sexton PM, Summers RJ. The Relaxin Family Peptide Receptor 3 (RXFP3) activates ERK1/2 through a PKC dependent mechanism. Mol Pharmacol. 2007;71:1618–29.
van der Westhuizen ET, Christopoulos A, Sexton PM, Wade JD, Summers RJ. H2 Relaxin Is a Biased Ligand Relative to H3 Relaxin at the Relaxin Family Peptide Receptor 3 (RXFP3). Mol Pharmacol. 2010;77:759–72.
Wilkinson TN, Speed TP, Tregear GW, Bathgate RAD. Evolution of the relaxin-like peptide family. BMC Evol Biol. 2005;5(14):1471–2148.
Zhu J, Kuei C, Sutton S, Kamme F, Yu J, Bonaventure P, et al. Identification of the domains in RXFP4 (GPCR142) responsible for the high affinity binding and agonistic activity of INSL5 at RXFP4 compared to RXFP3 (GPCR135). Eur J Pharmacol. 2008;590(1–3):43–52.

Resistance to Inhibitors of Cholinesterase 8

► Ric-8

ret-GC

► Guanylyl Cyclase Receptors

Retinoic Acid Receptors (RARA, RARB, and RARC)

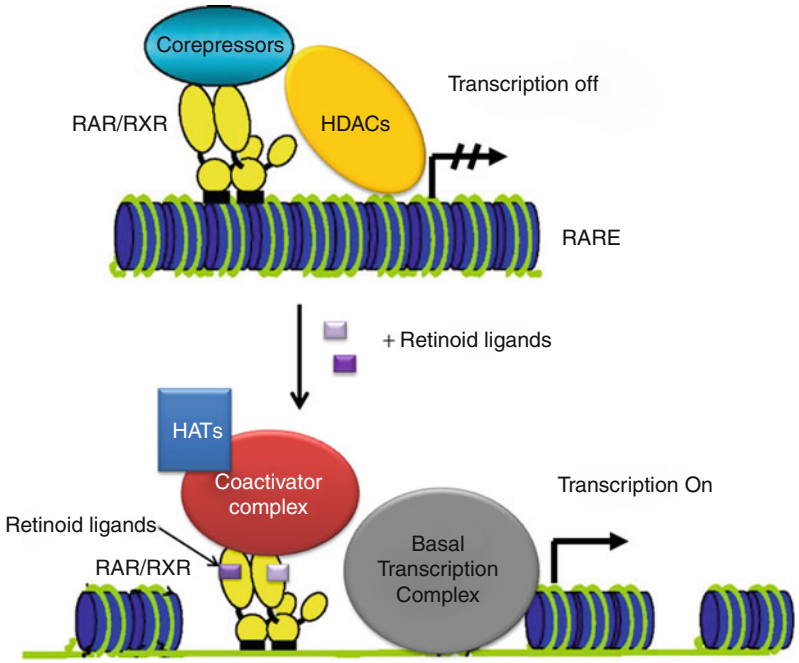
Scott A. Busby and Thomas P. Burris
Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, FL, USA

Synonyms

NR1B1; NR1B2; NR1B3; RARα; RARβ; RARγ

Historical Background

The retinoic acid receptors (RARs) are ligand-dependent transcription factors that belong to the NR1B subtype of the nuclear receptor (NRs) superfamily and have broad roles in development, cell growth and survival, vision, spermatogenesis, inflammation, and neural patterning. These receptors act in *trans* mainly as heterodimers with retinoid X receptors (RXRs). The actions of RARs are stimulated by the binding of cognate natural ligands (all *trans* retinoic acid and 9-*cis* retinoic acid) as well as a number of synthetic ligands. In the presence or absence of ligand, RAR/RXR heterodimers associate with retinoic acid DNA response elements (RAREs) present in the promoter or enhancer regions of target genes. When no ligand is present, corepressor proteins and histone deacetylases interact with the receptor DNA complexes and prevent transcription from occurring. When retinoid ligands are present, they bind and activate the RARs by initiating a conformation more favorable to the association of coactivator proteins and subsequent recruitment of histone acetyltransferases (HATs) and the components of the basal transcriptional machinery that initiate transcription of the RARE (Fig. 1). These consensus site DNA sequences generally consist of two directly repeated half sites of AGGTCA separated by two or five base pair spacers (DR2 or DR5 elements). Due to



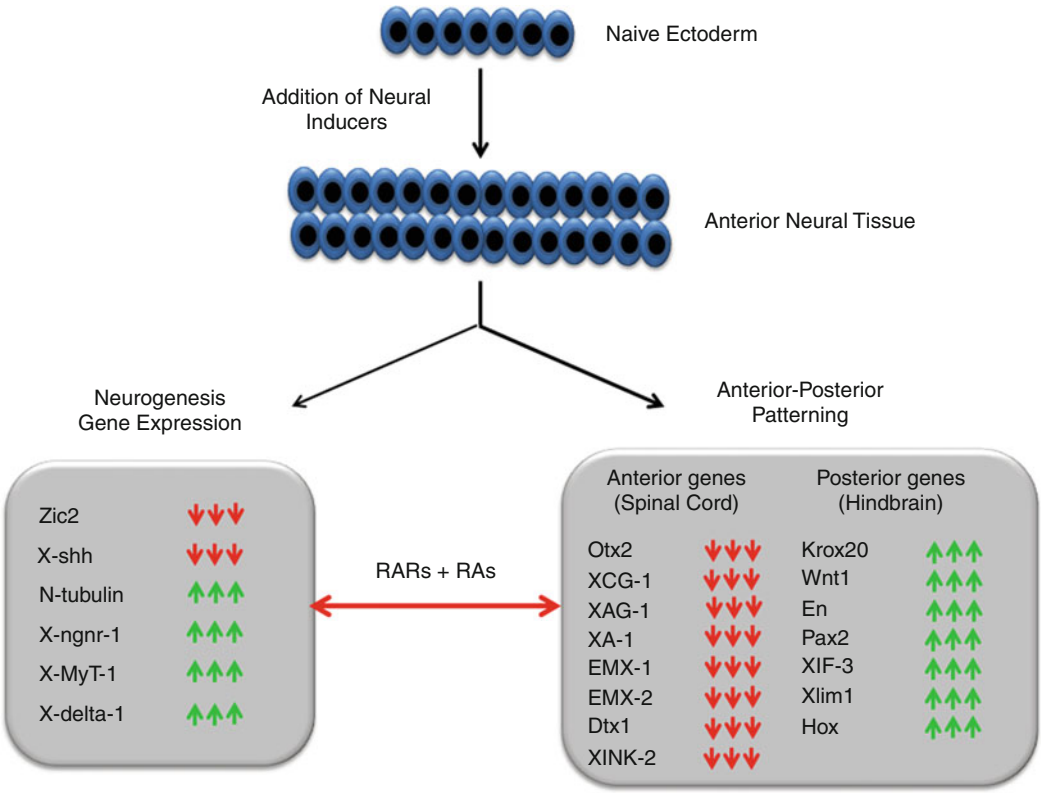
Retinoic Acid Receptors (RARA, RARB, and RARC), Fig. 1 Activation model of RARs. In the presence or absence of ligand, RAR/RXR heterodimers associate with retinoic acid response elements (RAREs) present in the promoter or enhancer regions of target genes. When no ligand is present, corepressor proteins and histone deacetylases interact with the receptor DNA complexes and prevent transcription from occurring. When retinoid ligands are present, they bind and activate the RARs by initiating a conformation more favorable to the association of coactivator proteins and subsequent recruitment of histone acetyltransferases (HATs) and the components of the basal transcriptional machinery to initiate transcription

their broad action in diverse cell and tissue populations, RARs are essential signaling proteins for basic and clinical research.

Role of RARs in Embryonic Development

Research from the 1950s implicated vitamin A deficiencies as a cause for a number of congenital malformations and defects observed in the development of animals. These results were known long before the discovery of retinoic acids as the most biologically active forms of vitamin A. Later research then investigated the role of retinoic acid in early embryonic development and in the early 1990s led to the discovery of the retinoic acid receptors (RARs). Since then much work has been done to determine the role that RARs play in embryonic development.

Genetic studies were conducted in the early 1990s to investigate the role of all three RAR isoforms in embryonic development by generating single and double RAR mutant mice. While mice with a mutation deleting only one RAR isoform contained certain developmental abnormalities, they were still viable, indicating a certain redundancy among isoforms (Mark et al. 2009). However, RAR double null mutants, containing genetic deletions of any two of the RAR isoforms, died in utero or at birth due to severe developmental deficits mainly in the vertebrae, brain, and limbs indicating the importance of the expression of the RARs on the formation of these structures. The same deficits were observed by a number of investigators where various components of retinoic acid signaling were knocked out such as retinaldehyde-synthesizing enzyme RDH10 (Sandell et al. 2007), RA-synthesizing enzyme RALDH2 (Halilagic et al. 2007), or RALDH3 (Dupe et al. 2003). In addition, treatment of wild-type animals with synthetic pan-specific RAR antagonists also produced the same defects (Kochhar et al. 1998; Wendling et al. 2001). This demonstrated that not only expression of RARs was essential for normal development



Retinoic Acid Receptors (RARA, RARB, and RARC), Fig. 2 RAR-mediated RA signaling mediates neurogenesis and anterior-posterior patterning of central nervous system. Diagram showing RAR-mediated up- and downregulation of genes involved in both neurogenesis and anterior-posterior which are vital in the development of neurons, spinal cord, and hindbrain

but that the receptors required activation by retinoic acid ligands to mediate their important roles in developmental signaling programs.

Using combined strategies of selective RAR isoform knockouts and mutations in the RA signaling pathways, much has been learned about the crucial roles that RARs play in specific stages of organ and brain development. For instance, RARs are involved in formation of a number of limb structures and control the antero-posterior axis of the limbs (Dupe et al. 1999). Moreover, RAR-mediated RA signaling is responsible for both neurogenesis as well as the anterior-posterior patterning of the developing central nervous system through a complex mechanism of gene activation and repression by RARs (Maden 2002) (Fig. 2). Several studies have shown that RARs are required for the formation of a number of eye structures as well as histogenesis and physiological apoptosis in the retina. Moreover, it has been discovered that RARs play important roles in cardiac development, respiratory system development, as well as the formation of important structures in the kidneys and urogenital tract.

While much has been learned about the important roles played by the RARs in embryonic development, research is now shifting toward elucidating the roles played by these receptors during the postnatal development. To do this, new strategies have emerged to allow for the selective mutation of the retinoid receptors in specific cell types so as to further understand the functions of these receptors in the postnatal animal as it develops and grows (Metzger and Chambon 2001; Metzger et al. 2003).

Role of RARs in Regulating Cell Proliferation and Cancer

In addition to the effects that retinoic acids and the RARs have on developmental pathways, a large

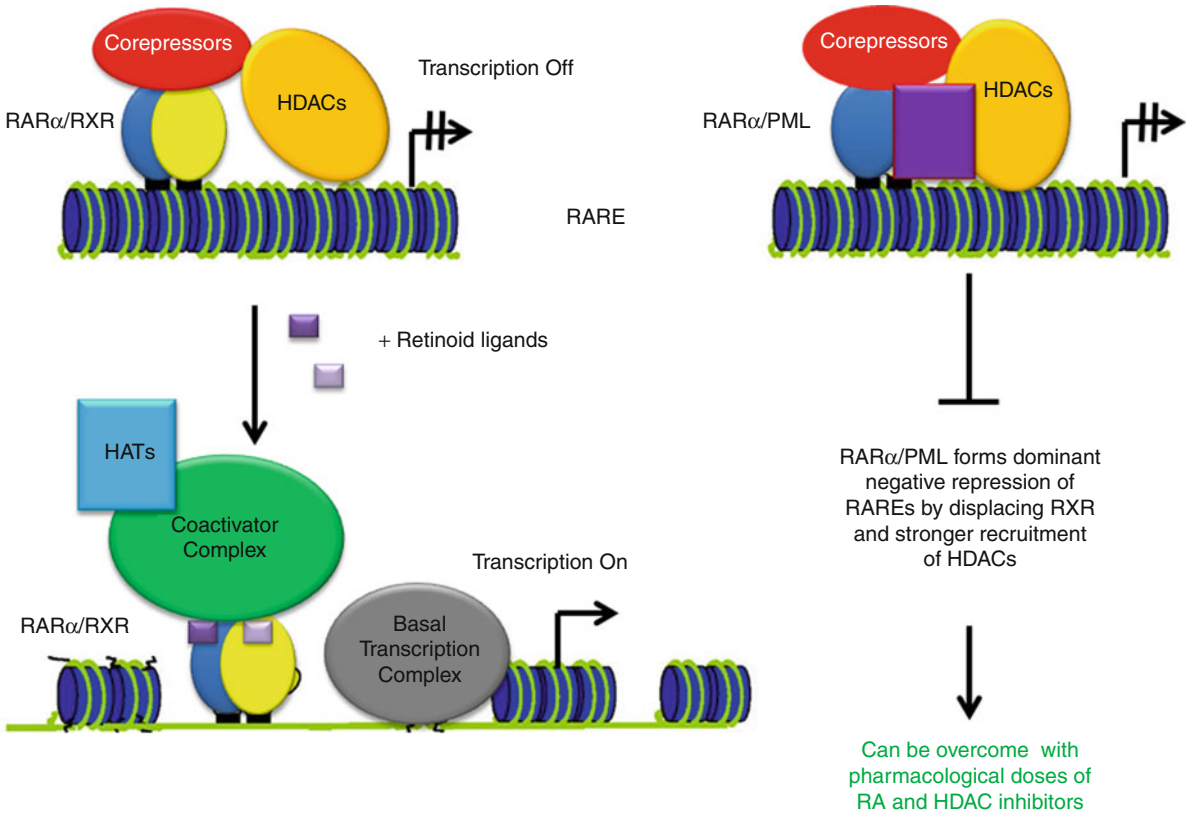
amount of evidence has emerged implicating RARs in the control of cell-cycle pathways and cellular proliferation. In normal cells, retinoic acids generally inhibit cell-cycle progression by instituting a block in the G1 phase of the cell cycle (Mongan and Gudas 2007). Of all the RAR isoforms, these effects are mostly mediated by RARβ_2 following binding and activation by retinoic acids (Faria et al. 1999). Several studies have shown in a number of cell types that activation of RARβ_2 leads to the transactivation of several genes involved in cell-cycle arrest such as $p21^{CIP1}$ and $p27^{KIP1}$ (Li et al. 2004; Suzui et al. 2004). In addition to activating cell-cycle arrest proteins, RARs also mediate both the downregulation of mRNA expression as well as protein ubiquitination and degradation for both the Cyclin D and E families which prevents progression of the cell cycle from the G1 to S phase (Tang and Gudas 2011). Moreover, RARs induce apoptosis following binding of retinoids in a number of cell types as a guard against tumor formation. Retinoic acid binds to RARα and induces apoptosis in both acute lymphoblastic leukemia cells as well as myeloid leukemia cell lines (Chikamori et al. 2006; Luo et al. 2009). In addition, it has been reported that RARγ induces apoptosis upon binding to retinoids in both skin keratinocytes as well as pancreatic adenocarcinoma cells (Hatoum et al. 2001; Pettersson et al. 2002). Finally, RARβ_2 has been implicated in the induction of apoptosis in breast cells. Taken together, these observations have provided clear evidence that RARs play key roles in the regulation of cell-cycle progression and cell growth as well as apoptosis and therefore when RAR-mediated signaling pathways are disturbed, there can be major implications for the development and progression of cancer.

One of the most well-studied examples of aberrant RAR signaling leading to cancer is acute promyelocytic leukemia (APL). It has been shown in a number of reports that APL is the result of a genetic rearrangement of the RARα gene that fuses it to the promyelocytic leukemia gene (PML) or other PML-related genes (Tang and Gudas 2011). These PML/RARα fusion proteins lead to dramatic increases in expression of both HDACs and DNA methyltransferases that cause reductions in gene expression for retinoid-regulated genes such as those involved in differentiation (Fig. 3). As previously stated, under normal conditions retinoid-regulated genes regulate cellular proliferation mainly through inhibiting cell-cycle progression and promoting cellular differentiation. When the expression of these genes is reduced, the regulatory controls on these functions are lost resulting in uncontrolled proliferation. In addition to inducing epigenetic silencing, the PML/RARα fusion protein can also repress important RAR target genes such as RARβ_2 itself by binding the RAR response element (RARE) on the RARβ_2 promoter and recruiting corepressors such as NuRD that keep RARβ_2 from being expressed.

The loss of retinoic acid signaling is not restricted to APL and in fact reduction in expression of both RARα and RARβ_2 has been shown to occur in several types of cancer such as embryonal carcinomas, acute myeloid leukemia, and breast cancer (Mongan and Gudas 2007; Altucci et al. 2007). In contrast to APL where RARα is mutated into the PML/RARα fusion protein, most cancer cell types do not contain mutated RARs but rather have dramatically downregulated expression of these receptors. Understanding the underlying mechanisms behind the silencing of RARs in cancer cells has been a major focus of recent research and several mechanisms have been uncovered. For example, it was reported that the RARβ_2 promoter in many cancer cell types is silenced by hypermethylation at CpG regions of its promoter. In addition, corepressors such as PRAEME, meningioma 1, acinus-S', HACE1, and SMRT have all been shown to inhibit expression of either RARβ_2 or RAR response genes either due to overexpression of these corepressors or a greater affinity for the RARβ_2 response elements concomitant to changes caused by aberrant AKT signaling in a variety of cancer cell types (Tang and Gudas 2011). In contrast, repression of RAR coactivator expression has also been observed to downregulate expression of RARs in neuroblastoma cells. Taken together, these reports indicate that a multitude of mechanisms are at work in various cancer cell types that all result in the repression of RARs and their downstream target genes. Regardless of mechanism, the end result is an absence of RAR-mediated balances between differentiation and proliferation and demonstrates the vital roles RARs play in the progression of cancer.

Development of RAR Ligands for Use as Therapeutics

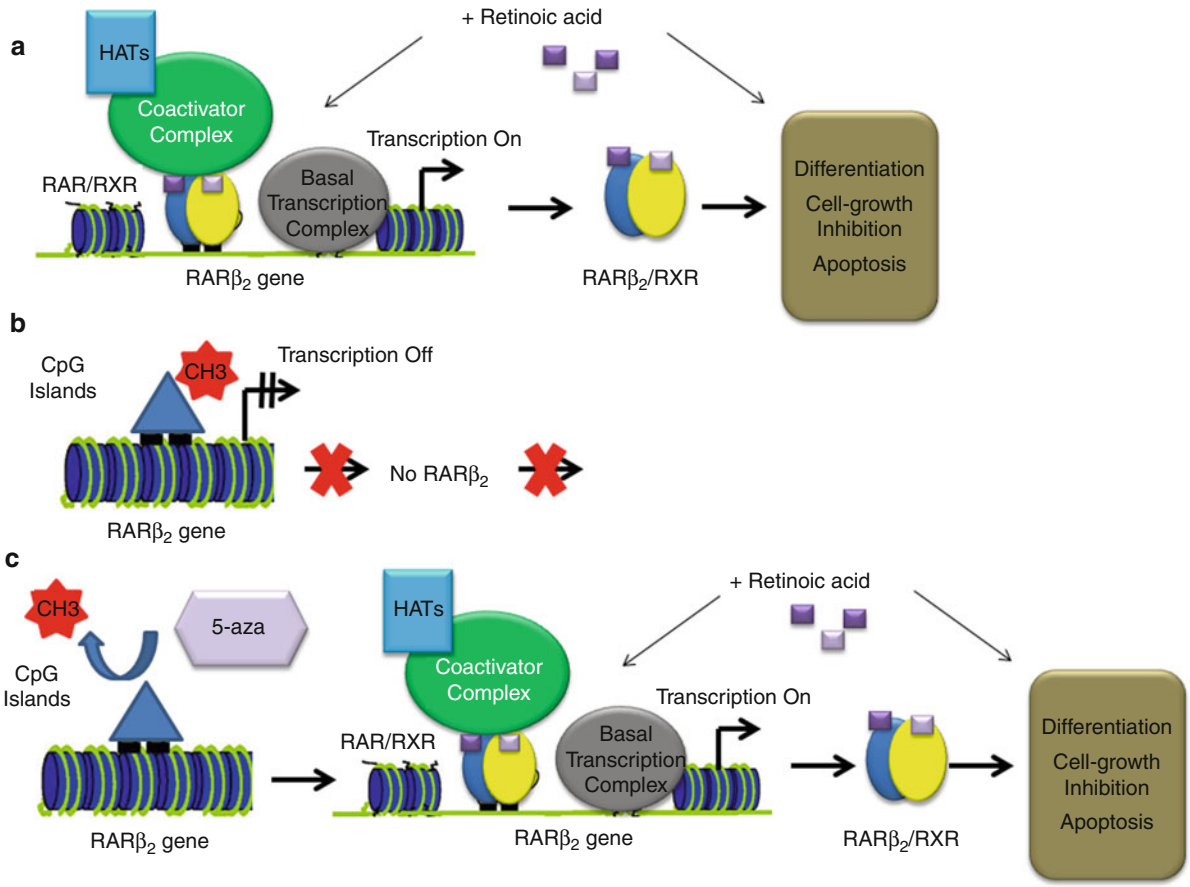
Given the correlation between the reduction in RAR-mediated retinoic acid signaling and the progression of



Retinoic Acid Receptors (RARA, RARB, and RARC), Fig. 3 Model of aberrant repression of RAR gene activation by the PML/RARα fusion protein. Schematic comparing the ligand-dependent activation of RAR response genes in normal tissues expressing the wild-type RARα/RXRα heterodimer compared to the APL model where that is replaced by the PML/RARα fusion protein. The PML/RARα fusion protein forms a dominant negative repressive complex onto the RAREs due to the lack of RXRα and the enhanced recruitment of HDACs to the PML portion of the fusion protein. Reversing this phenomonon requires HDAC inhibitors or excess amounts of retinoic acids

a number of cancers, therapies have been developed to treat cancer patients with natural retinoids such as all-trans retinoic acid (ATRA) to induce differentiation and cell growth arrest. This strategy has been extremely successful in the treatment of APL as pharmacological doses of retinoic acid stimulate irreversible differentiation of leukemic cells into granulocytes. Moreover, it has been reported that pharmacological doses of retinoic acid also trigger growth arrest and differentiation of leukemia stem cells known as leukemia-inducing cells (LICs) (Tang and Gudas 2011). When combined with other apoptosis-inducing chemotherapeutic drugs such as anthracyclins, retinoic acid treatment reverses gene silencing and leads to induced cell death of the cancer cells curing 70–80% of APL patients (Altucci et al. 2007). This treatment is successful since the expression of the PML/RXRα fusion protein is high and the RARα portion of this protein contains a functioning ligand-binding domain and coactivator recruitment site that allows for the retinoic acid-mediated activation of a number of RARα genes that stimulate differentiation.

Differentiation therapy involving treatment with natural retinoids has been developed for many cancers such as breast, ovarian, renal, head and neck, melanoma, and prostate. However, the success of this approach has been much less successful in these other types of cancers where the expression of RAR genes themselves are downregulated by events such as DNA methylation of their promoters as previously discussed. Combination therapies have been adopted with some success to overcome these limitations with the coadministration of HDAC inhibitors and DNMTase inhibitors in addition to retinoic acid



Retinoic Acid Receptors (RARA, RARB, and RARC), Fig. 4 Schematic model of the benefit of combination therapy to reverse the repression of RARβ$_2$ expression in various cancers. (**a**) Normal tissue where RAR/RXRs regulate expression of RARβ$_2$ which is vital for the balance between cell proliferation and differentiation and inducing apoptosis when necessary (**b**) Tumor tissue where hypermethylation of CpG islands on the promoter of RARβ$_2$ prevent its expression and in turn repress genes involved in regulation of cellular proliferation and apoptosis leading to the cancer phenotype. (**c**) Treatment of tumors with combinations of retinoic acid and other inhibitors such as DNA methyltransferase inhibitors (DNMTase inhibitor) that first remove the detrimental hypermethylation and then restore normal expression of RARβ$_2$ which is activated by retinoic acids

(Tang et al. 2009). This strategy first reverses the repressive effects of protein acetylation and DNA methylation on RAR gene expression and then once expressed, provides the natural agonist to activate RAR target genes to induce growth arrest and apoptosis (Fig. 4).

While cell differentiation therapies using high levels of natural retinoids such as ATRA have proven to be very successful in the treatments of some cancers, there are significant drawbacks to their therapeutic use. Retinoids are powerful teratogens that at pharmacological concentrations can induce congenital defects and toxicity in all vertebrate species. In addition, there are some cancers such as prostate cancer where ATRA and other synthetic retinoid agonists are not effective in inducing growth arrest and/or apoptosis. Moreover, a common feature of many cancers is the development of resistance to the growth inhibitory effects of retinoids limiting the utility of these therapies. Even APL, which responds well to differentiation therapy, has several variants that display retinoid resistance and does not respond to this therapy. For these reasons, efforts have been underway to develop new types of synthetic ligands for RAR that can promote the positive effects of retinoids without the detrimental side effects.

A number of synthetic retinoids have been developed as potential therapeutics for a variety of cancers.

These are often referred to as atypical retinoids or retinoid-related molecules because they are based on the retinoic acid structure and have been shown to bind and transactivate RARs. Many of these compounds have been approved for the treatment of a number of diseases such as cancer, acne, and psoriasis (Altucci et al. 2007). The majority of these atypical retinoids are RAR agonists; however, there have been some RAR antagonists that have also been synthesized. In some cancers such as prostate cancer, pan-specific antagonists of RAR such as AGN194310 demonstrated much more significant anti-proliferative and pro-apoptotic effects than any RAR natural or synthetic agonist. In fact a number of synthetic molecules known as the retinoid-related molecules such as MX781, AGN 194310, and ST1926 have demonstrated potent anti-proliferative activities against large panels of human tumor cells (de Lera et al. 2007). Until recently, all of the synthetic retinoid-related molecules reported that directly bind and modulate RAR activity share structural similarities to the natural agonist retinoic acid. This means that while some have proven efficacious in the treatments of a number of important cancers, they could still be susceptible to the same limitations regarding retinoid resistance as the natural retinoids. Interestingly, a recent report has identified the first synthetic non-retinoid, non-acid RAR modulator that binds and activates all three isoforms of RAR (Busby et al. 2011). Synthetic structures such as these may provide the basis for novel chemical scaffolds of non-retinoid, non-acid RAR modulators that may be developed that are potent and efficacious toward restoring RAR signaling while at the same time overcome the challenges of toxicity and resistance seen with use of natural retinoids such as ATRA.

Summary

The retinoic acid receptors (RARs) are ligand-dependent transcription factors that belong to the NR1B subtype of the nuclear receptor (NR) superfamily. RARs are ligand-dependent transcription factors that bind to retinoids, the most potent biologically active forms of vitamin A, and heterodimerize with the rexinoid X receptor (RXR) to regulate many genes involved in the regulation of cellular growth and differentiation. RARs play significant roles in a number of developmental cascades from formation of limbs and organs to the central nervous system. In addition, all three RAR isoforms are instrumental in the control of a cellular growth through the inhibition of the cell cycle. That combined with the activation of genes involved in differentiation provides multiple pathways that RARs regulate cellular growth. Given these critical roles in cellular growth, it is not surprising that a great deal of evidence has emerged that either mutations or reductions in RAR expression are correlated with a number of cancers. This has led to the development of differentiation therapies alone or in combination with other types of drugs to restore RAR-mediated retinoic acid signaling in a number of cancers. Due to the potential toxicity and emergence of retinoid resistance in some cancers, synthetic retinoid-related molecules have been developed including one novel non-acid non-retinoid chemical scaffold that may provide safer, more efficacious ways to treat cancer by restoring normal RAR-mediated RA signaling. Further understandings of the roles of the various RAR isoforms in the progression of cancer and how to modulate the activities of RARs may provide important clues to develop novel therapies to treat cancer.

References

Altucci L, Leibowitz MD, Ogilvie KM, de Lerea AR, Gronemeyer H. RAR and RXR modulation in cancer and metabolic disease. Nat Rev Drug Discov. 2007;6(10):793–810.

Busby SA, Kumar N, Kuruvilla DS, Istrate MA, Conkright JJ, Wang Y, Kamenecka TM, Cameron MD, Roush WR, Burris TP, Griffin PR. Identification of a novel non-retinoid pan inverse agonist of the retinoic acid receptors. ACS Chem Biol. 2011;6(6):618–27.

Chikamori K, Hill JE, Grabowski DR, Zarkhin E, Grozav AG, et al. Downregulation of topoisomerase IIβ in myeloid leukemia cell lines leads to activation of apoptosis following all-trans retinoicacid–induced differentiation/growth arrest. Leukemia. 2006;20:1809–18.

de Lera AR, Bourguet W, Altucci L, Gronemeyer H. Design of Selective Nuclear receptor modulators: RAR and RXR as a case study. Nat Rev Drug Discov. 2007;6:811–20.

Dupe V, Ghyselinck NB, Thomazy V, Nagy L, Davies PJ, Chambon P, Mark M. Essential roles of retinoic acid signaling in interdigital apoptosis and control of BMP-7 expression in mouse autopods. Dev Biol 1999;208:30–43.

Dupe V, Matt N, Garnier JM, Chambon P, Mark M, Ghyselinck NB. A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment. Proc Natl Acad Sci USA. 2003;100:14036–41.

Faria TN, Mendelsohn C, Chambon P, Gudas LJ. The targeted disruption of both alleles of RARbeta(2) in F9 cells results in the loss of retinoic acid-associated growth arrest. J Biol Chem. 1999;274(38):26783–8.
Halilagic A, Ribes V, Ghyselinck NB, Zile MH, Dolle P, Studer M. Retinoids control anterior and dorsal properties in the developing forebrain. Dev Biol. 2007;303:362–75.
Hatoum A, El-Sabban ME, Khoury J, Yuspa SH, Darwiche N. Overexpression of retinoic acid receptors α and γ into neoplastic epidermal cells causes retinoic acid–induced growth arrest and apoptosis. Carcinogenesis. 2001;22:1955–63.
Kochhar DM, Jiang H, Penner JD, Johnson AT, Chandraratna RA. The use of a retinoid receptor antagonist in a new model to study vitamin A-dependent developmental events. Int J Dev Biol. 1998;42:601–8.
Li R, Faria TN, Boehm M, Nabel EG, Gudas LJ. Retinoic acid causes cell growth arrest and an increase in p27 in F9 wild type but not in F9 retinoic acid receptor β2 knockout cells. Exp Cell Res. 2004;294:290–300.
Luo P, Lin M, Lin M, Chen Y, Yang B, He Q. Function of retinoid acid receptor α and p21 in all-trans-retinoic acid–induced acute T-lymphoblastic leukemia apoptosis. Leuk Lymphoma. 2009;50:1183–9.
Maden M. Retinoid signaling in the development of the central nervous system. Nat Rev Neurosci. 2002;3:843–53.
Mark M, Ghyselinck NB, Chambon P. Function of retinoic acid receptors during embryonic development. Nucl Recept Signal. 2009;7:1–15. doi:10.1621/nrs.07002.
Metzger D, Chambon P. Site- and time-specific gene targeting in the mouse. Methods 2001;24:71–80.
Metzger D, Indra AK, Li M, Chapellier B, Calleja C, Ghyselinck NB, Chambon P. Targeted conditional somatic mutagenesis in the mouse: temporally-controlled knock out of retinoid receptors in epidermal keratinocytes. Methods Enzymol 2003;364:379–408.
Mongan NP, Gudas LJ. Diverse actions of retinoid receptors in cancer prevention and treatment. Differentiation. 2007;75(9):853–70.
Pettersson F, Dalglesh AG, Bissonnette RP, Colston KW. Retinoids cause apoptosis in pancreatic cancer cells via activation of RARγ and altered expression of Bcl-2/Bax. Br J Cancer. 2002;87:555–61.
Sandell LL, Sanderson BW, Moiseyev G, Johnson T, Mushegian A, Young K, Rey JP, Ma JX, Staehling-Hampton K, Trainor PA. RDH10 is essential for synthesis of embryonic retinoic acid and is required for limb, craniofacial, and organ development. Genes Dev. 2007;21:1113–24.
Suzui M, Shimizu M, Masuda M, Lim JT, Yoshimi N, Weinstein IB. Acyclic retinoid activates retinoic acid receptor βand induces transcriptional activation of p21CIP1 in HepG2 human hepatoma cells. Mol Cancer Ther. 2004;3:309–16.
Tang XH, Gudas LJ. Retinoids, retinoic acid receptors and cancer. Annu Rev Pathol Mech Dis. 2011;6:345–64.
Tang XH, Albert M, Scognamiglio T, Gudas LJ. A DNA methyltransferase inhibitor and alltransretinoic acid reduce oral cavity carcinogenesis induced by the carcinogen 4-nitroquinoline 1-oxide. Cancer Prev Res. 2009;2:1100–10.
Wendling O, Ghyselinck NB, Chambon P, Mark M. Roles of retinoic acid receptors in early embryonic morphogenesis and hindbrain patterning. Development. 2001;128:2031–8.

RGS 12/Regulator of G-Protein Signaling 12 (AGS6)

► Activators of G-Protein Signaling (AGS)

RGS Protein Family

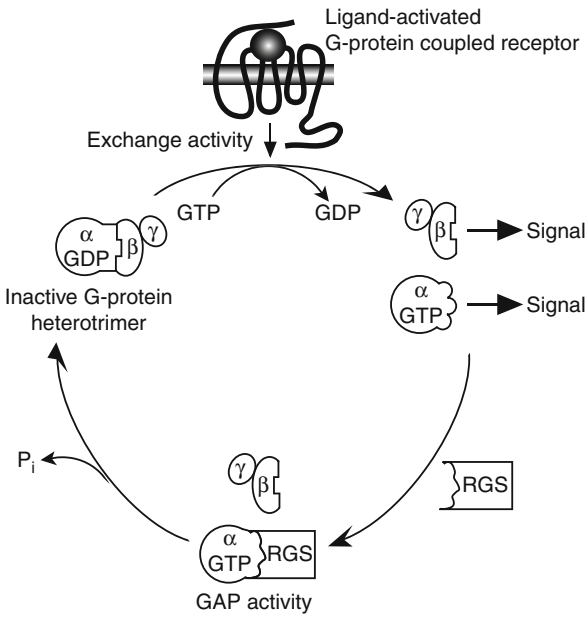
David P. Siderovski and Adam J. Kimple
Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Synonyms

Gα GAPs; Regulators of G-protein signaling; RGS proteins

Historical Background

Signal transduction by G protein–coupled receptors (GPCRs) was considered for many years (Gilman 1987) to be a three-component system: the cell-surface receptor to receive external input from hormones and neurotransmitters, the heterotrimeric G protein to transduce this input to the intracellular compartment by its structural changes upon the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP), and effector proteins (such as ► adenylyl cyclase, phospholipase C, and ion channels) to propagate the signal forward as changes in cell membrane potential and/or intracellular second messenger levels. However, for many physiological responses mediated by GPCRs, including the visual response controlled by the photoreceptor, rhodopsin (Arshavsky and Pugh 1998), intracellular signaling was known to be far shorter in duration than the time observed for the isolated components to revert to ground state in vitro (i.e., the time required for the heterotrimeric G-protein α subunit to hydrolyze GTP and return to its GDP-bound, inactive state). A critical fourth component to this system was discovered to be a large family of "regulators of G-protein signaling" proteins, also known as RGS proteins (Willard et al. 2008), that dramatically accelerate GTP hydrolysis by Gα subunits and thereby hasten signal termination (Fig. 1).



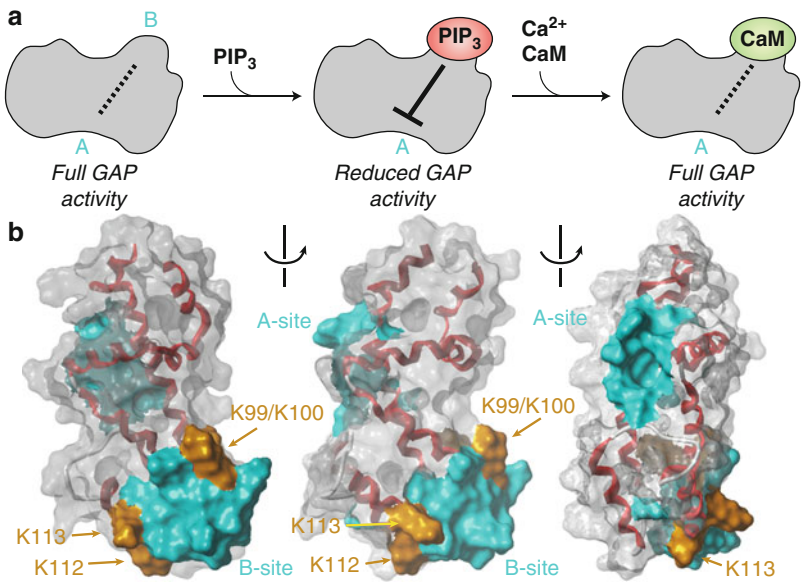
RGS Protein Family, Fig. 1 *Role of RGS proteins in GPCR signaling as negative regulators*. Ligand-activated GPCRs act as guanine nucleotide exchange factors for the inactive, GDP- and Gβγ-bound Gα subunit. The resultant release of GDP, and subsequent binding of the more abundant GTP, leads to a conformational change within Gα, eliminating the high-affinity binding site for Gβγ. The GTP-bound Gα and released Gβγ subunits are then able to bind effector proteins to propagate intracellular signaling. The intrinsic GTP hydrolysis activity of Gα subunits is greatly accelerated by the binding of RGS proteins, leading to the release of inorganic phosphate (P_i) and reassembly of the inactive Gα·GDP/Gβγ heterotrimer

One of the earliest reports of cloning a human RGS protein-encoding gene was that of *G0S8* (now known as *RGS2*) in 1990. Trapped as one of a number of putative "G0/G1-switch genes" from mitogen-treated, primary human T-lymphocytes (Siderovski et al. 1990), *G0S8*/*RGS2* was subsequently observed to encode a protein with sequence similarity to kinases specific for activated GPCRs (e.g., β-adrenergic receptor kinase, ▶ rhodopsin kinase) and to the yeast "supersensitivity-to-phermone" protein SST2 (Siderovski et al. 1994). Functional complementation of yeast deficient in SST2 by overexpression of the human *G0S8*/*RGS2* gene (Siderovski et al. 1996) provided one of the first clues that this emergent gene family encoded negative regulators of signal transduction acting downstream of GPCR activation; other groups working in disparate systems came to the same conclusion contemporaneously (Druey et al. 1996; Koelle and Horvitz 1996). These reports were quickly followed by a definitive demonstration of the biochemical activity underlying the negative regulatory function of RGS proteins: namely, acceleration of the intrinsic GTPase activity of Gα subunits using purified proteins and radiolabeled GTP (Berman et al. 1996a).

RGS Protein Activities

The signature enzymatic activity of RGS proteins is the acceleration of GTP hydrolysis by activated, GTP-bound Gα subunits. This acceleration can be made on certain GTPase-deficient Gα mutants as well (e.g., point mutation to the arginine, such as Arg-178 of $G\alpha_{i1}$, that helps stabilize the γ-phosphate leaving group) but not on classical, GTPase-dead, Gln-to-Leu Gα mutants (e.g., $G\alpha_{i1}$ Q204L) (Berman et al. 1996a). Following discovery of this signature biochemical activity of RGS proteins, a crystal structure of RGS4 bound to $G\alpha_{i1}$ in a transition state-mimetic form was reported (Tesmer et al. 1997). The ability to observe the interaction between a RGS protein and its Gα target to high-resolution solidified early speculation that RGS proteins employ solely their highly conserved, ~120 amino-acid "RGS domain" to stabilize Gα in its transition state along the path to GTP hydrolysis (Berman et al. 1996b); this "GTPase-accelerating protein" or "GAP" mechanism is distinctly different from that exhibited by the GAPs of Ras-superfamily GTPases. The GAP activity of certain RGS proteins, including RGS4, is thought to be modulated in a cellular context by the binding of phosphatidylinositol head-groups or calmodulin to a "B-site," within the RGS domain but distinct from the Gα-binding interface (or "A-site") (Fig. 2); engagement of the B-site with phosphatidylinositol-3,4,5-trisphosphate (PIP_3) is considered to inhibit A-site GAP function in an allosteric fashion, whereas the binding of calmodulin (in a calcium-dependent manner) to the B-site removes the inhibitory influence of PIP_3 (Tu and Wilkie 2004).

Observations of RGS protein overexpression leading to accelerated *on-rates* of GPCR signal transduction without affecting response sensitivity or amplitude have been presented in the literature



RGS Protein Family, Fig. 2 *Predicted structural determinants of the allosteric control over RGS protein GAP activity.* (**a**) For some RGS proteins, the binding of the RGS domain "B-site" with phosphatidylinositol-3,4,5-trisphosphate (PIP_3) is thought to allosterically inhibit the GTPase-accelerating activity of the RGS domain Gα-binding "A-site"; in a calcium-dependent fashion, the binding of calmodulin to the B-site is thought to remove the inhibitory influence of PIP_3 on A-site GAP function. (**b**) Visualization of the predicted functional sites within the RGS domain of RGS4 (Protein Data Bank id 1AGR) responsible for allosteric control of GAP activity. Highlighted (orange/dark grey) regions depict lysines thought to be required for PIP_3 binding, while solid surface (cyan/light grey) areas depict the proposed A- and B-sites. The alpha-helical secondary structure that comprises the conserved RGS domain fold is displayed in red/black as ribbon tracing within the translucent surface rendering of the domain. Rotation about the vertical axis by 90° and 180° are shown in consecutive panels from left to right

(e.g., Doupnik et al. 1997) as paradoxical findings that run counter to expectations that RGS protein GAP activity should only serve to accelerate the off-rate of GPCR signaling and, thereby, blunt signaling. It is possible that RGS proteins contribute more than just GAP activity to the functioning of the GPCR/G–protein/effector axis, especially since many RGS proteins contain multiple protein/protein-interaction domains in addition to the signature RGS domain (see below). More recently, by combining Gα mutations that accelerate intrinsic GTPase activity and that eliminate sensitivity to RGS domain GAP activity (while preserving all other Gα functions), it has been definitively demonstrated that accelerated GTP hydrolysis alone is sufficient to elicit observations of increased signaling onset and recovery times (Lambert et al. 2010). This finding, however, does not exclude the possibility that conventional ("GAP-active") RGS proteins and/or other RGS domain-containing proteins exhibit additional functional effects on GPCR-initiated signal transduction in a cellular context.

The Conventional RGS Protein Subfamilies

The "conventional" members of the RGS protein family (Table 1) exhibit Gα-directed GAP activity and have been numbered from RGS1 to RGS21 (excluding RGS15, which turned out to be RGS3). The majority of these proteins target Gα subunits of the $G\alpha_i$ and $G\alpha_q$ subfamilies (Soundararajan et al. 2008), albeit with notable exceptions as described below. These proteins have been divided into subfamilies based on overall protein architecture and RGS domain sequence similarity (Willard et al. 2008). The R4-subfamily is the largest by membership, consisting of RGS1, -2, -3, -4, -5, -8, -13, -16, -18, and -21, yet the smallest by individual protein size; most members merely consist of the ~120 amino-acid RGS domain with short N- and C-terminal polypeptide extensions (e.g., RGS21 is only 152 amino acids in length). An exception to this small size is the R4-subfamily member RGS3, given that alternative isoforms of this protein are expressed that include

RGS Protein Family, Table 1 The conventional RGS proteins

Family	Name	GenBank locus	UniProt id	Entrez gene id	Distinguishing characteristic(s)
R4	RGS1	NM_002922	Q08116	5996	Implicated in multiple sclerosis
R4	RGS2	NM_002923	P41220	5997	Selective for $G\alpha_q$; modulator of anxiety and vasoconstrictor signaling
R4	RGS3	NM_144489	P49796	5998	Isoforms can contain PDZ and C2 domains
R4	RGS4	NM_005613	P49798	5999	Associated with susceptibility to schizophrenia
R4	RGS5	NM_003617	O15539	8490	Expressed in pericytes; associated with neovascularization
R7	RGS6	NM_004296	P49758	9628	Potential modulator of parasympathetic activation in heart
R7	RGS7	NM_002924	P49802	6000	Implicated in CNS opioid and muscarinic acetylcholine signaling
R4	RGS8	NM_033345	P57771	85397	Directly binds GPCR loops (or indirectly via spinophilin); may control stable cell-surface GPCR expression
R7	RGS9	NM_003835	O75916	8787	Key deactivator of retinal phototransduction cascade
R12	RGS10	NM_002925	O43665	6001	Phosphorylation and palmitoylation control nuclear localization and Gα substrate selectivity
R7	RGS11	NM_183337	O94810	8786	Modulator of retinal ON-bipolar cell light response
R12	RGS12	NM_198229	O14924	6002	Contains PDZ, PTB, RBD, and GoLoco domains; scaffold for Ras/Raf/MAPK cascade
R4	RGS13	NM_002927	O14921	6003	Modulator of GPCR signaling in mast cells / allergic responses
R12	RGS14	NM_006480	O43566	10636	Contains RBD and GoLoco domains; scaffold for Ras/Raf/MAPK cascade
R4	RGS16	NM_002928	O15492	6004	Feeding and fasting controls expression in periportal hepatocytes
RZ	RGS17	NM_012419	Q9UGC6	26575	Implicated in lung tumorigenesis
R4	RGS18	NM_130782	Q9NS28	64407	Expressed in leukocytes, megakaryocytes, and platelets
RZ	RGS19	NM_005873	P49795	10287	Implicated in Wnt/β-catenin signaling
RZ	RGS20	NM_170587	O76081	8601	Modulator of mu-opioid receptor signaling
R4	RGS21	NM_001039152	Q2M5E4	431704	Expressed in lingual taste buds

N-terminal PDZ and C2 domains. Within the R4-subfamily, RGS2 is unique in acting as a potent GAP solely on $G\alpha_q$ subfamily members (and not $G\alpha_i$ subunits) in vitro (Kimple et al. 2009), although inhibition of Gi-coupled GPCR signaling can be observed in a cellular context upon RGS2 overexpression (Ingi et al. 1998). RZ-subfamily members (RGS17, -19, and -20) are also small polypeptides but are distinct from the R4-subfamily in containing cysteine-rich N-termini thought to be reversibly palmitoylated for differential subcellular trafficking. As the name suggests, RZ-subfamily members have particular selectivity for $G\alpha_z$ subunits, although this is not exclusive, and binding of (and GAP activity on) $G\alpha_i$ subunits is also manifested (e.g., Soundararajan et al. 2008).

R7-subfamily members (RGS6, -7, -9, and -11) are known to play key roles in the regulation of various neuronal processes such as nociception, motor control, reward behavior, and vision. These four proteins share an expression pattern biased to neuronal tissues, as well as a unique multi-domain protein architecture composed of DEP (*D*ishevelled/*E*GL-10/*P*leckstrin) and GGL (*G*-*g*amma-*l*ike) domains present N-terminal to a central RGS domain. The DEP domain mediates interaction with unique membrane anchor proteins R7BP and R9AP, whereas the GGL domain (as its name implies) binds a neuronal-specific Gβ subunit, Gβ5, to form an obligate dimeric configuration akin to conventional Gβ/Gγ subunits (Snow et al. 1998). While the R12-subfamily member RGS10 consists of little more than an RGS domain, the other two members of this subfamily (RGS12 and RGS14) share elaborate multi-domain architectures. C-terminal to their RGS domains, both RGS12 and RGS14, possess a tandem repeat of Ras-binding domains (RBDs) and a single GoLoco motif; the first of the two RBDs binds selectively to activated H-Ras (Willard et al. 2007), whereas the GoLoco motif is known to bind $G\alpha_i$ subunits in their GDP-bound inactive state (Kimple et al. 2002). Unlike RGS14, RGS12 also possesses N-terminal PDZ and PTB domains which play important

roles in the functional organization of an H-Ras-Raf-MAPK signaling cascade required for nerve growth factor (NGF)-mediated axonogenesis by dorsal root ganglion neurons (Willard et al. 2007).

Other RGS Domain-Containing Proteins

There are an equivalent number of "nonconventional" RGS proteins (Table 2) that, while possessing the highly conserved nine alpha-helical structure of the RGS domain (Tesmer et al. 1997; Soundararajan et al. 2008), either have already been identified in other functional contexts or have yet to be identified as bona fide Gα-directed GAPs. With respect to the latter situation, AKAP-10 (also known as D-AKAP2) and RGS22 possess more than one RGS domain, but to date neither have been convincingly shown to bind to (nor accelerate the GTPase activity of) Gα subunits; this is also true of three sorting nexins (SNX13, -14, and -25) and the RA-subfamily members (Axin, Axin2) that each possess a single, central RGS domain of poorly characterized or controversial Gα-modulatory function.

As previously mentioned, early reports of the discovery of the RGS protein family highlighted the presence of an N-terminal RGS domain within the known family of serine/threonine kinases (GRK1 to 7; Table 2) that are specific for activated GPCRs (Siderovski et al. 1994; Siderovski et al. 1996); subsequent examination of this N-terminal RGS domain within GRK2 revealed in vitro $G\alpha_q$ binding selectivity and a cellular function in inhibiting Gq-coupled GPCR signaling, albeit with little (if any) $G\alpha_q$-directed GAP activity. N-terminal RGS domains were also identified in guanine nucleotide exchange factors for the small GTPase RhoA (i.e., the GEF-subfamily of RGS proteins; namely, p115-RhoGEF/ARHGEF1, PDZ-RhoGEF/ARHGEF11, and LARG/ARHGEF12). This identification helped to explain the ability of $G_{12/13}$-coupled GPCRs to activate RhoA in a cellular context; therefore, the GEF-subfamily of RGS proteins

RGS Protein Family, Table 2 Other proteins containing RGS domain(s)

Family	Name	GenBank locus	UniProt id	Entrez gene id	Distinguishing characteristic(s)
	AKAP10	NM_007202	O43572	11216	Contains 2 RGS domains which interact with Rab4 and Rab11 GTPases
	RGS22	NM_015668	Q9BYZ4	26166	Contains 3 RGS domains; specifically expressed in testes
SNX	SNX13	NM_015132	Q9Y5W8	23161	Also known as RGS-PX1; controversial report of $G\alpha_s$-directed GAP activity
SNX	SNX14	NM_153816	Q9Y5W7	57231	Also known as RGS-PX2
SNX	SNX25	NM_031953	Q9H3E2	83891	Sorting nexin-25; speculated to bind phosphatidylinositols with its PX domain
RA	Axin	NM_003502	O15169	8312	Involved in Wnt signaling; component of β-catenin destruction complex
RA	Axin2	NM_004655	Q9Y2T1	8313	Also known as conductin; regulator of centrosome cohesion
GRK	GRK1	NM_002929	Q15835	6011	Also known as rhodopsin kinase
GRK	GRK2	NM_001619	P25098	156	Also known as β-adrenergic receptor kinase-1 (βARK1); RGS domain binds activated $G\alpha_{q/11}$
GRK	GRK3	NM_005160	P35626	157	Also known as β-adrenergic receptor kinase-2 (βARK2)
GRK	GRK4	NM_182982	P32298	2868	Linked to genetic and acquired hypertension
GRK	GRK5	NM_005308	P34947	2869	Modulator of NFκB signaling via IκBα interaction
GRK	GRK6	NM_002082	P43250	2870	Involved in phosphorylation and desensitization of CXCR4
GRK	GRK7	NM_139209	Q8WTQ7	131890	Involved in cone phototransduction
GEF	ARHGEF1	NM_004706	Q92888	9138	Also known as p115 RhoGEF; $G\alpha_{12/13}$-dependent exchange factor for RhoA GTPase
GEF	ARHGEF11	NM_014784	O15085	9826	Also known as PDZ-RhoGEF
GEF	ARHGEF12	NM_015313	Q9NZN5	23365	Also known as LARG or "leukemia-associated RhoGEF"

are emblematic of RGS domain-containing proteins that serve as effectors (i.e., propagating the signal forward) even while they also serve as GAPs for their upstream activators (i.e., activated $G\alpha_{12}$·GTP and $G\alpha_{13}$·GTP subunits).

Summary

Originally discovered as negative regulators of GPCR signal transduction owing to their Gα-directed GAP activity, the RGS proteins are now appreciated to possess multifaceted functions in cellular signaling networks. These multiple functions can arise from elaborate, multiple protein-domain architectures, unique binding partners, and their individual abilities to coordinate and/or modulate other signal transduction components, such as small Ras-superfamily GTPases. Unique expression patterns and Gα-binding selectivities of the RGS proteins underlie their individual involvement in distinct physiological and pathophysiological phenomena. What remains to be determined is whether RGS proteins can be selectively inhibited by small molecules and, even more speculatively, whether their activity could be enhanced by small molecules that usurp normal allosteric control over the Gα-binding A-site.

References

Arshavsky VY, Pugh Jr EN. Lifetime regulation of G protein-effector complex: emerging importance of RGS proteins. Neuron. 1998;20(1):11–4.

Berman DM, Wilkie TM, Gilman AG. GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. Cell. 1996a;86(3):445–52.

Berman DM, Kozasa T, Gilman AG. The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. J Biol Chem. 1996b;271(44):27209–12.

Doupnik CA, Davidson N, Lester HA, Kofuji P. RGS proteins reconstitute the rapid gating kinetics of gbetagamma-activated inwardly rectifying K+ channels. Proc Natl Acad Sci USA. 1997;94(19):10461–6.

Druey KM, Blumer KJ, Kang VH, Kehrl JH. Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. Nature. 1996;379(6567):742–6.

Gilman AG. G proteins: transducers of receptor-generated signals. Annu Rev Biochem. 1987;56:615–49.

Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, et al. Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. J Neurosci. 1998;18(18):7178–88.

Kimple RJ, Kimple ME, Betts L, Sondek J, Siderovski DP. Structural determinants for GoLoco-induced inhibition of nucleotide release by Galpha subunits. Nature. 2002;416(6883):878–81.

Kimple AJ, Soundararajan M, Hutsell SQ, Roos AK, Urban DJ, Setola V, et al. Structural determinants of G-protein alpha subunit selectivity by regulator of G-protein signaling 2 (RGS2). J Biol Chem. 2009;284(29):19402–11.

Koelle MR, Horvitz HR. EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins. Cell. 1996;84(1):115–25.

Lambert NA, Johnston CA, Cappell SD, Kuravi S, Kimple AJ, Willard FS, et al. Regulators of G-protein signaling accelerate GPCR signaling kinetics and govern sensitivity solely by accelerating GTPase activity. Proc Natl Acad Sci USA. 2010;107(15):7066–71.

Siderovski DP, Blum S, Forsdyke RE, Forsdyke DR. A set of human putative lymphocyte G0/G1 switch genes includes genes homologous to rodent cytokine and zinc finger protein-encoding genes. DNA Cell Biol. 1990;9(8):579–87.

Siderovski DP, Heximer SP, Forsdyke DR. A human gene encoding a putative basic helix-loop-helix phosphoprotein whose mRNA increases rapidly in cycloheximide-treated blood mononuclear cells. DNA Cell Biol. 1994;13(2):125–47.

Siderovski DP, Hessel A, Chung S, Mak TW, Tyers M. A new family of regulators of G-protein-coupled receptors? Curr Biol. 1996;6(2):211–2.

Snow BE, Krumins AM, Brothers GM, Lee SF, Wall MA, Chung S, et al. A G protein gamma subunit-like domain shared between RGS11 and other RGS proteins specifies binding to Gbeta5 subunits. Proc Natl Acad Sci USA. 1998;95(22):13307–12.

Soundararajan M, Willard FS, Kimple AJ, Turnbull AP, Ball LJ, Schoch GA, et al. Structural diversity in the RGS domain and its interaction with heterotrimeric G protein alpha-subunits. Proc Natl Acad Sci USA. 2008;105(17):6457–62.

Tesmer JJ, Berman DM, Gilman AG, Sprang SR. Structure of RGS4 bound to AlF4–activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. Cell. 1997;89(2):251–61.

Tu Y, Wilkie TM. Allosteric regulation of GAP activity by phospholipids in regulators of G-protein signaling. Meth Enzymol. 2004;389:89–105.

Willard MD, Willard FS, Li X, Cappell SD, Snider WD, Siderovski DP. Selective role for RGS12 as a Ras/Raf/MEK scaffold in nerve growth factor-mediated differentiation. EMBO J. 2007;26(8):2029–40.

Willard MD, Willard FS, Siderovski DP. The superfamily of 'regulator of G-protein signaling' (RGS) proteins. In: Bradshaw R, Dennis E, editors. Handbook of cell signaling. 2nd ed. San Diego: Elsevier; 2008.

RGS Proteins

► RGS Protein Family

RGS13

Zhihui Xie and Kirk M. Druey
Laboratory of Allergic Diseases, National Institute of Allergy and infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Synonyms

Regulator of G-protein Signaling 13

Historical Background

Regulator of G-protein signaling (RGS) is a protein superfamily discovered in the mid-1990s (Druey et al. 1996; Watson et al. 1996; Hunt et al. 1996). Since that time more than 30 members in the family have been identified. The major physiological function of RGS molecules is to negatively modulate G-protein coupled receptor (GPCR)-mediated signaling and biology (Bansal et al. 2007; Neitzel and Hepler 2006; Thompson et al. 2008). ▶ RGS13 was first identified in 1996 and is one of the smallest molecules in the family (Druey et al. 1996). Full length human RGS13 cDNA was cloned and deposited into gene bank in 1997 by Chatterjee and Fisher. In 2002, the first functional studies of human RGS13 were reported. RGS13 was shown to inhibit muscarinic M1- and M2 receptor-induced ▶ MAP kinase activation (Johnson and Druey 2002). Concurrently, a separate study characterized mouse RGS13, which was cloned from B cells. RGS13 expression was demonstrated in germinal center regions of mouse spleen and shown to inhibit chemokine receptor CXCR4 and CXCR5-mediated signaling pathways, suggesting a function of RGS13 in B cell migration and trafficking (Shi et al. 2002).

More recently, GPCR-independent functions of RGS13 were revealed. RGS13 suppressed IgE receptor-induced mast cell degranulation through interaction with the p85 subunit of ▶ phosphoinositide 3-kinase (Bansal et al. 2008b). Mice deficient in RGS13 have enhanced IgE-mediated anaphylaxis after antigen challenge. RGS13 was also shown to regulate cAMP-dependent pathways. RGS13 bound phosphorylated cAMP responsive element binding protein (▶ CREB) in the nucleus and inhibited CREB-mediated gene transcription (Xie et al. 2008).

Expression Pattern

RGS13 has very restricted tissue distribution expressed mainly in B cells (Shi et al. 2002), mast cells (Bansal et al. 2008b), and enteroendocrine cells (Xie et al., unpublished observation). Central nervous system expression of RGS13 mRNA has been observed in rat brain by in situ hybridization (Grafstein-Dunn et al. 2001), but not in the human brain (Larminie et al. 2004). The expression level of RGS13 may vary considerably under physiological or pathological conditions. For example, although RGS13 is highly expressed in germinal center B cells and Burkitt lymphoma, it is absent in mantle cell lymphoma (Islam et al. 2003). Upregulated RGS13 mRNA has been reported in malignant T cells from acute T cell leukemia (ATL) patients by gene expression profiling (Pise-Masison et al. 2009); however, it was not detected in normal human tonsil T cells or in Jurkat and MOLT-4 T cell lines (Shi et al. 2002). In BXD2 autoimmune mice, increased IL-17 production is associated with elevated RGS13 mRNA expression and the suppression of B cell chemotactic responses to the chemokine CXCL12 (Hsu et al. 2008). Restricted tissue distribution and apparent disease relevance make RGS13 an excellent target for drug development.

Regulation of RGS13 Expression

Similar to many RGS molecules, RGS13 expression is relatively low in quiescent cells, which may be necessary for allowing adequate GPCR signaling to maintain cell homeostatic functions. Incubation of bone marrow–derived mast cells (BMMCs) with IgE-antigen for 24 h results in a four- to fivefold increase in RGS13 mRNA and protein (Bansal et al. 2008a), which may have a negative feedback role to amplify the inhibitory effect of RGS13 on antigen-mediated mast cell degranulation. In other words RGS13 may desensitize mast cells to antigen stimulation. Interestingly exposure of human LAD2 mast cells to cAMP decreases RGS13 mRNA quantities (Xie et al. 2010), suggesting that RGS13 expression is differentially regulated by distinct ligands or signaling pathways.

In human tonsillar B lymphocytes, anti-► CD40 antibody augments RGS13 mRNA expression (Shi et al. 2002), suggesting a role of RGS13 in B cell activation. Consistent with its expression in splenic germinal center B cells, human Burkitt lymphoma cell lines Ramos, HS-Sultan, and Raji express abundant RGS13, as do immunized mouse spleen B cells (Shi et al. 2002). Notably, the tumor suppressor ► p53 inhibits RGS13 mRNA transcription in mast cells by binding to its promoter region (Iwaki et al. 2011).

RGS13 is also regulated at the posttranslational level. RGS13 protein undergoes proteasome-mediated degradation. Phosphorylation of RGS13 on Thr41 by protein kinase A (PKA) protects it from degradation, resulting in elevated steady-state RGS13 protein levels (Xie et al. 2010), which could promote the inhibitory function of RGS13 on transcription factor CREB.

Biological Functions

GPCR-Dependent Functions

RGS proteins interact with α subunit of the heterotrimeric G-protein in its activated (GTP-bound) state, leading to increased intrinsic GTPase activity of Gα. This GTPase activating protein (GAP) activity increases the rate of GTP hydrolysis, which hastens deactivation/termination of GPCR signaling and functions (Patel 2004). RGS13 interacts with the α subunit of Gi and Gq, but not ► Gs, accelerating the GTPase activity of Gα (7,8). MAP kinase Erk1/2 activation stimulated by Gq-coupled muscarinic M2 receptor is significantly inhibited by overexpression of RGS13 in human embryonic kidney (HEK) 293 T cells (Johnson and Druey 2002). Overexpression of RGS13 in Chinese Hamster Ovary (CHO) cells inhibited the Gi-coupled chemokine receptor CXCR4-mediated cell migration (Shi et al. 2002).

SiRNA-mediated knockdown of endogenous RGS13 in HS-Sultan cells results in enhanced Ca^{2+} flux and chemotaxis induced by the chemokines CXCL12 and CXCL13, which utilize Gi-coupled GPCRs CXCR4 and CXCR5, respectively (Han et al. 2006). Depletion of RGS13 in the human mast cell lines LAD2 and HMC-1 by shRNA increases degranulation evoked by the GPCR ligand sphingosine-1-phosphate (S1-P), and greater Ca^{2+} mobilization in response to several GPCR ligands including C5a, adenosine, and S1P (Bansal et al. 2008a).

GPCR-Independent Functions

In recent years, GPCR-independent cellular functions of RGS13 have been characterized, which do not involve classical RGS13 GAP activity. Cross-linking of the IgE receptor FcεRI by antigen on mast cells activates signaling molecules including Syk kinase, phospholipase Cγ, ► LAT, and PI3 Kinase, leading to mast cell degranulation and cytokine production (Gilfillan and Tkaczyk 2006). Mice lacking RGS13 displayed enhanced systemic and local cutaneous anaphylactic responses when challenged with IgE/antigen (Bansal et al. 2008b). BMMCs from $Rgs13^{-/-}$ mice degranulated much more than those from wild-type littermates. Interestingly cytokine generation by BMMC after IgE/antigen stimulation were not affected by RGS13 protein deficiency, indicating that RGS13 specifically regulates IgE-mediated mast cell degranulation. Further analysis revealed that RGS13 binds to the p85 subunit of PI3 kinase enzyme, which is a critical downstream effector in the degranulation pathways. RGS13 inhibited formation of an FcεRI-associated p85-► Gab2-Grb2 signaling complex, which is required for mast cell degranulation (Bansal et al. 2008b). Reconstitution of RGS13 deficient BMMC with a GAP-inactive RGS13 mutant suppressed antigen-stimulated degranulation similar to wild-type RGS13, indicating that RGS13 GAP activity is not required for its inhibition of mast cell function (Bansal et al. 2008b).

A separate set of studies led to the discovery that although RGS13 does not interact with Gαs, it inhibits Gs-mediated signaling through the β2-adrenergic receptor downstream of the G protein (Johnson and Druey 2002). Further studies revealed that PKA activation induced by Gs-coupled β2-adrenergic receptor stimulation leads to RGS13 accumulation in the nucleus. RGS13 binds to the phosphorylated transcription factor CREB in the presence of its co-activator CREB-binding protein (CBP). Binding of RGS13 to phosphorylated CREB eventually inhibited CREB transactivation, resulting in decreased CREB target gene expression (Xie et al. 2008).

Summary

RGS13 acts as a signaling modulator, playing critical roles in both GPCR-dependent and GPCR-independent cellular processes. The high expression

of RGS13 in murine germinal center B cells and its suppression of chemokine-induced B cell migration suggests a potential role of RGS13 in adaptive immune responses. Altered expression of RGS13 in conditions of autoimmunity or malignancy suggests disease relevance. In the light of negative regulatory roles of RGS13 in mast cell functions, upregulated RGS13 expression in mast cells repeatedly exposed to antigen could increase inhibition of IgE/antigen-mediated mast cell degranulation and/or anaphylactic responses. Mast cell degranulation, which leads to release of granular contents such as histamine, plays a key role in many diseases including allergy, asthma, mastocytosis, and anaphylaxis. Therefore, RGS13 could be a potential therapeutic target, especially given its limited tissue distribution. Since most of the studies conducted thus far have been in mice or in murine cell lines, further investigations of human subjects, such as expression patterns of RGS13 in health and disease, will be of utmost importance.

Acknowledgments This study was supported by the Intramural Research Program of the NIH, NIAID.

References

Bansal G, Druey KM, Xie Z. Pharmacol Ther. 2007;116:473–95.
Bansal G, Xie Z, Rao S, Nocka KH, Druey KM. Nat Immunol. 2008a;9:73–80.
Bansal G, DiVietro JA, Kuehn HS, Rao S, Nocka KH, Gilfillan AM, Druey KM. J Immunol. 2008b;181:7882–90.
Druey KM, Blumer KJ, Kang VH, Kehrl JH. Nature. 1996;379:742–6.
Gilfillan AM, Tkaczyk C. Nat Rev Immunol. 2006;6:218–30.
Grafstein-Dunn E, Young KH, Cockett MI, Khawaja XZ. Brain Res Mol Brain Res. 2001;88:113–23.
Han JI, Huang NN, Kim DU, Kehrl JH. J Leukoc Biol. 2006;79:1357–68.
Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, Le TV, Lorenz RG, Xu H, Kolls JK, Carter RH, Chaplin DD, Williams RW, Mountz JD. Nat Immunol. 2008;9:166–75.
Hunt TW, Fields TA, Casey PJ, Peralta EG. Nature. 1996;383:175–7.
Islam TC, Asplund AC, Lindvall JM, Nygren L, Liden J, Kimby E, Christensson B, Smith CI, Sander B. Leukemia. 2003; 17:1880–90.
Iwaki S, Lu Y, Xie Z, Druey KM. J Biol Chem. 2011; epublished ahead of print.
Johnson EN, Druey KM. J Biol Chem. 2002;277:16768–74.
Larminie C, Murdock P, Walhin JP, Duckworth M, Blumer KJ, Scheideler MA, Garnier M. Brain Res Mol Brain Res. 2004;122:24–34.
Neitzel KL, Hepler JR. Semin Cell Dev Biol. 2006;17:383–9.
Patel TB. Pharmacol Rev. 2004;56:371–85.
Pise-Masison CA, Radonovich M, Dohoney K, Morris JC, O'Mahony D, Lee MJ, Trepel J, Waldmann TA, Janik JE, Brady JN. Blood. 2009;113:4016–26.
Shi GX, Harrison K, Wilson GL, Moratz C, Kehrl JH. J Immunol. 2002;169:2507–15.
Thompson MD, Cole DE, Jose PA. Methods Mol Biol. 2008;448:77–107.
Watson N, Linder ME, Druey KM, Kehrl JH, Blumer KJ. Nature. 1996;383:172–5.
Xie Z, Geiger TR, Johnson EN, Nyborg JK, Druey KM. Mol Cell. 2008;31:660–70.
Xie Z, Yang Z, Druey KM. J Mol Cell Biol. 2010;2:357–65.

RGS9 Anchor Protein (R9AP)

► R7BP/R9AP

Rho Guanine Nucleotide Exchange Factor 25

► ARHGEF25

Rho Kinase

► ROCK Kinases

Rho-Associated Coiled Coil Kinase

► ROCK Kinases

Rho-Associated Kinase

► ROCK Kinases

Rho-Associated Protein Kinase

► ROCK Kinases

RhoC (RHOC)

Nicolas Reymond[1], Francisco M. Vega[2] and Anne J. Ridley[1]
[1]Randall Division of Cell and Molecular Biophysics, King's College London, London, UK
[2]Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Synonyms

ARH9; ARHC; Ras homolog gene family, member C

Historical Background

RhoC was originally identified, together with its homologues RhoA and RhoB, as a Ras-related small GTPase (Madaule and Axel 1985). RhoA, RhoB, and RhoC together comprise the Rho subfamily of small GTPases characterized by their high homology within the Rho GTPase family. The human *rhoc* gene is on chromosome 1p13.1-p21. The *rhoc* gene is proposed to originate from a duplication of *rhoa* during evolution (Boureux et al. 2007). RhoA and RhoC are 93% identical at the protein level; the divergence is mainly concentrated in the so-called hypervariable region of the proteins at the C-terminus. Although multiple Rho subfamily GTPases exist in many eukaryotic organisms, specific RhoC orthologs do not exist outside vertebrates (Boureux et al. 2007). This together with the fact that, like RhoA, when RhoC is overexpressed in cells it induces the formation of actin stress fibers means that its specific functions were not investigated until relatively recently. It was originally described to regulate reorganization of the actin cytoskeleton and cell shape, attachment, and motility in a similar way to RhoA. The focus of attention shifted when it was discovered that RhoC but not RhoA specifically contributes to cancer metastasis (Clark et al. 2000). Since then many studies have investigated the different functions of RhoA, RhoB, and RhoC in cell biology and disease processes.

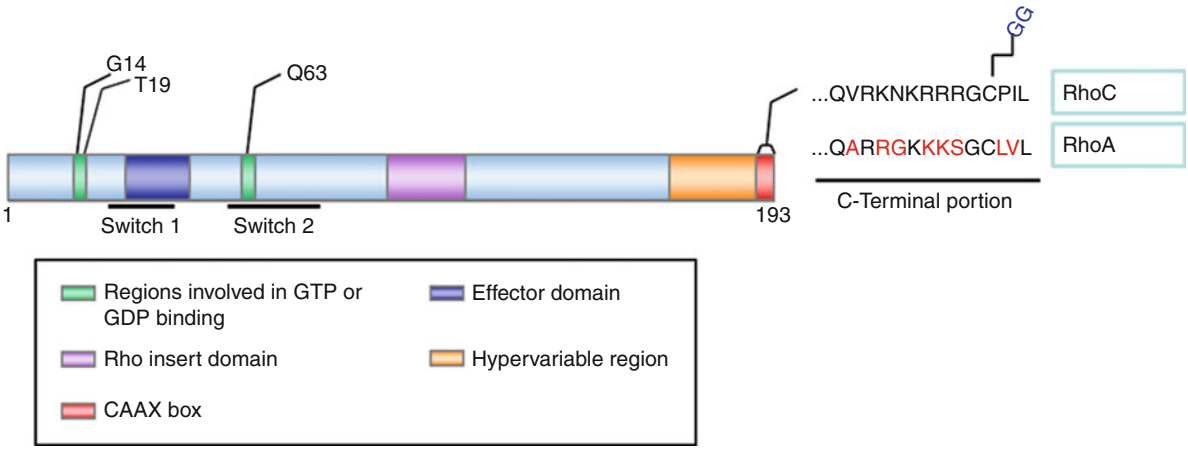
Tools for the Study of RhoC Function

Classic tools for the study of Rho GTPases, including RhoC, took advantage of some conserved amino acids essential for GTP hydrolysis to make dominant negative (T19N substitution) or constitutively active (G14V or Q63L substitutions) forms of the protein (Fig. 1; Wheeler and Ridley 2004). However, these mutations have the disadvantage that they probably do not allow the functional specificity of closely related Rho GTPases like RhoA and RhoC to be explored. Bacterial toxins, like *Clostridium Botulinum* exoenzyme C3 transferase or Toxin B, efficiently inhibit Rho activity by covalently modifying the protein in key residues, but again they target all Rho subfamily proteins and, although they were central to establishing the role of these proteins in actin cytoskeletal organization, they cannot be used to study RhoC-specific functions. More recently, RNA interference (RNAi) has been used to differentiate the functions for RhoA, RhoB, and RhoC in cells. This has shown that RhoA and RhoC regulate cytoskeletal dynamics, cell morphology, migration, and invasion in different ways (Bellovin et al. 2006; Wu et al. 2010; Bravo-Cordero et al. 2011; Vega et al. 2011).

Spatio-temporal regulation of Rho GTPase activity is crucial to cell migration and invasion. The combined use of live-cell imaging and fluorescence biosensors is now helping to decifer how the activity of RhoC and other Rho GTPases is tightly regulated on a subcellular level. For example, using a RhoC -specific biosensor in cancer cells, dynamic RhoC activation in invadopodia has been visualized (Bravo-Cordero et al. 2011).

Regulation of RhoC Activity

RhoC is a GTPase that act as a molecular switch, cycling between a GDP-bound inactive state and a GTP-bound active state. When active it interacts with effector proteins to regulate a variety of different processes (Wheeler and Ridley 2004). Its activation is positively regulated by guanine nucleotide exchange factors (GEFs), which stimulate the exchange of GDP for GTP on the protein, and negatively regulated by GTPase-activating proteins (GAPs), which stimulate its intrinsic GTPase activity. RhoC is also posttranslationally modified at the C-terminus by prenylation with the addition of a geranylgeranyl group (Fig. 1). This modification allows its anchorage to membranes and is likely to be essential for its biological function. Similar to several other Rho GTPases, RhoC associates with Rho GTPase dissociation inhibitor proteins (RhoGDIs),



RhoC (RHOC), Fig. 1 RhoC domain structure and features. Schematic of RhoC protein showing the different domains and residues important for its activity and used to create dominant negative and constitutively active mutations as described in the text. The C-terminal 14 amino acids, including the CAAX box, and comparison with the equivalent region of RhoA is shown. *Red* amino acids indicate nonconserved residues. GG depicts the prenylation site; following geranylgeranlylation the last three amino acids are cleaved off

which sequester the protein in the cytoplasm by interacting with the C-terminal geranylgeranyl group. These modes of regulation are shared with the other Rho proteins, RhoA and RhoB. The different functions of RhoC compared to RhoA and RhoB are thought to be achieved through RhoC-specific GEFs and/or GAPs and by its binding to specific effector proteins.

Most regulators of Rho subfamily proteins have only been tested on RhoA, and based on the sequence similarity with RhoC would be predicted to act on both proteins. The ones that have been specifically tested on RhoC are the GEFs Tim and Scambio, the GAPs p190RhoGAP, GRAF, p50RhoGAP and Myr5 and the 3 RhoGDI proteins (Bos et al. 2007). Few regulators have been compared for their activity on RhoA versus RhoC. One of the few examples of a specific regulator comes from studies of the RhoGEF ARHGEF3 (also known as XPLN), which acts on RhoA and RhoB but not RhoC, but no RhoC-selective GEFs or GAPs have been described so far (Arthur et al. 2002; Bos et al. 2007). A report described enhanced RhoC activity compared to RhoA in pancreatic carcinoma cells which correlated with an increase of RhoC membrane localization (Dietrich et al. 2009); this might reflect differential regulation of Rho isoform interaction with RhoGDIs.

RhoC expression is ubiquitous but its levels are variable between different tissues. Overexpression of *RhoC* has been reported in a variety of pathological conditions, particularly in more aggressive metastatic cancers, and this indicates that its expression is regulated and plays a role in its physiological function. A newly discovered way in which *RhoC* expression and activity can be regulated in cells is by the action of noncoding microRNAs. In breast cancer, for example, the overexpression of the microRNA miRNA-10b induces the upregulation of RhoC by inhibiting the translation of the messenger RNA encoding HOXD10, and this in turn promotes invasion and metastasis (Ma et al. 2007). In squamous cell carcinoma, the downregulation of microRNA-138 induces metastasis by reducing direct *RhoC* mRNA degradation (Jiang et al. 2010).

Some Rho proteins are subject to posttranslational modifications that regulate their stability or activity. Notably, the residue Ser188 present in RhoA that is subject to protein kinase A and protein kinase G phosphorylation is not conserved in RhoC (Ellerbroek et al. 2003). RhoA is also ubiquitinated and thereby targeted for proteasomal degradation, but RhoC does not appear to be regulated similarly (Chen et al. 2009). It is possible that as-yet-identified residues in RhoC could be subject to phosphorylation, ubiquitination, or other posttranslational modifications.

RhoC Effectors

Because RhoA, B, and C possess a high level of identity at the protein level, they share many common downstream effectors (Wheeler and Ridley 2004).

The affinity of these interactions may vary due to their amino acid sequence differences. These interactions all involve the Rho switch I and II regions (see Fig. 1) and Rho-binding domains (RBDs) of their effectors, which include Rho-associated kinase (▶ ROCK-1 and-2), Protein kinase N (PKN1-3, also known as PRKs), Citron kinase, mDia1-3, Rhotekin, and Rhophilin-1. RhoC and RhoA also interact with Phospholipase C ε (PLC-ε) via its catalytic core. RhoC, but not RhoA, has recently been reported to bind to the Formin-like family members FMNL2 and FMNL3 (Kitzing et al. 2010; Vega et al. 2011). RhoC also binds to IQGAP1 (Wu et al. 2011).

RhoC Functions in Tumorigenesis

Formation of Metastases

RhoC expression and activity is often increased in cancer and correlates with progression, metastasis formation and therefore a poor prognosis for patients (Vega and Ridley 2008). Increased *RhoC* expression in cancer was first identified in a screen for genes upregulated in melanoma metastases (Clark et al. 2000). *RhoC* expression was subsequently found to be upregulated in a variety of cancers including prostate cancer, breast cancer, gastric cancer, ovarian cancer, bladder cancer, hepatocellular cancer, pancreatic ductal adenocarcinoma, non-small cell lung carcinoma (NLCLC), oesophageal squamous cell carcinoma, head and neck squamous cell carcinoma, and skin squamous cell carcinoma (Karlsson et al. 2009). RhoC has been shown to play a causal role in metastasis in animal models. Initial studies found that overexpression of dominant and negative forms of RhoC correlated with the formation or the inhibition of experimental lung metastases, respectively (Clark et al. 2000). Subsequently, it was shown using RhoC-null mice that RhoC was dispensable for breast cancer initiation and growth but confirmed that RhoC is critical for formation of metastases (Hakem et al. 2005). The inhibition of RhoC has since been described to reduce cancer cell invasion and metastasis in several in vitro and in vivo cancer models. RhoC is now proposed to be a marker for poor prognosis in many different cancers.

No genomic or somatic mutations in human cells have been described for RhoC. Somatic mutations have been reported in the RhoA and RhoC downstream effector ▶ ROCK-1 (Lochhead et al. 2010).

Migration and Invasion

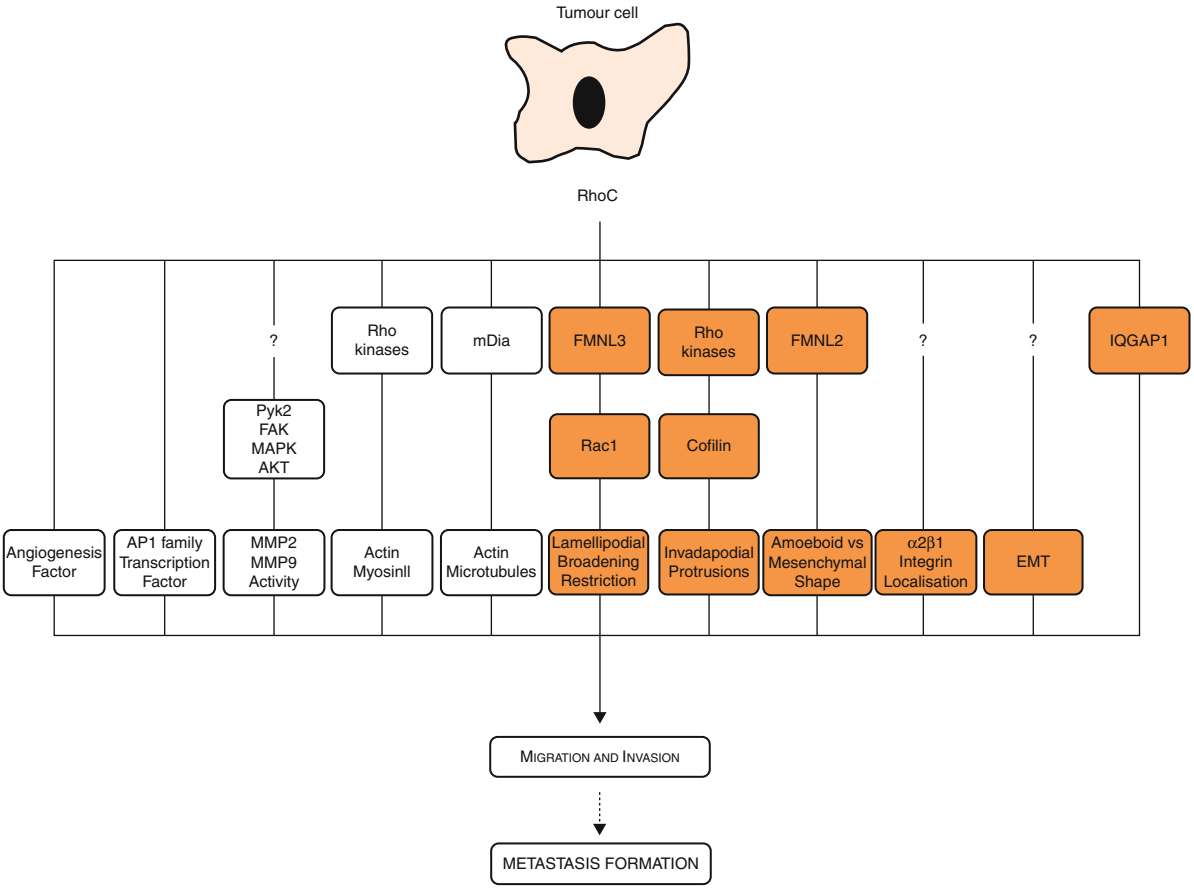
Recent studies show that RhoC has a unique role in cell migration, distinct from RhoA, which could underlie its specific contribution to cancer cell invasion and metastasis (Fig. 2). For example, *RhoC* expression is increases during colon carcinoma cell epithelial-mesenchymal transition (EMT) and regulates EMT-induced migration (Bellovin et al. 2006). RhoC promotes polarized cell migration and invasion by controlling cell spreading and Rac1 activation around the cell periphery hence restricting lamellipodial broadening (Vega et al. 2011). RhoC regulates breast cancer cell adhesion to the extracellular matrix and motility and invasion by modulating the expression and co-localization of α2 and β1 integrins on collagen I (Wu et al. 2011). RhoC is also implicated in the degradation of extracellular matrix as it is involved in the formation of matrix-degrading invadopodia in cancer cells: an active ring of RhoC restricts Cofilin activity and focuses invadopodial protrusion and matrix degradation (Bravo-Cordero et al. 2011). In addition, RhoC coordinates prostate cancer cell invasion in vitro by activating the protein kinases Pyk2, FAK, MAPK, and AKT, which results in activation of the matrix-degrading metalloproteinases 2 and 9 (MMP2 and MMP9; Fig. 2) (Iiizumi et al. 2008). RhoC is also involved in the transcriptional program that controls the TGFß1-induced switch from cohesive to single-cell motility in breast cancer cells (Giampieri et al. 2009).

Angiogenesis

RhoC can stimulate the production of pro-angiogenic factors by breast cancer cells (Merajver and Usmani 2005). RhoC is a downstream effector of vascular endothelial growth factor (VEGF) in endothelial cells and cancer cells. RhoC is thus essential for VEGF-mediated angiogenesis induced by hepatocellular carcinoma cells (Wang et al. 2008). These pro-tumoral functions could potentiate the vascularization of tumors that express RhoC and also may facilitate cancer cell intravasation and extravasation during tumor metastasis.

Proliferation and Apoptosis Resistance

Contradictory studies have been reported concerning the role of RhoC in cancer cell proliferation and apoptosis resistance, which remain to be clarified. On the one hand, RNAi-mediated suppression of RhoC in



RhoC (RHOC), Fig. 2 RhoC functions in cancer. Schematic of RhoC protein showing the different functions of RhoC in cancer and its downstream effectors. Noncolored functions and interactors are shared with RhoA while *orange*-colored functions and interactors are RhoC-specific. Note the *arrow* between migration/invasion and metastasis formation is a *dotted line* because not all of the RhoC effectors have been tested in vivo

hepatocellular carcinoma cells showed that RhoC does not regulate cancer cell proliferation in mice and that depletion of RhoC in endothelial cells does not affect their apoptosis (Wang et al. 2008). On the other hand, RhoC depletion in human gastric carcinoma cells was reported to inhibit proliferation and increase apoptosis in vitro (Sun et al. 2007); and RhoC promoted human oesophageal squamous cell carcinoma and breast cancer cell proliferation in mice in vivo (Faried et al. 2006).

RhoC Regulates Transcription Factors

RhoA is well known to regulate transcription through actin-dependent and actin-independent effects on a variety of transcription factors (Jaffe and Hall 2005). Recent evidence indicates that RhoC also plays a role in transcriptional regulation (Fig. 2). RhoC is induced in melanoma cells by the transcriptional regulator ETS-1. RhoC then indirectly stabilizes the AP-1 family transcription factor c-Jun through the actin cytoskeleton (Spangler et al. 2011). c-Jun is an oncogene which is a critical mediator of tumor development.

Summary

RhoC is a member of the small family of Rho GTPases that is very closely related to RhoA and RhoB. It is best known for its role in cell migration regulation and control of actin cytoskeleton dynamics. Although RhoC was originally considered to act similarly to RhoA and share the same partners and functions, there is now good evidence that RhoC has unique functions in cells and is probably regulated by specific

partners. Recently, some RhoC-specific downstream effectors have been described and the analysis of these interactions gave new insights into RhoC function in cells. This is reflected by studies in mouse models, where RhoC has distinct functions to RhoA in cancer metastasis. RhoC is upregulated in many types of cancer and is a critical regulator of cancer progression and metastasis formation. It is likely to be useful as a prognostic marker in many types of cancer and could be a possible target in cancer therapy. The understanding of RhoC function in cells and organisms will be advanced in the future by the use of biosensors and fluorescence resonance energy transfer methods to decipher where and when RhoC is active and interacts with its partners.

References

Arthur WT, Ellerbroek SM, et al. XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. J Biol Chem. 2002;277(45):42964–72.

Bellovin DI, Simpson KJ, et al. Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma. Oncogene. 2006;25(52):6959–67.

Bos JL, Rehmann H, et al. GEFs and GAPs: critical elements in the control of small G proteins. Cell. 2007;129(5):865–77.

Boureux A, Vignal E, et al. Evolution of the Rho family of ras-like GTPases in eukaryotes. Mol Biol Evol. 2007;24(1):203–16.

Bravo-Cordero JJ, Oser M, et al. A novel spatiotemporal RhoC activation pathway locally regulates cofilin activity at invadopodia. Curr Biol. 2011;21(8):635–44.

Chen Y, Yang Z, et al. Cullin mediates degradation of RhoA through evolutionarily conserved BTB adaptors to control actin cytoskeleton structure and cell movement. Mol Cell. 2009;35(6):841–55.

Clark EA, Golub TR, et al. Genomic analysis of metastasis reveals an essential role for RhoC. Nature. 2000;406(6795):532–5.

Dietrich KA, Schwarz R, et al. Specific induction of migration and invasion of pancreatic carcinoma cells by RhoC, which differs from RhoA in its localisation and activity. Biol Chem. 2009;390(10):1063–77.

Ellerbroek SM, Wennerberg K, et al. Serine phosphorylation negatively regulates RhoA in vivo. J Biol Chem. 2003;278(21):19023–31.

Faried A, Faried LS, et al. RhoA and RhoC proteins promote both cell proliferation and cell invasion of human oesophageal squamous cell carcinoma cell lines in vitro and in vivo. Eur J Cancer. 2006;42(10):1455–65.

Giampieri S, Manning C, et al. Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. Nat Cell Biol. 2009;11(11):1287–96.

Hakem A, Sanchez-Sweatman O, et al. RhoC is dispensable for embryogenesis and tumor initiation but essential for metastasis. Genes Dev. 2005;19(17):1974–9.

Iiizumi M, Bandyopadhyay S, et al. RhoC promotes metastasis via activation of the Pyk2 pathway in prostate cancer. Cancer Res. 2008;68(18):7613–20.

Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 2005;21:247–69.

Jiang L, Liu X, et al. Downregulation of the Rho GTPase signaling pathway is involved in the microRNA-138-mediated inhibition of cell migration and invasion in tongue squamous cell carcinoma. Int J Cancer. 2010;127(3):505–12.

Karlsson R, Pedersen ED, et al. Rho GTPase function in tumorigenesis. Biochim Biophys Acta. 2009;1796(2):91–8.

Kitzing TM, Wang Y, et al. Formin-like 2 drives amoeboid invasive cell motility downstream of RhoC. Oncogene. 2010;29(16):2441–8.

Lochhead PA, Wickman G, et al. Activating ROCK1 somatic mutations in human cancer. Oncogene. 2010;29(17):2591–8.

Ma L, Teruya-Feldstein J, et al. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature. 2007;449(7163):682–8.

Madaule P, Axel R. A novel ras-related gene family. Cell. 1985;41(1):31–40.

Merajver SD, Usmani SZ. Multifaceted role of Rho proteins in angiogenesis. J Mammary Gland Biol Neoplasia. 2005;10(4):291–8.

Spangler B, Kappelmann M et al. ETS-1/RhoC signaling regulates the transcription factor c-Jun in melanoma. Int J Cancer. 2011

Sun HW, Tong SL, et al. RhoA and RhoC -siRNA inhibit the proliferation and invasiveness activity of human gastric carcinoma by Rho/PI3K/Akt pathway. World J Gastroenterol. 2007;13(25):3517–22.

Vega FM, Ridley AJ. Rho GTPases in cancer cell biology. FEBS Lett. 2008;582(14):2093–101.

Vega FM, Fruhwirth G, et al. RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. J Cell Biol. 2011;193(4):655–65.

Wang W, Wu F, et al. RhoC is essential for angiogenesis induced by hepatocellular carcinoma cells via regulation of endothelial cell organization. Cancer Sci. 2008;99(10):2012–18.

Wheeler AP, Ridley AJ. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. Exp Cell Res. 2004;301(1):43–9.

Wu M, Wu ZF, et al. Characterization of the roles of RHOC and RHOA GTPases in invasion, motility, and matrix adhesion in inflammatory and aggressive breast cancers. Cancer. 2010;116(11 Suppl):2768–82.

Wu Y, Chen YC, et al. RhoC protein stimulates migration of gastric cancer cells through interaction with scaffold protein IQGAP1. Mol Med Rep. 2011;4(4):697–703.

Rhodopsin Kinase

► G-Protein-Coupled Receptor Kinase 1 (GRK1)

RhoK

▶ G-Protein-Coupled Receptor Kinase 1 (GRK1)

Rh-Related Antigen

▶ CD47

Ribosomal Protein S6 Kinase, 90kD, Polypeptide 5

▶ MSK1

Ribosomal Protein S6 Kinase, Polypeptide 5

▶ MSK1

RIBP

▶ SH2D2A

Ric-8

Gregory G. Tall
Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY, USA

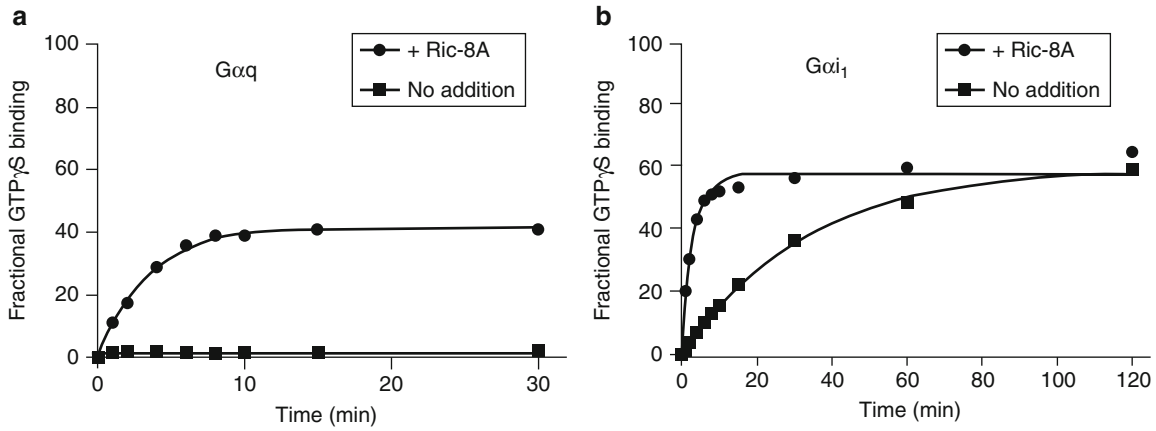
Synonyms

Resistance to inhibitors of cholinesterase 8; Ric-8A; Ric-8B; Synembryn

Historical Background

Discovery of Ric-8 proteins: The *Caenorhabditis elegans* RIC-8 gene and a homologous mouse gene that was later termed Ric-8A (or a synembryn) were discovered by Miller and Rand using a genetic screen to obtain *C. elegans* mutants that were resistant to the inhibitor of cholinesterase, aldicarb (Miller et al. 1996). Aldicarb treatment of wild-type worms leads to neurotoxic accumulation of postsynaptic acetylcholine and subsequent death. Ric mutants lived in the presence of aldicarb because they contained gene defects that restored normal acetylcholine levels, primarily by decreasing neurotransmitter secretion or release. Through epistasis analyses, the gene complementing the ric-8 mutant allele was predicted to elicit action upstream of or parallel to the gene encoding G protein α q in a diacylglycerol-dependent synaptic-vesicle-priming pathway (Miller et al. 2000a). Miller and Rand also first showed that centrosome (spindle pole) movements in the dividing *C. elegans* zygote were perturbed in ric-8 mutants, a phenotype shared by G protein αo(i) mutants (Miller and Rand 2000b).

Ric-8 proteins were first linked physically to ▶ G protein α subunits when two mammalian Ric-8 homologues were identified by the ability to bind Gαo and Gαs directly in yeast two-hybrid screens and in purified protein–protein binding and functional assays (Tall et al. 2003). The two homologues are the products of separate genes and were named mammalian Ric-8A and Ric-8B. Ric-8A binds Gαi, Gαq, and Gα12/13 class G protein α subunits, whereas Ric-8B binds the Gαs, Gαq, and Gα12/13 classes. Preliminary experiments demonstrating a preferred interaction of Ric-8A with the GDP-bound form of G protein α led to the hypothesis that Ric-8A might alter G protein catalysis by serving as an alternative guanine nucleotide dissociation inhibitor (GDI), or a non-receptor guanine nucleotide exchange factor (GEF). Purified Ric-8A stimulated intrinsic G protein α GDP release dramatically leading to accelerated, observed GTP(γS) binding kinetics (G protein activation) (Fig. 1). The mechanism of Ric-8A-stimulated guanine nucleotide exchange was elucidated. Ric-8A initially interacted with GDP-bound G alpha and stimulated rapid GDP release. In the absence of GTP, Ric-8A formed a stable nucleotide-free transition state complex with Gα. Addition of GTP and magnesium dissociated this complex to produce free Ric-8A and activated Gα-GTP. Currently, a biochemical characterization of Ric-8B has not been made. Ric-8B is predicted to be a Gαs and Gαq-class GEF based on



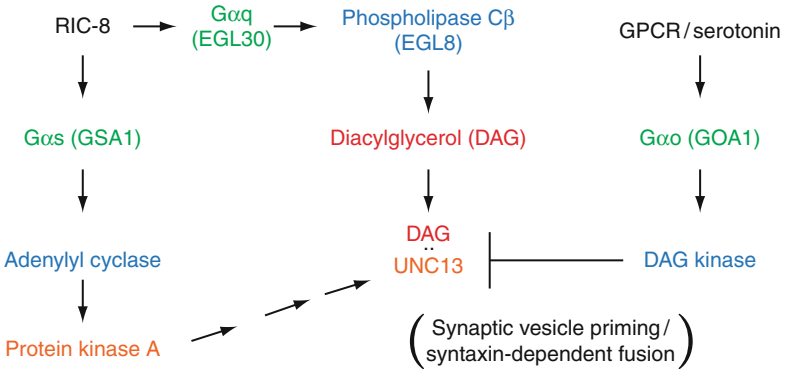
Ric-8, Fig. 1 Ric-8A is a Gα subunit GEF and accelerates the kinetics of (**a**) Gαq and (**b**) $G\alpha i_1$ GTPγS binding. Purified Gα subunits (200 nM) were incubated in reactions containing radiolabeled GTPγS with (*squares*), or without (*circles*) purified Ric-8A (200 nM). At the indicated times, Gα and its bound nucleotide were trapped on nitrocellulose filters and the amount bound GTPγS was measured by scintillation counting. This research was originally published in the *Journal of Biological Chemistry* (4) © the American Society for Biochemistry and Molecular Biology

its Gα binding preferences. Since these initial observations of Ric-8 function, the field has endeavored to understand how Ric-8 GEF activity regulates G protein function in cells. Many of the predicted Ric-8 physiological functions have been acquired from genetic studies in model organisms. These include functional interactions with varied G protein species to regulate neurotransmission, asymmetric cell division, olfaction, and G protein residence at the plasma membrane.

C. elegans RIC-8 and Gαq/Gαs/Gαo modulate neurotransmission. The original function ascribed to the *C. elegans* RIC-8 gene was made by its identification in the ric mutant screen designed to isolate mutants of genes whose products positively regulated neurotransmission (Miller et al. 1996). Gαq is one other such gene. Various genetic and biochemical tests confirmed that ric-8 mutants acted epistatically (upstream) to other Gαq-pathway-dependent synaptic-vesicle-priming defects. Gαq gain of function alleles, diacylglycerol (DAG) kinase mutants (deregulates DAG by phosphorylation), or application of phorbol esters (DAG analogs) all suppressed the ric-8 mutant neurotransmission defect (Miller et al. 2000a). These and other collective observations indicated that the RIC-8 protein likely acted upstream of Gαq to promote neurotransmission through phospholipase Cβ-dependent DAG production and subsequent UNC13 stimulation (see Fig. 2). UNC13 is a DAG binding protein/sensor that interacts with the membrane in a DAG-dependent manner. UNC13 also interacts with syntaxin proteins and may regulate SNARE-dependent vesicle fusion.

Ric-8 was later shown to be epistatic to Gαs through genetic suppression experiments. Gain of function mutants in Gαs, ▶ adenylyl cyclase, or protein kinase A individually suppressed the ric-8 paralysis defect (Reynolds et al. 2005; Schade et al. 2005). This showed that *C. elegans* RIC-8 gene action intersected with a second G protein, Gαs, to provide a cAMP second messenger input into the neurotransmitter release process. Collectively the *C. elegans* RIC-8 gene acts upstream of at least Gαq and Gαs and may maintain both G protein signaling pathways in activated states. It is not understood precisely how RIC-8 acted upon these divergent G proteins. The proposed role was gathered from the observation that mammalian Ric-8A acted as a Gαq GEF and directly activated these G proteins in a manner apart from the action of G protein–coupled receptors (GPCRs).

Ric-8 and G protein αi control of (asymmetric) cell division: Asymmetric cell division (ACD) is a process by which stem cells or progenitor cells divide such that the two daughter cells adopt unique characteristics and cell fate determinants during division (intrinsic ACD), or acquire these characteristics after division by influence of the surrounding tissue/niche (extrinsic ACD). ACD allows one daughter cell to retain stem/progenitor potential and the other to adopt a committed fate. Ric-8 and members of the G protein αi class of



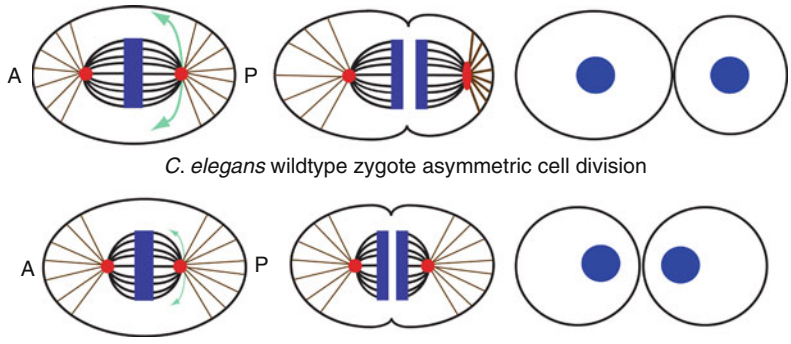
Ric-8, Fig. 2 Proposed pathways depicting *C. elegans* RIC-8 control of Gαq- and Gαs-dependent neurotransmission (*Arrows*: activation, *Bars*: inhibition). Genetic experiments showed that the requirement of the RIC-8 gene for neurotransmission was manifested before the action of the Gαq and Gαs genes. G proteins αq and αs provide divergent inputs to regulate neurotransmitter release through the activation of phospholipase Cβ and adenylyl cyclase, respectively. These two pathways converge to modulate UNC13 function/localization and regulation of synaptic vesicle priming. G protein αo provides an inhibitory input into the pathway, perhaps through diacylglycerol kinase that is regulated by a serotonin responsive GPCR. Gαo regulation was not necessarily thought to involve RIC-8 (5–7)

G proteins have an essential role in directing intrinsic asymmetric-, and perhaps normal-cell division. Gαi regulation in this context is thought to be GPCR-independent, as Gαi-family members reside not only on the plasma membrane, but on intracellular mitotic structures including spindle poles (centrosomes), spindle microtubules, and the cytokinesis midbody (Blumer et al. 2006).

The protein machinery that works with Gαi to control cell division is conserved and consists of components discovered in worms, flies, and mammals. Gαi, Ric-8(A), and orthologous GoLoco- or GPR-domain containing proteins, GPR1/2, PINS, or LGN/AGS3 in mammals are required for asymmetric cell division (ACD) of multiple cell types, including human adult progenitor cells. These and other conserved proteins in the pathway, including NuMA/Lin5/MUDs receive signals from cell polarity determinants to differentially regulate the strength of pulling forces on the two sets of aster microtubules during mitosis (for review and a comprehensive account of primary references, see Siderovski and Willard (2005), Siller and Doe (2009), Wilkie and Kinch (2005), and Yu et al. (2006)). This force differential pulls the entire mitotic spindle and metaphase plate as a unit toward one side of the cell. The asymmetric location of the metaphase plate marks the position of the cleavage plane for cytokinesis. Figure 3 depicts in cartoon format, the original observations demonstrating that the Ric-8, Gαi/o, and GPR1/2 genes were required for *C. elegans* zygote ACD. When these genes were mutated individually, zygotic cell division proceeded symmetrically, leading to developmental catastrophe and death at gastrulation.

The majority of the genetic and cell biological work delineating the influence of this nontraditional G protein pathway on mitotic spindle dynamics has been learned from ACD model systems including the *C. elegans* zygote, *Drosophila* neuroblast, and sensory organ precursor cell. It is clear that the network also operates in mammals to direct *both* symmetric and ACD because homologous and orthologous components of the pathway influence keratinocyte (skin) progenitor cell ACD (LGN, NuMA), neural precursor development (LGN, AGS3, G protein subunits) (for review of primary literature see Siller and Doe (2009)), and division of COS and HeLa cells (Gαi, AGS3) (Blumer et al. 2006; Cho and Kehrl 2007). Recently, Ric-8A and Gαi were shown to recruit LGN, NuMA, and the dynein microtubule motor to the plasma membrane of mitotic HeLa cells. Perturbation of Ric-8A expression, or its interaction with Gαi resulted in defective orientation of the HeLa mitotic spindle to the substratum and aberrant cell division (Woodard et al. 2010).

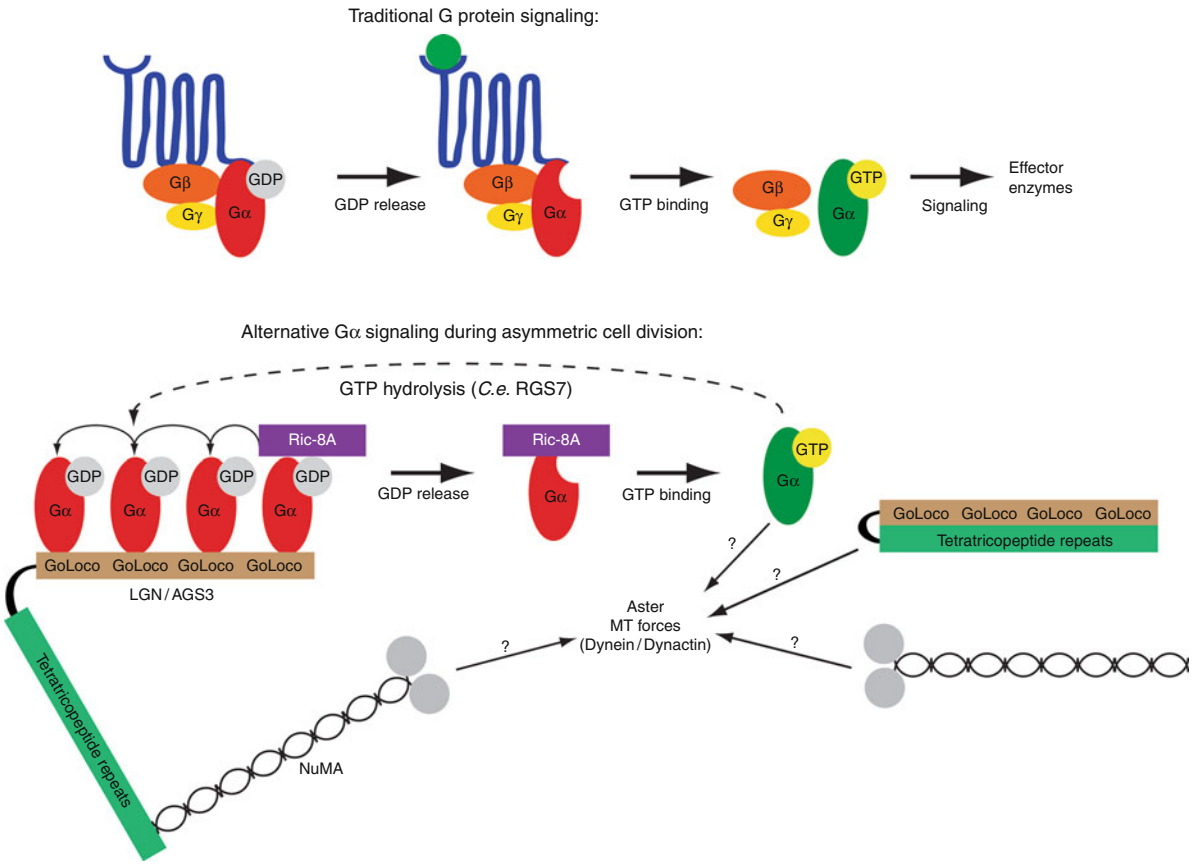
A biochemical demonstration of Ric-8A, Gαi, and GoLoco protein concerted function accounts for how Ric-8 GEF activity could supplant the lack of apparent GPCR-mediated G protein activation in ACD and provides one cellular context for the GoLoco component



Ric-8, Fig. 3 *C. elegans* zygote asymmetric cell division is perturbed in ric-8, gαo, or gpr1/2 mutants. Zygote polarity is determined by the site of sperm entry and designates the posterior (*P*), and anterior (*A*) sides of the zygote. During prophase the mitotic spindle (*black microtubule bundles*) rotates 90° (not depicted) to orient the spindle poles (*red color*) along the anterior and posterior axis of the zygote. Spindle rotation is an aster microtubule (*brown color*)-dependent process and is slowed in ric-8, gαo, or gpr1/2 mutants. Chromosomes (*blue color*) are shown aligned at the metaphase plate with intact mitotic spindles. The distance between spindle poles is reduced in ric-8, gαo, or gpr1/2 mutants. During meta/anaphase, the posterior spindle pole flattens and adopts a violent up and down rocking motion in relation to the A-P axis (*green arrow*) as it and the entire mitotic spindle is pulled toward the posterior side of the zygote by forces generated from the posterior aster microtubules. Overall aster microtubule force and the force differential between the anterior and posterior aster microtubules are considerably weaker in ric-8, gαo, or gpr1/2 mutants. The mutants exhibit a very weak rocking motion of the posterior spindle pole and reduced movement of either spindle pole toward the cell cortex. As chromosome segregation occurs, the cytokinetic cleavage plane appears asymmetrically in wild-type zygotes, closer to the posterior side. Ric-8, gαo, and gpr1/2 mutants lack asymmetrically oriented spindles and subsequently divide symmetrically

as an atypical guanine nucleotide dissociation inhibitor (GDI). In conditions where traditional GPCR signaling is turned off, Gβγ subunits serve as a Gα protein GDI and prevent GDP release. During nontraditional G protein signaling in mitosis and ACD, LGN (GoLoco) binds plasma membrane-bound Gαi-GDP subunits as a GDI. The nuclear mitotic apparatus protein (NuMA) is recruited in allosteric fashion to a binding site on LGN that becomes available when LGN is bound to Gαi (Du and Macara 2004). NuMA is not active in this complex with respect to its incapacity to participate in direct interactions with microtubules (Du et al. 2002). However, complexed NuMA may interact with the dynein microtubule motor complex (Siller and Doe 2009). Ric-8A dissociated a purified Gαi/GoLoco/NuMA complex by removing Gαi-GDP from LGN or AGS3 (GoLoco) through stimulation of nucleotide exchange and production of free Gαi-GTP (Tall and Gilman 2005; Thomas et al. 2008). Once Gαi was dissociated from LGN, the ability of NuMA to bind GoLoco domains was decreased and NuMA was released. Whether the intact GoLoco protein complex or any of the released species (NuMA, GoLoco ortholog, and/or Gαi-GTP) regulate aster microtubule pulling forces to direct spindle asymmetry is not completely clear and is an area of considerable interest. It is unlikely that a static, plasma membrane–bound Gα-GDP:GoLoco complex is the sole form of the G protein responsible for force generation, as rounds of nontraditional G protein guanine nucleotide consumption seem to be required (Wilkie and Kinch 2005). Gαi-GTP is inactivated by RGS GTPase-activating-proteins, which presumably resets the system for another cycle of Ric-8-mediated activation (Hess et al. 2004). Important unresolved questions remain. Does Ric-8(A) actually perform this function in cells and dissociate Gαi/GoLoco/NuMA complexes in the context of aster microtubule force regulation? What is the precise ordering of molecular events that occur downstream of Ric-8A, the intact GoLoco complex, or the dissociated Gαi-GTP, GoLoco, and/or NuMA species that directly signal to the dynein motor complex force generator (Fig. 4)?

Ric-8B and Gαolf regulation of olfaction: The second mammalian Ric-8 homologue was named Ric-8B after it was discovered in yeast two-hybrid screens intended to uncover Gαs-interacting proteins (Tall et al. 2003; Klattenhoff et al. 2003). Mammalian Ric-8/synembryn was renamed Ric-8A at this time. Ric-8A and Ric-8B share ~40% overall amino acid identity.



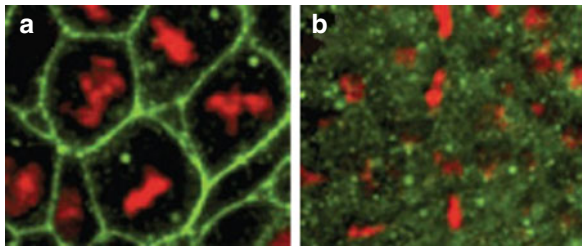
Ric-8, Fig. 4 Biochemical model comparing traditional GPCR-mediated activation of Gαβγ trimers and alternative Ric-8(A) activation of Gαi/GoLoco/NuMA complexes. In the traditional G protein signaling paradigm, inactive G protein trimers are bound to GDP. Gβγ serves as a GDI and prevents Gα subunit GDP release in the absence of GPCR activating ligand (●). Gβγ is also obligate for ligand-receptor complex stimulation of G protein GTP for GDP nucleotide exchange of the Gα subunit. Activated Gα-GTP and Gβγ transduce signals to downstream effector enzymes. In the proposed alternative Gα signaling model that regulates aster microtubule force generation during ACD, Gα-GDP is bound to the alternative GDI GoLoco. A mammalian GoLoco ortholog, LGN, contains four carboxyl-terminal GoLoco domains, and an amino-terminal tetratricopeptide repeat region that binds NuMA. Ric-8A binds Gαi-GDP, and in the process dissociates GDP from Gαi, and Gαi processively from GoLoco domains. GTP then rapidly binds the Ric-8A:Gα nucleotide-free complex producing free Ric-8A and Gα-GTP. As a consequence of Gα:GoLoco dissociation, NuMA is released from LGN and could be available to participate in enhanced interactions with microtubules or microtubule motors. Elucidation of the complete repertoire of molecules, or direct events that result in aster microtubule force generation is not entirely clear (10). Evidence in favor of G protein α i catalytic cycle involvement in this process comes from the finding that mutants of *C. elegans* RGS7 (an activator of G protein GTP hydrolysis) have many opposed phenotypes to ric-8, gαo, and gpr1/2 mutants during ACD (19)

The amino-terminal ~400 amino acids are more divergent (34% identity) and have been predicted to be highly α-helical in content and consist of weakly scoring Armadillo repeats (Wilkie and Kinch 2005). Ric-8A and Ric-8B share greater amino acid identity between the carboxyl-terminal 130–160 amino acids (~56% identity). No obvious protein sub-domains are present in the predicted α-helical Ric-8 carboxyl-termini, but the region in Ric-8B is alternatively spliced.

Ric-8B binds all G protein alpha classes in vitro with the exception of the Gαi-class, and unlike Ric-8A, uniquely binds members of the Gαs-family (Tall et al. 2003; Klattenhoff et al. 2003). Evidence supports a role for Ric-8B in the regulation of Gαolf during olfaction (Von Dannecker et al. 2006). Gαolf is the olfactory/brain-specific Gαs homologue that activates adenylyl cyclase to stimulate olfactory nerve firing. Malnic and colleagues portrayed functional differences between two expressed Ric-8B splice variants

(full length, FL and deleted exon 9, Δ9). Overexpression of Ric-8BFL, but not Ric-8BΔ9 enhanced Gαolf-dependent adenylyl cyclase activation (Von Dannecker et al. 2006). When Gαolf, Gβ, Gγ, odorant receptors, receptor co-factors, and Ric-8BFL, but not Ric-8BΔ9, were co-transfected into a heterologous system (HEK cells), functional odorant receptor coupling was achieved. Application of odorants activated Gαolf-dependent signaling (Von Dannecker et al. 2006; Zhuang and Matsunami 2007). The unresolved question raised from these studies is: Does Ric-8B promote odorant receptor signaling because it activates Gαolf/Gαs as a guanine nucleotide exchange factor, or because it facilitates functional Golf membrane expression in the heterologous system, thereby enhancing Golf coupling to odorant receptors? Overexpression of Ric-8BFL did increase the amount of overexpressed Golf in a crude membrane fraction (Kerr et al. 2008). It was later reported that the single copy of RIC-8 in *Xenopus* appeared to stimulate mammalian Gαs GTPγS binding, although the intrinsic rate of Gαs GTPγS binding reported in this study was negligible (Romo et al. 2008). A positive demonstration of mammalian Ric-8B protein function as a Gαs-class GEF has not been made as of yet (Nagai et al. 2010). Ric-8B is hypothesized to be a Gαs and Gαq-class GEF since it interacts with these subunits in vitro, and by analogy (and homology) to the described activities of Ric-8A. Ric-8A is a GEF for all Gα subunits that it can bind.

Ric-8 proteins support heterotrimeric G protein membrane expression: An alternative hypothesis of Ric-8 protein function was proposed from work performed on the sole copy of the *Drosophila* and *C. elegans* RIC-8 genes. Attenuation of *Drosophila* RIC-8 expression caused nearly complete and pleotropic loss of multiple Gα subunits and the major Gβ (and presumably Gγ) subunit from the plasma membrane (for review and primary literature references see Matsuzaki (2005)). *Drosophila* Gαi (Fig. 5), Gαo, and Gβ13F subunits were expressed in the cytosol or vesiculated compartments of *Drosophila* embryonic epithelial cells derived from larvae possessing both a maternal and zygotic P-element disruption of RIC-8. Overall steady-state expression levels of the mis-localized Gαi and Gβ were reduced in the absence of RIC-8. These findings were corroborated at the same time in *C. elegans* when cortical Gpa16 (a Gαi homologue) localization was attenuated

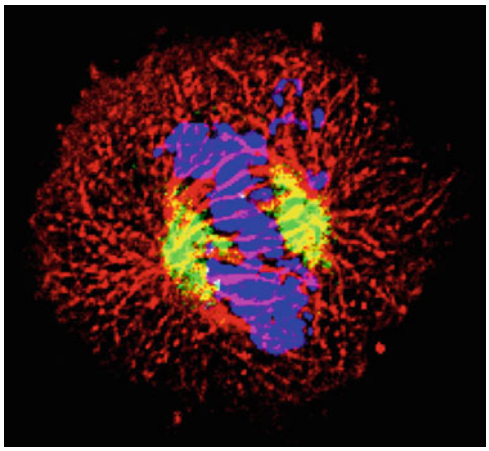


Ric-8, Fig. 5 *Drosophila* Gαi was mis-localized and not expressed on the plasma membrane when RIC-8 was P-element disrupted. Epithelial cells from (**a**) wild-type or (**b**) ric-8 mutant *Drosophila* embryos were stained with an anti-Gαi antibody (*Green*) and a DNA stain (*Red*) (Adapted by permission from Macmillan Publishers Ltd: Nature Cell Biology, (28). 2005)

in mitotic ric-8 mutant embryos (Afshar et al. 2005). More recently, RNAi-mediated reduction of mammalian Ric-8B resulted in reduced Gαs expression, and Ric-8B overexpression protected Gαs from ubiquitin-mediated degradation (Nagai et al. 2010). The provocative hypothesis arising from these findings is that the function of Ric-8 protein(s) may be to serve as key factors required for G protein subunit biosynthesis, or trafficking to, or retention at the plasma membrane. It is known that cytosolic G proteins are less stable than membrane-bound G proteins, so a shift of G proteins from the membrane to the cytosol in the absence of Ric-8 would be realized as a reduction in G protein steady-state expression levels. Aside from the finding that Ric-8B overexpression reduced levels of ubiquitinated Gαs, there is little understanding of the mechanism by which Ric-8-proteins mediate G protein membrane localization.

Summary

The hypothesis that Ric-8 proteins are required for efficient G protein membrane expression provides a potential explanation, in part, for the findings that Ric-8 proteins seem to exert affects upstream of divergent G protein signaling pathways (i.e., Gαq, synaptic transmission, Gαi, cell division, Gαolf olfaction, etc.). The one observation that cannot be explained intuitively by the idea that Ric-8 proteins are simply factors required for G protein expression is that Ric-8(A) is a mitotic spindle (pole) binding protein (Fig. 6 and Woodard et al. 2010; Hess et al. 2004). This result suggests that Ric-8A might participate as



Ric-8, Fig. 6 Ric-8A localizes proximally to the mitotic spindle poles. HeLa cells were transfected with YFP-Ric-8A (*Yellow-green*) and stained with DAPI (*Blue*) and by immunofluorescence with anti-α-tubulin monoclonal antibody (*Sigma*) and an anti-mouse Alexafluor-568 antibody. The tricolor images were captured using a Zeiss epifluorescence microscope with appropriate filters and subjected to nearest-neighbors deconvolution using Slidebook 4.0 software (unpublished observations)

a non-receptor GEF to activate intracellular Gαi-class G proteins that are localized on mitotic structures, including the mitotic spindle.

There are multiple regulatory points where Ric-8 proteins could act to promote or stabilize G protein membrane expression. Ric-8 proteins might act during G protein biosynthesis in positive fashion to promote stable membrane expression, or they could exert a protective role upon mature membrane–bound G proteins as these G proteins cycle between the plasma membrane and endo-membranes. Biosynthetic (forward) trafficking of G proteins to the plasma membrane requires two basic events: (1) G protein heterotrimer assembly on the endoplasmic reticulum (ER) and/or Golgi and (2) covalent attachment of lipid moieties to Gα and Gγ subunits (Marrari et al. 2007). Chaperone proteins including phosducin-like protein-1 (PhLP) and DRIP78 bind nascent Gβ and Gγ proteins, respectively, prior to Gβγ dimer formation (Dupre et al. 2007; Lukov et al. 2005). No component is known that binds nascent Gα after its synthesis, and aids its assembly into G protein trimers. Ric-8 proteins could fulfill such a role. In the absence of Ric-8 expression, Gα subunits not assembled into trimers would not reach the plasma membrane and would be more susceptible to degradation.

Ric-8 proteins could also potentially function in a capacity to counteract G protein downregulation by promoting G protein subunit recycling to the membrane. Debate exists whether G protein subunits become "solubilized" from membranes or clustered into membrane micro-domains upon activation by hormone receptors. Ric-8A and Ric-8B reside predominantly in the cytosol. Perhaps Ric-8 proteins function to "scavenge" cytosolic Gα subunits to restore membrane association. Upon experimental perturbation of Ric-8 protein expression, Gα subunits might slowly "leach off" the membrane over time due to tonic GPCR stimulation. If the pace of Gα biosynthesis does not keep up, this would be realized as a reduction in steady-state G protein expression. G proteins also cycle between the plasma membrane and Golgi membranes, perhaps as a means to receive reversible palmitoylation at the Golgi. Heterotrimeric G protein retrograde movement to the Golgi occurs at a rate faster than that predicted of a vesicular-transport-mediated event ($t_{1/2}$ <1 min) (Chisari et al. 2007; Tsutsumi et al. 2009). As such, G proteins were predicted to transit diffusively through the cytosol to the Golgi. It is not entirely clear whether G proteins transit as heterotrimers, or as free Gα or Gβγ species. If the latter, one could easily envision that an escort factor would be required to transit Gα (and Gβγ) to the proper intracellular membranes lest it signal inappropriately during transport. Small G proteins including Ras and Rab homologues share this type of membrane cycling principle. Rab GTPases utilize a combination of soluble GDI, GEF, and "escort" factors to shuttle unlipidated Rabs throughout the cell, present Rabs to the lipidation machinery, and target the lipidated Rabs to the correct cellular membrane compartment (Ali and Seabra 2005).

Given the known in vitro biochemical function of Ric-8 proteins as soluble G protein α subunit GEFs, it is a challenge, although conceivable, to envision a role for Ric-8 protein involvement in any one of these G protein subunit trafficking or biosynthetic processes. In this context, Ric-8 "GEF activity" could serve simply as a mechanism to regulate Gα:Ric-8 binding/dissociation. In this highly prospective role, Ric-8 would bind Gα subunits (either nascent chains, or mature membrane Gα) and traffic/escort the Ric-8:Gα nucleotide-free complex to the proper destination (the Golgi, or Gβγ on the ER or plasma membrane). At the destination, Ric-8 would be

invoked to stimulate GTP binding to Gα and release an activated-Gα species. Gα would hydrolyze its bound GTP, return to the inactive state, and reassociate with Gβγ or GoLoco.

References

Afshar K, Willard FS, Colombo K, Siderovski DP, Gonczy P. Cortical localization of the Gα protein GPA-16 requires RIC-8 function during *C. elegans* asymmetric cell division. Development. 2005;132(20):4449–59.

Ali BR, Seabra MC. Targeting of Rab GTPases to cellular membranes. Biochem Soc Trans. 2005;33:652–6.

Blumer JB, Kuriyama R, Gettys TW, Lanier SM. The G-protein regulatory (GPR) motif-containing Leu-Gly-Asn-enriched protein (LGN) and Giα3 influence cortical positioning of the mitotic spindle poles at metaphase in symmetrically dividing mammalian cells. Eur J Cell Biol. 2006;85(12): 1233–40.

Chisari M, Saini DK, Kalyanaraman V, Gautam N. Shuttling of G Protein subunits between the plasma membrane and intracellular membranes. J Biol Chem. 2007;282(33): 24092–8.

Cho H, Kehrl JH. Localization of Giα proteins in the centrosomes and at the midbody: implication for their role in cell division. J Cell Biol. 2007;178(2):245–55.

Du Q, Macara IG. Mammalian pins is a conformational switch that links NuMA to heterotrimeric G proteins. Cell. 2004;119(4):503–16.

Du Q, Taylor L, Compton DA, Macara IG. LGN blocks the ability of NuMA to bind and stabilize microtubules: a mechanism for mitotic spindle assembly regulation. Curr Biol. 2002;12(22):1928–33.

Dupre DJ, Robitaille M, Richer M, Ethier N, Mamarbachi AM, Hebert TE. Dopamine receptor-interacting protein 78 acts as a molecular chaperone for Gγ subunits before assembly with Gβ. J Biol Chem. 2007;282(18):13703–15.

Hampoelz B, Hoeller O, Bowman SK, Dunican D, Knoblich JA. Drosophila Ric-8 is essential for plasma-membrane localization of heterotrimeric G proteins. Nat Cell Biol. 2005;7(11):1099–105.

Hess HA, Roper J-C, Grill SW, Koelle MR. RGS-7 completes a receptor-independent heterotrimeric G protein cycle to asymmetrically regulate mitotic spindle positioning in *C. elegans*. Cell. 2004;119(2):209–18.

Kerr DS, Von Dannecker LEC, Davalos M, Michaloski JS, Malnic B. Ric-8B interacts with Gαolf and Gγ13 and colocalizes with Gαolf, Gβ1 and Gγ13 in the cilia of olfactory sensory neurons. Mol Cell Neurosci. 2008;38:341–8.

Klattenhoff C, Montecino M, Soto X, Guzmen L, Romo X, de los Angeles Garcia M, et al. Human brain synembryn interacts with Gsα and Gqα and is translocated to the plasma membrane in response to isoproterenol and carbachol. J Cell Physiol. 2003;195(2):151–7.

Lukov GL, Hu T, McLaughlin JN, Hamm HE, Willardson BM. Phosducin-like protein acts as a molecular chaperone for G protein βγ dimer assembly. EMBO J. 2005;24(11):1965–75 [10.1038/sj.emboj.7600673].

Marrari Y, Crouthamel M, Irannejad R, Wedegaertner PB. Assembly and trafficking of heterotrimeric G proteins. Biochemistry. 2007;46(26):7665–77 [doi: 10.1021/bi700338m].

Matsuzaki F. Drosophila G-protein signaling: intricate roles for Ric-8? Nat Cell Biol. 2005;7(11):1047–9 [10.1038/ncb1105-1047].

Miller KG, Rand JB. A role for RIC-8 (Synembryn) and GOA-1 (Goα) in regulating a subset of centrosome movements during early embryogenesis in *Caenorhabditis elegans*. Genetics. 2000b;156(4):1649–60.

Miller KG, Alfonso A, Nguyen M, Crowell JA, Johnson CD, Rand JB. A genetic selection for *Caenorhabditis elegans* synaptic transmission mutants. Proc Natl Acad Sci. 1996;93(22):12593–8.

Miller KG, Emerson MD, McManus JR, Rand JB. RIC-8 (Synembryn): a novel conserved protein that is required for Gqα signaling in the *C. elegans* nervous system. Neuron. 2000a;27:289–99.

Nagai Y, Nishimura A, Tago K, Mizuno N, Itoh H. Ric-8B stabilizes the alpha subunit of stimulatory G protein by inhibiting its ubiquitination. J Biol Chem. 2010;285(15):11114–20.

Reynolds NK, Schade MA, Miller KG. Convergent, RIC-8-dependent Gα signaling pathways in the *Caenorhabditis elegans* synaptic signaling network. Genetics. 2005;169(2):651–70.

Romo Ximena, Pasten P, MartÌnez S, Soto X, Lara P, Arellano ARd, et al. xRic-8 is a GEF for Gsα and participates in maintaining meiotic arrest in *Xenopus laevis* oocytes. J Cell Physiol. 2008;214(3):673–80.

Schade MA, Reynolds NK, Dollins CM, Miller KG. Mutations that rescue the paralysis of *Caenorhabditis elegans* ric-8 (Synembryn) mutants activate the Gαs pathway and define a third major branch of the synaptic signaling network. Genetics. 2005;169(2):631–49.

Siderovski DP, Willard FS. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci. 2005;1(2):51–66.

Siller KH, Doe CQ. Spindle orientation during asymmetric cell division. Nat Cell Biol. 2009;11(4):365–74 [10.1038/ncb0409-365].

Tall GG, Gilman AG. Resistance to inhibitors of cholinesterase 8A catalyzes release of Gαi-GTP and nuclear mitotic apparatus protein (NuMA) from NuMA/LGN/Gαi-GDP complexes. Proc Natl Acad Sci. 2005;102(46):16584–9.

Tall GG, Krumins AM, Gilman AG. Mammalian Ric-8A (Synembryn) is a heterotrimeric Gα protein guanine nucleotide exchange factor. J Biol Chem. 2003;278(10):8356–62.

Thomas CJ, Tall GG, Adhikari A, Sprang SR. RIC-8A catalyzes guanine nucleotide exchange on Gαi 1 bound to the GPR/GoLoco exchange inhibitor AGS3. J Biol Chem. 2008;2008: M802422200.

Tsutsumi R, Fukata Y, Noritake J, Iwanaga T, Perez F, Fukata M. Identification of G protein α subunit-palmitoylating enzyme. Mol Cell Biol. 2009;29(2):435–47.

Von Dannecker LEC, Mercadante AF, Malnic B. Ric-8B promotes functional expression of odorant receptors. PNAS. 2006;103(24):9310–4.

Wilkie TM, Kinch L. New roles for Gα and RGS proteins: communication continues despite pulling sisters apart. Curr Biol. 2005;15:R843–954.

Woodard GE, Huang N-N, Cho H, Miki T, Tall GG, Kehrl JH. Ric-8A and Giα recruit LGN, NuMA, and Dynein to the cell cortex to help orient the mitotic spindle. Mol Cell Biol. 2010;30(14):3519–30.
Yu F, Kuo CT, Jan YN. Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. Neuron. 2006;51(1):13–20.
Zhuang H, Matsunami H. Synergism of accessory factors in functional expression of mammalian odorant receptors. J Biol Chem. 2007;282:15284–93.

Ric-8A

▶ Ric-8

Ric-8B

▶ Ric-8

Rin (Ras-Like Protein in Neurons)

Weikang Cai, Jennifer L. Rudolph and Douglas A. Andres
Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY, USA

Synonyms

Small G-protein; Small GTPase; Small GTP-binding protein

Historical Background

Ras superfamily small GTP (guanosine triphosphate)-binding proteins function as molecular switches, responding to extra- and intracellular stimuli to control the activity of diverse signaling cascades. To date, over 150 different small GTPases have been identified and are classified into six distinct subfamilies: Ras (Rat sarcoma), Rho (Ras homolog gene family), Rab (Ras-related GTP-binding protein), ARF (ADP-ribosylation factor), Ran (Ras-related nuclear protein), and RGK (Rad/Gem/Kir family), based upon both sequence homology and the regulation of common cellular functions (Colicelli 2004). Rin (Ras-like protein in neurons), along with Rit (Ras-like protein in many tissues) and *Drosophila* Ric (Ras-related protein which interacted with calmodulin), comprise the Rit subfamily of Ras-related small GTPases (Lee et al. 1996). Rin is expressed exclusively within neurons and has been characterized as regulating neuronal differentiation by controlling distinct signaling cascades (Lee et al. 1996; Shi et al. 2005, 2008; Spencer et al. 2002). Rin contributes to nerve growth factor (NGF)-mediated neurite outgrowth via ERK (extracellular signal regulated MAP kinase) and p38 MAP kinase pathways (Shi et al. 2005), whereas in pituitary adenylate cyclase-activating polypeptide 38 (PACAP38)-mediated neuronal differentiation, Rin controls the cAMP (cyclic adenosine monophosphate) /PKA (protein kinase A) signaling cascade (Shi et al. 2008). In addition, Rin is involved in Brn-3a and Plexin signaling (Calissano and Latchman 2003; Hartwig et al. 2005). Rin also associates with the polarity protein PAR6 and has been found to display modest transforming ability (Hoshino et al. 2005).

Biochemical Characterization of Rin GTPase

Rin was originally isolated from mouse retina, and both Northern analysis and in situ hybridization studies indicated that it is expressed solely in neurons (Lee et al. 1996). Rin possesses most of the common characteristics of Ras subfamily members, including a high degree of amino acid conservation within the five Ras core regions (the G1-G5 domains) which are essential for GTP binding and hydrolysis (Colicelli 2004). However, Rin also contains a number of unique features including calmodulin (CaM) binding (Harrison et al. 2005), the lack of prenylation consensus sites or other lipidation signals within the C-terminus, and an effector domain that differs from other Ras subfamily proteins (Lee et al. 1996). These features have generated speculation that Rin might regulate important aspects of neuronal growth and differentiation (Hoshino and Nakamura 2003; Shi et al. 2005, 2008; Spencer et al. 2002).

Recombinant Rin has been shown to specifically bind guanine nucleotides (guanosine triphosphate or guanosine diphosphate, GTP/GDP) in the presence of

Mg^{2+} and to exhibit low intrinsic GTPase activity (Shao et al. 1999). Surprisingly, the guanine nucleotide dissociation rates for purified Rin protein are significantly different when compared with the majority of Ras-related GTPases. Rin displays higher k_{off} values for GTP than GDP (Shao et al. 1999). These GTP dissociation rates are five- to tenfold faster than most Ras-like GTPases. Since the cellular concentration of GTP is much higher than that of GDP, these biochemical studies suggest that a relatively high percentage of Rin may remain in the GTP-bound state under basal conditions. Despite these unique biochemical properties, the available data indicate that Rin functions as a nucleotide-dependent molecular switch, and as predicted, only a very low in vivo level of GTP-bound Rin is found in unstimulated cells (Shi et al. 2008; Spencer et al. 2002). Therefore, these in vitro studies do not appear to accurately represent Rin activity in the normal cellular environment. It is possible that the presence of cellular regulatory proteins contribute to these differences.

Rin Cellular Distribution and Trafficking

For the majority of Ras-related GTPases, association with specific cellular membranes is essential for their biological activities (Colicelli 2004). Conserved C-terminal cysteine-rich motifs are used to direct covalent modification by isoprenoid lipids (prenylation). Prenylation is the initial step in the attachment of these proteins to the cytoplasmic leaflets of a variety of cellular organelles. However, specific membrane localization often requires additional targeting signals, provided either by a cluster of basic amino acids or the palmitoylation of internal cysteine residues (Heo et al. 2006). For example, adjacent to the CAAX prenylation motif ("C" is Cysteine, "A" is an aliphatic amino acid, and "X" is variable), H-Ras and N-Ras have a cysteine residue for palmitoylation, directing the protein to the plasma membrane via the Golgi apparatus. In contrast, K-Ras4A has a lysine-rich polybasic motif, which leads the protein to the plasma membrane more rapidly through a Golgi-independent route (Colicelli 2004).

Surprisingly, analysis of an over-expressed GFP (green fluorescent protein)-tagged protein indicates that Rin primarily localizes to the nucleus, although a detectable amount of GFP protein is also found at the plasma membrane in these studies (Heo et al. 2006). Investigation of the Rin C-terminus has provided key insights into this unique subcellular distribution. Distinct from the majority of G-proteins, the Rit and RGK family GTPases lack a CAAX motif, and instead contain a polybasic cluster at the C-terminus (Heo et al. 2006). Interestingly, sequence analysis of the Rin polybasic motif reveals a canonical nuclear localization signal (NLS) (K-K/R-x-K/R). Additionally, Heo et al. have demonstrated the importance of the polybasic C-terminal region in targeting the protein to the plasma membrane via interactions with phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$) lipids (Heo et al. 2006). Thus, Rin appears capable of shuttling between the nucleus and plasma membrane in a PIP-lipid dependent fashion, allowing for a novel cellular trafficking pattern, which may contribute to the physiological function of Rin.

The C-terminus of Rin has also been reported to direct Ca^{2+}-dependent interactions with calmodulin (CaM) (Hoshino and Nakamura 2002; Lee et al. 1996). Although CaM binding has been shown to be important for neurite outgrowth mediated by Rin (Hoshino and Nakamura 2003), the exact signaling mechanism as well as the physiological significance of CaM binding still needs to be elucidated. The Rin homolog *Drosophila* Ric was originally identified as a CaM binding protein, and genetic studies suggest that CaM association contributes to the regulation of Ric signaling (Harrison et al. 2005).

Regulation of Rin Activity

Like most Ras GTPases, Rin is activated by exchanging GDP with GTP in the nucleotide binding pocket, which results in conformational changes that expose the effector domain and promote the recruitment of proteins responsible for signal transduction cascade activation. This classic GTPase cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which are responsible for the release of bound GDP and the induction of intrinsic GTPase activity, respectively. However, to date no Rin-specific GEFs or GAPs have been identified.

Several Rin point mutations have been reported to block intrinsic GTPase activity or GTP/GDP

exchange, leading to a protein locked in the active or inactive state, respectively. Mutation of Gln78 to Leu (Rin^{Q78L}), equivalent to the oncogenic Ras^{Q61L} mutation, causes complete inhibition of GTP hydrolysis, resulting in a constitutively active Rin mutant. Meanwhile, the Rin^{S34N} mutation, equivalent to dominant-negative Ras^{S17N}, is predominantly GDP-bound (Shao et al. 1999), and disrupts signal transduction when over-expressed (Shi et al. 2005). Both of these mutations have proved useful in the analysis of Rin GTPase function.

Rin Effectors

The G2 domain of Ras GTPases is known as the "effector domain" and serves as the primary region of interaction responsible for directing downstream effector protein binding following G-protein activation. Rin shares a conserved effector domain (HDPTIEDAY) with Rit, and this domain is evolutionarily conserved within the subfamily, as there is only a single amino acid substitution in the *Drosophila* Ric effector loop (HDPTIEDSY). In addition, the Rin effector domain shares seven out of nine residues with the Ras effector loop (YDPTIEDSY) (Shao et al. 1999). This high rate of conservation has led to the suggestion that Rin is likely to control distinct, but perhaps partially overlapping, downstream signaling pathways compared to Ras.

A variety of proteins have been implicated as candidate Rin effectors using yeast two-hybrid screens (Shao et al. 1999). These studies first identified the Ras-binding domain (RBD) from the Raf kinases as binding partners for constitutively active Rin^{Q78L}. Recent in vivo co-immunoprecipitation analyses in pheochromocytoma cells (PC6) support these initial findings by demonstrating that active Rin preferentially associates with B-Raf, suggesting that B-Raf functions as a valid downstream target of Rin (Shi et al. 2005). However, whether B-Raf directly binds Rin in vivo, thus acting as a true effector, has yet to be determined. Rin also interacts with the Ras interacting domains (RID) of two Ral exchange factors, RalGDS and RLF, in yeast two-hybrid screens suggesting that Rin may regulate Ral GTPase signaling (Shao et al. 1999), although this hypothesis has not been formally tested. While Rin appears capable of associating with a number of known Ras effectors, in agreement with Rin's distinct G2 domain sequence, not all known Ras effectors demonstrate Rin binding. For example, active Rin does not bind to RIN1 or p110, the catalytic subunit of PI3K (Shao et al. 1999). More interestingly, Rin directly associates with the PDZ domain of PAR6, a cell polarity-regulating molecule, in a GTP-dependent fashion. This interaction requires an intact Rin G2 effector domain, suggesting that PAR6 may represent the first authentic Rin effector that does not also associate with Ras (Hoshino et al. 2005).

The identification of effectors for Ras-like small GTPases is not only important for understanding their physiological functions, but the minimum GTPase binding domain of select effector proteins have been adapted for use in pull-down activity assays. In the case of Rin, both the Raf-RBD and the RalGDS-RID have been used to examine in vivo Rin activity (Hoshino and Nakamura 2002; Spencer et al. 2002).

Functions of Rin GTPase

Rin can be activated by a variety of extracellular stimuli, including NGF, epidermal growth factor (EGF), ionomycin, and tocopherol acetate (Hoshino and Nakamura 2002; Spencer et al. 2002), suggesting that Rin controls the activation of a variety of signaling pathways and cellular functions. Indeed, a significant amount of work has been performed to understand the physiological role of Rin GTPase-mediated signaling.

Hoshino et al. first demonstrated that GTP-bound Rin associated with the PDZ domain of PAR6 (Hoshino et al. 2005), which has previously been shown to bind the GTP-bound active forms of the Rho family GTPases Rac and Cdc42 (Qiu et al. 2000). In contrast, despite the high homology to Rin, Rit associates in a GTP-independent fashion with PAR6C, one of the three PAR6 isoforms, which is preferentially expressed in the brain. Unlike Rin/PAR6 binding, the Rit/PAR6C interaction does not require an intact PAR6C PDZ domain (Rudolph et al. 2007). These differences suggest distinct mechanisms in Rit/Rin-mediated PAR6 signaling. More importantly, these same studies suggested that Rin can promote the formation of a larger Rin-PAR6-Rac/Cdc42 ternary complex scaffolded by PAR6 (Hoshino et al. 2005). Enlightened by the role of Rac/Cdc42 in tumorigenesis, Hoshino and colleagues have further explored the role of Rin in cell transformation and

demonstrated that the Rin-PAR6-Rac/Cdc42 complex was capable of enhancing the formation of foci in NIH-3T3 cells (Hoshino et al. 2005). Although the lack of endogenous Rin expression in fibroblasts complicates the interpretation of this study, these data suggest that Rin may function in tumorigenesis. Moreover, since PAR6 is known to contribute to the regulation of axon specification (Shi et al. 2003), it is possible that the Rin-PAR6-Rac/Cdc42 complex plays a pivotal role in axonal/dendritic growth.

The exclusive expression of Rin in neurons indicates a critical role for Rin in the central nervous system. Indeed, over-expression of a constitutively active Rin mutant alone is capable of inducing neurite outgrowth in PC6 cells (Hoshino and Nakamura 2003; Shi et al. 2005), suggesting a physiological role for Rin signaling in neuronal differentiation and development. shRNA-mediated knockdown of Rin dramatically reduces NGF-mediated neurite outgrowth in PC6 cells, suggesting that Rin is a pivotal component of NGF signaling by regulating both ERK and p38 MAPK pathways (Shi et al. 2005). Further detailed mechanistic studies have revealed that Rin activates ERK through B-Raf. Rin selectively regulates the p38α MAPK isoform in vitro; however, the mechanism of Rin-mediated p38 activation has yet to be determined (Shi et al. 2005). The identification of a Rin-PAR6-Rac/Cdc42 complex suggests a putative pathway (Hoshino et al. 2005), since Rac and Cdc42 are known to serve as upstream regulators of p38 MAPK signaling (Shin et al. 2005).

PACAP38 potently induces neuronal differentiation through the activation of PACR1, a heterotrimeric G-protein-coupled receptor (GPCR). Rin is activated downstream of PACAP38 and appears to play a critical role in PACAP38-mediated neuronal differentiation. Typically, PACAP38-mediated PACR1 activation results in stimulation of a $G\alpha_s$-adenylate cyclase (AC)-cAMP signaling cascade. However, PACAP38-dependent Rin activation is mediated by Src downstream of $G\alpha_{s/i}$ and functions upstream of the cAMP/PKA signaling cascade, which in turn regulates heat shock protein 27 (HSP27) phosphorylation (Shi et al. 2008). HSP27 serves as a chaperone to facilitate correct protein folding, has been reported to regulate cytoskeleton stability, and has known roles in neuronal morphology and survival signaling (Stetler et al. 2009). Indeed, RNAi-mediated silencing of HSP27 is sufficient to block PACAP38- and Rin^{Q78L}-induced neurite outgrowth (Shi et al. 2008), further supporting the notion that HSP27 serves as a critical downstream target of Rin in neuronal differentiation.

Rin has also been found to associate with the intracellular domain of Plexin B3, a member of the Plexin family of semaphorin receptors (Hartwig et al. 2005). Semaphorins are secreted or membrane-bound proteins that provide critical guidance signals to redirect or inhibit axonal growth (Vanderhaeghen and Cheng 2010). Given the ability of Rin to promote neurite outgrowth, an intriguing possibility is that Rin signaling contributes to Plexin B3-mediated axonal extension/retraction. Indeed, Plexin B1 has been shown to function as a GAP, down-regulating R-Ras GTPase activity to induce axonal retraction (Oinuma et al. 2004). Despite the report of the direct interaction of Rin with Plexin B3, it remains to be determined whether Plexin B3 functions as a RinGAP.

Calissano et al. used a yeast two-hybrid screen to identify a putative interaction between Rin and the N-terminus of Brn-3a, a transcriptional factor widely expressed in the peripheral nervous system. Surprisingly, Brn-3a preferentially associated with GDP-bound Rin (Calissano and Latchman 2003), and only GDP-bound Rin was shown to induce Brn-3a-mediated gene transcription. However, unstimulated neurons contain high basal levels of GDP-Rin, and additional studies are needed to determine whether transient in vivo fluctuations in GDP-Rin levels contribute to Brn-3a-mediated neuronal gene transcription.

Summary

To our knowledge, Rin is the only Ras subfamily GTPase to be expressed exclusively in neurons. In keeping with this unique expression pattern, in vitro studies indicate that Rin serves as a critical mediator of pheochromocytoma cell neurite outgrowth, acting to couple diverse extracellular stimuli (e.g., NGF, PACAP38) to the activation of ERK and p38 MAPK pathways, cAMP/PKA cascade signaling, and the PAR6-Rac/Cdc42 polarity pathway. In addition, preliminary reports suggest that Rin contributes to both Plexin- and Brn-3a-mediated neuronal differentiation. Despite this progress, much remains to be elucidated concerning the molecular mechanisms that govern these diverse actions. To date, our understanding of Rin function comes from

over-expression and RNAi silencing studies in mammalian cell lines, and future studies must investigate the neuronal effects of Rin ablation making use of genetically engineered Rin null animals. A deeper understanding of in vivo Rin regulation, together with the identification of additional Rin effector proteins, will be critical to define the physiological function of this novel neuronal regulatory protein.

Acknowledgments This work was supported by Public Health Service grant NS045103 from the National Institute of Neurological Disorders and Stroke and 2P20 RR020171 from the National Center for Research Resources.

References

Calissano M, Latchman DS. Functional interaction between the small GTP-binding protein Rin and the N-terminal of Brn-3a transcription factor. Oncogene. 2003;22(35):5408–14.

Colicelli J. Human RAS superfamily proteins and related GTPases. Sci STKE. 2004;2004(250):RE13.

Harrison SM, Rudolph JL, Spencer ML, Wes PD, Montell C, Andres DA, et al. Activated RIC, a small GTPase, genetically interacts with the Ras pathway and calmodulin during Drosophila development. Dev Dyn. 2005;232(3):817–26.

Hartwig C, Veske A, Krejcova S, Rosenberger G, Finckh U. Plexin B3 promotes neurite outgrowth, interacts homophilically, and interacts with Rin. BMC Neurosci. 2005;6:53.

Heo WD, Inoue T, Park WS, Kim ML, Park BO, Wandless TJ, et al. PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane. Science. 2006;314(5804):1458–61.

Hoshino M, Nakamura S. The Ras-like small GTP-binding protein Rin is activated by growth factor stimulation. Biochem Biophys Res Commun. 2002;295(3):651–6.

Hoshino M, Nakamura S. Small GTPase Rin induces neurite outgrowth through Rac/Cdc42 and calmodulin in PC12 cells. J Cell Biol. 2003;163(5):1067–76.

Hoshino M, Yoshimori T, Nakamura S. Small GTPase proteins Rin and Rit Bind to PAR6 GTP-dependently and regulate cell transformation. J Biol Chem. 2005;280(24):22868–74.

Lee CH, Della NG, Chew CE, Zack DJ. Rin, a neuron-specific and calmodulin-binding small G-protein, and Rit define a novel subfamily of ras proteins. J Neurosci. 1996;16(21):6784–94.

Oinuma I, Ishikawa Y, Katoh H, Negishi M. The semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. Science. 2004;305(5685):862–5.

Qiu RG, Abo A, Steven Martin G. A human homolog of the *C. elegans* polarity determinant Par-6 links Rac and Cdc42 to PKCzeta signaling and cell transformation. Curr Biol. 2000;10(12):697–707.

Rudolph JL, Shi GX, Erdogan E, Fields AP, Andres DA. Rit mutants confirm role of MEK/ERK signaling in neuronal differentiation and reveal novel Par6 interaction. Biochim Biophys Acta. 2007;1773(12):1793–800.

Shao H, Kadono-Okuda K, Finlin BS, Andres DA. Biochemical characterization of the Ras-related GTPases Rit and Rin. Arch Biochem Biophys. 1999;371(2):207–19.

Shi SH, Jan LY, Jan YN. Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. Cell. 2003;112(1):63–75.

Shi GX, Han J, Andres DA. Rin GTPase couples nerve growth factor signaling to p38 and b-Raf/ERK pathways to promote neuronal differentiation. J Biol Chem. 2005;280(45):37599–609.

Shi GX, Jin L, Andres DA. Pituitary adenylate cyclase-activating polypeptide 38-mediated Rin activation requires Src and contributes to the regulation of HSP27 signaling during neuronal differentiation. Mol Cell Biol. 2008;28(16):4940–51.

Shin I, Kim S, Song H, Kim HR, Moon A. H-Ras-specific activation of Rac-MKK3/6-p38 pathway: its critical role in invasion and migration of breast epithelial cells. J Biol Chem. 2005;280(15):14675–83.

Spencer ML, Shao H, Tucker HM, Andres DA. Nerve growth factor-dependent activation of the small GTPase Rin. J Biol Chem. 2002;277(20):17605–15.

Stetler RA, Gao Y, Signore AP, Cao G, Chen J. HSP27: mechanisms of cellular protection against neuronal injury. Curr Mol Med. 2009;9(7):863–72.

Vanderhaeghen P, Cheng HJ. Guidance molecules in axon pruning and cell death. Cold Spring Harb Perspect Biol. 2010;2(6):a001859.

RIN = *R*as and Rab *IN*teractor

► RIN Family Proteins (RIN1, RIN2, and RIN3)

RIN Family Proteins (RIN1, RIN2, and RIN3)

John Colicelli, Pamela Y. Ting and Christine Janson
Department of Biological Chemistry, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Synonyms

RIN = *R*as and Rab *IN*teractor

Historical Background

The RIN1 and RIN2 genes were first identified in a selection for expressed human cDNAs that suppress

phenotypes associated with oncogenic RAS mutations in a yeast model system (Colicelli et al. 1991). Subsequent analysis demonstrated that RIN1 binds specifically to activated (GTP-bound) human HRAS (Han and Colicelli 1995; Han et al. 1997), suggesting that RIN1 is a downstream effector of RAS family proteins. RIN2 and RIN3 also bind to activated HRAS (Rodriguez-Viciana et al. 2004). All three RIN proteins contain a carboxy terminal RAS association (RA) domain that mediates this interaction (Colicelli (2004)).

It was subsequently reported that RIN1 encodes a VPS9-related guanine nucleotide exchange factor (GEF) function specific for the RAB5 family of early endocytosis GTPase (Tall et al. 2001). RIN2 and RIN3 also encode VPS9-type GEF domains upstream of the RA domain. This suggests that RIN family proteins are RAS effectors that promote RAB5 function, connecting RAS activation to receptor endocytosis.

Another defining feature of RIN proteins is their amino terminal SRC homology 2 (SH2) domain. The RIN1 SH2 domain mediates binding to activated receptor tyrosine kinases (Barbieri et al. 2003), which likely facilitates endocytosis and downregulation of these receptors. The RIN2 and RIN3 SH2 domains show some species variation (discussed below).

RIN1 also binds and activates ABL1 and ABL2 tyrosine kinases (Cao et al. 2008; Hu et al. 2005). This capacity requires an amino terminal fragment of RIN1, which may not be functionally conserved in RIN2 and RIN3.

RIN Family: Evolution and General Properties

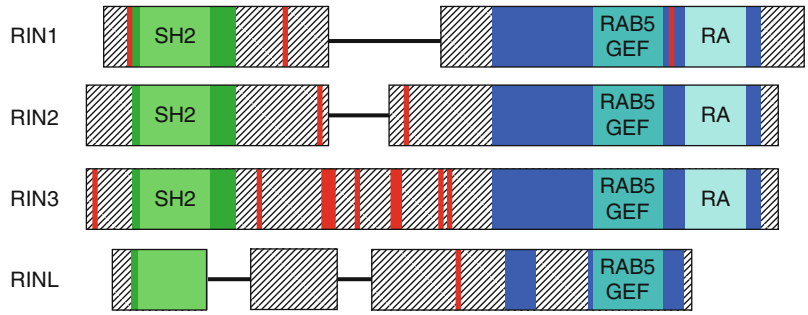
The RIN family of genes likely evolved from a progenitor represented in arthropods by the fruit fly *Drosophila melanogaster* gene Sprint (Szabo et al. 2001) and in echinoderms by the sea urchin *Strongylocentrotus purpuratus* gene annotated as Rin2L (www.spbase.org). The fruit fly and sea urchin gene products show strong conservation with vertebrate RIN proteins in the amino terminal SH2 domain and the carboxy terminal GEF domain. However, the fruit fly and sea urchin RIN proteins show no significant alignment with vertebrate RIN proteins in the central region or in the RA domain. This suggests that the gene expansion that gave rise to a RIN family in vertebrates was accompanied by the development of RAS effector capability. A recently described vertebrate RINL gene (Woller et al. 2011) encodes a protein that aligns with RIN1-3 but has no RA domain (Fig. 1), and may represent the vestige of a pre-vertebrate RIN gene.

Specific properties of each are described in the following sections.

RIN1

RIN1, the defining member of the RIN protein family, is a RAS effector that couples cell signaling with receptor trafficking and cytoskeletal remodeling. It is localized to the cytoplasm and the plasma membrane. Binding of RIN1 to RAS is GTP-dependent and mediated by the carboxy-terminal region of RIN1 (Han et al. 1997). RIN1 overexpression suppresses fibroblast transformation by $HRAS^{G12V}$, likely by competing with RAS effectors that promote mitosis and suppress apoptosis (Wang et al. 2002). RIN1 localizes in cytoplasmic and membrane compartments, and this partition is regulated through a 14-3-3 interaction. Phosphorylation of RIN1-Ser^{351} by ▶ PKD enhances 14-3-3 binding, and mutation of this residue shifts RIN1 localization to the plasma membrane (Wang et al. 2002). This result suggests that 14-3-3 binding reduces access to RAS proteins, which are membrane tethered.

RIN1 encodes a VPS9 subfamily GEF domain that mediates endosome fusion and receptor endocytosis through activation of RAB5 family proteins (Tall et al. 2001). RIN1 preferential associates with and activates the RAB5A isoform (Chen et al. 2009). The SH2 domain of RIN1 binds to activated (tyrosine phosphorylated) EGFR to promote receptor downregulation (Barbieri et al. 2003). Through a proline-rich domain, RIN1 may recruit STAM2, a component of the ESCRT (endosomal sorting complex required for transport) machinery, and facilitate trafficking of ubiquitinated EGFR to lysosomes (Kong et al. 2007). RIN1 also promotes internalization of the TGF-β receptor through RAB5 activation. In this case, however, the result is increased signaling through SMAD2/3 and the transcription repressor SNAI1 (Hu et al. 2008). The contribution of RIN1 to growth factor receptor internalization and signaling may account for the observed silencing of RIN1 in breast tumor cells (Milstein et al. 2007) and enhanced expression in non-small cell lung adenocarcinoma cells (Tomshine et al. 2009).



RIN Family Proteins (RIN1, RIN2, and RIN3), Fig 1 RIN Protein Family in Vertebrates. Conserved domains include SRC Homology 2 (SH2), Guanine nucleotide Exchange Factor (RAB5 GEF) and RAS Association (RA). Regions of sequence conservation are shown in green and blue. Lines indicate gaps in alignment. Red bars are used to denote proline-rich motifs common to SH3 ligands ([RKHYFW]xxPxxP and PxxPx[RK]) and WW domain ligands (PPxY and PPPPP)

Other downstream effectors of RIN1 are the ABL family non-receptor tyrosine kinases. The ABL SH3 domain mediates binding to a proline-rich motif in RIN1, leading to ABL-mediated phosphorylation of RIN1-Y^{36} and subsequent association with the ABL SH2 domain (Han et al. 1997; Afar et al. 1997). Binding of RIN1 to ABL relieves autoinhibition and stimulates ABL tyrosine kinase activity (Cao et al. 2008; Hu et al. 2005). As a positive regulator of ABL activity, RIN1 regulates actin remodeling. Mammary epithelial cells from $Rin1^{-/-}$ mice show extensive peripheral-actin networks, enhanced attachment to fibronectin, and increased cell motility (Hu et al. 2005). RIN1 potentiates the catalytic and transforming activity of the BCR-ABL1 fusion oncogene, and RIN1 over-expression increases BCR-ABL1 mediated leukemogenesis in a mouse model system (Afar et al. 1997). Importantly, the $ABL1^{T315I}$ mutant resistant to therapeutic kinase inhibitors remains responsive to positive regulation by RIN1 (Cao et al. 2008), and *Rin1*$^{-/-}$ bone marrow cells were refractory to transformation by BCR-$ABL1^{T315I}$ (Thai et al. 2011). These results suggest that the RIN1::ABL1 interaction may be a drugable vulnerability of oncogenic ABL fusion proteins.

Rin1 is most strongly expressed in mature forebrain neurons, with moderate expression in hematopoietic and epithelial cells (Dzudzor et al. 2010). This restricted expression of Rin1 is mediated in part by SNAI1. The Rin1 promoter sequence also contains a consensus recognition site for the transcription repressor REST. Deletion of this region surprisingly led to a reduction in reporter gene expression, suggesting that elements in this region enhance expression and may be positively regulating Rin1 expression in neuronal cells (Dzudzor et al. 2010).

$Rin1^{-/-}$ mice are viable and fertile and show no gross morphological abnormalities. However, they have elevated amygdala LTP (long-term potentiation) and enhanced fear conditioning, suggesting that Rin1 normally acts as a negative regulator of synaptic plasticity in this region (Dhaka et al. 2003). In addition, $Rin1^{-/-}$ mice are deficient in conditioned fear extinction and latent inhibition (Bliss et al. 2010). $Rin1^{-/-}$ mice have normal hippocampal-dependent learning, as well as normal motor learning, anxiety, and exploratory behavior, suggesting that $Rin1^{-/-}$ mice may be a useful model for studying neuropsychiatric conditions such as PTSD (post-traumatic stress disorder).

RIN2

The RIN2 gene is widely expressed in mouse, based on analysis of mRNA levels ((Kajiho et al. 2003), BioGPS.gnf.org). The SH2, GEF, and RA domains first characterized in RIN1 are well conserved in RIN2, which has demonstrable guanine nucleotide exchange activity on RAB5 (Saito et al. 2002) and RAS interaction properties (Rodriguez-Viciana et al. 2004). However, RIN2 gene products in primates (human and chimpanzee) differ from their orthologs in other vertebrates (cow, dog, mouse, opossum, chicken, frog, and fish) in two notable aspects. First, the amino termini of primate RIN2 proteins extend about 50 residues beyond other vertebrate RIN2 gene products. Second, an arginine residue critical for the phosphotyrosine binding function of SH2 domains (mouse Rin2: $FLVR^{122}$) is instead a histidine in primate RIN2 (human RIN2: $FLVH^{171}$).

Loss of function mutations in RIN2 are associated with two related human connective tissue disorders referred to as MACS (Basel-Vanagaite et al. 2009) and RIN2 Syndrome (Syx et al. 2010).

RIN3

RIN3 was first described as a novel RAB5 guanine nucleotide exchange factor, isolated from a human leukocyte cDNA library based on a yeast two-hybrid screen for $RAB5B^{Q79L}$ interacting proteins (Kajiho et al. 2003).

RIN3 contains the GEF, RA, and SH2 domains conserved throughout the RIN gene family. It demonstrates guanine nucleotide exchange activity for RAB5 (Kajiho et al. 2003) and associates with activated RAS (Rodriguez-Viciana et al. 2004). The SH2 domain sequence of RIN3, like that of RIN2, shows a curious divergence at the FLVR motif. The sequences of most vertebrates, including human, encode the arginine residue critical for phosphotyrosine binding. But several species (pig, cow, cat, rat, and mouse) have a cysteine substitution at this key position, implying that RIN3 in these organisms may function somewhat differently.

RIN3 also associates with BIN1 (a.k.a. amphyphisin II), a membrane-bending protein involved in endocytosis (Kajiho et al. 2003). RIN3 can translocate to RAB5 positive endosomes and deletion of the RA domain caused RIN3 to be constitutively located to endocytic vesicles, suggesting that the RA domain has an autoinhibitory effect on RIN3's endosomal localization (Yoshikawa et al. 2008).

In mice, RIN3 expression is highly enriched in mast cells with lower expression levels in other hematopoietic tissues including lymph node, bone, and T cells (http://symatlas.gnf.org). Protein levels in established human cell lines also indicate enrichment in mast cells (CJ and JC, 2011).

Summary

RIN1-3 proteins connect RAS signal transduction with RAB5 activation and receptor endocytosis. RIN1 has a special role in ABL tyrosine kinase regulation, with a likely contribution to cytoskeleton remodeling. A mouse knockout model suggests RIN1 involvement in stimulation-induced signal transduction in multiple cell types. Mouse models of RIN2 and RIN3 deficiencies have not yet been described.

There are several outstanding questions to be answered regarding the biochemistry of RIN proteins. Particularly curious is a species-specific divergence in the SH2 domain arginine residue required for phosphotyrosine binding. Does this imply an alternate function for these SH2 domains? Further study is also needed to identify the full range of protein partners, and to determine the location and consequence of these interactions.

Evidence for the involvement of RIN proteins in human pathologies is just beginning to emerge. RIN1 appears to collaborate in tumorigenesis at multiple levels in ways that are complex and cell type specific. In addition, the fear learning and extinction phenotypes of $Rin1^{-/-}$ mice suggest that reduced RIN1 function in forebrain neurons could contribute to post-traumatic stress disorder. The correlation of RIN2 deficiency with connective tissue disorders provides another clear indication that RIN proteins play diverse and essential roles in human physiology.

References

Afar DE, Han L, McLaughlin J, Wong S, Dhaka A, Parmar K, et al. Regulation of the oncogenic activity of BCR-ABL by a tightly bound substrate protein RIN1. Immunity. 1997;6(6):773–82.

Barbieri MA, Kong C, Chen PI, Horazdovsky BF, Stahl PD. The SRC homology 2 domain of Rin1 mediates its binding to the epidermal growth factor receptor and regulates receptor endocytosis. J Biol Chem. 2003;278(34):32027–36.

Basel-Vanagaite L, Sarig O, Hershkovitz D, Fuchs-Telem D, Rapaport D, Gat A, et al. RIN2 deficiency results in macrocephaly, alopecia, cutis laxa, and scoliosis: MACS syndrome. Am J Hum Genet. 2009;85(2):254–63.

Bliss JM, Gray EE, Dhaka A, O'Dell TJ, Colicelli J. Fear learning and extinction are linked to neuronal plasticity through Rin1 signaling. J Neurosci Res. 2010;88(4):917–26.

Cao X, Tanis KQ, Koleske AJ, Colicelli J. Enhancement of ABL kinase catalytic efficiency by a direct binding regulator is independent of other regulatory mechanisms. J Biol Chem. 2008;283(46):31401–7.

Chen PI, Kong C, Su X, Stahl PD. Rab5 isoforms differentially regulate the trafficking and degradation of epidermal growth factor receptors. J Biol Chem. 2009;284(44):30328–38.

Colicelli J. Human RAS superfamily proteins and related GTPases. Sci STKE. 2004;2004(250):RE13.

Colicelli J, Nicolette C, Birchmeier C, Rodgers L, Riggs M, Wigler M. Expression of three mammalian cDNAs that interfere with RAS function in Saccharomyces cerevisiae. Proc Natl Acad Sci USA. 1991;88(7):2913–7.

Dhaka A, Costa RM, Hu H, Irvin DK, Patel A, Kornblum HI, et al. The Ras effector Rin1 modulates the formation of aversive memories. J Neuroscience. 2003;23:748–57.

Dzudzor B, Huynh L, Thai M, Bliss JM, Nagaoka Y, Wang Y, et al. Regulated expression of the Ras effector Rin1 in forebrain neurons. Mol Cell Neurosci. 2010;43(1):108–16.
Han L, Colicelli J. A human protein selected for interference with Ras function interacts directly with Ras and competes with Raf1. Mol Cell Biol. 1995;15(3):1318–23.
Han L, Wong D, Dhaka A, Afar D, White M, Xie W, et al. Protein binding and signaling properties of RIN1 suggest a unique effector function. Proc Natl Acad Sci USA. 1997;94(10):4954–9.
Hu H, Bliss JM, Wang Y, Colicelli J. RIN1 is an ABL tyrosine kinase activator and a regulator of epithelial-cell adhesion and migration. Curr Biol. 2005;15(9):815–23.
Hu H, Milstein M, Bliss JM, Thai M, Malhotra G, Colicelli J. Integration of TGFbeta and RAS signaling silences a RAB5 GEF and enhances growth factor-DIrected cell migration. Mol Cell Biol. 2008;28:1573–83.
Kajiho H, Saito K, Tsujita K, Kontani K, Araki Y, Kurosu H, et al. RIN3: a novel Rab5 GEF interacting with amphiphysin II involved in the early endocytic pathway. J Cell Sci. 2003;116(Pt 20):4159–68.
Kong C, Su X, Chen PI, Stahl PD. Rin1 interacts with signal-transducing adaptor molecule (STAM) and mediates epidermal growth factor receptor trafficking and degradation. J Biol Chem. 2007;282(20):15294–301.
Milstein M, Mooser CK, Patel A, Hu H, Fejzo M, Slamon DJ, et al. RIN1 is a breast tumor suppressor gene. Cancer Res. 2007;67:11510–6.
Rodriguez-Viciana P, Sabatier C, McCormick F. Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Mol Cell Biol. 2004;24(11):4943–54.
Saito K, Murai J, Kajiho H, Kontani K, Kurosu H, Katada T. A novel binding protein composed of homophilic tetramer exhibits unique properties for the small GTPase Rab5. J Biol Chem. 2002;277(5):3412–8.
Syx D, Malfait F, Van Laer L, Hellemans J, Hermanns-Le T, Willaert A, et al. The RIN2 syndrome: a new autosomal recessive connective tissue disorder caused by deficiency of Ras and Rab interactor 2 (RIN2). Hum Genet. 2010;128(1):79–88.
Szabo K, Jekely G, Rorth P. Cloning and expression of sprint, a Drosophila homologue of RIN1. Mech Dev. 2001; 101(1–2):259–62.
Tall GG, Barbieri MA, Stahl PD, Horazdovsky BF. Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev Cell. 2001;1:73–82.
Thai M, Ting PY, McLaughlin J, Cheng D, Muschen M, Witte ON, et al. ABL fusion oncogene transformation and inhibitor sensitivity are mediated by the cellular regulator RIN1. Leukemia. 2011;25(2):290–300.
Tomshine JC, Severson SR, Wigle DA, Sun Z, Beleford DA, Shridhar V, et al. Cell proliferation and epidermal growth factor signaling in non-small cell lung adenocarcinoma cell lines are dependent on Rin1. J Biol Chem. 2009; 284(39):26331–9.
Wang Y, Waldron RT, Dhaka A, Patel A, Riley MM, Rozengurt E, et al. The RAS effector RIN1 directly competes with RAF and is regulated by 14–3–3 proteins. Mol Cell Biol. 2002;22(3):916–26.
Woller B, Luiskandl S, Popovic M, Prieler BE, Ikoge G, Mutzl M, et al. Rin-like, a novel regulator of endocytosis, acts as guanine nucleotide exchange factor for Rab5a and Rab22. Biochim Biophys Acta. 2011;1813(6):1198–210.
Yoshikawa M, Kajiho H, Sakurai K, Minoda T, Nakagawa S, Kontani K, et al. Tyr-phosphorylation signals translocate RIN3, the small GTPase Rab5-GEF, to early endocytic vesicles. Biochem Biophys Res Commun. 2008;372(1): 168–72.

RING Finger Protein 85 (RNF85)

▶ TRAF6

RIP140 (Receptor-Interacting Protein 140)

▶ nrip1 (Nuclear Receptor-Interacting Protein 1)

RK

▶ G-Protein-Coupled Receptor Kinase 1 (GRK1)

RLPK

▶ MSK1

RLSK

▶ MSK1

ROCK I

▶ ROCK Kinases

ROCK II

▶ ROCK Kinases

ROCK Kinases

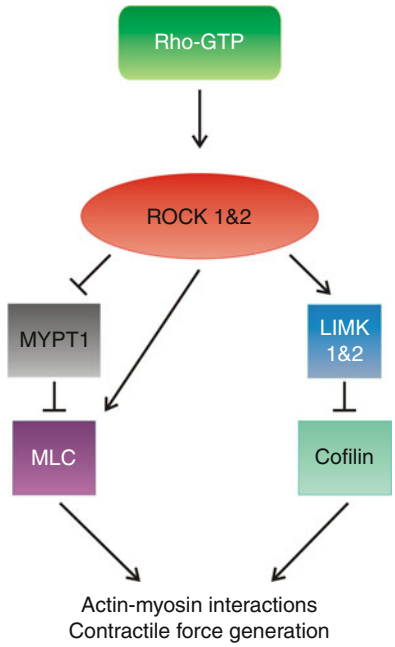
Michael S. Samuel[1] and Michael F. Olson[2]
[1]Centre for Cancer Biology, SA Pathology, Adelaide, SA, Australia
[2]Molecular Cell Biology Laboratory, Beatson Institute for Cancer Research, Glasgow, UK

Synonyms

p160ROCK; Rho-associated coiled coil kinase; Rho-associated kinase; Rho-associated protein kinase; Rho kinase; ROCK I; ROCK II; ROCK: ROK kinase; ROCK1; ROCK2; ROKα; ROKβ

Historical Background

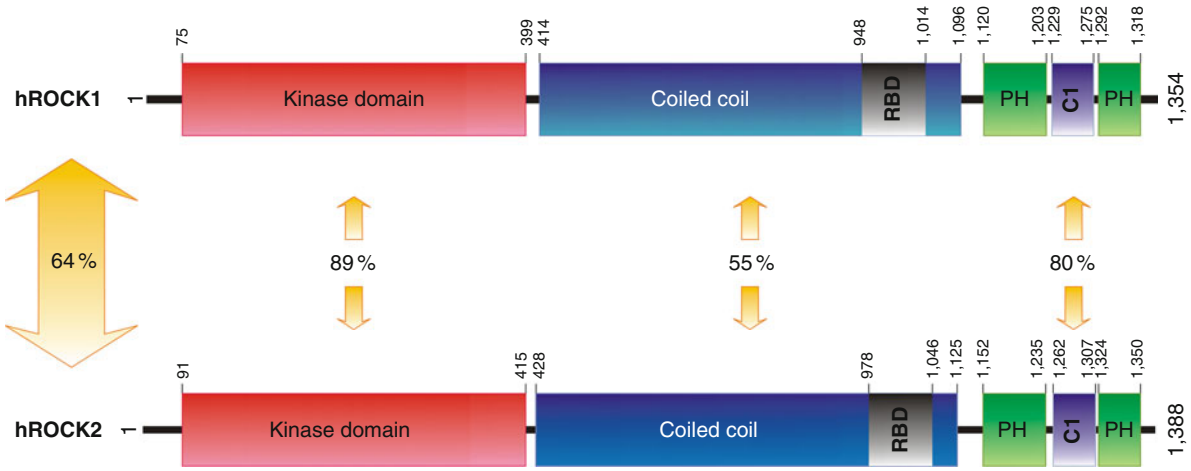
The small molecular weight GTP-binding proteins of the Rho family, which comprises 22 proteins including RhoA, Rac1, and Cdc42, were originally isolated based on their high degree of homology with the Ras proto-oncogenes (Rho = Ras homologue). Following their identification, a variety of approaches were used to isolate interacting proteins that might convey signals downstream to regulate numerous biological processes. One approach was to use GTP-loaded RhoA as a high-affinity reagent to fish for interacting proteins, and the ROCK kinases were identified in this way (Leung et al. 1995; Ishizaki et al. 1996; Matsui et al. 1996). A number of different names have been used to describe the two kinases and early reports were sometimes not careful in accurately describing which isoforms were actually used in the studies. However, the official gene names have been set as ROCK1 and ROCK2. The function of ROCK kinases downstream of active RhoA was quickly determined to be the regulation of actin-myosin contractile force generation (Fig. 1), mediated via the phosphorylation of a number of substrates including ▶ LIMK (which in turn phosphorylates cofilin and inactivates its actin-severing function), myosin light chain (MLC), and the myosin-binding subunit of the MLC phosphatase (MYPT1). In cultured cells, ROCK activation leads to the formation of actin stress fibers and focal adhesions. The discovery of the selective ROCK inhibitor Y27632 (Uehata et al. 1997) rapidly advanced the understanding of ROCK contributions to numerous biological functions.



ROCK Kinases, Fig. 1 ROCK pathways leading to increased actin–myosin contractility. Active GTP-bound Rho associates with ROCK and increases specific activity with the consequence of increased phosphorylation of proteins including the MYPT1 myosin-binding subunit of the myosin light chain phosphatase. ROCK phosphorylation of MYPT1 affects both its substrate binding and catalytic activity, resulting in inhibition of myosin light chain dephosphorylation. MLC has also been reported to be a directly phosphorylated and activated by ROCK. ROCK phosphorylation of LIMK1 and LIMK2 increases their specific activity, resulting in phosphorylation and inactivation of cofilin family proteins. Cofilin phosphorylation inhibits its filamentous actin-severing activity. The sum total of these events is stabilization of filamentous actin and increased actin–myosin contractility

Structure and Function of ROCK Kinases

The ROCK kinases are both 160 kDa serine-threonine kinases with identical domain organization. The primary sequence of the kinase domains places ROCK in the AGC family, their closest relatives being the myotonic dystrophy kinase (DMPK) and the myotonic dystrophy kinase-related CDC42-binding kinases (MRCK). As depicted in Fig. 2, the ROCK kinases are composed of an N-terminal kinase domain,



ROCK Kinases, Fig. 2 ROCK functional domains. Common functional domains in human ROCK1 and ROCK1 with the positions of starting and ending residues. The percentage identities between matched regions were determined by pairwise BLAST comparisons. *RBD* Rho-Binding Domain; *PH* Pleckstrin Homology domain; *C1* Protein kinase C conserved region 1 (Not to scale)

a coiled-coil region that contains the Rho-binding domain (RBD), and a pleckstrin homology (PH) domain that is split into two portions on either side of a cysteine-rich C1 domain. The high degree of homology between the ROCK1 and ROCK2 kinase domains suggests that they probably phosphorylate the same substrates and experimental data bears out this conclusion. Differences in substrate phosphorylation or biological functions observed in vivo likely result from different sub cellular localization and/or protein/protein interactions. Although there are no three dimensional structures of either entire protein, crystal structures of the kinase domains alone or liganded with various small molecule inhibitors have been reported. These structural studies have revealed some novel aspects of the kinase domain. In comparison to other related kinases, there are extensions to both N-terminal and C-terminal ends that promote dimerization. Also, unlike most other AGC kinases, phosphorylation in the kinase activation loop or hydrophobic motifs does not appear to be required for ROCK activation. Instead, dimerization appears to allow the kinase to adopt an active conformation without the need for phosphorylation. NMR structural information for the PH and C1 domains of ROCK2 has also been reported (Wen et al. 2008). Activity of the kinase domain is autoinhibited by the C-terminus, which can be relieved through binding to RhoA, B, or C (Leung et al. 1995; Ishizaki et al. 1996; Matsui et al. 1996) or through proteolytic cleavage (Coleman et al. 2001; Sebbagh et al. 2005). Multiple sites in the C-terminus appear to interact with the kinase domain to regulate activity (Lochhead et al. 2010). Interestingly, a single Proline to Serine amino acid substitution within the first portion of the split PH domain was sufficient to activate ROCK1, suggesting that the conformation of the C-terminal region is important for kinase regulation (Lochhead et al. 2010). ROCK1 activity may be inhibited by another Rho family member RhoE (Riento et al. 2003), which in turn can be antagonized by the kinase PDK1 in a manner independent of its catalytic activity (Pinner and Sahai 2008). Given the key role that ROCK kinases play in promoting contractile force generation, it is not surprising that they have been found to make important contributions to fundamental processes including: motility, adhesion, cytokinesis, apoptosis, phagocytosis, smooth muscle contraction, and neurite retraction.

ROCK in Development

Genetic deletion of either ROCK1 (Shimizu et al. 2005) or ROCK2 (Thumkeo et al. 2003) in mice resulted in similar phenotypes, which helped reveal that a major function of the ROCK kinases in vivo is the regulation of epithelial cell motility. The homozygous deletion of ROCK1 still allowed for the birth of mice at the expected Mendelian ratios, indicating that

there were no major problems during growth and development in utero but newborns had defects in eyelid and ventral body wall closure that gave rise to eyes-open at birth (EOB) and omphalocele (organs such as the liver and gut not being contained with the abdomen) phenotypes, respectively. EOB and omphalocele were also observed in homozygous ROCK2 knockout mice, but in this case a sub-Mendelian incidence of ROCK2$^{-/-}$ mice resulted from defects in the placental labyrinth layer, causing decreased blood flow to developing ROCK2$^{-/-}$ embryos (Thumkeo et al. 2003, 2005). Given these results, it seems consistent that ROCK1$^{+/-}$; ROCK2$^{+/-}$ double heterozygous mice also exhibited EOB and omphalocele, indicating that both kinases contribute to the same actin-driven movement and reorganization of epithelial sheets for eyelid and ventral body wall closure (Thumkeo et al. 2005). That being said, homozygous ROCK1 knockout mice were also independently generated, showing no obvious phenotypic differences from wild-type littermates, which suggests that strain background differences may contribute to the penetrance of the ROCK1 deficient phenotype (Zhang et al. 2006).

ROCK in Disease

The ready availability of potent and selective ROCK inhibitors has made it possible to examine whether ROCK kinases are involved in a wide variety of pathological conditions, including cancer, hypertension and cardiovascular disease, neuronal degeneration, kidney failure, asthma, glaucoma, osteoporosis, erectile dysfunction, and insulin resistance. However, the areas that have attracted the most research effort are cancer, hypertension/cardiovascular disease, and glaucoma.

Interest in ROCK as a cancer target stems from its wide range of activities that contribute to the growth and progression of tumors including proliferation, survival, and metastasis (reviewed in [Wickman et al. 2010]). Early studies revealed that Rho GTPs are over-expressed in a variety of tumors, consistent with increased signaling through the ROCK pathway being a contributory factor. In addition, elevated ROCK expression has been reported in bladder (Kamai et al. 2003) and testicular cancers (Kamai et al. 2002) and correlates with poor survival (Kamai et al. 2003). Large scale sequencing efforts directed at the identification of genetic alterations in human cancers revealed a number of activating somatic ROCK1 mutations in human tumors and tumor cell lines (Lochhead et al. 2010). A current concept is that it would be advantageous to develop ROCK2 selective inhibitors for the treatment of cancer to avoid the pronounced hypotension that is a side effect of ROCK1 inhibition.

The connection between ROCK and hypertension was revealed with the development of ROCK selective inhibitors. Inhibition of ROCK with Y-27632 and related compounds was shown to relieve hypertension in rats by inhibiting the calcium sensitization of smooth muscle contraction. Since that time, numerous studies have built on these observations to show that ROCK activity mediates increased smooth muscle contraction principally via modulation of MLC phosphorylation. In particular, ROCK appears to contribute to aberrant vascular contraction, for example, during coronary vasospasm, cerebral vasospasm following subarachnoid hemorrhage, pulmonary hypertension, and Raynaud's phenomenon, a condition in which the blood supply to distal extremities such as fingers and toes is decreased to the point of numbness or pain and which is the result of vasospasms.

Glaucoma is a disease in which damage to the optic nerve progressively leads to impaired vision and possibly blindness. One way that optic nerve damage may occur is through elevated intraocular pressure. ROCK inhibition helps to relieve this pressure by increasing aqueous outflow by reducing MLC phosphorylation in cells lining the trabecular meshwork. Significant research by pharmaceutical companies in this area has resulted in several promising clinical trials.

Within the nervous system, ROCK has been shown to be an important trigger of neuronal growth cone collapse and neurite withdrawal. As a result, there has been considerable interest in the possibility that ROCK inhibition would actually promote neurite outgrowth and dendrite formation. Possible applications for this include recovery from spinal cord injury by assisting the re-establishment of neural connections across the lesion and Alzheimer's disease treatment through the decreased production of amyloid precursor protein.

ROCK and Stem Cell Survival

When human embryonic stem cells (hESC) are dissociated or plated at low density they often undergo

apoptotic death. Chemical biology screens to identify agents that would promote survival identified the ROCK selective inhibitor Y-27632 as a particularly potent agent. Mechanistic studies revealed that ROCK-mediated actin–myosin contraction makes epiblast derived hESCs die during low density growth and validate the use of inhibitors of ROCK or actin–myosin contractility for the propagation and genetic manipulation of hESCs for eventual therapeutic use (Samuel and Olson 2010).

ROCK Inhibitors

1. HA-1077 (fasudil) and hydroxyfasudil have been in clinical use in Japan for cerebral vasospasm since 1995 (Olson 2008). Since it has been used for such a long period, there is positive post-marketing safety data, which has encouraged trials for a number of indications, including angina, acute ischemic stroke, cerebral blood flow, stable angina pectoris, coronary artery spasm, heart failure associated vascular resistance and constriction, pulmonary arterial hypertension, essential hypertension, atherosclerosis, and aortic stiffness (Olson 2008).
2. Y-27632 was the first published selective ROCK inhibitor (Uehata et al. 1997) and its ready availability has made it the inhibitor of choice. Although this inhibitor is not strictly specific for ROCK kinases, ROCK1 and ROCK2 siRNA experiments have not revealed significant off-target effects in cells.
3. H-1152 was developed as an improved version of HA-1077 with greater ROCK selectivity over PKA and PKC. Although also readily available from commercial sources, H-1152 is less often used than HA-1077 to corroborate results from experiments in which Y-27632 was used, despite the improved selectivity.
4. SLx-2119 was developed as a potential cancer therapeutic initially, but more recently has gone into preclinical development for the treatment of metabolic disease.

A number of studies have highlighted the fact that no inhibitor is entirely specific. Therefore, greater robustness can be built into studies that make use of ROCK inhibitors if a number of additional conditions were satisfied including:

1. Structurally unrelated inhibitors should produce the same biological effects at concentrations that produce equivalent kinase inhibition. The lowest effective doses should be used to reduce off-target effects.
2. Dose-response experiments to establish rank order of potency for a set of inhibitors, i.e., the most potent ROCK inhibitors should be the most effective if a biological response is mediated by ROCK.
3. Examination of the relationship between ROCK inhibitor dose, substrate phosphorylation and biological endpoint.
4. Where possible additional methods should be used to inhibit ROCK function, such as RNAi-mediated knockdown.

Although there is no doubt that inhibitors are useful and convenient research tools, care should be taken in interpreting the results. The substantial knowledge base of the biological functions of ROCK has been made possible due to the ready availability of such inhibitors. Their greatest utility is actually in excluding a possible involvement of ROCK in specific biological responses when there are adequate positive controls in place.

Summary

The most important and central cellular function of ROCK kinases is to regulate morphology, largely through actin–myosin contractility. Due to its profound influence on morphology and contractility, ROCK directly influences numerous activities, such as cytokinesis, adhesion, motility, endothelial barrier function, and membrane blebbing. In addition, via direct or indirect pathways, the ROCK kinases also influence biological processes including gene transcription, proliferation, regulation of cell size, and survival. There has been considerable interest in ROCK kinases as potential therapeutic targets for cancer, hypertension/cardiovascular disease, and glaucoma, and a number of potent and selective inhibitors have been discovered. In fact, one ROCK inhibitor fasudil has been used clinically in Japan for a number of years for the treatment of cerebral vasospasm. Although there is a substantial literature on ROCK function, largely generated due to the availability of pharmacological inhibitors, greater knowledge at the tissue and organismal levels will result from conditional knock-out and conditional-activation mouse models (Samuel et al. 2009). In vivo experiments

using these types of genetically modified models will validate the role of ROCK in various pathological conditions, and will highlight additional indications for the use of ROCK inhibitors. Although quite a few ROCK substrates have been identified and well-characterized, recent phosphoproteomic studies have identified a large number of previously unknown ROCK substrates. As a result, a significant opportunity awaits to characterize the biological outcomes of ROCK-mediated phosphorylation on these novel substrates and ultimately to determine their possible contributions to human disease.

References

Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol. 2001;3:339–45.

Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, et al. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 1996;15:1885–93.

Kamai T, Arai K, Sumi S, Tsujii T, Honda M, Yamanishi T, et al. The rho/rho-kinase pathway is involved in the progression of testicular germ cell tumour. BJU Int. 2002;89:449–53.

Kamai T, Tsujii T, Arai K, Takagi K, Asami H, Ito Y, et al. Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res. 2003;9:2632–41.

Leung T, Manser E, Tan L, Lim L. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem. 1995;270:29051–4.

Lochhead PA, Wickman G, Mezna M, Olson MF. Activating ROCK1 somatic mutations in human cancer. Oncogene. 2010;29:2591–8.

Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, et al. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J. 1996;15:2208–16.

Olson MF. Applications for ROCK kinase inhibition. Curr Opin Cell Biol. 2008;20:242–8.

Pinner S, Sahai E. PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE. Nat Cell Biol. 2008;10:127–37.

Riento K, Guasch RM, Garg R, Jin B, Ridley AJ. RhoE binds to ROCK I and inhibits downstream signaling. Mol Cell Biol. 2003;23:4219–29.

Samuel MS, Olson MF. Dying alone: a tale of Rho. Cell Stem Cell. 2010;7:135–6.

Samuel MS, Munro J, Bryson S, Forrow S, Stevenson D, Olson MF. Tissue selective expression of conditionally-regulated ROCK by gene targeting to a defined locus. Genesis. 2009;47:440–6.

Sebbagh M, Hamelin J, Bertoglio J, Solary E, Breard J. Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner. J Exp Med. 2005;201:465–71.

Shimizu Y, Thumkeo D, Keel J, Ishizaki T, Oshima H, Oshima M, et al. ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles. J Cell Biol. 2005;168:941–53.

Thumkeo D, Keel J, Ishizaki T, Hirose M, Nonomura K, Oshima H, et al. Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. Mol Cell Biol. 2003;23:5043–55.

Thumkeo D, Shimizu Y, Sakamoto S, Yamada S, Narumiya S. ROCK-I and ROCK-II cooperatively regulate closure of eyelid and ventral body wall in mouse embryo. Genes Cells. 2005;10:825–34.

Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature. 1997;389:990–4.

Wen W, Liu W, Yan J, Zhang M. Structure basis and unconventional lipid membrane binding properties of the PH-C1 tandem of Rho kinases. J Biol Chem. 2008;283:26263–73.

Wickman GR, Samuel MS, Lochhead PA, Olson MF. The Rho-regulated ROCK kinases in cancer. In: van Golen K, editor. The Rho GTPases in cancer. New York: Springer; 2010. p. 163–92.

Zhang YM, Bo J, Taffet GE, Chang J, Shi J, Reddy AK, et al. Targeted deletion of ROCK1 protects the heart against pressure overload by inhibiting reactive fibrosis. FASEB J. 2006;20:916–25.

ROCK: ROK kinase

► ROCK Kinases

ROCK1

► ROCK Kinases

ROCK2

► ROCK Kinases

ROKα

► ROCK Kinases

ROKβ

▶ ROCK Kinases

ROS (Reactive Oxygen Species)

▶ p38 MAPK Family of Signal Transduction Proteins

Rotamase Pin1

▶ Pin1

RP11-175O19.1

▶ LCoR

RPN8

James P. Brody
Department of Biomedical Engineering, University of California, Irvine, CA, USA

Synonyms

PSMD7; S12

Background

The gene *PSMD7* encodes the protein RPN8, also known as S12. RPN8, the human homologue of Mov-34, is a non-ATPase component of the 19S regulatory complex (Dubiel et al. 1995). Two 19S regulatory complexes bind to each end of the 20S proteasome to form the 26S proteasome.

The Proteasome

The proteasome plays an important role in the cell, but its mechanism of action is not well understood. Although the proteasome was first isolated in 1979 (DeMartino and Goldberg 1979), the essential role the proteasome plays within a cell was not realized until 1990 (Fujiwara et al. 1990). The proteasome is the main component in the intracellular protein degradation pathway. This pathway was once thought to be a relatively unimportant part of the cell, but is now recognized to play an important role in regulating the lifetime of cellular proteins (Spataro et al. 1998).

Degradation is an important process within the cell (Glickman and Ciechanover 2002). The ultimate state of a cell is governed by the set of cellular proteins that exist within it. Cellular proteins are regulated both through creation (transcription/translation) and through degradation (ubiquitin–proteasome pathway). The degradation of proteins is just as important as the creation of them when studying regulatory processes within the cell. Since the understanding of the proteasome is immature, fundamental studies on assembly, activity, and control within the proteasome may well reveal important information about the complex and, in turn, the regulation of cellular proteins.

The proteasome plays an important role in regulating protein concentrations inside eukaryotic cells through the ubiquitin–proteasome pathway (Hochstrasser 1995). Ubiquitin is a small (76 amino acid) protein that may be specifically conjugated to lysine residues on a protein. This ubiquitin conjugation signals the proteasome to degrade the protein into short polypeptides. Proteins are ubiquitinated through a process that involves three classes of enzymes called E1, E2, and E3 (Weissman 2001). First an E1 enzyme activates the ubiquitin by modifying the C-terminal glycine residue. Simultaneously, an E3 enzyme binds to the target protein. Finally, an E2 enzyme transfers the activated ubiquitin to the E3/target complex, resulting in a ubiquitin tagged target protein that will be degraded by the 26S proteasome in eukaryotic cells. There are many (dozens) of each class of enzyme, and the combination of E2/E3 enzyme is thought to give specificity to the degradation (Glickman and Ciechanover 2002).

The eukaryotic proteasome consists of several components. The entire complex is known as the 26S proteasome. The 26S complex is composed of a single 20S proteasome (total mass 700 kDa, composed of 28 subunits) and two 19S multi-subunit complexes. The 20S proteasome has a cylindrical shape, and the 19S complexes form caps for the two ends of the 20S proteasome (Orlowski and Wilk 2000). The activity of

the proteasome can be modified by the substitution of different subunits. In cultured cells, stimulation by gamma interferon changes the composition of the proteasome (Tanka 1994). A sub-complex known as the 11S regulator replaces the entire 19S sub-complex. Three of the 28 proteins in the 20S proteasome are replaced with completely different proteins. It is thought that these changes enhance the production of polypeptides suitable for MHC class I antigen presentation. This form of the proteasome is called the immunoproteasome (Van den Eynde and Morel 2001).

A heterogeneous population of proteasomes exists within cells. A structural study using transmission electron microscopy of individual 26S proteasomes isolated from both *Drosophila melanogaster* and *Xenopus laevis* revealed that the 19S cap has an extraordinary flexible attachment to the 20S proteasome core (Walz et al. 1998). This study also revealed that one portion of the 19S cap has at least four different conformations. The heterogeneous population implies that the proteasome is used as a general framework for protein degradation machinery.

The standard model of the proteasome's protein degradation mechanism works like this: a protein binds to the 19S subunit at one end of the proteasome, the protein is unfolded and fed into the 20S proteasome, the protein is cleaved inside the 20S barrel, and the cleavage products pass out the other end of the proteasome. The model is based upon structural (Walz et al. 1998; Unno et al. 2002) and kinetic measurements (Stein et al. 1996; Akopian et al. 1997; Kisselev et al. 1998; Nussbaum et al. 1998; Peters et al. 2002) of the proteasome. However, there are other models consistent with this data. For instance, some (Nussbaum et al. 1998) suggest that degradation products may exit through the sides of the 20S proteasome. The structural data (Walz et al. 1998; Unno et al. 2002) makes it clear that the 20S proteasome is symmetric, with no preferred direction.

Polyubiquitinated proteins can be degraded by the proteasome. The 26S proteasome is a large (approximately 2,000 kDa) complex composed of three different components. The core component, called the 20S proteasome is a barrel-shaped protease, with the active sites protected inside the barrel. The 20S core is regulated through several mechanisms. A complex termed the 19S binds to the top and bottom of the 20S core to form the 26S proteasome (Fukunaga et al. 2010; Isono et al. 2004). The 19S complex is thought to recognize, bind, and unfold poly ubiquitinated proteins. These proteins are then fed into the barrel of the 20S proteasome, where they are degraded into polypeptide chains. Other complexes, such as the PA28 complex, can also bind to the 20S core, and these are thought to regulate the proteasome in different ways (Bochtler et al. 1999; Wigley et al. 1999).

Structure

Several domains and motifs for RPN8 have been identified. These include the C-terminal KEKE motif, a putative site of protein–protein interaction (Realini et al. 1994), the Jun activation-domain binding protein (JAB) domain, originally described as a regulator of transcription, the MPR1p and PAD1p N-terminal (MPN) domain which, along with surrounding sequence, has been shown to be important for pairing with S13 (Rpn11/POH1) (Fu et al. 2001), and possibly even weak homology to a MAPKK activation loop motif (Seeger et al. 1998).

The RPN8 MPN domain has been crystallized and the crystal structure has been solved. This structure showed that the MPN domain contains a metalloprotease fold, as expected, but this metalloprotease fold is surprisingly unable to coordinate a metal ion (Sanches et al. 2007).

Localization

RPN8 is cytosolic and localized around the nucleus. This was determined using an antibody raised against amino acids 1–205 of recombinant RPN8 to localize the 26S proteasome in human JU77 mesothelioma cells (Seeger et al. 1998). Furthermore, phosphorylated RPN8 was found by using an anti-RPN8 antibody that reacted with both the phosphorylated and unphosphorylated forms from 26S proteasomes immunoprecipitated from human L-132 cells (Mason et al. 1998). These data identified phosphorylated RPN8 migrating above 50 kDa, whereas unphosphorylated RPN8 migrates at 40 kDa (Braun et al. 1999).

Roles in Human Disease

The proteasome has been implicated in multiple diseases. Defects in proteasome activity are associated

with chronic human neurodegenerative diseases. Protein aggregates are found in some patients with Alzheimer's (Ciechanover and Brundin 2003), Parkinson's (Dauer and Przedborski 2003), and Huntington's (Rubinsztein 2006) diseases. These aggregates are thought to form when the proteasome is unable to keep up with the amount of ubiquitinated protein being produced by the cell. The ubiquitin–proteasome pathway of protein degradation is also the target of cancer-related deregulation. Proteasome inhibitors form a new class of anticancer drugs and are under study for use in pancreatic, colon, lung, breast, prostate, and ovarian cancers (Voorhees and Orlowski 2006). One proteasome inhibitor, Velcade, has been approved by the FDA for treatment of multiple myeloma.

Certain mutations in the gene BRCA1 are known to lead to a predisposition to developing breast cancer. These cases account for less than 10% of all breast cancers. The protein product of BRCA1 exhibits E3 (ubiquitin protein ligase) activity. Furthermore, it was shown that cancer-predisposing mutations in BRCA1 also lead to a loss of this E3 activity (Ruffner et al. 2001). This clearly implicates the ubiquitin–proteasome pathway as a potential mechanism in the development of breast cancer. Other defects leading to the aberrant regulation of the ubiquitin–proteasome pathway (e.g., E1, E2, E3, or proteasome subunits) may be responsible for a greater percentage of breast carcinomas.

RPN8/PSMD7 is one of a cohort of 231 genes whose expression levels were significantly associated with clinical outcome in breast cancer patients (van't Veer et al. 2002).

In cell line studies, RPN8 appears to be functionally related to transformed cells. RPN8 was posttranslationally modified in six normal breast epithelial cell lines, but not four transformed cell lines. Rpn8 was unique among proteasome subunits in this characteristic. Modified RPN8 has identical mass, but different isoelectric points then unmodified RPN8, see Fig. 3 in (Thompson et al. 2004). Modified RPN8 does not associate with the 26S proteasome, but does associate with a separate high molecular weight complex, see Fig. 5 in (Thompson et al. 2004). Finally, modified RPN8 is localized to the nuclei of normal cells, whereas unmodified RPN8 is only present in the cytoplasm.

The differential RPN8 protein expression and nuclear localization observed between the normal and cancer cell lines suggests that RPN8 has multiple functions associated with normal and cancer phenotypes. For instance, while mRNA levels are essentially the same between normal and cancer cell lines, the difference in levels of modified RPN8 is great. Thus, mechanisms that modulate posttranslational modifications of RPN8 in normal cells are disrupted in each of the cancer cell lines studied. Furthermore, no differences exist in the protein expression pattern of six other proteasome subunits between the ten cell lines, with the exception of S10a, lending significance to the differences observed with RPN8.

Summary

In summary, RPN8 exists in normal cell lines with at least two different posttranslational modifications, but in transformed cell lines with only one. The modified form of RPN8 does not associate with the 26S proteasome, but does associate with the immunoproteasome. These data suggest a differential nuclear function of modified and unmodified RPN8 in cancer cells.

References

Akopian TN, Kisselev AF, Goldberg AL. Processive degradation of proteins and other catalytic properties of the proteasome from *Thermoplasma acidophilium*. J Biol Chem. 1997;272(3):1791–8.

Bochtler M, Ditzel L, Groll M, Hartmann C, Huber R. The proteasome. Annu Rev Biophys Biomol Struct. 1999;28:295–317.

Braun BC, Glickman M, Kraft R, Dahlmann B, Kloetzel PM, Finley D, Schmidt M. The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat Cell Biol. 1999;1(4):221–6.

Ciechanover A, Brundin P. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron. 2003;40(2):427–46.

Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron. 2003;39(6):889–909.

DeMartino GN, Goldberg AL. Identification and partial purification of an ATP-stimulated alkaline protease in rat liver. J Biol Chem. 1979;254(10):3712–15.

Dubiel W, Ferrell K, Dumdey R, Standera S, Prehn S, Rechsteiner M. Molecular cloning and expression of subunit 12: a non-MCP and non-ATPase subunit of the 26 S protease. FEBS Lett. 1995;363(1–2):97–100.

Fu H, Reis N, Lee Y, Glickman MH, Vierstra RD. Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J. 2001;20(24):7096–107.

Fujiwara T, Tanaka K, Orino E, Yoshimura T, Kumatori A, Tamura T, Chung CH, Nakai T, Yamaguchi K, Shin S. Proteasomes are essential for yeast proliferation. cDNA cloning and gene disruption of two major subunits. J Biol Chem. 1990;265(27):16604–13.

Fukunaga K, Kudo T, Toh-e A, Tanaka K, Saeki Y. Dissection of the assembly pathway of the proteasome lid in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun. 2010;396(4):1048–53.

Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev. 2002;82(2):373–428.

Hochstrasser M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr Opin Cell Biol. 1995;7:215–23.

Isono E, Saeki Y, Yokosawa H, Toh-e A. Rpn7 is required for the structural integrity of the 26 s proteasome of *Saccharomyces cerevisiae*. J Biol Chem. 2004;279(26):27168–76.

Kisselev AF, Akopian TN, Goldberg AL. Range of sizes of peptide products generated during degradation of different proteins by archaeal proteasomes. J Biol Chem. 1998;273(4):1982–9.

Mason GG, Murray RZ, Pappin D, Rivett AJ. Phosphorylation of ATPase subunits of the 26S proteasome. FEBS Lett. 1998;430(3):269–74.

Nussbaum AK, Dick TP, Keilholz W, Schirle M, Stevanovic S, Dietz K, Heinemeyer W, Groll M, Wolf DH, Huber R, Rammensee H-G, Schild H. Cleavage motifs of the yeast 20s proteasome β subunits deduced from digests of enolase 1. Proc Natl Acad Sci USA. 1998;95:12504–9.

Orlowski M, Wilk S. Catlaytic activities of the 20 s proteasome, a multicatalytic proteinase complex. Arch Biochem Biophys. 2000;383(1):1–16.

Peters B, Janek K, Kuckelkorn U, Holzhutter H-G. Assessment of proteasomal cleavage probabilities from kinetic analysis of time-dependent product formation. J Mol Biol. 2002;318:847–62.

Realini C, Rogers SW, Rechsteiner M. KEKE motifs. Proposed roles in protein-protein association and presentation of peptides by MHC class I receptors. FEBS Lett. 1994;348(2):109–13.

Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. 2006;443(7113):780–6.

Ruffner H, Joazeiro CA, Hemmati D, Hunter T, Verma IM. Cancer-predisposing mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity. Proc Natl Acad Sci USA. 2001;98(9):5134–9.

Sanches M, Alves BSC, Zanchin NIT, Guimares BG. The crystal structure of the human mov34 mpn domain reveals a metal-free dimer. J Mol Biol. 2007;370(5):846–55.

Seeger M, Kraft R, Ferrell K, Bech-Otschir D, Dumdey R, Schade R, Gordon C, Naumann M, Dubiel W. A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. FASEB J. 1998;12(6):469–78.

Spataro V, Norbury C, Harris AL. The ubiquitin-proteasome pathway in cancer. Br J Cancer. 1998;77(3):448–55.

Stein RL, Melandri F, Dick L. Kinetic characterization of the chymotryptic activity of the 20S proteasome. Biochemistry. 1996;35:3899–908.

Tanka K. Role of proteasomes modified by interferon-gamma in antigen processing. J Leukoc Biol. 1994;56(5):571–5.

Thompson HGR, Harris JW, Lin L, Brody JP. Identification of the protein ZIBRA, its genomic organization, regulation and expression in breast cancer cells. Exp Cell Res. 2004;295(2):448–59.

Unno M, Mizushima T, Morimoto Y, Tomisugi Y, Tanaka K, Yasuoka N, Tsukihara T. The structure of the mammalian 20S proteasome at 2.75Å resolution. Structure. 2002;10:609–18.

Van den Eynde BJ, Morel S. Differential processing of class-i-restricted epitopes by the standard proteasome and the immunoproteasome. Curr Opin Immunol. 2001;13(2):147–53.

van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415(6871):530–6.

Voorhees PM, Orlowski RZ. The proteasome and proteasome inhibitors in cancer therapy. Annu Rev Pharmacol Toxicol. 2006;46:189–213.

Walz J, Erdmann A, Kania M, Typke D, Koster AJ, Baumeister W. 26s proteasome structure revealed by three dimensional electron microscopy. J Struct Biol. 1998;121:19–29.

Weissman AM. Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol. 2001;2(3):169–78.

Wigley WC, Fabunmi RP, Lee MG, Marino CR, Muallem S, DeMartino GN, Thomas PJ. Dynamic association of proteasomal machinery with the centrosome. J Cell Biol. 1999;145(3):481–90.

Rps6ka5

► MSK1

RPT

Julie A. Maupin-Furlow and Hugo V. Miranda
Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, USA

Synonyms

Rpt, *R*egulatory *p*article *t*riple-A (Rpt) subunits of 26S proteasomes; triple-A (or AAA) is a family of proteins within the AAA+ superfamily of ATPases associated with various cellular activities

Historical Background

*R*egulatory *p*article *t*riple-A (Rpt) subunits are associated with the ubiquitin-proteasome system, a central mechanism of protein breakdown with a rich historical background. Aaron Ciechanover, Avram Hershko, and Irwin Rose were awarded the 2004 Nobel Prize in Chemistry for their "discovery of ubiquitin-mediated protein degradation" by proteasomes and have outlined some of this history in reviews (e.g., Ciechanover 2010). The term "proteasome" was first coined by Arrigo et al. (1988) for a "large alkaline multifunctional protease" known to mediate energy-dependent intracellular protein breakdown, once it was clear that this protease was related to the ring-shaped "prosomes" of unknown function commonly found in eukaryotic cells. The Rpt nomenclature was later proposed by Finley et al. (1998) to designate the subunits of the yeast 26S proteasome that cluster to the *A*TPases *a*ssociated with various cellular *a*ctivities (AAA) family of the AAA+ superfamily (Wollenberg and Swaffield 2001). AAA+ proteins are required for the degradation of folded proteins by self-compartmentalized proteases such as proteasomes.

Proteasomes and Rpt Subunits

Protein degradation by proteasomes is a major regulatory mechanism in eukarya, archaea, and actinobacteria. Proteasomes can degrade short-lived proteins that control cell division, apoptosis, DNA repair, information processing (transcription and translation), and other cellular processes (Navon and Ciechanover 2009). Proteasomes are also important in maintaining protein quality by destroying improperly synthesized, foreign, and damaged proteins (Schrader et al. 2009).

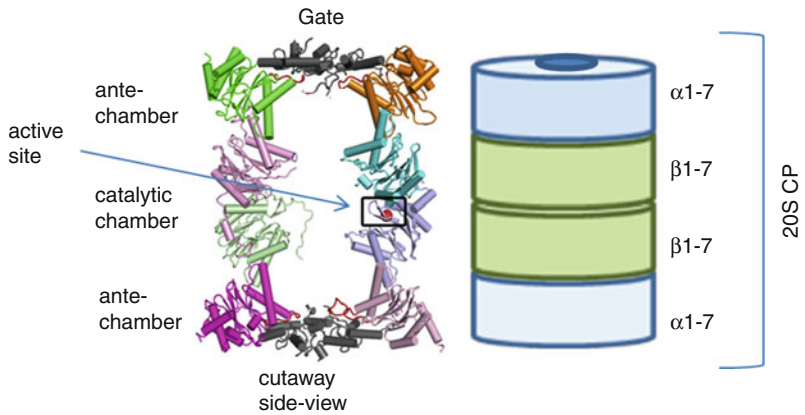
All proteasomes are composed of a 20S core particle (CP) lined on its interior with proteolytic active sites that are sequestered away from the cytosol (Stadtmueller and Hill 2011) (Fig. 1). CPs are barrel-like and formed from four-stacked heptameric rings of α- and β-type subunits. The α-type subunits form the outermost rings, while the β-type subunits form the inner two rings. The N-terminal tails of α-type subunits can gate the pores on each end of CPs and limit substrate access to the proteolytic active sites formed by β-type subunits.

CPs associate with various regulators (Stadtmueller and Hill 2011). In eukarya, CPs bind 19S regulatory particles (RPs) to form an energy-dependent protease known as the 26S proteasome (Fig. 2). Substrates of 26S proteasomes include proteins covalently conjugated to chains of ubiquitin (Ub) through a process termed ubiquitylation (Ravid and Hochstrasser 2008). The primary roles of 19S RPs are to recognize, unfold, and translocate substrate proteins to the catalytic central chamber of CPs for protein degradation. In yeast, the 19S RP can be separated into base and lid subcomplexes by deletion of the gene encoding the *r*egulatory *p*article *n*on-ATPase Rpn10 subunit (Tomko and Hochstrasser 2011). The base subcomplex is composed of three Rpn subunits (Rpn1, Rpn2, and Rpn13) and six proteins termed *r*egulatory *p*article *t*riple-A subunits (Rpt1-6) that cluster to the AAA family of the AAA+ superfamily. Rpt1-6 form a heterohexameric ring that directly interacts with 20S CPs and are arranged in Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5 order in 26S proteasomes (Tomko and Hochstrasser 2011). The lid subcomplex is composed of nine other Rpn subunits and harbors the deubiquitylating enzyme Rpn11 that removes polyubiquitin chains from substrate proteins.

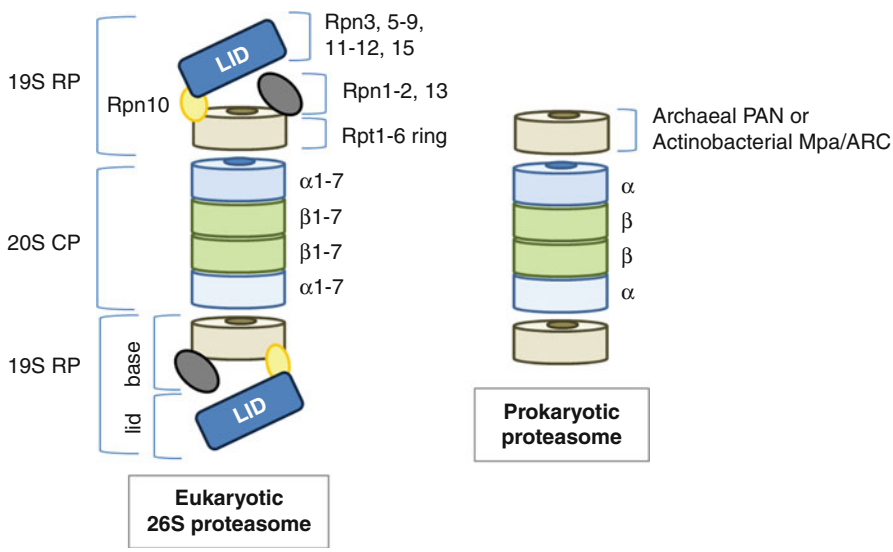
Archaea and actinobacteria synthesize proteasomes that are relatively simple in subunit complexity, yet these proteases share many basic structural and functional features with eukaryal 26S proteasomes (Bar-Nun and Glickman 2011). Like eukarya, the CPs and proteasomal AAA+ proteins of archaea and actinobacteria can associate together in vitro and catalyze the energy-dependent degradation of folded proteins (Fig. 2). The prokaryotic AAA+ proteins are often hexameric rings formed from a single protein including the archaeal proteasome-associated nucleotidase (PAN) and actinobacterial AAA ATPase forming ring-shaped complexes (ARC) or mycobacterium proteasome ATPase (Mpa). The archaeal PAN is closely related to the eukaryal Rpt subunits, while the actinobacterial ARC/Mpa proteins are divergent members of the AAA family. It has been proposed that the six different types of eukaryal Rpt proteins (Rpt1-6) evolved from a single archaeal PAN ancestor, which over time was duplicated and diversified (Wollenberg and Swaffield 2001).

Rpt and Related AAA+ Proteins in Proteolysis

While the hydrolysis of peptide bonds is exergonic, ATP binding and hydrolysis by AAA+ proteins (or domains) is needed to fuel the regulated degradation



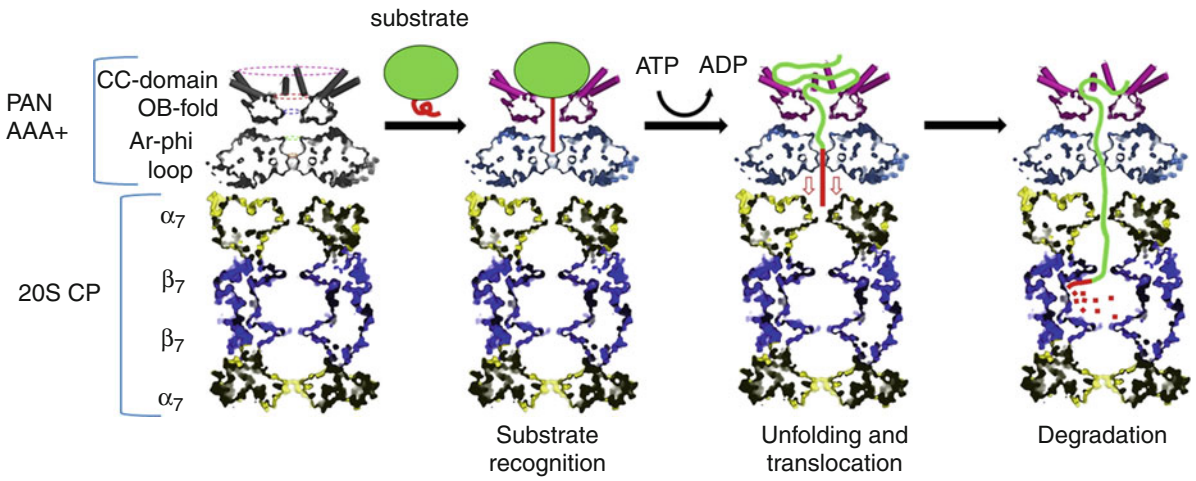
RPT, Fig. 1 20S proteasomes. The central proteolytic component of all proteasomes is a cylindrical 20S core particle (CP) formed from four-stacked heptameric rings. The outer rings are of α-type subunits and inner rings are of β-type subunits. The N-terminal tails of α-type subunits gate substrate access to the central channel that connects three chambers (two antechambers and one catalytic chamber). The proteolytic active sites are sequestered from the cytosol and formed by the N-terminal threonine residues of β-type subunits that are exposed by autocatalytic processing of the β-type proteins during complex assembly (Modified with permission from Stadtmueller and Hill (2011))



RPT, Fig. 2 ATP-dependent proteasomes. Proteasomal CPs can associate on each end with heptameric rings of AAA+ proteins including eukaryal Rpt1-6, archaeal PAN, and actinobacterial ARC (or Mpa). In eukaryotes, Rpt1-6 are the ATPase subunits of the 19S regulatory particles (RPs) that together with CPs form 26S proteasomes. In yeast, the 19S RP can be dissociated into lid and base subcomplexes by deletion of the *RPN10* gene

of folded proteins by self-compartmentalized proteases such as 26S proteasomes. The AAA+ rings can bind substrate proteins, open the gates of the protease chamber (closed-gates limit substrate access to the proteolytic active sites), unfold substrate proteins, and facilitate the translocation of protein substrates into the proteolytic center of the protease.

Subdomain X-ray crystal and cryo-electron microscopy structures of proteasomal AAA+ proteins are now available and provide valuable insight into how this group of ATPases might function at the atomic level (Bar-Nun and Glickman 2011). Details on the related bacterial AAA+ proteases such as ClpXP and HslUV have also guided proteasomal models (Sauer and Baker 2011). In general, proteasomal ATPases have a central channel that is coaxial to the 20S CP channel (Fig. 3). On the distal face of the proteasomal ATPase ring are six protruding and paired N-terminal coiled-coil (CC) domains that are required for substrate binding. Moving down the ATPase channel, one finds a conserved interdomain region with an oligonucleotide-binding (OB)-fold domain that is



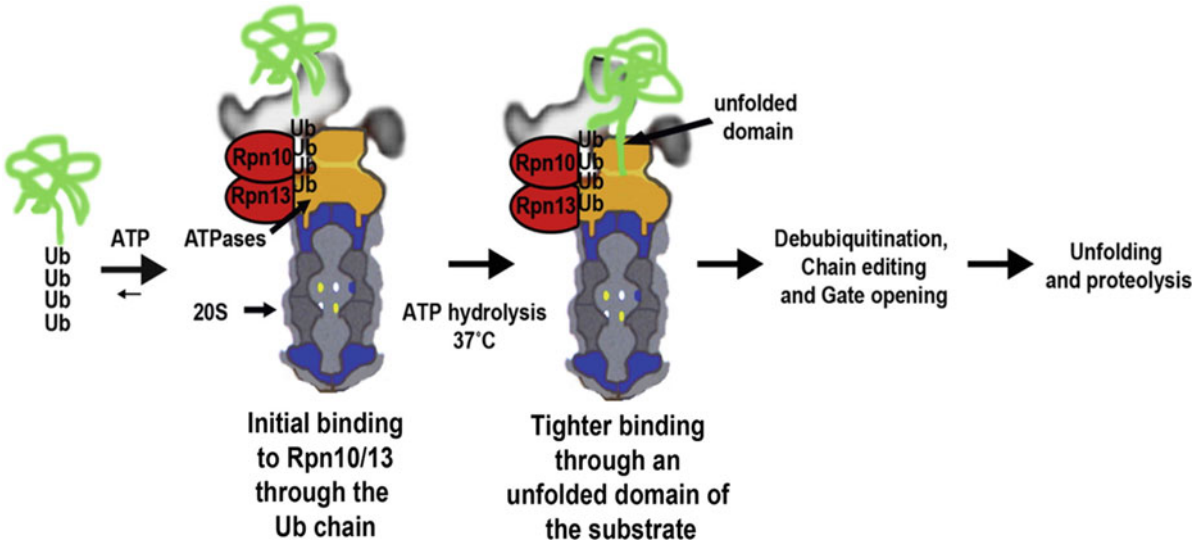
RPT, Fig. 3 Model of proteasome-mediated degradation of folded proteins. Model is based on archaeal PAN, a close relative of the Rpt1-6 subunits of eukaryal 26S proteasomes. Substrate proteins are thought to be unfolded on the distal face of the proteasomal ATPase, translocated through the central ATPase channel, and ultimately reach the central chamber of CPs for destruction. The N-terminal coiled-coil (CC) domain, oligonucleotide-binding (OB)-fold domain, and aromatic-hydrophobic (Ar-phi) loop discussed in text are indicated (Modified with permission from Zhang et al. (2009))

important for AAA+ ring formation. The OB-fold domain also appears to serve as a rigid entryway for substrates that traverse the ATPase channel. The archaeal PAN and eukaryal Rpt1-6 proteins have a single OB-fold (as depicted in Fig. 3), while the actinobacterial ARC/Mpa has a double OB-fold that forms two separate ring-like structures within the ATPase channel. The C-terminal AAA+ domain mediates cycles of ATP hydrolysis and harbors a highly conserved aromatic-hydrophobic (Ar-phi) loop within its channel. The Ar-phi loop is common to energy-dependent AAA+ proteases and is thought to grab onto hydrophobic tails of substrates that may extend into the ATPase channel. Once bound, cycles of ATP hydrolysis around the ATPase ring are thought to mediate conformational changes in the Ar-phi loop that result in the tugging and unfolding of the substrate protein. The rigid OB-fold pore is believed to serve as a platform for this unfolding process. Thus, the protein substrate is thought to be unfolded and translocated through the ATPase and into the CP for destruction.

Many of the proteasomal ATPases (e.g., archaeal PAN and eukaryal Rpt2, 3 and 5) have C-terminal hydrophobic-tyrosine-any residue (HbYX) motifs that bind to pockets between the α-type subunits of CPs and induce CP gate opening. The proteasome-associated regulator Blm10 and α-ring assembly factor Pba1-Pba2 also have penultimate tyrosine (or phenylalanine) residues that appear important for binding within the α/α-intersubunit interface (Stadtmueller and Hill 2011; Kusmierczyk et al. 2011).

Substrate Recognition by Proteasomes

Protein degradation is a highly regulated process. In order to ensure that only the desired proteins are degraded, eukaryotes and prokaryotes have evolved various strategies for identifying proteins for degradation. Specific degradation signals or "degrons" within the protein often initiate the process of proteolysis. Degrons range from the phosphorylation or glycosylation status of a protein to the exposure of destabilizing N- or C-termini (Ravid and Hochstrasser 2008). In eukaryotic cells, the ubiquitylation system often recognizes these degrons and results in the covalent attachment of polyubiquitin (Ub) chains. While all seven lysine residues of Ub can form chains, Ub chains linked through Lys48 commonly target proteins for destruction by 26S proteasomes (Xu et al. 2009). These ubiquitylated proteins are thought to first bind the Rpn10 and Rpn13 subunits of 26S proteasomes by a mechanism stimulated by ATP binding to the Rpt subunits (Peth et al. 2010) (Fig. 4). Next, Rpt-mediated ATP hydrolysis is thought to unfold and expose unstructured portions of the substrate protein for tighter binding to 26S proteasomes. Ultimately, this is thought to ready the substrate for the editing or



RPT, Fig. 4 26S proteasome-mediated degradation of ubiquitylated proteins. Initially, the 26S proteasomal subunits Rpn10 and 13 (Ub receptors) bind the Ub chain of ubiquitylated substrate proteins by a mechanism that is stimulated by ATP binding. In the next phase, ATP is hydrolyzed by the Rpt subunits and an unfolded domain of the substrate protein is exposed, resulting in tighter binding to 26S proteasomes. After these binding events, deubiquitylation, Ub-chain editing, substrate unfolding, CP gate opening, translocation, and proteolysis can occur (Modified with permission from Peth et al. 2010)

removal of polyUb chains by deubiquitylases. The Rpt subunits would unfold and translocate the protein while opening the CP gates for protein degradation.

In actinobacteria and archaea, the covalent attachment of small protein modifiers to substrate proteins appears to be linked to proteasome-mediated proteolysis– much like the process of ubiquitylation in eukaryotic cells. It has recently been shown in *Mycobacterium tuberculosis* that a small protein termed Pup can covalently modify proteins at lysine residues (Burns and Darwin 2010). While the mechanism of pupylation differs from ubiquitylation and the disordered structure of Pup is not like the highly ordered beta-grasp fold of Ub, Pup can target proteins for proteasome-mediated degradation (Burns and Darwin 2010). Interestingly, the mycobacterial proteasomal AAA+ Mpa (required for cellular resistance to reactive nitrogen intermediates) binds and induces formation of an alpha helix within Pup and is required for degradation of pupylated substrates by proteasomes (Burns and Darwin 2010). The haloarchaeon *Haloferax volcanii* can also attach small archaeal protein modifiers or SAMPs to substrate proteins (Humbard et al. 2010). However, unlike Pup, the archaeal SAMPs have a predicted beta-grasp fold structure similar to Ub and require the presence of an E1 Ub activating homolog (UbaA) for attachment (Miranda et al. 2011). Other enzyme (E2 Ub conjugating and E3 Ub ligase) homologs for the attachment of SAMPs to protein targets have yet to be identified. Sampylation has not been directly linked to targeting proteins for proteasome-mediated degradation. However, the sampylome accumulates in strains deficient in the synthesis of the proteasomal Rpt-like PAN-A and CP α1 subunits, suggesting sampylation may trigger proteins for degradation by proteasomes. However, green fluorescent protein (GFP) derivatives with hydrophobic C-terminal tails that are not modified by sampylation or other types of posttranslational modification are degraded in vitro by proteasomal CPs in the presence of archaeal PAN and hydrolyzable ATP (Navon and Goldberg 2001). Therefore, by an ATP-dependent mechanism, Rpt-like proteins can bind and unfold non-sampylated protein substrates with exposed hydrophobic regions. Interestingly, in eukaryotic cells, the chaperonin-like activities of the proteasomal Rpt proteins appear to also serve non-proteolytic roles (Kodadek 2010).

Summary

Proteins of the AAA+ superfamily that include eukaryal Rpt, archaeal PAN, and actinobacterial

Mpa/ARC are structurally related and important in protein degradation. These proteasomal ATPases can unfold proteins, open CP gates, and translocate proteins for ultimate destruction within the self-compartmentalized 20S CP structure that protects cells from uncontrolled proteolysis. In eukarya, Rpt1-6 associate with non-ATPase subunits in complexes such as the 19S RP to form 26S proteasomes but also appear to serve non-proteolytic roles. Whether the archaeal PAN and actinobacterial Mpa/ARC are similar to eukaryal Rpt in their association with proteasomal CPs and other non-ATPase factors in vivo remains an exciting area of research.

Acknowledgments This work was funded in part by grants from the National Institutes of Health (GM57498) and the Department of Energy Office of Basic Energy Sciences (DE-FG02-05ER15650).

References

Arrigo AP, Tanaka K, Goldberg AL, Welch WJ. Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalian cells (the proteasome). Nature. 1988;331(6152):192–4. doi:10.1038/331192a0.

Bar-Nun S, Glickman MH. Proteasomal AAA-ATPases: structure and function. Biochim Biophys Acta. 2011. doi:10.1016/j.bbamcr.2011.07.009.

Burns KE, Darwin KH. Pupylation versus ubiquitylation: tagging for proteasome-dependent degradation. Cell Microbiol. 2010;12:424–31.

Ciechanover A. The ubiquitin system: historical perspective. Proc Am Thorac Soc. 2010;7:11–2. doi:10.1513/pats.200908-095JS.

Finley D, Tanaka K, Mann C, Feldmann H, Hochstrasser M, Vierstra R, Johnston S, Hampton R, Haber J, Mccusker J, Silver P, Frontali L, Thorsness P, Varshavsky A, Byers B, Madura K, Reed SI, Wolf D, Jentsch S, Sommer T, Baumeister W, Goldberg A, Fried V, Rubin DM, Toh-e A, et al. Unified nomenclature for subunits of the *Saccharomyces cerevisiae* proteasome regulatory particle. Trends Biochem Sci. 1998;23:244–5. doi:10.1016/S0968-0004(98)01222-5.

Humbard MA, Miranda HV, Lim JM, Krause DJ, Pritz JR, Zhou G, Chen S, Wells L, Maupin-Furlow JA. Ubiquitin-like small archaeal modifier proteins (SAMPs) in *Haloferax volcanii*. Nature. 2010;463:54–60.

Kodadek T. No Splicing, no dicing: non-proteolytic roles of the ubiquitin-proteasome system in transcription. J Biol Chem. 2010;285:2221–6.

Kusmierczyk AR, Kunjappu MJ, Kim RY, Hochstrasser M. A conserved 20S proteasome assembly factor requires a C-terminal HbYX motif for proteasomal precursor binding. Nat Struct Mol Biol. 2011;18:622–9. doi:10.1038/nsmb.2027.

Miranda HV, Nembhard N, Su D, Hepowit N, Krause DJ, Pritz JR, Phillips C, Söll D, Maupin-Furlow JA. E1- and ubiquitin-like proteins provide a direct link between protein conjugation and sulfur transfer in archaea. Proc Natl Acad Sci USA. 2011;108:4417–22.

Navon A, Ciechanover A. The 26S proteasome: from basic mechanisms to drug targeting. J Biol Chem. 2009;284:33713–8. doi:10.1074/jbc.R109.018481.

Navon A, Goldberg AL. Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. Mol Cell. 2001;8:1339–49. doi:10.1016/S1097-2765(01)00407-5.

Peth A, Uchiki T, Goldberg AL. ATP-dependent steps in the binding of ubiquitin conjugates to the 26S proteasome that commit to degradation. Mol Cell. 2010;40(4):671–81. doi:10.1016/j.molcel.2010.11.002.

Ravid T, Hochstrasser M. Diversity of degradation signals in the ubiquitin-proteasome system. Nat Rev Mol Cell Biol. 2008;9:679–90.

Sauer RT, Baker TA. AAA+ proteases: ATP-fueled machines of protein destruction. Annu Rev Biochem. 2011;80:587–612.

Schrader EK, Harstad KG, Matouschek A. Targeting proteins for degradation. Nat Chem Biol. 2009;5:815–22. doi:10.1038/nchembio.250.

Stadtmueller BM, Hill CP. Proteasome activators. Mol Cell. 2011;41:8–19. doi:10.1016/j.molcel.2010.12.020.

Tomko Jr RJ, Hochstrasser M. Order of the proteasomal ATPases and eukaryotic proteasome assembly. Cell Biochem Biophys. 2011;60:13–20.

Wollenberg K, Swaffield JC. Evolution of proteasomal ATPases. Mol Biol Evol. 2001;18:962–74.

Xu P, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J, Rush J, Hochstrasser M, Finley D, Peng J. Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell. 2009;137:133–45.

Zhang F, Wu Z, Zhang P, Tian G, Finley D, Shi Y. Mechanism of substrate unfolding and translocation by the regulatory particle of the proteasome from *Methanocaldococcus jannaschii*. Mol Cell. 2009;34:485–96. doi:10.1016/j.molcel.2009.04.022.

Rpt, Regulatory particle triple-A (Rpt) Subunits of 26S Proteasomes

► RPT

RPTPe

► PTPe (RPTPe and Cyt-PTPe)

RRAD

► Ras-Related Associated with Diabetes

RSK (p90 Ribosomal S6 Kinase)

Philippe P. Roux
Department of Pathology and Cell Biology, Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, Montreal, QC, Canada

Synonyms

90 kDa ribosomal S6 kinase; p90RSK

Historical Background

The p90 ribosomal S6 kinase (RSK) family comprises four mammalian Ser/Thr kinases (RSK1-4) (Anjum and Blenis 2008; Carriere et al. 2008). The first RSK family member was identified as a kinase activity in maturating *Xenopus laevis* oocytes that phosphorylated the 40S ribosomal subunit protein S6 (rpS6) (Erikson and Maller 1985, 1986). Although the p70 ribosomal S6 kinases 1 and 2 (S6K1 and S6K2) were later shown to be the predominant S6 kinases operating in somatic cells (Blenis et al. 1991; Chung et al. 1992), RSK1 and RSK2 were found to phosphorylate rpS6 in response to ▶ MAP kinase pathway activation (Cohen et al. 2007; Roux et al. 2007). Interestingly, whereas S6K1/2 were found to phosphorylate all sites on rpS6 (Ser235, Ser236, Ser240, and Ser244), RSK1 and RSK2 were shown to specifically phosphorylate Ser235 and Ser236 (Roux et al. 2007). The role of this specific regulation is unknown, but some evidence suggests that rpS6 phosphorylation may be involved in fine-tuning the cellular response elicited by some growth factors (Meyuhas 2008). At the moment, little is known about specific and overlapping functions of the RSK isoforms, but the recent identification of specific RSK inhibitors (Cohen et al. 2005; Smith et al. 2005; Sapkota et al. 2007), the use of RNA interference, and the generation of mouse knockouts should help shed light on the contribution of each RSK isoform.

Structure of the RSK Isoforms

The RSK isoforms are 73–80% identical to each other and are mostly divergent in their amino- and carboxyl-termini sequences. The structure of RSK is complex and comprises two functionally distinct kinase domains, a linker region, and N- and C-terminal tails. While the N-terminal-kinase domain (NTKD) shares homology with kinases of the AGC family (PKA, PKG, PKC), the C-terminal kinase domain (CTKD) is homologous to the calcium/calmodulin-dependent protein kinases (CaMKs). It is thought that, during evolution, the genes for two different protein kinases have fused, generating a single polypeptide capable of receiving an upstream activating signal from the Ras/MAPK pathway to its CTKD and transmitting, with high efficiency and fidelity, an activating input to its NTKD. Thus, the CTKD appears to be only involved in autophosphorylation of RSK, resulting in its activation, and the NTKD is responsible for downstream substrate phosphorylation (Bjorbaek et al. 1995). The C-terminal tail contains an ERK1/2 docking motif, known as the D domain, and interaction of RSK with ERK1/2 was shown to depend on a short motif consisting of Leu-Arg-Gln-Arg-Arg (Smith et al. 1999; Roux et al. 2003). Finally, the C-terminal tail of all RSK isoforms contains a type 1 PDZ domain-binding motif, consisting of Thr-Xaa-Leu, where Xaa is any amino acid. This motif was shown to be functional with at least some PDZ domain-containing proteins (Thomas et al. 2005), but more will need to be done to fully determine its role and biological significance.

Activation Mechanisms

The RSK isoforms contain six phosphorylation sites that are responsive to mitogenic stimulation. Mutational analysis revealed that four of these sites (Ser221, Ser352, Ser369, and Thr562 in mouse RSK1) are essential for RSK activation (Dalby et al. 1998). Of these, Ser221 (located in the NTKD activation loop), Ser352 (turn motif), and Ser369 (hydrophobic motif) are located within sequences highly conserved in other AGC kinases (Newton 2003). The current model of RSK activation suggests that ERK and RSK form an inactive complex in quiescent cells that is mediated by the D domain on RSK (Hsiao et al. 1994; Zhao et al. 1996). After mitogenic stimulation, ERK1/2 phosphorylates Thr562 in the activation loop of the CTKD (Sutherland et al. 1993) and possibly Thr348 and Ser352 in the linker region

between the two kinase domains (Dalby et al. 1998). Activation of the CTKD leads to autophosphorylation at Ser369 (Vik and Ryder 1997), which creates a docking site for phosphoinositide-dependent protein kinase 1 (PDK1) (Frodin et al. 2000). In turn, PDK1 phosphorylates Ser221 in the activation loop of the NTKD (Jensen et al. 1999; Richards et al. 1999) and, along with phosphorylated Ser352 and Ser369, promotes an intramolecular allosteric mechanism that allows the NTKD to phosphorylate downstream substrates (Frodin et al. 2002). RSK also autophosphorylates at a C-terminal residue that releases ERK1/2 binding, presumably to allow these kinases to find their respective substrates throughout the cell (Roux et al. 2003).

The process of RSK activation is closely linked to ERK1/2 activity, and MEK1/2 inhibitors (U0126, PD98059, PD184352) have been widely used to study RSK function. Recently, three different classes of RSK inhibitors targeting the NTKD (SL0101 and BI-D1870) or the CTKD (FMK) have been identified (Cohen et al. 2005; Smith et al. 2005; Sapkota et al. 2007). While BI-D1870 and SL-0101 are competitive inhibitors with respect to ATP, FMK (fluoromethylketone) is an irreversible inhibitor that covalently modifies the CTKD of RSK1, RSK2, and RSK4. These compounds have been tested against a panel of protein kinases and found to be relatively specific for the RSK isoforms (Bain et al. 2007). There are alternate mechanisms of activation for RSK, but these appear to be cell type- and context-specific. One of these involves tyrosine phosphorylation by ▶ Src, which was shown to stabilize the interaction between ERK and RSK, and thereby increase the rate at which RSK becomes activated (Kang et al. 2008). Another mechanism involves the regulation of the hydrophobic motif of RSK by the related enzymes, MAPK-activated protein kinases 2 and 3 (▶ MK2/3), which can facilitate RSK activation upon stimulation of the stress-responsive p38 MAPK pathway (Zaru et al. 2007).

Biological Functions

RSK seems to be a multifunctional ERK effector because it participates in various cellular processes (Cargnello and Roux 2011). Although a number of RSK functions can be deduced from the nature of its substrates, data from many groups point toward roles for the RSKs in nuclear signaling, cell cycle progression and cell proliferation, cell growth and protein synthesis, and cell migration and cell survival. RSK was found to regulate several transcription factors, including SRF, c-Fos, and Nur77. On the basis of its substrates, RSK seems to have important functions in cellular growth control and proliferation. RSK may stimulate cell cycle progression through the regulation of immediate early gene products, such as c-Fos, which promotes the expression of cyclin D1 during the G0/G1 transition to S phase. RSK may also promote proliferation by regulating cell-growth-related protein synthesis. Indeed, RSK was found to regulate the tumor suppressor TSC2 (Roux et al. 2004), and thereby promote ▶ mTOR signaling. RSK has also been shown to regulate cell survival. RSK phosphorylates and inhibits the pro-apoptotic proteins Bad and ▶ DAPK, thereby promoting survival in response to mitogenic stimulation (Shimamura et al. 2000; Anjum et al. 2005). Many additional RSK substrates have been identified through the years, but at this point, very little is known regarding isoform specificity. Whereas more substrates have been identified for RSK2 than any other RSK isoforms, most studies have not determined isoform selectivity. Therefore, many known substrates of RSK2 may be shared by different RSK family members and more effort will be necessary to assess potential overlapping functions.

Physiological Functions

An important clue into the physiological roles of the RSK isoforms came from the finding that inactivating mutations in the *Rps6ka3* gene (which encodes RSK2) were the cause of Coffin-Lowry Syndrome (CLS) (Trivier et al. 1996). CLS is an X-linked mental retardation syndrome characterized in male patients by psychomotor retardation and facial, hand, and skeletal malformations (Pereira et al. 2010). *Rps6ka3* mutations are extremely heterogeneous and lead to loss of phosphotransferase activity in the RSK2 kinase, most often because of premature termination of translation. It was shown that individuals with CLS consistently presented markedly reduced total brain volume, with cerebellum and hippocampus volumes being particularly impacted in CLS patients. The physiological role of RSK2 was also studied in the mouse through the generation of a deletion model. These mice were

shown to have deficiencies in learning and cognitive functions, as well as having poor coordination compared to wild-type littermates (Dufresne et al. 2001; Poirier et al. 2007). The exact cause for these phenotypes remains unknown, but a recent study demonstrated that shRNA-mediated RSK2 depletion perturbs the differentiation of neural precursors into neurons and maintains them as proliferating radial precursor cells (Cargnello and Roux 2011). Evidently, more experimentation using RSK2-deficient animals will be required to fully understand the developmental role of RSK2 in the nervous system.

Mice deficient in RSK2 expression also develop a progressive skeletal disease, called osteopenia, due to cell-autonomous defects in osteoblast activity (David et al. 2005). Both c-Fos and ATF4 transcription factors were shown to be critical RSK2 substrates involved in these effects in osteoblasts (Cargnello and Roux 2011). In addition, RSK2 knockout mice are approximately 15% smaller than their wild-type littermates, with a specific loss of white adipose tissue that is accompanied by reduced serum levels of the adipocyte-derived peptide, leptin. RSK1/RSK2/RSK3 triple knockout mice are viable, but no other information regarding their phenotype has yet been reported (Cargnello and Roux 2011). The *Rps6ka6* gene (that codes for RSK4) is located on chromosome X and was suggested to be involved in nonspecific X-linked mental retardation, but definitive evidence remains to be provided. Interestingly, deletion of *Drosophila* RSK was found to result in defects in learning and conditioning (Putz et al. 2004), but whether these deficits result from the specific loss of RSK activity will require further investigation.

Summary

Many studies have now clearly established RSK as an important effector of the Ras/MAPK pathway, and it is becoming increasingly evident that deregulation of RSK signaling has a significant role in several human diseases (Romeo and Roux 2011). Recent studies have expanded the repertoire of biological functions linked to the RSK family of protein kinases, ranging from the regulation of transcription, translation, and protein stability to the control of cell survival, cell motility, cell growth, and proliferation. It has become clear that other AGC family members such as Akt and S6K have similar functions and often share similar protein targets. These findings emphasize the importance of a tight and intricate regulation by more than one pathway of cellular processes that are important for cell growth and cell survival. Ongoing systems approaches that aim to identify RSK phosphorylation targets using mass spectrometry should expand our understanding of RSK signaling and function. The generation of isoform-specific mouse knockouts will similarly help resolve the distinct functionality of these protein kinases. Combined, these studies will help to identify additional targets for therapeutic intervention in diseases that are associated with inappropriate signaling downstream of Ras and Raf, and reveal novel targets for biomarker development for disease detection.

References

Anjum R, Blenis J. The RSK family of kinases: emerging roles in cellular signalling. Nat Rev Mol Cell Biol. 2008;9:747–58.

Anjum R, Roux PP, Ballif BA, Gygi SP, Blenis J. The tumor suppressor DAP kinase is a target of RSK-mediated survival signaling. Curr Biol. 2005;15:1762–7.

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. Biochem J. 2007;408:297–315.

Bjorbaek C, Zhao Y, Moller DE. Divergent functional roles for p90rsk kinase domains. J Biol Chem. 1995;270:18848–52.

Blenis J, Chung J, Erikson E, Alcorta DA, Erikson RL. Distinct mechanisms for the activation of the RSK kinases/MAP2 kinase/pp 90rsk and pp70–S6 kinase signaling systems are indicated by inhibition of protein synthesis. Cell Growth Differ. 1991;2:279–85.

Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. 2011;75:50–83.

Carriere A, Ray H, Blenis J, Roux PP. The RSK factors of activating the Ras/MAPK signaling cascade. Front Biosci. 2008;13:4258–75.

Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell. 1992;69:1227–36.

Cohen MS, Zhang C, Shokat KM, Taunton J. Structural bioinformatics-based design of selective, irreversible kinase inhibitors. Science. 2005;308:1318–21.

Cohen MS, Hadjivassiliou H, Taunton J. A clickable inhibitor reveals context-dependent autoactivation of p90 RSK. Nat Chem Biol. 2007;3:156–60.

Dalby KN, Morrice N, Caudwell FB, Avruch J, Cohen P. Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. J Biol Chem. 1998;273:1496–505.

David JP, Mehic D, Bakiri L, Schilling AF, Mandic V, Priemel M, Idarraga MH, Reschke MO, Hoffmann O, Amling M, Wagner EF. Essential role of RSK2 in c-Fos-dependent osteosarcoma development. J Clin Invest. 2005;115:664–72.

Dufresne SD, Bjorbaek C, El-Haschimi K, Zhao Y, Aschenbach WG, Moller DE, Goodyear LJ. Altered extracellular signal-regulated kinase signaling and glycogen metabolism in skeletal muscle from p90 ribosomal S6 kinase 2 knockout mice. Mol Cell Biol. 2001;21:81–7.

Erikson E, Maller JL. A protein kinase from Xenopus eggs specific for ribosomal protein S6. Proc Natl Acad Sci USA. 1985;82:742–6.

Erikson E, Maller JL. Purification and characterization of a protein kinase from Xenopus eggs highly specific for ribosomal protein S6. J Biol Chem. 1986;261:350–5.

Frodin M, Jensen CJ, Merienne K, Gammeltoft S. A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1. EMBO J. 2000;19:2924–34.

Frodin M, Antal TL, Dummler BA, Jensen CJ, Deak M, Gammeltoft S, Biondi RM. A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. EMBO J. 2002;21:5396–407.

Hsiao K-M, Chou S-Y, Shih S-J, Ferrell Jr JE. Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer *in vivo*. Proc Natl Acad Sci USA. 1994;91:5480–4.

Jensen CJ, Buch MB, Krag TO, Hemmings BA, Gammeltoft S, Frodin M. 90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1. J Biol Chem. 1999;274:27168–76.

Kang S, Dong S, Guo A, Ruan H, Lonial S, Khoury HJ, Gu TL, Chen J. Epidermal growth factor stimulates RSK2 activation through activation of the MEK/ERK pathway and src-dependent tyrosine phosphorylation of RSK2 at Tyr-529. J Biol Chem. 2008;283:4652–7.

Meyuhas O. Physiological roles of ribosomal protein S6: one of its kind. Int Rev Cell Mol Biol. 2008;268:1–37.

Newton AC. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem J. 2003;370:361–71.

Pereira PM, Schneider A, Pannetier S, Heron D, Hanauer A. Coffin-Lowry syndrome. Eur J Hum Genet. 2010;18:627–33.

Poirier R, Jacquot S, Vaillend C, Soutthiphong AA, Libbey M, Davis S, Laroche S, Hanauer A, Welzl H, Lipp HP, Wolfer DP. Deletion of the Coffin-Lowry syndrome gene Rsk2 in mice is associated with impaired spatial learning and reduced control of exploratory behavior. Behav Genet. 2007;37:31–50.

Putz G, Bertolucci F, Raabe T, Zars T, Heisenberg M. The S6KII (rsk) gene of Drosophila melanogaster differentially affects an operant and a classical learning task. J Neurosci. 2004;24:9745–51.

Richards SA, Fu J, Romanelli A, Shimamura A, Blenis J. Ribosomal S6 kinase 1 (RSK1) activation requires signals dependent on and independent of the MAP kinase ERK. Curr Biol. 1999;9:810–20.

Romeo Y, Roux PP. Paving the way for targeting RSK in cancer. Expert Opin Ther Targets. 2011;15:5–9.

Roux PP, Richards SA, Blenis J. Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. Mol Cell Biol. 2003;23:4796–804.

Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci USA. 2004;101:13489–94.

Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N, Blenis J. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. J Biol Chem. 2007;282:14056–64.

Sapkota GP, Cummings L, Newell FS, Armstrong C, Bain J, Frodin M, Grauert M, Hoffmann M, Schnapp G, Steegmaier M, Cohen P, Alessi DR. BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms in vitro and in vivo. Biochem J. 2007;401:29–38.

Shimamura A, Ballif BA, Richards SA, Blenis J. Rsk1 mediates a MEK-MAP kinase cell survival signal. Curr Biol. 2000;10:127–35.

Smith JA, Poteet-Smith CE, Malarkey K, Sturgill TW. Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. J Biol Chem. 1999;274:2893–8.

Smith JA, Poteet-Smith CE, Xu Y, Errington TM, Hecht SM, Lannigan DA. Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. Cancer Res. 2005;65:1027–34.

Sutherland C, Campbell DG, Cohen P. Identification of insulin-stimulated protein kinase-1 as the rabbit equivalent of rskmo-2: identification of two threonines phosphorylated during activation by mitogen-activated protein kinase. Eur J Biochem. 1993;212:581–8.

Thomas GM, Rumbaugh GR, Harrar DB, Huganir RL. Ribosomal S6 kinase 2 interacts with and phosphorylates PDZ domain-containing proteins and regulates AMPA receptor transmission. Proc Natl Acad Sci USA. 2005;102 (42):15006–11.

Trivier E, De Cesare D, Jacquot S, Pannetier S, Zackai E, Young I, Mandel JL, Sassone-Corsi P, Hanauer A. Mutations in the kinase Rsk-2 associated with Coffin-Lowry syndrome. Nature. 1996;384:567–70.

Vik TA, Ryder JW. Identification of serine 380 as the major site of autophosphorylation of Xenopus pp 90rsk. Biochem Biophys Res Commun. 1997;235:398–402.

Zaru R, Ronkina N, Gaestel M, Arthur JS, Watts C. The MAPK-activated kinase Rsk controls an acute Toll-like receptor signaling response in dendritic cells and is activated through two distinct pathways. Nat Immunol. 2007;8:1227–35.

Zhao Y, Bjorbaek C, Moller DE. Regulation and interaction of pp 90(rsk) isoforms with mitogen-activated protein kinases. J Biol Chem. 1996;271:29773–9.

Rsk-Like (RSKL)

► MSK1

Rsk-Like Protein Kinase (RLPK)

▶ MSK1

Rtef-1

▶ Tead

R-Type

▶ Voltage-Gated Calcium Channels: Structure and Function (CACNA)

RXFP1: LGR7

▶ Relaxin Family Peptide Receptors (RXFP) 1 and 2

RXFP2: LGR8

▶ Relaxin Family Peptide Receptors (RXFP) 1 and 2

RXFP3: GPCR135, SALPR

▶ Relaxin Family Peptide Receptors (RXFP) 3 and 4

RXFP4: GPCR142

▶ Relaxin Family Peptide Receptors (RXFP) 3 and 4

Ryanodine Receptor

▶ Ryanodine Receptor (RyR)

Ryanodine Receptor (RyR)

Filip Van Petegem and Kelvin Lau
Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

Synonyms

Calcium Release Channel; Ryanodine Receptor; Ryanodine-sensitive Ca^{2+} Release Channels; Sarcoplasmic Reticulum Calcium Release Channels

Historical Background

The contraction of cardiac and skeletal muscles requires the release of Ca^{2+} from the sarcoplasmic reticulum (SR) through specialized membrane proteins. The ion channels responsible for the release were not identified until the late 1980s. Ryanodine is an alkaloid found in the South American plant *Ryania speciosa*, and has insecticidal activity. This compound was found to bind to the elusive Ca^{2+} release channel, which was subsequently named the Ryanodine Receptor (RyR) (Meissner 1986). Early biochemical evidence first showed that the channel exists as a homotetramer, with each monomer having a mass of ~550 kDa. This makes RyRs the largest known ion channels at ~2.2 MDa (Lai et al. 1988). They were shown to be located in the SR with a "foot" region that spans the gap between the transverse tubule and the SR. Work on SR vesicles showed that Ca^{2+} release can be observed from these channels and that they could be modulated by various small molecules like ATP, Mg^{2+}, and Ca^{2+}. Recordings made in planar lipid bilayers showed that RyRs are permeable only to small inorganic and organic cations and are completely impermeable to anions.

Mammalian organisms contain three known RyR isoforms. In 1989, Takeshima et al. reported the first primary sequence and cloning of cDNA of RyR derived from rabbit skeletal muscle (RyR1) (Takeshima et al. 1989). The following year, the group of MacLennan published the cloning of the human skeletal muscle RyR and rabbit cardiac muscle isoform (RyR2) (Otsu et al. 1990). A third isoform

(RyR3), distinct from both the cardiac and skeletal muscle isoforms, was originally cloned from brain tissue (Hakamata et al. 1992). The genes of all the isoforms are located on different chromosomes in humans. RyR1 is found on chromosome 19q13.2 spanning 104 exons. The gene encoding RyR2 is on chromosome 1q43 (102 exons) and RyR3 is encoded on 15q13.3-14 (103 exons). In addition to mammalian organisms, RyRs have also been identified in nonmammalian vertebrates with two isoforms, RyRα (homologous to RyR1) and RyRβ (similar to RyR3). Moreover, RyR genes have also been identified in invertebrates including *Caenorhabditis elegans* and *Drosophila melanogaster*.

Overall Function

RyRs are large (~2.2 MDa) ion channels that release Ca^{2+} from the endoplasmic (ER) or sarcoplasmic reticulum (SR) upon opening. In doing so, they play a major role in the contraction of both cardiac and skeletal muscle. Their expression in many cell types suggests they are involved in many diverse processes that are Ca^{2+} dependent. Three different isoforms have been found in mammalian organisms (RyR1, RyR2, RyR3). They are highly similar with amino acid sequence identities of ~70%. There are several divergent regions between the RyRs, with the greatest dissimilarity found near the C-terminus. RyR1 is known as the skeletal muscle form, whereas RyR2 is often referred to as the cardiac isoform. However, all three isoforms are expressed in many diverse cell types. RyRs display significant homology to another intracellular Ca^{2+} release channel, the inositol-1,4,5-trisphosphate receptor.

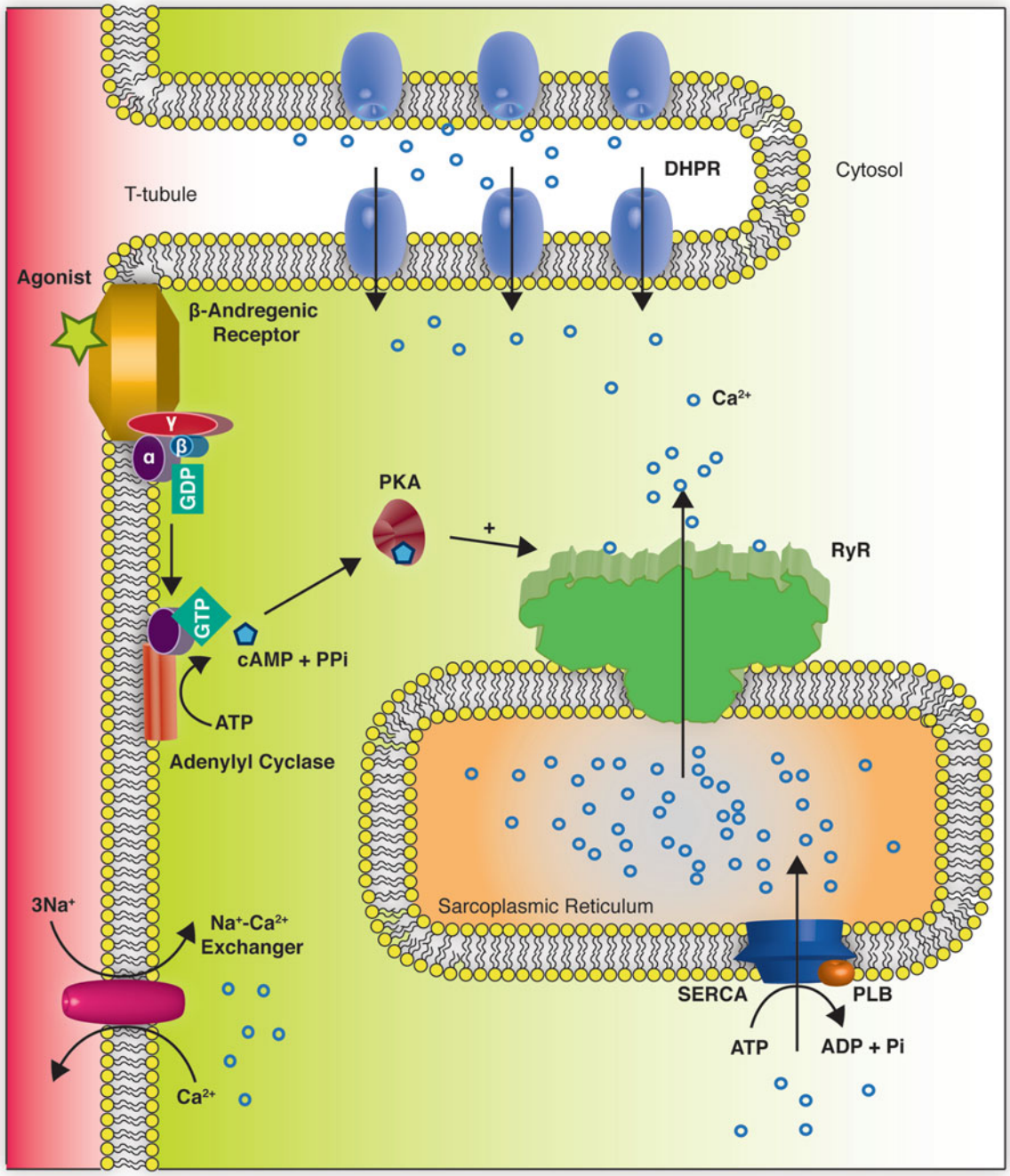
The primary signal to trigger the opening of RyRs is Ca^{2+}. This is especially relevant in cardiac myocytes, in a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (Fig. 1). In this scheme, depolarization of the plasma membrane down the T-tubules leads to opening of L-type voltage-gated Ca^{2+} channels, resulting in the influx of Ca^{2+} from the extracellular space. This signal is detected by RyR2, which binds Ca^{2+}, opens and releases more Ca^{2+} from the SR. The RyR thus acts as a signal amplifier, by detecting small elevations in Ca^{2+} concentrations and increasing the signal. In addition, RyRs can be stimulated by rising Ca^{2+} levels in the ER lumen, in a process known as store overload induced Ca^{2+} release (SOICR) (Jiang et al. 2004). When Ca^{2+} levels in the cytoplasm rise too high, or when the lumenal Ca^{2+} levels drop too low, several mechanisms cause the channel to shut down.

A different situation occurs in skeletal muscle. Whereas RyR1 is also affected by Ca^{2+} levels, it can, in addition, detect conformational changes in the skeletal muscle variant of the voltage-gated Ca^{2+} channel ($Ca_V1.1$). It is thought that there is a direct link between an intracellular loop of $Ca_V1.1$, and the cytoplasmic face of RyR1(Block et al. 1988; Tanabe et al. 1990). Conformational changes in $Ca_V1.1$ can then directly cause structural changes in RyR1 in the absence of an initial Ca^{2+} signal.

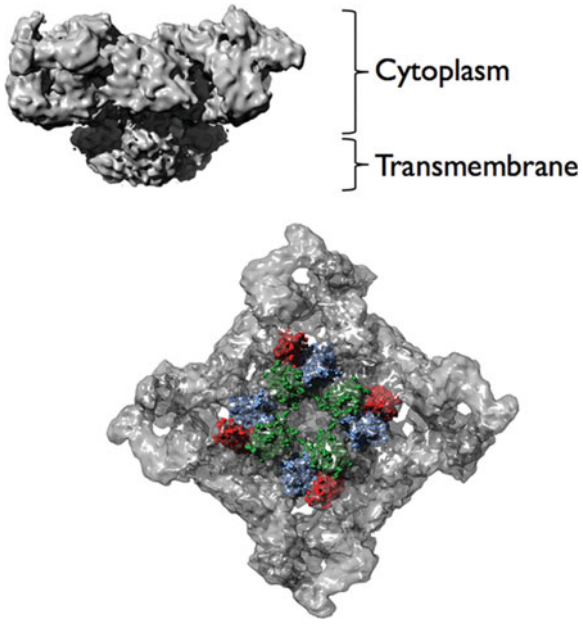
The importance of RyRs is evident from several knockout (KO) studies. RyR1 double KO mice die immediately after birth (Takeshima et al. 1994), whereas an RyR2 KO is embryonically lethal (Takeshima et al. 1998). RyR3 KO mice survive, but have impaired learning abilities (Balschun et al. 1999).

Structure

RyRs have been studied using both low- and high-resolution structural methods. Most notably, several cryo-electron microscopy (cryo-EM) studies have yielded high-quality images of the entire RyR1 isoform in the closed state, with a maximum reported resolution of 9.6 Å (Ludtke et al. 2005). This study, as well as many others on RyR2 and RyR3, revealed that RyRs display a mushroom-like shape (Fig. 2). The bulk of the protein (~90%) is located in the cytoplasm, with the remainder forming the transmembrane segments and ER lumenal portions. The cytoplasmic portion is often referred to as a "foot," and consists of several tubes and holes. This creates a maximum amount of exposed surface for the binding of auxiliary proteins and ligands. At least five α helices can be observed per monomer in the cryo-EM maps, but there is more volume to accommodate additional helices or possibly even β strands (Ludtke et al. 2005). In addition to the closed state architecture, cryo-EM studies have revealed the structure of RyR1 in the open state at 10.2 Å (Samso et al. 2009). Upon opening, the channel seems to undergo large conformational changes in the cytoplasmic region,



Ryanodine Receptor (RyR), Fig. 1 An overview of processes involved in RyR activation in cardiac myocytes. Particular voltage-gated calcium channels known as dihydropyridine receptors (*DHPR*) allow an influx of Ca^{2+} upon the arrival of an action potential. Through *CICR*, the RyR releases Ca^{2+} from the sarcoplasmic reticulum, triggering muscle contraction. Also shown is a PKA-dependent phosphorylation pathway which activates RyR upon β-adrenergic stimulation. The Sodium-Calcium Exchanger pumps remove one Ca^{2+} ion for 3 Na^{+} in return. The Sarco/endoplasmic Reticulum Ca^{2+}-ATPase (*SERCA*) allows for reuptake of Ca^{2+} from the cytosol. It is regulated by phospholamban (*PLB*) which is also a substrate target for PKA



Ryanodine Receptor (RyR), Fig. 2 (*Top*) A cryo-EM structure of RyR1 at 9.6 Å (Ludtke et al. 2005), as viewed from the side. A clear majority of the channel is located in the cytoplasm while only a fraction is embedded within the sarcoplasmic reticulum membrane. (*Bottom*) Top view of the RyR1 cryo-EM map, also showing the crystal structure of the first three N-terminal domains of RyR1, docked into the map (Tung et al. 2010). Shown are domains A (*blue*), B (*green*), and C (*red*). The domains form a four-fold symmetric vestibule of the channel that faces the cytoplasm. The RyR displays many tubes and holes for the binding of modulatory ligands and proteins

along with motions in the pore-forming part of the transmembrane region. The RyR is therefore a bona fide allosteric protein, whereby motions in the pore are coupled to regulatory domains in the cytoplasmic portion.

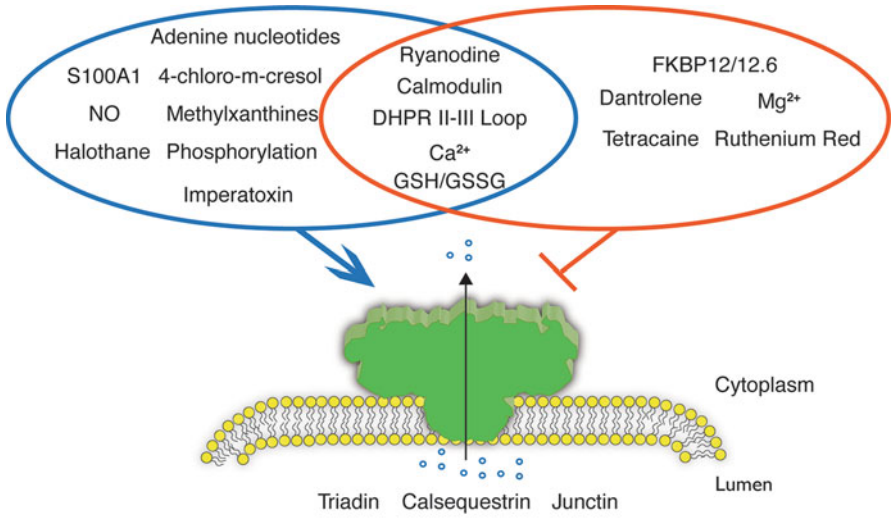
High resolution studies have been limited to individual domains of the RyR. The largest portion includes ~550 amino acids at the N-terminus of RyR1 (Fig. 2). This region folds up as three individual domains, including two β trefoil domains (domains A and B), and a bundle of five α helices (domain C). The three domains are located near the center at the cytoplasmic face, and connect to the corresponding domains in the neighboring subunits (Tung et al. 2010). These domains are predicted to undergo relative motions during opening and closing of the channel, thus being allosterically coupled to the pore region.

Protein and Ligand Interactions

As structural studies have shown, the majority of the volume of RyRs is exposed in the cytoplasm. The large surface area serves as a docking platform for numerous proteins and small molecules that are able to bind and modulate RyR function (Fig. 3). In addition, proteins in the ER lumen can also associate with the RyR. The precise sites of action for many of these interactions are still to be discovered.

Among the notable binding partners of RyRs is Calmodulin (CaM), a Ca^{2+}-binding protein that can provide Ca^{2+}-dependent feedback to RyRs and either stimulate or inhibit the channel depending on Ca^{2+} levels and the precise isoform. FK506-binding proteins (FKBPs) are known to bind tightly to RyRs and stabilize their closed-states. FKBP12 primarily associates with RyR1, while FKBP12.6 has the highest affinity for RyR2. Experimental evidence suggests that removal of FKBP results in subconductance states (Ahern et al. 1997). The ER lumen contains calsequestrin (CASQ), a Ca^{2+}-binding protein that can bind multiple Ca^{2+} ions and oligomerize. Together with the integral membrane proteins junctin and triadin, CASQ is thought to report on luminal Ca^{2+}-levels by providing feedback to the channel (Gyorke and Terentyev 2008). A peculiar interaction is present in skeletal muscle, where RyR1 is thought to interact directly with a protein in the plasma membrane, the voltage-gated calcium channel ($Ca_V1.1$) (Block et al. 1988; Tanabe et al. 1990). This allows for a direct link that couples electrical signals in the plasma membrane to Ca^{2+} release from the SR.

RyRs are the target of phosphorylation and dephosphorylation events. Enzymes implicated in this cycle include Protein Kinase A (PKA), which is anchored via an A kinase anchoring protein (AKAP), Protein Kinase G, protein phosphatases PP1 and PP2A, and the kinase CaMKII. Phosphorylation by both PKA and CaMKII has been found to increase the open probability of the channels, although a lot of controversy exists around the exact effect and mechanism (Bers 2004). Because RyRs are phosphorylated by PKA, they are under the control of β-adrenergic receptors, through a pathway involving G proteins, adenylate cyclase, cAMP, and PKA (Fig. 1). In addition to the proteins mentioned here, RyRs have been found to be modulated by many other binding partners (Fig. 3) (Kushnir and Marks 2010).



Ryanodine Receptor (RyR), Fig. 3 Multiple effectors modulate RyRs. Multiple agonists (*blue*) and antagonists (*red*) are known to affect both RyR isoforms. Some modulators have dual effects, depending on their concentration or specific RyR isoform. Modulation of RyRs can occur from either the cytoplasmic or lumenal side

Several small molecule ligands are also known to affect RyRs (Fig. 3). The receptor is sensitive to Ca^{2+}, in addition to other small cations. Notably, caffeine has a low-affinity binding site on the RyR and stimulates Ca^{2+} release. Ryanodine, a plant alkaloid that was originally found to affect the Ca^{2+} release channel, has dual effects on the channel. It stabilizes subconductance states at lower concentrations, but becomes inhibitory at higher levels (Lai et al. 1989). Various adenosine nucleotides are able to activate RyRs with ATP being the most potent. In addition, RyRs are sensitive to molecules present during oxidative stress such as NO.

Disease

Because Ca^{2+} is a very potent intracellular second messenger, it is not surprising that mutations in RyR genes can cause serious conditions. So far, only mutations in RyR1 and RyR2 have been associated with disease phenotypes (Betzenhauser and Marks 2010). The most commonly associated phenotypes are shown below.

Point mutations in RyR1 can lead to Malignant Hyperthermia (MH), Central Core Disease (CCD), and Multi Mini Core Disease (mmCD). MH manifests itself as severe rises in body temperatures upon the administration of volatile anesthetics, or under conditions of heat stress. CCD and mmCD are disorders with variable pathologies, but they are usually associated with cores of metabolically inactive tissue in the center of muscle fibers. Very often this results in muscle weakness.

In cardiac muscle, mutations in RyR2 are a cause of sudden cardiac death, mostly associated with two types of cardiac arrhythmias, catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2). CPVT is associated with bidirectional VT, usually triggered by emotional or physical stress. ARVD2 results in the replacement of ventricular tissue with fibrofatty deposits. In addition, idiopathic ventricular fibrillation can be the result of loss of RyR2 function.

The bulk of the mutations characterized so far cause a gain of function, resulting in increased or prolonged Ca^{2+} signals in the cytoplasm. In cardiac myocytes, this may activate the Na^+/Ca^{2+} exchanger, situated in the plasma membrane, which exchanges one Ca^{2+} for 3 Na^+ ions. The net influx of additional positive charges causes a delayed after depolarization (DAD), underlying the arrhythmogenic nature of the mutations. In skeletal muscle, the activation of RyR1 by halogenated anesthetics can cause a massive release of Ca^{2+}, leading to activation of Ca^{2+} ATPases. This depletes the ATP reserve, driving the cell in a hypermetabolic state which causes acidosis and a lethal increase in temperature (Betzenhauser and Marks 2010).

Disease mutations in RyR1 and RyR2 seem to cluster in three different regions of the receptor genes (hot spots). These three locations in both receptors match, suggesting important functional roles for those portions. They are located near the N-terminus, in a central domain region, and a C-terminal

region covering the transmembrane segments. A high-resolution structure of the N-terminal hot spot shows that most mutations affect domain–domain interactions (Tung et al. 2010). As these domains likely move during opening of the channel, weakening their interfaces is thought to facilitate the opening, causing inadvertent leak of Ca^{2+} into the cytoplasm.

Summary

Ryanodine Receptors (RyRs) are large ion channels, located in the membrane of the ER, that release Ca^{2+} upon stimulation. Three isoforms are found in mammalian organisms. All form homotetrameric assemblies of up to 2.2 MDa in size, and have a very large cytoplasmic portion that contains phosphorylation targets and docking sites for multiple small molecules and protein binding partners. Ca^{2+} is the primary ligand that triggers opening, and the channel can sense both cytoplasmic and lumenal Ca^{2+} levels. They play a prime role in excitation-contraction coupling, whereby they amplify the signal generated by voltage-gated calcium channels. Mutations are known to cause devastating diseases originating either in skeletal or cardiac muscle, and most of them are responsible for a gain of function, leading to increased Ca^{2+} release.

References

Ahern GP, Junankar PR, Dulhunty AF. Subconductance states in single-channel activity of skeletal muscle ryanodine receptors after removal of FKBP12. Biophys J. 1997;72(1):146–62.

Balschun D, Wolfer DP, Bertocchini F, Barone V, Conti A, Zuschratter W, et al. Deletion of the ryanodine receptor type 3 (RyR3) impairs forms of synaptic plasticity and spatial learning. EMBO J. 1999;18(19):5264–73.

Bers DM. Macromolecular complexes regulating cardiac ryanodine receptor function. J Mol Cell Cardiol. 2004; 37(2):417–29.

Betzenhauser MJ, Marks AR. Ryanodine receptor channelopathies. Pflugers Arch. 2010;460(2):467–80.

Block BA, Imagawa T, Campbell KP, Franzini-Armstrong C. Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. J Cell Biol. 1988;107(6 Pt 2):2587–600.

Gyorke S, Terentyev D. Modulation of ryanodine receptor by luminal calcium and accessory proteins in health and cardiac disease. Cardiovasc Res. 2008;77(2):245–55.

Hakamata Y, Nakai J, Takeshima H, Imoto K. Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. FEBS Lett. 1992; 312(2–3):229–35.

Jiang D, Xiao B, Yang D, Wang R, Choi P, Zhang L, et al. RyR2 mutations linked to ventricular tachycardia and sudden death reduce the threshold for store-overload-induced Ca2+ release (SOICR). Proc Natl Acad Sci U S A. 2004; 101(35):13062–7.

Kushnir A, Marks AR. The ryanodine receptor in cardiac physiology and disease. Adv Pharmacol. 2010;59:1–30.

Lai FA, Erickson HP, Rousseau E, Liu QY, Meissner G. Purification and reconstitution of the calcium release channel from skeletal muscle. Nature. 1988;331(6154):315–9.

Lai FA, Misra M, Xu L, Smith HA, Meissner G. The ryanodine receptor-Ca2+ release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. J Biol Chem. 1989;264(28):16776–85.

Ludtke SJ, Serysheva II, Hamilton SL, Chiu W. The pore structure of the closed RyR1 channel. Structure. 2005;13(8): 1203–11.

Meissner G. Ryanodine activation and inhibition of the Ca2+ release channel of sarcoplasmic reticulum. J Biol Chem. 1986;261(14):6300–6.

Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH. Molecular cloning of cDNA encoding the Ca2+ release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J Biol Chem. 1990;265(23):13472–83.

Samso M, Feng W, Pessah IN, Allen PD. Coordinated movement of cytoplasmic and transmembrane domains of RyR1 upon gating. PLoS Biol. 2009;7(4):e85.

Takeshima H, Nishimura S, Matsumoto T, Ishida H, Kangawa K, Minamino N, et al. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature. 1989;339(6224):439–45.

Takeshima H, Iino M, Takekura H, Nishi M, Kuno J, Minowa O, et al. Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. Nature. 1994;369(6481):556–9.

Takeshima H, Komazaki S, Hirose K, Nishi M, Noda T, Iino M. Embryonic lethality and abnormal cardiac myocytes in mice lacking ryanodine receptor type 2. EMBO J. 1998;17(12):3309–16.

Tanabe T, Beam KG, Adams BA, Niidome T, Numa S. Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. Nature. 1990; 346(6284):567–9.

Tung CC, Lobo PA, Kimlicka L, Van Petegem F. The amino-terminal disease hotspot of ryanodine receptors forms a cytoplasmic vestibule. Nature. 2010;468(7323):585–8.

Ryanodine-Sensitive Ca^{2+} Release Channels

► Ryanodine Receptor (RyR)

S

S100 Calcium-Binding Protein

► S100 Proteins

S100 Proteins

Brian R. Dempsey, Anne C. Rintala-Dempsey and Gary S. Shaw
Department of Biochemistry, The University of Western Ontario, London, ON, Canada

Synonyms

S100 calcium-binding protein

S100 Protein Family Members

S100A1, S100A2, S100A3, S100A4, S100A5, S100A6, S100A7, S100A8, S100A9, S100A10, S100A11, S100A12, S100A13, S100A14, S100A15, S100A16, S100B, S100P, S100G, S100Z, trichohylin, filaggrin, filaggrin-2, cornulin, repetin

Historical Background/General: S100 Family

S100 proteins were first discovered in 1965 by Moore as a major protein fraction (0.6% of total soluble protein) isolated from bovine brain (Moore 1965). The protein was given the name S100 due to its high solubility in saturated ammonium sulfate. Later experiments showed the S100 protein fraction constituted two different dimeric species comprised of two β protomers (S100B) or an α, β heterodimer (Isobe et al. 1977). Early members of the S100 protein family were frequently given suffixes based on their localization or molecular size and included S100P (placental), S100C (cardiac or calgizzarin), p11 (11 kDa), and MRP8/MRP14 (myeloid regulatory proteins, 8 and 14 kDa). In 1993, initial genetic studies showed that six of the S100 genes were clustered on chromosome 1q21 (Engelkamp et al. 1993), a number that has expanded since. Based on this observation most of the proteins were renamed according to the physical order they occupy on the chromosome. These include S100A1 (formerly S100α), S100A2 (formerly S100L), S100A10 (p11), S100A8/S100A14 (MRP8/MRP14). A few S100 proteins are found on other chromosomes including S100B (21q21). Currently there are 27 known S100 family members: S100A1-A18, S100B, S100G, S100P, S100Z, trichohylin, filaggrin, filaggrin-2, cornulin, and repetin (Table 1).

Role of S100 Proteins in Calcium Signaling

The S100 proteins are members of the EF-hand family of calcium-binding proteins that also includes calmodulin, ► recoverin, and troponin-C. The sequence similarity between S100 family members is very high ranging from 54% for distantly related family members such as S100B and S100A14 to 76% for S100B and S100A1, which are located in close proximity on the S100 phylogenetic tree (Marenholz et al. 2004). Each S100 protomer contains between 90 and 114 residues and two calcium-binding sites. The first

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

S100 Proteins, Table 1 Signaling functions of some S100 proteins

S100	Other names	Tissue(s)	Signaling function	Effectors[a]
S100A1	S100α, S100, S100A	Heart, skeletal muscle	Skeletal muscle contraction	RyR1
			Heart muscle contraction	RyR2, SERCA2a, PLB, F1-ATPase
			Neurotransmitter release	Synapsins I and II
			Tumor suppression	p53, MDM2
			(−) Differentiation	MyoD
			(+) Heat shock response	Hsp70/90, CyP40, FKBP52
			(+) Cell morphogenesis and proliferation	NDR kinase
			Ubiquitination	CacyBP/SIP
			Cytoskeletal dynamics	CapZ, MT, tubulin, F-actin, titin, GFAP, desmin, RAGE
S100A2	S100L, CaN19	Epithelial, mammary, lung, kidney, prostate, salivary, esophagus	(−) Heat shock response	HOP, CyP40, FKBP52
			(+) Tumor suppression	p53, MDM2
			Cytoskeletal dynamics	Tropomyosin
S100A3	S100E	Epidermis (hair follicles)	Differentiation and Ca^{2+} homeostasis	PAD3, S100A3 (tetramer)
S100A4	Metastasin (Mts1), fibroblast-specific protein (FSP1), 18A2 pEL98, p9Ka, 42A CAPL, calvasculin	Ubiquitous, placenta	Cytoskeletal dynamics	Myosin IIA, liprin β1, F-actin
			Tumor suppression	p53, MDM2, MetAP2, NFκB
			Inflammation	RAGE
			Cell migration	Annexin A2. MMP-2, E-cadherin
S100A5	S100D	Olfactory bulb, brain stem, spinal trigeminal tract	Inflammation	RAGE
S100A6	Calcyclin, CACY, 2A9, CABP, 5B10, PRA	Fibroblasts, epithelial, smooth muscle, brain	Cytoskeletal dynamics	Annexin A2, A6, and A11
			Heat shock response	Sgt1, HOP
			Regulation of nuclear transport	Importinα
			Tumor suppression	p53, MDM2, RAGE
			Ubiquitination	CacyBP/SIP
S100A7	Psoriasin, PSOR1	Epithelial, mammary, fetal, ear, fetal skin, fetal tongue	(+) Cell proliferation	Jab1
			Cell migration	RAGE
			Anti-microbial inflammation	RAGE
S100A8	Calgranulin A, CAGA, MRP8, P8, CGLA, MIF, NIF, L1Ag, MAC387, 60B8AG, CFAG, calprotectin	Epithelial and immune cells	Cytoskeletal dynamics	Tubulin, S100A9
			(−) Cell proliferation	CKI, CKII, S100A9, RAGE
			(−) Inflammation	NADPH oxidase, S100A9, arachidonic acid
S100A9	Calgranulin B, CAGB, MRP14, CGLB, MIF, NIF, L1Ag, MAC387, 60BAG, CFAG, calprotectin, P14	Epithelial and immune cells	Differentiation	NADPH oxidase, S100A8, RAGE
			(−) Inflammation	NADPH oxidase, S100A8, arachidonic acid
S100A10	p11, annexin II ligand, calpactin I Light polypeptide, ANX2LG, CLP11, ANX2L, p11, p10, 42 C, GP11, CAL1L	Lung, intestine, kidney	Cytoskeletal dynamics	F-actin
			Neuromodulation	5HT4, 5HT1B,
			(−) Regulation of macrophages	tPA, Plg
			Exocytosis	Annexin A2, VAMP2
			(−) Tumor suppression	RhoGAP

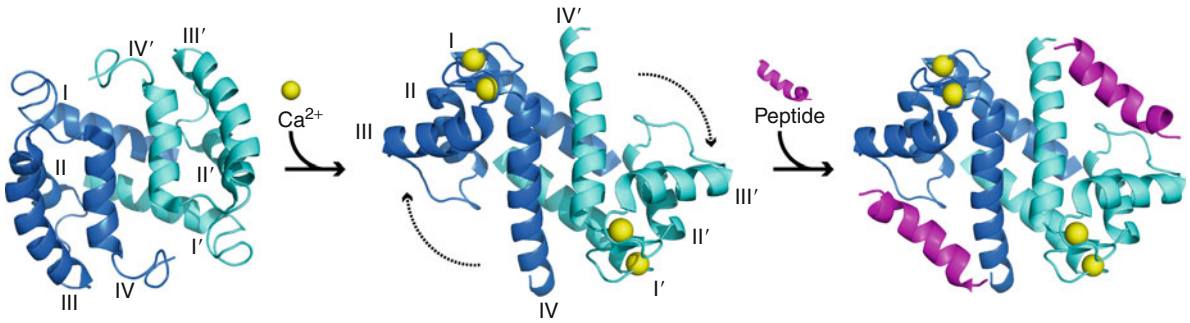
(*continued*)

Table 1 (continued)

S100	Other names	Tissue(s)	Signaling function	Effectors[a]
			Cell junction formation	Annexin A2, Cdc42
			Membrane receptor and channel presentation	Annexin A2, TRPV5, TRPV6, $NA_V1.8$, AHNAK, ASIC1a, TASK-1
S100A11	Calgizzarin, S100C, LN70	Smooth muscle, epidermal, Placenta, heart, lung, spleen, kidney, liver, bladder uterus	(−) Cell proliferation	Annexin A1, PLA2
			(+) Cell proliferation	RAGE
			Membrane repair	Annexin A1, annexin A2
			Tumor suppression	p53
			DNA damage response	Rad54b, nucleolin, PKCα
S100A12	Calgranulin C, CAAF1, CGRP p6, ENRAGE, calcitermin, MRP6	Immune cells	Inflammation	RAGE
			Ubiquitination	CacyBP/SIP
			Chemotaxis	GPCR
S100A13	N/A	Heart, skeletal muscle	Inflammation and angiogenesis	IL1α
			Neuroprotection and anti-necrosis	ProTα
			Mitogenesis	FGF1
S100A14	BCMP84, S100A15	Colon, thymus, kidney, liver, lung, small intestine	(+) Cell proliferation	RAGE
			(+) Apoptosis	RAGE
			Ca^{2+} homeostasis	Nucleobindin
S100A15	koebnerisin, S100A7L1, S100A7a	Epithelial, mammary	anti-microbial inflammation	GPCR
S100A16	S100F, DT1P1A7, MGC17528	Ubiquitous	(+) Cell proliferation	p53
S100B	S100β, NEF	Brain	(+) Cell survival	RAGE
			(+) Cell proliferation and (−) differentiation	FGF1
			Neuromodulation	D2R
			Cell migration	RAGE
			(−) Tumor suppression	p53, MDM2
			Cell morphogenesis and proliferation	NDR kinase
			Cytoskeletal dynamics	Annexin A6, CapZ, GFAP, MAG, MT, Caldesmon
S100P	N/A	Placenta, brain, spleen, lung	Cytoskeletal dynamics and cell migration	IQGAP1
			Inflammation	RAGE

Only S100 proteins with known signaling functions have been included in this table. Where possible (+) and (−) signs indicate the positive and negative regulation of the signaling function by the S100

[a]5-hydroxytryptamine receptor (5HT4/5HT1B), acid-sensing ion-channel-1a (ASIC1a), actin-capping protein (CapZ), c-jun activation domain binding, protein-1 (Jab1), casein kinase (CKI/CKII), cell division control protein 42 (Cdc42), cyclophillin 40 (CyP40), dopamine D2 receptor (D2R), FGF receptor-1 (FGFR1), fibroblast growth factor-1 (FGF1), G-protein coupled receptor (GPCR), glial fibrillary acidic protein (GFAP), heat shock protein (HSP), hsp70/hsp90 organizing protein (HOP), immunophilin (FKBP52), interleukin-1 (IL1α), kinesin light chain (KLC), matrix metalloproteinase-2 (MMP2), methionine aminopeptidas-2 (METAP2), microtubules (MT), mitochondrial ATPase (F1-ATPase), murine double minute (MDM2), mylein-associated glycoprotein (MAG), myogenic differentiation transciption factor (MyoD), nicotinamide adenine dinucleotide phosphate (NADPH), nuclear Dbf2-related protein kinase (NDR kinase), nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB), p38 mitogen activated protein kinase (MAPK), peptidylarginine deiminase type III (PAD3), phospholamban (PLB), phospholipase A2 (PLA2), protein kinase C (PKCα), prothymosin-α (ProTα), Ras GTPase-activating-like protein-1 (IQGAP1), receptor for advanced glycation end-products (RAGE), Rho GTPase-activating protein (RhoGAP), Ryanodine receptor (RyR1/RyR2), S100A6 binding protein/Siah 1 interacting protein (CacyBP/SIP), sarcoplasmic reticulum ATPase (SERCA2a), sodium voltage channel 1.8 (NAV1.8), synaptotagmin-1 (Syt1), tissue Plg activator (tPA), transient receptor potential channel (TRPV5/TRPV6), TWIK-related acid-sensitive K-1 (TASK-1), vesicle-associated membrane protein-2 (VAMP2), zymogen plasminogen (Plg)



S100 Proteins, Fig. 1 A general calcium-signaling mechanism for the S100 proteins. The three-dimensional structure of calcium-free S100B (*left*) comprises a symmetric dimer (*cyan, purple*) with each protomer possessing four α-helices (I-IV, I′-IV′) (Malik et al. 2008). Upon influx of calcium to the cytosol, the S100 protein binds calcium to two sites in each protomer (middle) forming the "open" conformation. In this structure, helix III (and III′) rearrange by approximately 100° compared to helix IV. This structural change exposes a shallow hydrophobic cleft used to recruit a target protein such as the N-terminus of NDR kinase (residues 72–87 shown) (*right*) that functions with S100B in regulation of cell proliferation and maintenance of cell morphology (Bhattacharya et al. 2003)

three-dimensional structure of an S100 protein was determined for calcium-free S100A6 (Potts et al. 1995). Subsequent to this, more than 40 structures of calcium-free, calcium-bound, and S100-protein complexes have been determined. These structures have shown the S100 protomer comprises two helix-loop-helix EF-hand motifs (Fig. 1). The first EF-hand comprises a 14-residue non-canonical calcium-binding site with weak calcium affinity sandwiched between two α-helices (I, II). The second EF-hand contains helices III and IV along with a 12-residue canonical calcium-binding site that typically has a higher affinity for the metal ion. The S100 dimer is formed through interactions between helices I and IV (I′, IV′) from each protomer (Fig. 1).

Calcium signaling is propagated by an S100 protein through its calcium-binding properties. In the resting cell, intracellular calcium levels are at around 100 nM. Due to the relatively low calcium affinity of S100 proteins, they are likely found in the calcium-free or apo state at resting cellular calcium concentrations. As a result of an extracellular signal from ligand-receptor binding, stress, or injury, calcium concentrations within the cell increase about tenfold, high enough to allow an S100 protein to bind two calcium ions per S100 protomer. The binding of calcium results in a conformational change of the S100 protein. The rearrangement causes helix III of each monomer of the S100 protein to swing outward resulting in a more "open" conformation (Fig. 1). For example, in S100B helix III rotates by more than 100° in relation to helix IV, exposing several hydrophobic residues at the helix III-IV interface (Drohat et al. 1998) while other S100 proteins open to varying degrees (Malik et al. 2008). The large, flat nature of this hydrophobic patch is used to recruit a considerable number of other proteins in the cell, giving rise to varied biological responses (Bhattacharya et al. 2004). Because the S100 dimer is symmetric, the net result of the calcium-binding event is exposure of two ligand-binding sites per molecule.

S100 proteins play a role in regulation of protein phosphorylation, enzyme activities, transcription factor activation, and cytoskeletal reorganization (Donato 2001; Santamaria-Kisiel et al. 2006). For example, S100B acts to modulate the phosphorylation activity of Ndr kinase, an important cell division enzyme, through binding of the autoinhibitory region of Ndr kinase (Fig. 1) (Bhattacharya et al. 2003). This in turn causes the release of the catalytic domain allowing autophosphorylation of key Ser/Thr residues, and full activation of the kinase as a key step in the regulation of cell division. Similarly, the heterodimeric S100A8/S100A9 protein inhibits the phosphorylation activity of the enzymes Casein Kinases I and II that are involved in cell-cycle control (Murao et al. 1990). S100A1, S100A2, S100A4, S100A8, S100A10, and S100B have been shown to bind to components of the cytoskeleton including tubulin, intermediate filaments, actin, and ▶ myosin modulating the assembly of these proteins. For example, the interaction between S100B and CapZ regulates actin filament extension. Binding of CapZ inhibits S100B from associating with, and

disassembling, microtubles and intermediate filaments (Sorci et al. 2000).

In some cases, S100 proteins can bind to targets in the absence of calcium in which case expression levels, cellular localization and, ultimately, availability of the target protein control their signaling role. In one particular case, substitutions in the calcium-binding sites of S100A10 prevent it from binding calcium, yet the protein retains an "open" conformation similar to other calcium-bound S100 proteins. In an interesting turn S100A10 interacts with partner proteins such as Annexin A2, which itself is a calcium-binding protein. It is the calcium-binding properties of Annexin A2 that regulate formation of a complex with S100A10 that is proposed to bridge phospholipid membranes in fusion and repair processes (Gerke and Moss 2002).

Expression and Tissue Specificity

The members of the S100 protein family are expressed in a wide variety of tissue types. Some of the S100 proteins appear to have ubiquitous expression (S100A11, S100A16) while others are expressed in only one or two specific tissue types (Table 1). The S100 proteins are typically expressed in the cytoplasm within cells but can undergo nuclear localization and even translocation to the extracellular matrix, through an unknown mechanism. Determining the expression profile for the S100 proteins is complicated due to their regulation by various cellular stimuli. For example, the expression of S100A8, S100A9, and S100A11 is altered due to infection or DNA damage. In addition, the S100 gene cluster is frequently rearranged in cancer cell types, often resulting in either increased or decreased S100 protein expression. One of the more unique and well-studied S100 proteins is S100B, which is expressed predominantly in glial cells of the brain. Recently the Dopamine D2 Receptor, which is found almost exclusively in brain tissue, was identified as a new S100B binding partner and it appears that S100B can enhance the dopamine signal (Liu et al. 2008). S100A1 is another S100 with a very specific tissue distribution. It is highly expressed in cardiac muscle cells and helps to regulate calcium cycling and contractile performance by binding to the ▶ Ryanodine Receptor (RyR) and the sarcoplasmic reticulum ATPase (SERCA2a). Interestingly, calcium-bound S100A1 competes at an overlapping binding site on the ▶ RyR with another EF-hand signaling protein calmodulin. The binding of S100A1 to ▶ RyR enhances calcium release and cell contraction in heart muscle while CaM binding results in inhibited ▶ RyR function (Treves et al. 1997).

Several S100 proteins are involved in the immune response. For example, S100A7 and S100A15 have been implicated in inflammation pathways leading to psoriasis and are highly expressed in epithelial cells. The S100A8/A9 heterodimer is expressed in immune cell types such as macrophages, neutrophils, and monocytes and are part of an inflammation pathway that is often involved in arthritis (Odink et al. 1987). For example, the expression of these proteins is regulated by transcriptional events such as the upregulation of mRNA of S100A8 and S100A9 in response to bacterial infection. Interestingly, the expression of S100A8, S100A9, and S100A7 has also been shown to be repressed by the transcription factors BRCA1 and c-▶ Myc, which are known as strong anticancer genes and appear to bind to the promoter elements of these S100 proteins. Not surprisingly, expression of the S100A7 gene has been shown to be DNA-damage inducible where it likely functions to regulate cell cycle progression.

Some S100 proteins have either been found in the extracellular matrix, or have been implicated in a signaling path through binding to the extracellular domain of the receptor for advanced glycation endproducts (RAGE). The RAGE receptor transduces extracellular stimuli leading to the activation of ▶ NF-κB and the release of proinflammatory cytokines as part of the innate immune response. Currently 12 different S100 proteins (S100A1, S100A4-S100A9, S100A11, S100A12, S100A14, S100B, S100P) have been found to interact with RAGE and elicit a variety of cellular responses. Interestingly, none of the S100 family members are expressed with signal peptides, which are normally required for transport across the cellular membrane through ER/golgi targeting. Therefore, S100 translocation is likely occurring through an alternate, but unidentified, mechanism.

Several S100 proteins experience altered localization in order to perform specific functions. For example, DNA damage (double-strand breaks and/or cell stress-calcium) results in the translocation of S100A11 to the nucleus by the nuclear transport protein nucleolin. Once in the nucleus, S100A11 competes with Sp1 for binding to nucleolin (Sakaguchi et al. 2003).

Free Sp1 results in changes in the transcription factor p21 and termination of DNA synthesis. Likewise, when a calcium signal is received, cytoplasmic S100A10 is targeted to the inner surface of the cell membrane by its binding partner Annexin A2.

S100 Proteins and Disease

Many S100 proteins and their signaling pathways are linked to a variety of diseases such as cancer (including: breast, prostate, bladder, lung, colorectal, melanomas), inflammatory conditions (including arthritis, psoriasis, Crohns/colitis), neurological disorders (including Alzheimer's, Parkinson's, schizophrenia), and cardiomyopathies.

A large number of S100 proteins are associated with cancers. Altered levels of expression of the S100 proteins have been linked to the disease and therefore are being used as biomarkers for the measurement of patient outcome. The majority of the S100 genes are located in a cluster on human chromosome 1q21, called the epidermal differentiation complex. This gene cluster is frequently rearranged in a wide array of cancers. This link between S100 proteins and cancer is likely due to the role that many of the proteins play in signaling paths involved in differentiation and proliferation of cells. In addition to their direct involvement in cancer, some of the S100 proteins are involved in cancer metastasis through their functioning in the regulation of cell motility. S100A4, S100A7, S100A12, S100B, and S100P are all involved in signaling pathways tied to cell migration and as a result are often linked to poor prognosis in cancer. S100 proteins have potential to be used as biomarkers for cancer screening. For example, overexpression of S100B correlates with reduced survival in malignant melanoma patients. Levels of S100B expression are being used in a clinical setting as a diagnostic marker for staging the melanoma, determining prognosis, evaluating treatment, and predicting relapse (Harpio and Einarsson 2004).

Several S100 proteins alter the activity of the tumor suppressor protein, ▶ p53 (Salama et al. 2008). Some S100 proteins, such as S100A4 and S100B, inhibit the phosphorylation of ▶ p53 by binding to its C-terminus. This leads to a decrease in its transcriptional activity and inhibition of its tumor-suppressor function. Alternatively, other S100 proteins, such as S100A2, have been found to upregulate ▶ p53 activity and increase the expression of pro-apoptotic genes. S100A4 overexpression has been linked to several cancers including bladder, breast, thyroid, lung, prostate, and colorectal cancers. S100A4 plays role in the metastasis of cancer cells by potentially altering cell motility through its interactions with F-actin and ▶ myosin and increasing invasiveness through the regulation of matrix metalloproteinase (MMP) activity. S100A7 is overexpressed in breast, bladder, and skin tumors and its elevated levels indicate poor prognosis and reduced survival. Enhancement of breast cancer cell survival mechanisms through an interaction of S100A7 with Jab1 has been shown. S100A8 and S100A9 form a heterodimer and together are upregulated in gastric, prostate, and colorectal cancers. In prostate tumor cells, S100A8/A9 were found to induce activation of the ▶ NF-κB pathway and cause phosphorylation of ▶ MAP Kinase through an extracellular interaction with RAGE. In some cases, S100 overexpression is related to tumor suppression rather than promotion. For example, S100A2 is expressed in the prostate, but is downregulated in prostate cancer. Similar observations have been found in breast cancer, with normal mammary epithelial cells expressing S100A2 but not tumor cells. Overexpression of S100A11 has been linked to tumor suppression in bladder and renal carcinomas.

S100B is found in the cells of the brain and has been linked to a number of neurological disorders such as Alzheimer's disease (AD). In the example of AD, S100B protein is found to be overexpressed in the astrocytes of affected individuals. Similarly, patients with schizophrenia also have increased serum concentrations of S100B protein. S100B has been shown to interact with the Dopamine D2 Receptor and can increase receptor signaling. The D2-Receptor is involved in neuromodulation and is closely tied to the above-mentioned disorders.

Downregulation of S100A1 results in a diseased heart and can lead to heart failure. S100A1 binds calcium during the calcium oscillations of heart cells increasing the contractile performance of the cardiomyocytes. The use of S100A1 gene therapy for the treatment of heart failure is approaching clinical trial stages. Keratinocytes overexpress S100A7 in the skin disorder, psoriasis. S100A8/S100A9 are secreted by neutrophils and play a role in inflammatory diseases such as inflammatory arthritis.

The S100A10/Annexin A2 complex controls the recruitment and function of ion channels including CFTR in cystic fibrosis.

Summary

First discovered in the brain, the S100 proteins are expressed in a wide variety of tissues. There are a large number of S100 family members and each has multiple binding partners and functions. The members of the S100 protein family are EF-hand calcium-binding proteins, which utilize calcium to propagate a response through a signaling pathway. Binding of calcium to the S100 protein causes a structural rearrangement, exposing a target-binding surface. This surface in most S100 proteins is large and can accommodate multiple targets, sometimes at the same time. Target binding results in a range of responses from inflammation and cytoskeletal reorganization to control of cell growth and tumor suppression. Altered expression of S100 proteins has been found in a number of human cancers and these proteins are becoming recognized as important signaling molecules in this disease. In addition, some S100 proteins such as S100B, are linked to neurological diseases including Parkinson's, Alzheimer's, and schizophrenia. Future research focusing on the in vivo identification of new target proteins will help expand the knowledge of S100 signaling pathways and the mechanisms these proteins use to recruit molecules. This in turn may allow some of the S100 proteins to become valuable pharmacological targets.

References

Bhattacharya S, Large E, Heizmann CW, Hemmings B, Chazin WJ. Structure of the Ca2+/S100B/NDR kinase peptide complex: insights into S100 target specificity and activation of the kinase. Biochemistry. 2003;42:14416–26.

Bhattacharya S, Bunick CG, Chazin WJ. Target selectivity in EF-hand calcium binding proteins. Biochim Biophys Acta. 2004;1742:69–79.

Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol. 2001;33:637–68.

Drohat AC, Baldisseri DM, Rustandi RR, Weber DJ. Solution structure of calcium-bound rat S100B (betabeta) as determined by nuclear magnetic resonance spectroscopy. Biochemistry. 1998;37:2729–40.

Engelkamp D, Schafer BW, Mattei MG, Erne P, Heizmann CW. Six S100 genes are clustered on human chromosome 1q21: identification of two genes coding for the two previously unreported calcium-binding proteins S100D and S100E. Proc Natl Acad Sci U S A. 1993;90:6547–51.

Gerke V, Moss SE. Annexins: from structure to function. Physiol Rev. 2002;82:331–71.

Harpio R, Einarsson R. S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. Clin Biochem. 2004;37:512–8.

Isobe T, Nakajima T, Okuyama T. Reinvestigation of extremely acidic proteins in bovine brain. Biochim Biophys Acta. 1977;494:222–32.

Liu Y, Buck DC, Neve KA. Novel interaction of the dopamine D2 receptor and the Ca2+ binding protein S100B: role in D2 receptor function. Mol Pharmacol. 2008;74:371–8.

Malik S, Revington M, Smith SP, Shaw GS. Analysis of the structure of human apo-S100B at low temperature indicates a unimodal conformational distribution is adopted by calcium-free S100 proteins. Proteins. 2008;73:28–42.

Marenholz I, Heizmann CW, Fritz G. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun. 2004;322:1111–22.

Moore BWA. soluble protein characteristic of the nervous system. Biochem Biophys Res Commun. 1965;19:739–44.

Murao S, Collart F, Huberman E. A protein complex expressed during terminal differentiation of monomyelocytic cells is an inhibitor of cell growth. Cell Growth Differ. 1990;1:447–54.

Odink K, Cerletti N, Bruggen J, Clerc RG, Tarcsay L, Zwadlo G, Gerhards G, Schlegel R, Sorg C. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. Nature. 1987;330:80–2.

Potts BCM, Smith J, Akke M, Macke TJ, Okazaki K, Hidaka H, Case DA, Chazin WJ. The structure of calcyclin reveals a novel homodimeric fold for S100 Ca^{2+}-binding proteins. Nat Struct Biol. 1995;2:790–6.

Sakaguchi M, Miyazaki M, Takaishi M, Sakaguchi Y, Makino E, Kataoka N, Yamada H, Namba M, Huh NH. S100C/A11 is a key mediator of Ca(2+)-induced growth inhibition of human epidermal keratinocytes. J Cell Biol. 2003;163:825–35.

Salama I, Malone PS, Mihaimeed F, Jones JL. A review of the S100 proteins in cancer. Eur J Surg Oncol. 2008;34:357–64.

Santamaria-Kisiel L, Rintala-Dempsey AC, Shaw GS. Calcium-dependent and -independent interactions of the S100 protein family. Biochem J. 2006;396:201–14.

Sorci G, Agneletti AL, Donato R. Effects of S100A1 and S100B on microtubule stability. An in vitro study using triton-cytoskeletons from astrocyte and myoblast cell lines. Neuroscience. 2000;99:773–783.

Treves S, Scutari E, Robert M, Groh S, Ottolia M, Prestipino G, Ronjat M, Zorzato F. Interaction of S100A1 with the Ca2+ release channel (ryanodine receptor) of skeletal muscle. Biochemistry. 1997;36:11496–503.

S12

► RPN8

S15

▶ Fibulins

S1P

▶ Sphingosine-1-Phosphate

SAMSN1 (SAM Domain, SH3 Domain, and Nuclear Localization Signal)

Yuan Xiao Zhu[1], A. Keith Stewart[1] and Jaime O. Claudio[2]
[1]Division of Hematology-Oncology, Mayo Clinic, Scottsdale, AZ, USA
[2]Toronto General Research Institute, University Health Network, Toronto Medical Discovery Tower, Toronto, ON, Canada

Synonyms

HACS1

Historical Background

The gene encoding SAMSN1 maps to human chromosome 21q11.2 in a region that is frequently disrupted by translocation events in hematopoietic malignancies (Mitelman et al. 1997). The full-length cDNA of human SAMSN1 was first identified and cloned in 2001 by two groups from myeloma cells and human cord blood–derived mast cells, named as HACS1 and Nash1, respectively (Claudio et al. 2001; Uchida et al. 2001). The predicted protein showed unique primary structure with a nuclear localization signal (NLS), a sterile alpha motif (SAM), and a Src homology 3 domain (SH3). SAMSN1 belongs to a novel family of scaffolding and adaptor proteins that includes SASH3/SLY/HACS2 (Beer et al. 2001) and SASH1/KIAA0790 (Zeller et al. 2003). The function of this novel family is not well characterized. The mouse, rat, and chimp orthologs have been identified, and unnamed protein homologs exist in chicken, zebrafish, and fly.

Expression of SAMSN1

SAMSN1 is expressed in hematopoietic tissues, particularly in B cells, macrophages, mast cells, and dendritic cells (Claudio et al. 2001). In embryonic mouse (Gitton et al. 2002; Reymond et al. 2002), it is highly expressed in blood vessels at 9.5–10.5 days post coitum (dpc). It is also expressed in the brain, future spinal cord, dorsal root ganglia, otocyst, eye, limb, heart, surface ectoderm, and bronchial arch, albeit at lower levels. At 14.5 dpc, weak and regionally restricted expression in the skin and homogenous weak expression in mesenchyme can be detected by in situ hybridization, but expression can no longer be detected in the brain or arterial and venous systems. In human, it is also expressed in the adult heart, kidney, placenta, and lung, and major expression can be detected in the bone marrow, peripheral blood, and immune tissues, including lymph node, spleen, and thymus. The Gene Expression Atlas database (http://www.ebi.ac.uk/gxa) shows human SAMSN1 is differentially expressed in 95 experiments, 40 organism parts, 100 disease states, 37 cell types, 27 cell lines, 13 compound treatments, and 20 other conditions.

Protein and Splice Variants

SAMSN1 encodes a 441 amino acid protein containing two domains frequently associated with signaling molecules. An SH3 motif is in the middle half of the protein and a SAM domain is located toward the C-terminal end. The SH3 motif shares 39% homology with the well-characterized adaptor protein Crk. There are three predicted consensus nuclear localization signals. A tyrosine kinase phosphorylation motif is predicted at amino acids 221–228. The orthologous mouse *Hacs1* gene is located on murine chromosome 16, which encodes a protein of 364 aa with an overall 87% homology to human SAMSN1 at protein level.

Two major splice variants have been detected in both human and mouse. The difference between these major variants is the N terminus, but both contain the SAM and SH3 domains. These two variants suggest

that there are two different promoters. In addition to truncation of the N terminus, six additional transcripts appear to differ by truncation of the 3' end and the presence or absence of, or differential use of, boundaries of eight cassette exons (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly).

Function of SAMSN1 in Immune Responses

An in vitro study and immunoblotting analysis showed SAMSN1 expression is upregulated in activated human B cells treated with interleukin (IL)-4, IL-13, CD40L, and anti-immunoglobulin (Ig) M. In murine spleen B cells, *Samsn1* can also be upregulated by IL-4, lipopolysaccharide, but not IL-13 (Zhu et al. 2004). SAMSN1 associates with tyrosine-phosphorylated proteins after B cell activation and binds in vitro to the inhibitory molecule paired Ig-like receptor B (PIR-B). Overexpression of *Samsn1* in murine spleen B cells resulted in a downregulation of the activation marker CD23 and enhancement of CD138 expression, IgM secretion, and *Xbp-1* expression. Silencing *SAMSN1* in a human B lymphoma cell line by small interfering ribonucleic acid did not significantly change IL-4-stimulated B cell proliferation.

In vivo study was performed by generating *Samsn1* gene knockout mice ($Samsn1^{-/-}$) (Wang et al. 2010). *Samsn1* knockout mice were viable and fertile and had normal bone marrow B cell development and normal splenic T cell and B cell populations. However, similar to PIR-B-deficient mice, $Samsn1^{-/-}$ mice have an increased peritoneal B1 cell population, increased phosphorylation of *Lyn* kinase in B cells, and augmented adaptive immune responses, suggesting that *Samsn1* plays an important role in suppression of adaptive immunity. Interestingly, SASH3/Sly, another member in the same family of SAMSN1, which has a 50% amino acid identity with SAMSN1 and also similarly expressed in lymphocytes, was found to play an opposite role to *Samsn1* (Beer et al. 2005). Mice expressing a defective SLY ($SLY1^{d}$) protein exhibit impaired immune responses and prolonged allograft survival.

SAMSN1 and Cancer

SAMSN1 protein was detected from different hematopoietic malignant cells, including myeloid leukemia, lymphoma, and multiple myeloma. The function of SAMSN1 in those cells remains unknown. SAMSN1 was suggested as a candidate tumor suppressor in a recent extensive screening of a large panel of lung cancer cell lines (Yamada et al. 2008). In another study, SASH1, another member in this family, has been described as a candidate tumor suppressor gene downregulated in breast cancer (Zeller et al. 2003). SASH1 was also reported to serve as a candidate tumor suppressor in colon cancer and associated with patient prognosis (Rimkus et al. 2006).

Summary

SAMSN1 is a member of a novel gene family of putative adaptors and scaffold proteins containing SH3 and SAM domains. Recent studies demonstrated SAMSN1 is a component of immune response and is upregulated and functions in activation of adaptive immunity. SAMSN1 was suggested as a candidate tumor repressor. Future studies will continue to investigate the functions of SAMSN1 in different normal cells and tumor cells, to identify the pathways, partners and mechanisms involved in SAMSN1-mediated biological or pathologic consequences.

References

Beer S, Simins AB, Schuster A, Holzmann B. Molecular cloning and characterization of a novel SH3 protein (SLY) preferentially expressed in lymphoid cells. Biochim Biophys Acta. 2001;1520(1):89–93.

Beer S, Scheikl T, Reis B, Hüser N, Pfeffer K, Holzmann B. Impaired immune responses and prolonged allograft survival in sly1 mutant mice. Mol Cell Biol. 2005;25(21):9646–60.

Claudio JO, Zhu YX, Benn SJ, Shukla AH, McGlade CJ, Falcioni N. Stewart AK HACS1 encodes a novel SH3-SAM adaptor protein differentially expressed in normal and malignant hematopoietic cells. Oncogene. 2001;20(38):5373.

Gitton Y, Dahmane N, Baik S, Ruiz I, Altaba A, Neidhardt L, et al. HSA21 expression map initiative. A gene expression map of human chromosome 21 orthologues in the mouse. Nature. 2002;420(6915):586–90.

Mitelman F, Mertens F, Johansson B. A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. Nat Genet. 1997;15:417–74.

Reymond A, Marigo V, Yaylaoglu MB, Leoni A, Ucla C, Scamuffa N, et al. Human chromosome 21 gene expression atlas in the mouse. Nature. 2002;420(6915):582–6.

Rimkus C, Martini M, Friederichs J, Rosenberg R, Doll D, Siewert JR, et al. Prognostic significance of downregulated expression of the candidate tumour suppressor gene SASH1 in colon cancer. Br J Cancer. 2006;95(10):1419–23.
Uchida T, Nakao A, Nakano N, Kuramasu A, Saito H, Okumura K, et al. Identification of nash1, a novel protein containing a nuclear localization signal, a sterile alpha motif, and an SH3 domain preferentially expressed in mast cells. Biochem Biophys Res Commun. 2001;288(1):137–41.
Wang D, Stewart AK, Zhuang L, Zhu Y, Wang Y, Shi C, et al. Enhanced adaptive immunity in mice lacking the immunoinhibitory adaptor Hacs1. FASEB J. 2010;24(3):947–56.
Yamada H, Yanagisawa K, Tokumaru S, Taguchi A, Nimura Y, Osada H, et al. Detailed characterization of a homozygously deleted region corresponding to a candidate tumor suppressor locus at 21q11-21 in human lung cancer. Genes Chromosom Cancer. 2008;47(9):810–8.
Zeller C, Hinzmann B, Seitz S, Prokoph H, Burkhard-Goettges E, Fischer J, et al. SASH1: a candidate tumor suppressor gene on chromosome 6q24.3 is downregulated in breast cancer. Oncogene. 2003;22(19):2972–83.
Zhu YX, Benn S, Li ZH, Wei E, Masih-Khan E, Trieu Y, et al. The SH3-SAM adaptor HACS1 is up-regulated in B cell activation signaling cascades. J Exp Med. 2004;200(6):737–47.

SAPKK3

▶ MKK6

SAPS (Src-Associated Adapter Protein with PH and SH3 Domains)

▶ SKAP-HOM

SARA

Constance E. Runyan and H. William Schnaper
Department of Pediatrics, Northwestern University, Chicago, IL, USA

Synonyms

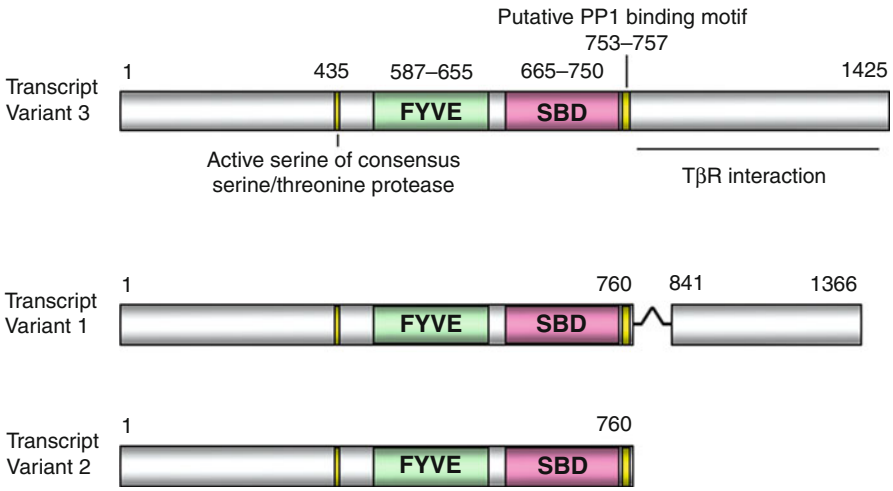
LOC9372; MADHIP, MAD homolog interacting protein; NSP, Novel serine protease; SMADIP, Smad interacting protein; ZFYVE9

Historical Background

The adaptor protein SARA was first identified as a novel serine protease-like molecule in human brain (Meckelein et al. 1998), and then later characterized as an important regulator of TGF-β1 signal transduction. SARA interacts with both the type I and type II TGF-β1 receptors (TβRI and TβRII), and contains a Smad-binding domain (SBD) as well as a double zinc finger FYVE domain that localizes SARA to endosomal subcellular compartments (Tsukazaki et al. 1998). SARA also contains a region homologous to the active site of trypsin-like serine proteases (Meckelein et al. 1998) and a binding motif for the catalytic subunit of type 1 serine/threonine protein phosphatase (PP1c) (Bennett and Alphey 2002). Three alternatively spliced transcripts encoding distinct isoforms have been found for this gene (Fig. 1).

SARA in TGF-β1 Signaling

The TGF-β superfamily consists of TGF-β1, TGF-β2, TGF-β3, activins, and bone morphogenic proteins (BMP). These proteins are widely expressed in virtually all mammalian cell types, as are their downstream signaling mediators, the Smad proteins. TGF-β signaling is initiated when ligand-bound TGF-β type II receptor (TβRII) binds to, and phosphorylates, the TGF-β type I receptor (TβRI) (Roberts et al. 1990; Piek et al. 1999; Shi and Massague 2003). This phosphorylation, in the TβRI cytoplasmic GS region, leads to its activation and its ability to activate the receptor-regulated Smads (R-Smads), Smad2, and Smad3, by C-terminal serine phosphorylation. Once phosphorylated, the R-Smads form a heteromultimeric complex with the common mediator (Co)-Smad (Smad4) and accumulate in the nucleus to regulate transcriptional responses (Roberts et al. 1990; Piek et al. 1999; Shi and Massague 2003). The inhibitory Smads, Smad6 and Smad7, compete with the R-Smads for binding to the activated receptors (Shi and Massague 2003). R-Smads and Smad4 contain a Mad Homology (MH)-1 domain, which confers transcriptional activity, a linker region, and an MH2 domain involved in protein-protein interactions with other transcription factors and co-activators (Piek et al. 1999; Shi and Massague 2003). Figure 2 shows a basic diagram of the Smad signaling cascade induced by TGF-β1. However, this



SARA, Fig. 1 Putative domain structure of the three known variants of SARA. FYVE; zinc finger domain, named after the four cysteine-rich proteins: Fab 1 (yeast orthologue of PIKfyve), YOTB, Vac 1 (vesicle transport protein), and EEA1, in which it has been found. SBD; Smad-binding domain

oversimplified pathway is clearly insufficient to explain the plethora of outcomes induced by TGF-β1. Numerous proteins have been identified that promote Smad expression, stability, activation, and assembly into transcription-regulatory complexes (Bottinger and Bitzer 2002). Among these proteins, recent research has demonstrated important roles for adaptor proteins such as SARA that control the localization of Smads and TβRs, and the interaction of Smads with the receptor complex.

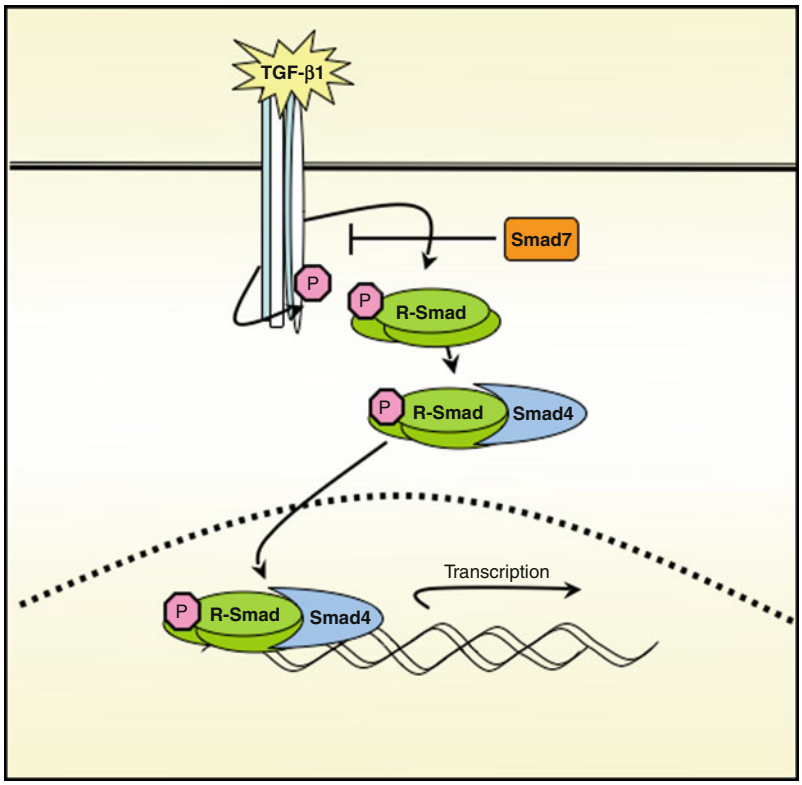
SARA was initially described as a recruitment factor, bringing R-Smads to the TβR complex for phosphorylation (Tsukazaki et al. 1998). In that study, SARA and Smad2 were described as being associated basally but separated upon TGF-β stimulation. However, a subsequent study found minimal basal association between SARA and Smad2, but a strong interaction after treatment with TGF-β (Runyan et al. 2005). SARA can associate basally with TβRI and TβRII (Tsukazaki et al. 1998). Therefore, it remains unclear whether SARA is predominantly localized with R-Smads or with TβRs in unstimulated cells.

The SBD of SARA can interact with the MH2 domain of either Smad2 or Smad3 (Tsukazaki et al. 1998) when over-expressed in cultured cell lines. However, examination of endogenous protein expression and interaction suggests that SARA preferentially binds Smad2 over Smad3 (Runyan et al. 2005). The ability of Smad3 to interact with SARA has been reported to be dispensable for Smad3 function (Goto et al. 2001; Runyan et al. 2009). In contrast, knockdown of SARA in HKC kidney proximal tubule cells results in a specific reduction in Smad2 expression, phosphorylation, nuclear translocation, and transcriptional activity (Runyan et al. 2009). In hepatic stellate cells, SARA expression is reduced during myofibroblastic differentiation and this reduced expression of SARA corresponds to a switch in predominant R-Smad activation by TGF-β from Smad2 to Smad3 (Liu et al. 2003).

In addition to promoting Smad2-dependent signaling, SARA may also function as a negative regulator of the TGF-β pathway. The *Drosophila melanogaster* homolog of SARA (Sara) binds the catalytic subunit of the type 1 serine/threonine protein phosphatase (PP1c), and mutations that disrupt this interaction result in hyper-phosphorylated type I receptor and inhibition of TGF-β-mediated signaling (Bennett and Alphey 2002). Later studies suggested that it was the Smad7-mediated antagonism of TβRI that was enhanced by the SARA PP1c interaction. Smad7 binds to the GADD34 regulatory subunit of the PP1 holoenzyme, which results in recruitment of the PP1c catalytic subunit to dephosphorylate the TβRI. This recruitment is enhanced by SARA interaction with PP1c (Shi 2004).

Endocytosis, SARA, and TGF-β1 Signaling

Tightly controlled localization of the TGF-β signaling components makes logistic sense as a modulator of the otherwise simple and linear Smad pathway. However, while roles for clathrin-mediated endocytosis (CME) as well as caveolar internalization have recently been proposed to impact the TGF-β pathway, receptor



SARA, Fig. 2 Basic TGF-β-Smad signaling pathway

trafficking in TGF-β-mediated Smad signaling is not completely understood.

CME initiates with formation of clathrin-coated vesicles via interaction of cytosolic proteins with components of the inner leaflet of the plasma membrane. Transmembrane receptors bind directly or indirectly to the heterotetrameric AP-2 adaptor complex which then interacts with the clathrin triskelion that polymerizes to form a basket-shaped lattice pulling the membrane into the cell. This invaginated clathrin-coated vesicle (CCV) is then internalized to the major sorting center of the cell, the early endosome. From the early endosome, internalized material can be transported back to the membrane through the recycling endosome, or routed to either the late endosome or to the trans-Golgi network. From either the late endosome (also referred to as a multi-vesicular body) or the trans-Golgi, internalized proteins may be redirected back to the early endosome, back to the membrane, or to the lysosome for degradation. These processes of endocytosis, trafficking, endosome fusion, and exocytosis (reviewed in (Mousavi et al. 2004; Miaczynska et al. 2004)) are controlled by diverse members of the Ras-like small G proteins called Rab GTPases. Like other GTPases, the Rab proteins cycle between an active, GTP-bound, and an inactive, GDP-bound state. Individual isoforms of the Rab proteins are localized to the surface of distinct, membrane-bound organelles. Rab4, Rab5, and Rab11 localize to early endosomes. However, Rab4 or Rab11 may also be found on the surface of perinuclear recycling endosomes. Rab7 and Rab9 localize to late endosomes and lysosomes, and Rab1 and Rab2 associate with vesicles mediating ER-to-Golgi transport (Seachrist and Ferguson 2003).

The FYVE domain in SARA directs its localization to Rab5-containing early endosomes. Within the early endosome, SARA interacts with Smad2 and the TβR complex. Other components that may be part of the SARA-containing endosome are the early endosome antigen 1 (EEA1), the cytoplasmic isoform of the promyelocytic leukemia tumor suppressor (cPML), and the hepatic growth factor-regulated tyrosine kinase substrate (Hrs).

EEA1 is a protein involved in the formation of the early endosome. cPML expression is induced by TGF-β1 and has been shown to be required for SARA association with Smad2 and for SARA and TβR accumulation in the early endosome (Lin et al. 2004).

Hrs is another FYVE domain-containing protein that regulates endosomal sorting and plays a critical role in the recycling and degradation of membrane receptors. Hrs binds to Smad2 and cooperates with SARA to stimulate activin-mediated signaling (Miura et al. 2000).

While the importance of internalization in dampening a signal through downregulation of receptors is well studied, endocytosis may also serve to propagate the signal, and this has led to the concept of a "signaling endosome" (Miaczynska et al. 2004; Benmerah 2004). The importance of endocytosis in TGF-β1 signaling has been described in numerous reports (Hayes et al. 2002; Penheiter et al. 2002; Runyan et al. 2005). Disruption of SARA endocytic localization through either expression of a mutant SARA lacking the FYVE domain (SARA/Δ1-664) (Tsukazaki et al. 1998) or inhibition of PI3P generation by wortmannin treatment (Itoh et al. 2002) causes a redistribution of SARA from punctate endocytic structures to the cytosol, and can prevent TGF-β1-mediated transcriptional responses.

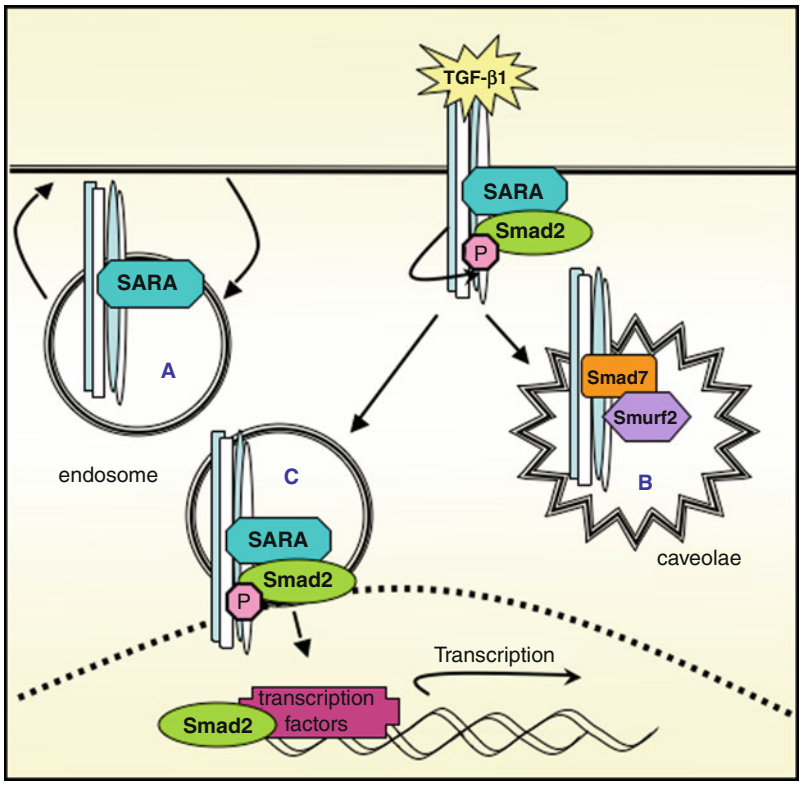
How the endosome specifically functions in TGF-β signaling is not known (Fig. 3). It may be that localization of the TβR to EEA1-positive, SARA-containing endosomes protects them from their alternative localization through the lipid raft-caveolar internalization pathway where they would interact with Smad7 and Smurf2 and be degraded (Di Guglielmo et al. 2003). Another possibility is that endosomal trafficking acts as a transport mechanism bringing the activated R-Smad in proximity of the nucleus. Smad2 can enter the nucleus through direct interaction with the nucleoporins CAN/Nup214 and Nup153, and these nucleoporins compete with SARA for the interaction with the hydrophobic corridor on the MH2 surface of Smad2 (Xu et al. 2002).

Putative Physiological Role of SARA

In developing tissues, morphogens can elicit concentration-dependent responses at very long ranges, and even small variations in their concentration can create very different effects. Because many signaling molecules are associated with intracellular membrane-bound compartments, these compartments, the signaling components, and their activation states need to be equally distributed between daughter cells during cell division. In the developing wing of *Drosophila melanogaster*, a specialized subset of Sara-endosomes, and the receptors therein, associate with the spindle machinery to segregate into the two daughter cells, thus allowing equal inheritance so that they retain the Dpp signaling levels of the mother cell (Bokel et al. 2006).

SARA has been reported to play a role in the signaling and remodeling events within the bone marrow niche where portions of the hematopoietic progenitor cells (HPC) membrane are actively endocytosed by osteoblasts and delivered to SARA-positive, signaling endosomes. In response to intercellular transfer, osteoblasts exhibit decreased Smad signaling and increased production of stromal-derived factor-1 (SDF-1), a chemokine responsible for HPC homing to bone marrow. Because TGF-β signaling can downregulate SDF-1, the transferred material within SARA-endosomes may sequester SARA away from its general cofactor function in Smad activation, allowing the osteoblast to produce more SDF-1 (Gillette et al. 2009).

SARA has also been reported to play a role in maintaining epithelial cell phenotype. During embryonic development, cells may initially have multiple characteristics but gradually differentiate into a more quiescent phenotype that has specialized functions. This process, known as mesenchymal-to-epithelial transition (MET), can be reversed in mature tissues in response to injury and attempted tissue repair in the process of epithelial-to-mesenchymal transition (EMT). Should such repair be inappropriate or misregulated, EMT can promote cancer progression and fibrosis. TGF-β is a well-established mediator of EMT, and at least in renal fibrosis, it may give rise to myofibroblast-like, matrix-producing cells. In a human kidney proximal tubule epithelial cell line, downregulation of SARA expression is a requirement for TGF-β-induced EMT. This depletion of SARA expression results in reduced Smad2 expression and activity via enhancement of Smad2 interaction with the ubiquitination factor Smurf2 (Runyan et al. 2009). Similar to EMT induced by TGF-β, SARA expression has been reported to decline during EMT induced by high glucose (Tang et al. 2010). The expression of SARA also declines during myofibroblast-like transition of hepatic stellate cells (Liu et al. 2003) and during progression of liver fibrosis (Tao et al. 2006). Therefore, SARA expression may be protective against fibrosis where EMT is a contributor.



SARA, Fig. 3 Possible roles for SARA and endocytosis in TGF-β signaling. (**a**) Through its ligand-independent binding to TβR, SARA may function to localize the TβRs to the endosome during constitutive receptor internalization and recycling. (**b**) Without CME, the receptor can be internalized into Smad7/Smurf2-containing caveolar compartments and be degraded. Therefore, SARA-mediated endosomal localization may protect the receptor from degradation. (**c**) After ligand binding and Smad2 recruitment, internalization of the TβR-SARA-Smad2 complex through CME may help transfer the active Smad through the cytosol to the nucleus

Other disease models suggest an increase in SARA expression. In asthma, damage to the bronchial epithelium results in release of TGF-β, which can suppress EGF-induced proliferation of epithelial cells, and higher levels of SARA expression are found when comparing epithelial cells from subjects with asthma to healthy controls (Semlali et al. 2008). Constitutive upregulation of SARA along with other components of the TGF-β signaling pathway has been found in rheumatoid arthritis compared to osteoarthritis synovial fibroblasts (Pohlers et al. 2007).

Summary

SARA is an adaptor protein, which interacts with TGF-β receptors and R-Smads and localizes to EEA1-containing, Rab5-positive early endosomes through clatherin-mediated endocytosis. While the precise role of SARA in TGF-β signaling is not fully characterized, it seems that SARA may preferentially modulate the Smad2-dependent response to TGF-β with little effect on the Smad3-mediated response. The importance of SARA's ability to localize to early endosomes has been demonstrated by a number of studies. However, the exact role of endocytosis in TGF-β signaling is not known. Although there are few studies linking SARA to a physiologic or disease process, it appears that SARA plays a negative role in fibrosis associated with its ability to suppress EMT and help maintain the intact epithelium.

References

Benmerah A. Endocytosis: signaling from endocytic membranes to the nucleus. Curr Biol. 2004;14:R314–16.

Bennett D, Alphey L. PP1 binds Sara and negatively regulates Dpp signaling in *Drosophila melanogaster*. Nat Genet. 2002;31(4):419–23.

Bokel C, Schwabedissen A, Entchev E, Renaud O, Gonzalez-Gaitan M. Sara endosomes and the maintenance of Dpp signaling levels across mitosis. Science. 2006;314(5802):1135–9.

Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. J Am Soc Nephrol. 2002;13(10):2600–10.

Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-β receptor signalling and turnover. Nat Cell Biol. 2003;5:410–21.

Gillette JM, Larochelle A, Dunbar CE, Lippincott-Schwartz J. Intercellular transfer to signalling endosomes regulates an ex vivo bone marrow niche. Nat Cell Biol. 2009;11(3):303–11.

Goto D, Nakajima H, Mori Y, Kurasawa K, Kitamura N, Iwamoto I. Interaction between Smad anchor for receptor activation and Smad3 is not essential for TGF-beta/Smad3-mediated signaling. Biochem Biophys Res Commun. 2001;281(5):1100–5.
Hayes S, Chawla A, Corvera S. TGF-β receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. J Cell Biol. 2002;158(7):1239–49.
Itoh F, Divecha N, Brocks L, Oomen L, Janssen H, Clalfat J, et al. The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-β/Smad signalling. Genes Cells. 2002;7:321–31.
Lin H, Bergmann S, Pandolfi PP. Cytoplasmic PML function in TGF-β signalling. Nature. 2004;431:205–11.
Liu C, Gaca MD, Swenson ES, Vellucci VF, Reiss M, Wells RG. Smads 2 and 3 are differentially activated by transforming growth factor-beta (TGF-beta) in quiescent and activated hepatic stellate cells. Constitutive nuclear localization of Smads in activated cells is TGF-beta-independent. J Biol Chem. 2003;278(13):11721–8.
Meckelein B, Marshall DC, Conn KJ, Pietropaolo M, Van Nostrand W, Abraham CR. Identification of a novel serine protease-like molecule in human brain. Brain Res Mol Brain Res. 1998;55(2):181–97.
Miaczynska M, Pelkmans L, Zerial M. Not just a sink: endosomes in control of signal transduction. Curr Opin Cell Biol. 2004;16(4):400–6.
Miura S, Takeshita T, Asao H, Kimura Y, Murata K, Sasaki Y, et al. Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol Cell Biol. 2000;20(24):9346–55.
Mousavi SA, Malerod L, Berg T, Kjeken R. Clthrin-dependent endocytosis. Biochem J. 2004;377(Pt 1):1–16.
Penheiter SG, Mitchell H, Garamszegi N, Edens M, Dore JJE, Leof EB. Internalization-dependent and -independent requirements for transforming growth factor β receptor signaling via the Smad pathway. Mol Cell Biol. 2002;22(13):4750–9.
Piek E, Heldin CH, ten Dijke P. Specificity, diversity and regulation by TGF-β superfamily signaling. FASEB J. 1999;13:2105–24.
Pohlers D, Beyer A, Koczan D, Wilhelm T, Thiesen HJ, Kinne RW. Constitutive upregulation of the transforming growth factor-beta pathway in rheumatoid arthritis synovial fibroblasts. Arthritis Res Ther. 2007;9(3):R59.
Roberts AB, Sporn MB. The transforming growth factor-βs. In: Sporn MB, Roberts AB, editors. Peptide growth factors and their receptors. Heidelberg: Springer; 1990. p. 419–72.
Runyan CE, Schnaper HW, Poncelet AC. The role of internalization in transforming growth factor beta1-induced Smad2 association with Smad anchor for receptor activation (SARA) and Smad2-dependent signaling in human mesangial cells. J Biol Chem. 2005;280(9):8300–8.
Runyan CE, Hayashida T, Hubchak S, Curley JF, Schnaper HW. Role of SARA (SMAD anchor for receptor activation) in maintenance of epithelial cell phenotype. J Biol Chem. 2009;284(37):25181–9.
Seachrist JL, Ferguson SSG. Regulation of G protein-coupled receptor endocytosis and trafficking by Rab GTPases. Life Sci. 2003;74(2–3):225–35.
Semlali A, Jacques E, Plante S, Biardel S, Milot J, Laviolette M, et al. TGF-beta suppresses EGF-induced MAPK signaling and proliferation in asthmatic epithelial cells. Am J Respir Cell Mol Biol. 2008;38(2):202–8.
Shi Y, Massague J. Mechanisms of TGF-β signaling from the cell membrane to the nucleus. Cell. 2003;113:685–700.
Shi W, Sun C, He B, Xiong W, Shi X, Yao D, Cao X. J of Cell Biol. 2004;164(2):291-300.
Tang W, Ling G, Sun L, Peng Y, Liu Y, Liu H, et al. SARA expression in high glucose induced epithelium to mesenchymal transition of proximal tubule cells. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2010;35(12):1230–5.
Tao YY, Cui HY, Liu CH. Dynamic characteristics of SARA during liver fibrogenesis in rats. Zhong Gan Zang Bing Za Zhi. 2006;14(12):909–13.
Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell. 1998;95(6):779–91.
Xu L, Kang Y, Col S, Massague J. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFβ signaling complexes in the cytoplasm and nucleus. Mol Cell. 2002;10:271–82.

Sarah

► Regulator of Calcineurin 1 (RCAN1)

Sarcoplasmic Reticulum Calcium Release Channels

► Ryanodine Receptor (RyR)

SARM

► Toll-like Receptor Adaptor Protein Family Members

Scalloped (Sd) (*D. Melanogaster*)

► Tead

SCYB10

► CXCL10

Serotonin

▶ The 5-HT_3 Receptor

Serotonin Receptor 2B

▶ 5-Hydroxytryptamine Receptor 2B

Sex Determining Region on the Y Chromosome

▶ Sry

sGC

▶ Guanylyl Cyclase Receptors

SH2 Domain-Containing Inositol Phosphatase

▶ SHIP

SH2D2A

Philip D. King
Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA

Synonyms

LAD; RIBP; TSAd

Historical Background

Adapter proteins participate in intracellular signal transduction processes through physical interaction with catalytically active binding partners resulting in the formation of signaling complexes. Incorporation of adapter proteins into these complexes is mediated by modular binding domains contained in the adapter itself or in the respective binding partner(s). Through incorporation into complexes, adapter proteins modulate the activity of catalytically active proteins, directly or indirectly. In a subtractive cDNA library screen of activated human peripheral blood CD8 T cells, a novel adapter protein was identified that was named T cell specific adapter protein (TSAd) (Spurkland et al. 1998). TSAd is also known as Src-homology 2 (SH2) domain protein 2A (SH2D2A), based upon the presence of an SH2 domain in the center of the linear sequence. Subsequently, murine TSAd was identified as an interacting partner of the Src-family protein tyrosine kinase (PTK), LCK, and the Tec family protein tyrosine kinases, RLK and ITK, in yeast two-hybrid screening experiments (Choi et al. 1999; Rajagopal et al. 1999). Hence, TSAd/SH2D2A is also known as LCK-associated adapter protein (LAD) and RLK/ITK-binding protein (RIBP).

Structure

SH2D2A resembles a classical adapter protein that contains a central SH2 domain and a carboxyl region with a conserved proline-rich stretch and tyrosine residues that are present in consensus phosphorylation motifs (Lapinski et al. 2009). The SH2 domain of SH2D2A recognizes phosphorylated tyrosine residues contained in other signaling proteins, and, visa versa, SH2 domains of other signaling proteins recognize phosphorylated tyrosine residues in the SH2D2A carboxyl region. In addition, interaction of SH2D2A with signaling proteins can be mediated by SH3 domain binding to the SH2D2A carboxyl region proline-rich stretch. The amino-terminal region of SH2D2A has no discernible structure or function.

Expression

SH2D2A is most strongly expressed in the T cell lineage and in natural killer (NK) cells (Lapinski et al. 2009). In T cells, expression levels are increased following T cell activation. SH2D2A is expressed in CD4 and CD8 T cells and comparably in CD4 T

helper 1 and T helper 2 cell lines. In part, expression of SH2D2A in T cells is regulated by a cyclic adenosine monophosphate (cAMP) response element that is present in the SH2D2A promoter (Dai et al. 2004). Other than T and NK cells, SH2D2A expression has also been noted in B cells, epithelial cells, and vascular endothelial cells (Lapinski et al. 2009).

Interacting Partners

As noted above, SH2D2A interacts physically with LCK, RLK, and ITK. Furthermore, for LCK, physical interaction has been demonstrated in primary T cells between cell endogenous proteins (Marti et al. 2006). Interaction is thought to involve PTK phosphorylation of SH2D2A carboxyl region tyrosine residues that are then bound by the PTK SH2 domain. In addition, the LCK SH3 domain binds to the SH2D2A proline-rich stretch, thus constituting another mechanism of interaction.

Ligands of the SH2D2A domain that have been identified include linker of activated T cells (LAT), vascular endothelial growth factor receptor 2 (VEGFR2), and valosin-containing protein (VCP) (Lapinski et al. 2009; Marti and King 2005; Marti et al. 2001; Matsumoto et al. 2005). In the first two examples, the SH2 domain binds directly to phosphorylated tyrosine residues in the respective target proteins. In the case of VCP, however, tyrosine phosphorylation likely results in a conformational change, which exposes a distinct interaction site upon VCP for SH2D2A SH2 domain recognition.

Other signaling proteins that SH2D2A physically interacts with include Gβ protein subunits of heterotrimeric G proteins and the mitogen-activated protein 3 kinase, MEKK2 (Park et al. 2007; Sun et al. 2001). However, the molecular basis for interaction with these proteins has not been clearly defined.

SH2D2A Function

SH2D2A promotes activation of LCK at the outset of T cell antigen receptor (TCR) signal transduction in T cells (Marti et al. 2006). TCR recognition of ligand results in local aggregation of LCK at the plasma membrane. For those LCK molecules that preexist in an open primed conformation, aggregation leads to an increase in LCK kinase activity, consequent to LCK-mediated phosphorylation of a positive regulatory tyrosine residue present in the LCK kinase domain (Palacios and Weiss 2004). Increased LCK kinase activity then results in SH2D2A tyrosine phosphorylation and subsequent interaction of SH2D2A with the LCK SH2 and SH3 domains. These interactions are important since LCK SH2 domain binding to a phosphorylated negative-regulatory tyrosine residue, positioned carboxyl to the LCK kinase domain, and LCK SH3 domain binding to an LCK proline stretch, present in a linker region between the SH3 domain and kinase domain, act to constrain this PTK in an inactive conformation (Palacios and Weiss 2004). Therefore, in binding to LCK, SH2D2A disrupts these intramolecular inhibitory interactions. Consequently, additional LCK molecules are driven into open primed conformations whereupon they may become fully activated by phosphorylation upon the kinase domain positive-regulatory tyrosine residue. In turn, this will result in further SH2D2A phosphorylation, leading to further LCK activation and so on.

The importance of SH2D2A in the activation of LCK during the course of TCR signal transduction is shown by the finding that TCR-induced activation of LCK is impaired in T cells from SH2D2A-deficient mice (Marti et al. 2006). Consequently, all downstream signaling events in T cells including activation of the ZAP-70 PTK, activation of the Ras-MAPK pathway, calcium mobilization, and cytokine synthesis are reduced in magnitude in SH2D2A-deficient T cells.

T cell migration induced by the chemokines CXC12 and CCL5 is also SH2D2A-dependent (Park et al. 2007). Interestingly, the function of SH2D2A in this response again appears to relate to a required role for SH2D2A in the activation of LCK and ZAP-70 in the chemokine-induced signaling pathway downstream of heterotrimeric G proteins.

SH2D2A may also have a nuclear role in T cells (Marti and King 2005; Marti et al. 2001). This conclusion is based upon the finding that SH2D2A is actively transported to the nucleus in T cells via a mechanism that involves SH2 domain recognition of VCP. The exact function of SH2D2A in the cell nucleus is unknown.

Outside of the T cell compartment, expression of SH2D2A has been shown to be required for VEGFR2-induced migration of blood vascular endothelial cells in vitro and for blood vessel angiogenesis toward

tumors in vivo (Matsumoto et al. 2005). Physical interaction of SH2D2A with the VEGFR2 cytoplasmic domain is necessary for both of these responses. In epithelial cell lines, SH2D2A physical interaction with MEKK2 is required for activation of the ERK5 big MAPK induced by the epidermal growth factor (EGFR), osmotic and oxidative stress (Sun et al. 2001, 2003).

SH2D2A-Deficient Mouse Model

SH2D2A-deficient mice are viable and initially healthy (Rajagopal et al. 1999). T cell development proceeds normally, and in young adult animals, numbers and ratios of peripheral T cell subsets are similar to those observed in control mice. However, with increasing age, SH2D2A-deficient mice show signs of systemic lupus-like autoimmunity (Drappa et al. 2003; Marti et al. 2005). Disease is associated with splenomegaly, increased numbers of activated memory phenotype T cells in spleen and lymph nodes, production of autoantibodies against DNA and other nuclear targets, glomerulonephritis, and leukocytic infiltration into nonlymphoid organs. Furthermore, young SH2D2A-deficient mice are highly susceptible to experimentally induced lupus-like autoimmune disease. The likely cause of autoimmunity in SH2D2A-deficient mice is impaired apoptotic death of autoreactive peripheral T cells upon recognition of self antigens (Drappa et al. 2003). The molecular basis for this impaired death is uncertain but likely relates to impaired cytokine production by SH2D2A-deficient T cells.

Summary

SH2D2A is an SH2 domain-containing signaling adapter protein that is strongly expressed in T lymphocytes. SH2D2A functions at the beginning of the TCR signal transduction cascade by promoting activation of the LCK Src-family PTK through competitive inhibition of LCK intramolecular inhibitory interactions. In addition, roles for SH2D2A in T cell migration, VEGFR2 signal transduction in endothelial cells, and activation of the big MAPK signaling pathway in epithelial cells have been described. SH2D2A-deficient mice are susceptible to the development of systemic lupus-like autoimmune disease, thus illustrating the importance of SH2D2A as a regulator of T cell tolerance to self antigens.

References

Choi YB, Kim CK, Yun Y. Lad, an adapter protein interacting with the SH2 domain of p56lck, is required for T cell activation. J Immunol. 1999;163:5242–9.

Dai KZ, Johansen FE, Kolltveit KM, Aasheim HC, Dembic Z, Vartdal F, Spurkland A. Transcriptional activation of the SH2D2A gene is dependent on a cyclic adenosine 5'-monophosphate-responsive element in the proximal SH2D2A promoter. J Immunol. 2004;172:6144–51.

Drappa J, Kamen LA, Chan E, Georgiev M, Ashany D, Marti F, King PD. Impaired T cell death and lupus-like autoimmunity in T cell-specific adapter protein-deficient mice. J Exp Med. 2003;198:809–21.

Lapinski PE, Oliver JA, Bodie JN, Marti F, King PD. The T-cell-specific adapter protein family: TSAd, ALX, and SH2D4A/SH2D4B. Immunol Rev. 2009;232:240–54.

Marti F, King PD. The p95-100 kDa ligand of the T cell-specific adaptor (TSAd) protein Src-homology-2 (SH2) domain implicated in TSAd nuclear import is p97 Valosin-containing protein (VCP). Immunol Lett. 2005;97:235–43.

Marti F, Lapinski PE, King PD. The emerging role of the T cell-specific adaptor (TSAd) protein as an autoimmune disease-regulator in mouse and man. Immunol Lett. 2005;97:165–70.

Marti F, Post NH, Chan E, King PD. A transcription function for the T cell-specific adapter (TSAd) protein in T cells: critical role of the TSAd Src homology 2 domain. J Exp Med. 2001;193:1425–30.

Marti F, Garcia GG, Lapinski PE, MacGregor JN, King PD. Essential role of the T cell-specific adapter protein in the activation of LCK in peripheral T cells. J Exp Med. 2006;203:281–7.

Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, Magnusson P, Wang L, Wikner C, Qi JH, Wernstedt C, et al. VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. EMBO J. 2005;24:2342–53.

Palacios EH, Weiss A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene. 2004;23:7990–8000.

Park D, Park I, Lee D, Choi YB, Lee H, Yun Y. The adaptor protein Lad associates with the G protein beta subunit and mediates chemokine-dependent T-cell migration. Blood. 2007;109:5122–8.

Rajagopal K, Sommers CL, Decker DC, Mitchell EO, Korthauer U, Sperling AI, Kozak CA, Love PE, Bluestone JA. RIBP, a novel Rlk/Txk- and itk-binding adaptor protein that regulates T cell activation. J Exp Med. 1999;190:1657–68.

Spurkland A, Brinchmann JE, Markussen G, Pedeutour F, Munthe E, Lea T, Vartdal F, Aasheim HC. Molecular cloning of a T cell-specific adapter protein (TSAd) containing an Src homology (SH) 2 domain and putative SH3 and phosphotyrosine binding sites. J Biol Chem. 1998;273:4539–46.

Sun W, Kesavan K, Schaefer BC, Garrington TP, Ware M, Johnson NL, Gelfand EW, Johnson GL. MEKK2 associates with the adapter protein Lad/RIBP and regulates the MEK5-BMK1/ERK5 pathway. J Biol Chem. 2001;276:5093–100.
Sun W, Wei X, Kesavan K, Garrington TP, Fan R, Mei J, Anderson SM, Gelfand EW, Johnson GL. MEK kinase 2 and the adaptor protein Lad regulate extracellular signal-regulated kinase 5 activation by epidermal growth factor via Src. Mol Cell Biol. 2003;23:2298–308.

SH3 Domain–Containing Proline-Rich Kinase

▶ MLK3

SHIP

Matthew D. Blunt[1] and Stephen G. Ward[2]
[1]Department of Pharmacy & Pharmacology, University of Bath, Bath, Somerset, UK
[2]Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, Somerset, UK

Synonyms

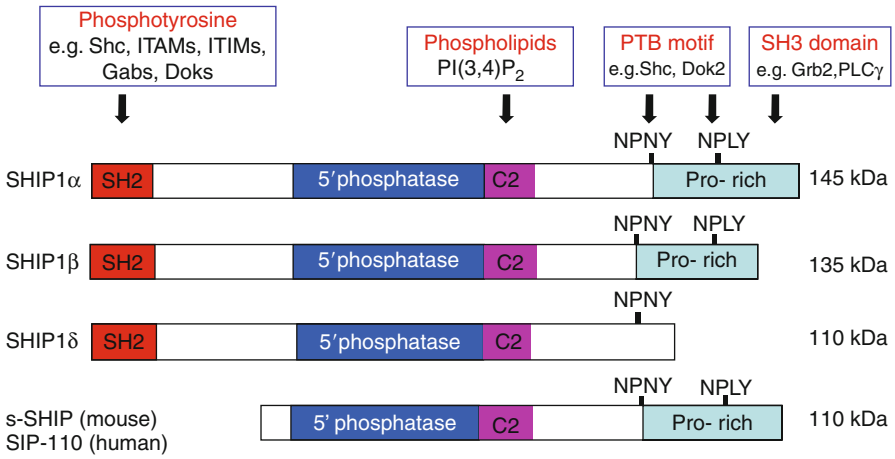
SHIP (Inositol polyphosphate-5-phosphatase, 145 kDa; Inpp5d; SH2 domain-containing inositol phosphatase)

Historical Background

SH2 domain-containing inositol phosphatase-1 (SHIP) was initially identified in 1994 as a tyrosine-phosphorylated protein after stimulation of blood cells by a broad number of cytokines and growth factors (Lioubin et al. 1994; Liu et al. 1994). It translocates to the plasma membrane after extracellular stimulation and hydrolyses the ▶ phosphoinositide 3-kinase (PI3K)-generated second messenger $PI(3,4,5)P_3$, to $PI(3,4)P_2$. As a result, SHIP is able to modulate $PI(3,4,5)P_3$-mediated signaling and hence the proliferation, differentiation, survival, activation, and migration of hematopoietic cells. The creation of germ-line $SHIP^{-/-}$ knockout mice in 1998 was instrumental in determining the role SHIP plays in the immune system (Helgason et al. 1998). Subsequent cell-restricted deletion in lymphoid and myeloid compartments some 10 years later, further advanced understanding of the intrinsic role of SHIP in a variety of immune cells (Leung et al. 2009).

Structure and Binding Partners of SHIP Family Members

SHIP is a 145 kDa protein encoded by the *INPP5D* gene on chromosome 2 (location 2q37.1) and is largely confined to hematopoietic cells. The protein is composed of 1,188 amino acids and possesses domains that mediate its interaction with other proteins on either side of its central catalytic domain. In addition to hydrolysing $PI(3,4,5)P_3$, SHIP can also dephosphorylate inositol-1,3,4,5-tetraphosphate, at least in vitro. SHIP possesses a centrally located catalytic domain responsible for the hydrolysis of the 5'-phosphate of the membrane phosphoinositide lipid $PI(3,4,5)P_3$, the major product of receptor-activated PI3K. A C2 domain adjacent to the catalytic domain has been identified and shown to be an allosteric- activating site when bound by SHIP's enzymatic product $PI(3,4)P_2$ (Hamilton et al. 2011). In addition, SHIP encodes multiple structural domains that facilitate protein–protein interactions and cellular re-localization upon receptor stimulation. The SH2 domain within SHIP interacts with proteins via the consensus amino acid sequence pY[S/Y][L/Y/M][L/M/I/V]. Through this SH2 domain, SHIP binds to the tyrosine phosphorylated forms of Shc, Doks, Gabs, CD150, platelet-endothelial cell adhesion molecule (PECAM), Cas, c-Cbl certain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and some immunoreceptor tyrosine-based activation motifs (ITAMs). Recruitment of SHIP by the phosphorylated ITIM in the cytoplasmic domain of FcγRIIB was shown to inhibit recruitment of PH-domain-containing proteins and prevent extracellular calcium influx, overall reducing transcription activation, and cytokine release. The proline-rich region of SHIP enables interaction with proteins encoding SH3 domains, such as Grb2, Src, Lyn and phospholipase-Cγ and is essential for SHIP function (Harris et al. 2008). The two NPXY motifs in SHIP can become tyrosine phosphorylated and bind the phosphotyrosine-binding domain (PTB) motifs in Shc, Dok1, and Dok 2. These structural domains of SHIP are able to support the



SHIP, Fig. 1 *Schematic representation of the SHIP protein isoforms*. The protein interaction motifs are indicated, along with their binding partners. The SH2 domain allows SHIP to bind proteins (such as those indicated) which express the sequence phospho-Y(Y/S/T)L(M/L). The 5' phosphatase domain catalyzes the conversion of $PI(3,4,5)P_3$ to $PI(3,4)P_2$. The C2 domain has been identified as a binding domain of the SHIP product $PI(3,4)P_2$ which acts to increase the catalytic activity of SHIP. The NPXY motifs when phosphorylated provide binding sites for proteins which express phosphotyrosine binding domains. The proline-rich domain (PR) allows SHIP to interact with SH3 domain-containing proteins

re-localization of SHIP from the cytosol to the plasma membrane, where its catalytic activity regulates $PI(3,4,5)P_3$ accumulation. In addition, these structural features and binding motifs allow SHIP to serve a scaffolding role for the recruitment of other proteins to the plasma membrane, independent of catalytic function. The interaction of SHIP with other proteins can also indirectly abrogate PI3K signaling since SH2 domain-mediated interactions with ITAM-containing adaptor proteins has been demonstrated to dislodge or prevent further recruitment of PI3K via the p85 subunit (Peng et al. 2010).

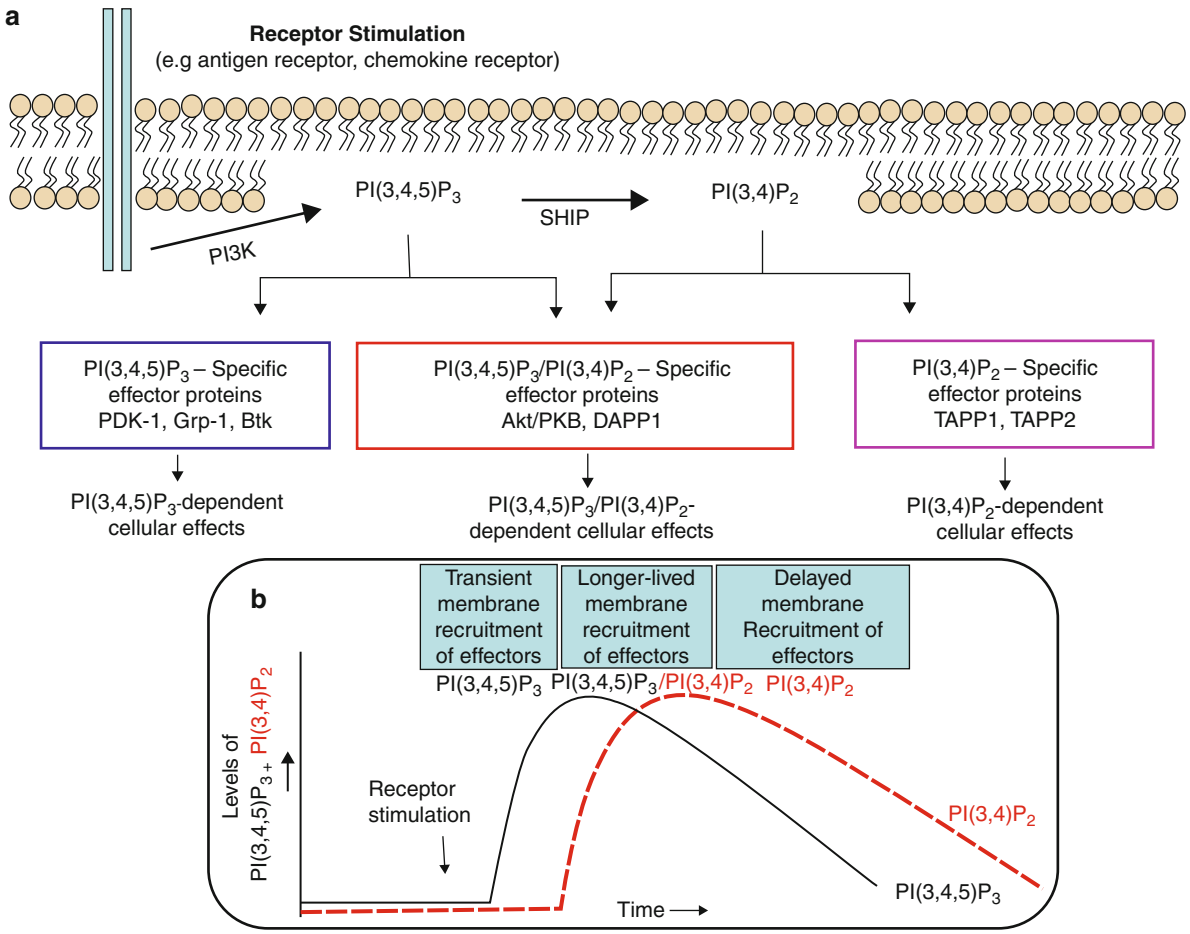
Splice Variants and Isoforms of SHIP

Multiple forms of SHIP have been reported with molecular weights of 145 (SHIPα),135 (SHIPβ), and 110 (SHIPδ) kDa (Fig. 1). In addition, other 130, 125, and 110 kDa forms of SHIP have been reported (Hamilton et al. 2011; Kerr 2011). The different forms of SHIP may arise from alternative mRNA splicing, protein degradation, or posttranslational modification such as phosphorylation. SHIP-2 is a 142 kDa protein that is highly homologous with the hematopoietic-specific SHIP, but is encoded by a different gene and exhibits distinct structural features as well as possibly having a broader phospholipid substrate specificity than SHIP, as it also hydrolyses $PI(4,5)P_2$ in vitro. SHIP-2 is expressed in both hematopoietic and non-hematopoietic tissues such as brain, skeletal muscle, heart, and to a lesser extent liver and kidney. The major role of SHIP-2 appears to be the negative regulation of insulin signaling in nonimmune cells (Ooms et al. 2009).

The different forms of SHIP exhibit different protein-binding profiles defined by the absence or presence of these distinct binding motifs that are described in more detail below (Fig. 1). For example, s-SHIP (expression of which is restricted to murine embryonic stem cells and primitive hematopoietic stem cells, but which is lost in lineage-committed hematopoietic cells) and its human homologue SIP-110, are truncated at the N-terminus and lack the SH2 domain which limits the repertoire of binding proteins available for interaction. For example, it cannot interact with Shc, but still interacts with Grb-2. Moreover, s-SHIP is mostly localized at the plasma membrane rather than the cytoplasm (Hamilton et al. 2011; Kerr 2011).

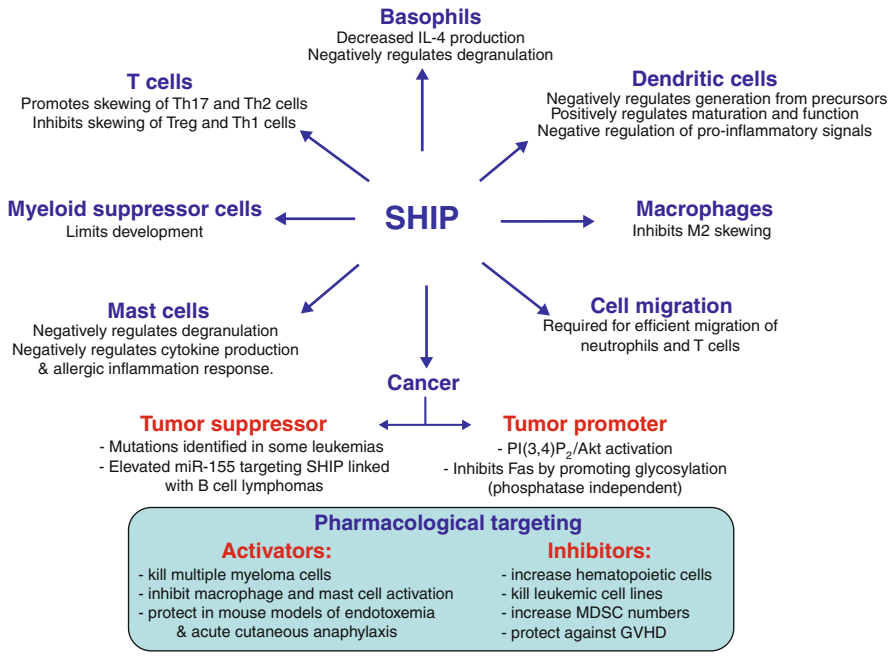
SHIP: A Checkpoint in PI3K-Dependent Signaling

The classical view of SHIP is that it acts to switch off PI3K-dependent signaling by degradation of $PI(3,4,5)P_3$ (Fig. 2). However, the metabolism of



SHIP, Fig. 2 *SHIP acts as a molecular "switch"*. SHIP catalyzes the conversion of the PI3K lipid product $PI(3,4,5)P_3$ to $PI(3,4)P_2$. Effector proteins which express PH domains are recruited and activated by these lipid second messengers at the cell surface membrane. PH-domains of proteins are able to discriminate between $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Examples of proteins which bind only $PI(3,4,5)P_3$, both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ or only $PI(3,4)P_2$ are shown (**a**). This diversity allows SHIP to both negatively regulate PI3K signaling and also to "switch" signal transduction pathways away from $PI(3,4,5)P_3$-dependent effectors toward $PI(3,4)P_2$-dependent effectors, in turn influencing kinetics and duration of recruitment of 3' phosphoinositide lipid binding proteins at the plasma membrane (depicted in **b**)

$PI(3,4,5)P_3$ by SHIP yields $PI(3,4)P_2$ which retains the phosphate grouping on the third position of the inositol ring and thus, may retain some signaling ability. Pleckstrin homology (PH) domains encoded in many proteins (e.g., Grp-1) bind exclusively to $PI(3,4,5)P_3$, whereas others such as that found in dual adaptor of phosphotyrosine and 3-phosphoinositides −1 (DAPP-1), can interact with both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Lemmon and Ferguson 2000). In addition, the tandem PH domain-containing protein TAPP-1 encodes PH domains that show selectivity toward $PI(3,4)P_2$ (Harris et al. 2008). The ability of PH domain-containing proteins to distinguish between different 3'-phosphoinositide lipids suggests that SHIP can act as a switch to redirect PI3K-dependent signaling toward a set of distinct effectors that are temporally and functionally separate from $PI(3,4,5)P_3$-dependent events. Thus, SHIP may function to fine-tune phosphoinositide signaling, rather than terminate it (Fig. 2). In this regard, SHIP promotes recruitment of the GTPase Irgm1 to sites of phagocytosis in macrophages via generation of $PI(3,4)P_2$, a critical step in maturation of the phagosome and engulfment of bacteria. $PI(3,4,5)P_3$ and $PI(3,4)P_2$ appear sequentially following agonist stimulation in many cell types including T lymphocytes, but show temporal overlap. Some cell types, notably



SHIP, Fig. 3 *SHIP regulates immune cell functions and influences tumor development and growth*. SHIP plays a key role in the generation of lymphocyte and myeloid subsets and maintaining a balance between inflammatory and regulatory cells. Thus, modulation of SHIP offers potential for therapeutic intervention in a range of inflammatory and autoimmune diseases as well as in cancer and transplantation settings

B lymphocytes and platelets, exhibit sustained $PI(3,4)P_2$ production, lasting for up to 45–60 min poststimulation (Harris et al. 2008).

Role of SHIP in the Immune System: Maintaining a Balance between Inflammatory and Regulatory Cells

SHIP is expressed ubiquitously in differentiated cells of the hematopoietic system, in endothelial cells, hematopoietic stem cells (HSC), and embryonic stem (ES) cells. Both SHIP and s-SHIP have been implicated in the biology of pluripotent and adult stem cells. More recently, it has been shown to play a key role in the function of the bone marrow microenvironment (the stem cell niche) (Kerr 2008). Largely as a consequence of hematopoietic-specific expression, SHIP has been extensively studied for its regulatory role in B cells, T cells, macrophages and mast cells. SHIP was first recognized as an important component of the inhibitory signaling pathway triggered by the IgG receptor FcγRIIB in mast cells and B cells (Ono et al. 1996). Once recruited to the plasma membrane by signaling complexes, its catalytic activity depletes $PI(3,4,5)P_3$ and prevents membrane localization of some PH domain-containing effectors. SHIP has also been implicated in signaling pathways triggered by cytokine, chemokine, antigen, and IgG engagement in a variety of immune cells (Harris et al. 2008). Additionally, SHIP plays an important regulatory role in establishing endotoxin tolerance in macrophages as well as regulating leucocyte polarization during migratory responses to chemoattractants (Hamilton et al. 2011; Harris et al. 2008; Kerr 2011).

Genetic analysis of SHIP mutant mice has revealed a pivotal role for SHIP in a wide variety of differentiated hematopoietic cell types. SHIP has been shown to play a role in regulating the receptor repertoire and cytolytic function of Natural Killer (NK) cells, B lymphocyte development and antibody production, the myeloid cell response to bacterial mitogens, development of marginal zone macrophages, lymph node recruitment of dendritic cells, and mast cell degranulation (Fig. 3). The negative regulatory role for SHIP in the immune system is best illustrated by the phenotype of SHIP null mice which develop progressive myeloid hyperplasia and myeloid infiltration in the lungs that leads to respiratory failure

and to a dramatic decrease of life span. The myeloid hyper-proliferation is caused by the combination of two factors. First, the myeloid cells and their precursors are more sensitive to growth factors and second, these cells show a decreased sensitivity to pro-apoptotic stimuli (Helgason et al. 1998). Analysis of the SHIP null mice has also established that SHIP plays a critical role in homeostasis of myeloid and lymphoid effector and regulatory cells. For example, SHIP deficient mice exhibit more myeloid-derived suppresser cells (MDSCs) than their wild type counterparts. SHIP also plays a role in regulating the balance of M1 macrophages (implicated in the first inflammatory response) and M2 macrophages (implicated in inflammatory response termination, tissue repair, regeneration and remodeling). SHIP deficiency leads to increased macrophage skewing toward M2 macrophages. This indicates that $PI(3,4,5)P_3$ drives macrophage progenitors toward an M2 phenoype and that SHIP blocks this skewing (Hamilton et al. 2011; Kerr 2011). Moreover, SHIP is essential for normal Th17 cell development and this lipid phosphatase plays a key role in the reciprocal regulation of Tregs and Th17 cells (Hamilton et al. 2011; Kerr 2011). Because of abnormalities in secretion of cytokines observed in SHIP-deficient mice, the root cause of an abnormality in SHIP-deficient mice could also be due to extrinsic effects on that cell type. Thus, analysis of cell type specific deletion of SHIP in vivo is ultimately required before one can conclusively determine which SHIP-deficient cell type causes a specific phenotype in mice with a germline homozygous mutation of SHIP. This is best illustrated by analysis of mice carrying a T cell-specific deletion of SHIP, which uncovered a regulatory role for SHIP in controlling Th1/Th2 bias and cytotoxic responses as a result of its inhibitory effect on T-bet expression. Mice with a T cell-specific deletion of SHIP revealed that they do not skew efficiently to a Th2 phenotype and display Th1-dominant immune responses in vitro and in vivo (Leung et al. 2009). This is in contrast to evidence from germ line $SHIP^{-/-}$ mice, which indicates that SHIP can also repress Th2 skewing by inhibiting IL-4 production from basophils (Hamilton et al. 2011).

SHIP in Cancer and Other Diseases: Opportunities for New Therapies

The PI3K-dependent signaling pathway has a well-established role in regulating cell survival, proliferation and differentiation (Manning and Cantley 2007). As such, the levels of $PI(3,4,5)P_3$ are tightly regulated not only by SHIP but also by the 3' lipid phosphatase ▶ PTEN (Harris et al. 2008). The importance of these lipid phosphatases is underlined by the fact that PTEN is frequently lost in many leukemias and immortalized leukemic cell lines (Hollander et al. 2011). SHIP expression is also frequently lost, downregulated, or mutated in many cancer cells including acute myeloid leukemia (AML). MicroRNAs (miRNAs) are recently discovered regulators of gene expression that have a role in the regulation of hematopoiesis, the immune response and inflammation. MicroRNAs can repress protein expression through their ability to bind directly to the 3'UTRs of specific genes and prevent translation of the protein products. Scanning of the SHIP 3' UTR revealed perfect sequence complementarity with the seed sequence of miR-155. Elevated levels of miR-155 and consequent diminished SHIP expression have been linked to B cell lymphomas (Costinean et al. 2009). In addition, it has been reported that oncogenic proteins including BCR/Abl, and Tax (implicated in chronic myelogenous leukemia and adult T cell leukemia/lymphoma, respectively), induce SHIP downregulation by a variety of mechanisms (Kerr 2011). Consistent with its role as a tumor suppressor, SHIP restricts development of MDSCs and Tregs. Thus, SHIP deficiency leads to an expansion of MDSCs and Tregs and hence, suppression of T cell immune responses and so, this may be another mechanism for increased tumorigenesis if SHIP expression is reduced. However, the role of SHIP in leukemia seems more complex than initially thought, since there is evidence that SHIP can actually support cancer cell survival as a small molecule inhibitor of SHIP induces apoptosis of multiple myeloma cells (Brooks et al. 2010). This is consistent with its production of $PI(3,4)P_2$ which is known to facilitate Akt activation and hence cell proliferation, survival, and tumorigenesis (Manning and Cantley 2007). Others have shown that SHIP inhibits CD95/APO-1/Fas-induced apoptosis in T cells by promoting CD95 glycosylation independently of its phosphatase activity (Charlier et al. 2010).

HSC proliferation and numbers are increased in $SHIP^{-/-}$ mice. Despite expansion of the compartment, SHIP deficient HSCs exhibit reduced capacity for long-term repopulation and home inefficiently to bone marrow. The role of SHIP in the biology of both

HSC and the hematopoietic stem cell niche, suggests that it may be a useful target for treatment of bone marrow failure syndromes caused by viruses, radiation, chemotherapy, or malignancy. As already mentioned, MDSCs are a type of immunoregulatory cell that can repress allogeneic T cell responses. A common complication arising after bone marrow transplantation is Graft-versus-host disease (GVHD) which involves priming of allogeneic T cells. Remarkably, SHIP deficient mice express more myeloid suppressor cells than their WT counterparts and accept allogeneic bone marrow grafts with a reduced incidence of GVHD (Ghansah et al. 2004; Kerr 2008).

The key regulatory role of SHIP has been exploited by several opportunistic pathogens that target these phosphatases in order to evade immune detection. Thus, lymphocytes are particularly sensitive to the cytolethal distending toxin subunit B (CdtB), an immunotoxin produced by *Actinobacillus actinomycetemcomitans* that can hydrolyse $PI(3,4,5)P_3$ to $PI(3,4)P_2$. Exposure to CdtB leads to cell cycle arrest and death by apoptosis. The lipid phosphatase activity of CdtB may therefore result in reduced immune function, facilitating chronic infection with *Actinobacillus* and other enteropathogens that express Cdt proteins (Shenker et al. 2007). The measles virus evades destruction by the immune system at least in part, by targeting negative regulation of PI3K/Akt signaling. It induces expression of SIP-110 which depletes the cellular $PI(3,4,5)P_3$ pools, suggesting that the threshold for activation signals leading to induction of T cell proliferation is raised (Avota et al. 2006).

The role of SHIP in regulating the development and function of various hematopoietic cells and evidence linking SHIP to cancer and other diseases, has led to the search for small molecules that are able to modulate activity and which may be useful as drugs. Several compounds have now been reported and have been shown to exert an anti-inflammatory effect in in vitro and in vivo models (Harris et al. 2008). Moreover, these molecules have been sucessfully used to kill multiple myeloma cells in vitro indicating that SHIP agonists could be effective anticancer agents. Small molecule inhibitors of SHIP have also been reported which, consistent with observations from SHIP deleted mice, led to increased myeloid suppressor cells, reduced ability of peripheral lymphoid tissues to prime myeloid-associated responses, and protected against GVHD. SHIP inhibitors increased levels of granulocytes, red blood cells, neutrophils, and platelets in mice and could therefore, have application to improve blood cell number in patients with myelodysplastic syndrome or myelosuppressive infection. A SHIP inhibitor also triggered the apoptosis of human acute myeloid leukemia cell lines, consistent with SHIP being anti-apoptotic under some circumstances (Brooks et al. 2010).

Summary

SHIP is largely confined to hematopoietic cells and modulates PI3K/Akt dependent signaling by hydrolysing the PI3K-generated second messenger $PI(3,4,5)P_3$, to $PI(3,4)P_2$. As a consequence, SHIP is able to modulate $PI(3,4,5)P_3$-mediated signaling and hence the proliferation, differentiation, survival, activation, and migration of hematopoeitic cells. SHIP possesses a centrally located catalytic domain responsible for the hydrolysis of the 5'-phosphate of the membrane phosphoinositide lipid $PI(3,4,5)P_3$. In addition, SHIP possesses multiple structural domains that facilitate protein–protein interactions and cellular re-localization upon receptor stimulation. These structural features and binding motifs allow SHIP to serve a scaffolding role for the recruitment of other proteins to the plasma membrane. Indeed, SHIP has been shown to exert several functional effects independently of its catalytic function. The ability of PH domain-containing proteins to distinguish between different 3'-phosphoinositide lipids suggests that SHIP can act as a switch to redirect PI3K signaling toward $PI(3,4)P_2$-dependent effectors that are temporally and functionally separate from $PI(3,4,5)P_3$-dependent events. Thus, SHIP may function to fine-tune phosphoinositide signaling, rather than terminate it. SHIP can regulate a variety of signaling pathways related to cytokine, chemokine, antigen, and IgG engagement in both lymphocytes and myeloid cells. It plays a key role in the generation of lymphocyte and myeloid subsets and maintaining a balance between inflammatory and regulatory cells. Additionally, SHIP plays an important regulatory role in establishing endotoxin tolerance in macrophages, as well as regulating leucocyte polarization during migratory responses to chemoattractants. The expression of SHIP and/or its activity is often targeted by pathogens, whilst SHIP expression is lost, downregulated or

mutated in many cancer cells. Thus, modulation of SHIP offers potential for therapeutic intervention in a range of inflammatory and autoimmune diseases as well as in cancer and transplantation settings.

References

Avota E, Harms H, Schneider-Schaulies S. Measles virus induces expression of SIP110, a constitutively membrane clustered lipid phosphatase, which inhibits T cell proliferation. Cell Microbiol. 2006;8:1826–1839.

Brooks R, Fuhler GM, Iyer S, Smith MJ, Park MY, Paraiso KH, Engelman RW, Kerr WG. SHIP1 inhibition increases immunoregulatory capacity and triggers apoptosis of hematopoietic cancer cells. J Immunol. 2010;184:3582–3589.

Charlier E, Conde C, Zhang J, Deneubourg L, Di Valentin E, Rahmouni S, Chariot A, Agostinis P, Pang PC, Haslam SM, Dell A, Penninger J, Erneux C, Piette J, Gloire G. SHIP-1 inhibits CD95/APO-1/Fas-induced apoptosis in primary T lymphocytes and T leukemic cells by promoting CD95 glycosylation independently of its phosphatase activity. Leukemia. 2010;24:821–832.

Costinean S, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, Ciarlariello D, Neviani P, Harb J, Kauffman LR, Shidham A, Croce CM. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein-beta are targeted by miR-155 in B cells of E{micro}-MiR-155 transgenic mice. Blood. 2009;114:1374–1382.

Ghansah T, Paraiso KHT, Highfill S, Desponts C, May S, McIntosh JK, Wang J-W, Ninos J, Brayer J, Cheng F, Sotomayor E, Kerr WG. Expansion of myeloid suppressor cells in SHIP-deficient mice represses allogeneic T cell responses. J Immunol. 2004;173:7324–7330.

Hamilton MJ, Ho VW, Kuroda E, Ruschmann J, Antignano F, Lam V, Krystal G. Role of SHIP in cancer. Exp Hematol. 2011;39:2–13.

Harris SJ, Parry RV, Westwick J, Ward SG. Phosphoinositide lipid phosphatases: natural regulators of phosphoinositide 3-kinase signaling in T lymphocytes. J Biol Chem. 2008;283:2465–2469.

Helgason CD, Damen JE, Rosten P, Grewal R, Sorensen P, Chappel SM, Borowski A, Jirik F, Krystal G, Humphries RK. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. Genes Dev. 1998;12:1610–1620.

Hollander MC, Blumenthal GM, Dennis PA. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. Nat Rev Cancer. 2011;11:289–301.

Kerr WG. A role for SHIP in stem cell biology and transplantation. Curr Stem Cell Res Ther. 2008;3:99–106.

Kerr WG. Inhibitor and activator: dual functions for SHIP in immunity and cancer. Ann N Y Acad Sci. 2011;1217:1–17.

Lemmon MA, Ferguson KM. Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem J. 2000;350:1–18.

Leung WH, Tarasenko T, Bolland S. Differential roles for the inositol phosphatase SHIP in the regulation of macrophages and lymphocytes. Immunol Res. 2009;43:243–251.

Lioubin MN, Myles GM, Carlberg K, Bowtell D, Rohrschneider LR. Shc, Grb2, Sos1, and a 150-kilodalton tyrosine-phosphorylated protein form complexes with Fms in hematopoietic cells. Mol Cell Biol. 1994;14:5682–5691.

Liu L, Damen JE, Cutler RL, Krystal G. Multiple cytokines stimulate the binding of a common 145-kilodalton protein to Shc at the Grb2 recognition site of Shc. Mol Cell Biol. 1994;14:6926–6935.

Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129:1261–1274.

Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. Nature. 1996;383:263–266.

Ooms LM, Horan KA, Rahman P, Seaton G, Gurung R, Kethesparan DS, Mitchell CA. The role of the inositol polyphosphate 5-phosphatases in cellular function and human disease. Biochem J. 2009;419:29–49.

Peng Q, Malhotra S, Torchia JA, Kerr WG, Coggeshall KM, Humphrey MB. TREM2- and DAP12-dependent activation of PI3K requires DAP10 and is inhibited by SHIP1. Sci Signal. 2010;3.

Shenker BJ, Dlakic M, Walker LP, Besack D, Jaffe E, LaBelle E, Boesze-Battaglia K. A novel mode of action for a microbial-derived immunotoxin: the cytolethal distending toxin subunit B exhibits phosphatidylinositol 3,4,5-triphosphate phosphatase activity. J Immunol. 2007;178:5099–5108.

sHsp-Kinase

► Mapkap Kinase 2/3 (MK2/3)

Sialophorin, Galactoglycoprotein

► CD43

Signal Transducers and Activators of Transcription

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

Sinc

► Prion Protein (PRNP)

SKAP2/SCAP2 (Src Kinase-Associated Phosphoprotein 2)

▶ SKAP-HOM

SKAP55 Homologue (Src Kinase-Associated Phosphoprotein of 55 kDa Homologue)

▶ SKAP-HOM

SKAP55-R (Src Kinase-Associated Phosphoprotein 55-Related Protein)

▶ SKAP-HOM

SKAP-HOM

Annegret Reinhold and Burkhart L. Schraven
Institute for Molecular and Clinical Immunology, Otto von Guericke University Magdeburg, Magdeburg, Germany

Synonyms

PRAP (Pyk2/RAFTK-associated protein); RA70 (Retinoic acid-induced protein 70); SAPS (Src-associated adapter protein with PH and SH3 domains); SKAP2/SCAP2 (Src kinase-associated phosphoprotein 2); SKAP55 homologue (Src kinase-associated phosphoprotein of 55 kDa homologue); SKAP55-R (Src kinase-associated phosphoprotein 55-related protein)

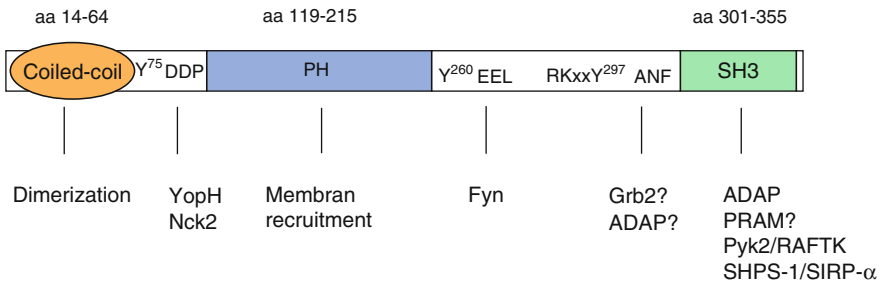
Historical Background

SKAP-HOM was originally described in 1998 as a protein homologous to the cytosolic adaptor protein SKAP55 (Marie-Cardine et al. 1998). Using the recombinant SH2 domain of the ▶ Src protein tyrosine kinase Fyn expressed as a GST fusion protein, SKAP55 as well as SKAP-HOM were purified from lysates of pervanadate-treated human T cells. Furthermore, it was demonstrated that SKAP-HOM acts as a substrate for the Src kinase Fyn and that it binds the adaptor molecule FYB/SLAP/▶ ADAP. At the same time, Liu et al. reported the identification and the molecular cloning of a protein termed SKAP55-R. Using the yeast two-hybrid system with FYB/SLAP/ADAP as bait, the SKAP55-related protein was identified (Liu et al. 1998). The murine homologue of human SKAP55-R was described in 2000 (Curtis et al. 2000). The authors reported that murine SKAP55-R plays a role in IL-6-induced myeloid cell differentiation and that overexpression of SKAP55-R inhibits growth of FDC-P1 myeloid cell line and primary hematopoietic progenitors. Independently, a retinoic acid-inducible protein was described, which is expressed during neuronal differentiation of mouse embryonal carcinoma cells. The protein, termed RA70, is highly homologous to SKAP55 and functions as an adaptor protein of the Src family kinases Fyn, Hck, and Lyn (Kouroku et al. 1998). Five years later, a novel adaptor protein, Pyk2/RAFTK-associated protein (PRAP), was identified using the yeast two-hybrid cloning system. PRAP specifically binds to Pyk2/RAFTK, thereby inhibiting Pyk2/RAFTK kinase activity. In this way, tyrosine phosphorylation of α-synuclein, a presynaptic protein that is involved in the pathogenesis of several neurodegenerative diseases is inhibited. Furthermore, PRAP was found to function as a substrate for Src family kinases such as Fyn (Takahashi et al. 2003).

Consistently, all researchers reported that unlike SKAP55, which is exclusively expressed in T cells, SKAP-HOM expression is ubiquitous.

Structural Organization

The cDNA of human SKAP-HOM encodes a 359 amino acid polypeptide which possesses a pleckstrin homology (PH) domain followed by a central region containing several tyrosine phosphorylation sites and a C-terminal SH3 domain. The tyrosine motif YEEL could serve as a putative binding site for the SH2 domain of Fyn. A second tyrosine motif YAN might represent a binding site for the SH2 domain of Grb2. SKAP-HOM shows the same overall structure as



SKAP-HOM, Fig. 1 Structure of SKAP-HOM and binding partners

SKAP55 except for an unique N-terminal coiled-coil domain, which is not present in SKAP55 (Fig. 1). The proteins show an overall 44% homology with the greatest conservation in the PH and the SH3 domains (Liu et al. 1998; Marie-Cardine et al. 1998). Given the size and the sequence similarity, it was hypothesized that SKAP55 and SKAP-HOM might originate from gene duplication. The chromosomal localization is chromosome 7 for human and chromosome 6 for the mouse gene, respectively. The identity in the amino acid sequence between mouse and human proteins is 91%.

Detailed structural and biochemical analysis including crystallization of the SKAP-HOM N-terminus revealed an unusual four-helix bundle dimerization domain. This dimerization domain mediates self-association and homodimerization in vivo. The isolated PH domain binds to phospholipids in vitro. The potential for allosteric inhibitory interactions between the dimerization domain and the PH domain suggests that SKAP-HOM exists in two states: a closed, docked state and an open free conformation capable of phosphoinositide binding. It was hypothesized that this phosphoinositide responsive molecular switch regulates subcellular targeting, thereby controlling SKAP-HOM function (Swanson et al. 2008).

SKAP55 binds to ADAP with high stoichiometry. In 2005, Huang and coworkers published the unexpected finding that ADAP-deficient Jurkat T cells also lack SKAP55 expression. It was shown that in the absence of ADAP, SKAP55 is rapidly proteolyzed and the half-life of the protein is decreased (Huang et al. 2005). This finding was further extended in ADAP-deficient mouse T cells where it was shown that both SKAP55 and SKAP-HOM are undetectable at protein level (Kliche et al. 2006). In contrast, SKAP-HOM-deficient T cells as well as SKAP55-deficient T cells show no changes in ADAP protein expression (Togni et al. 2005; Wang and Rudd 2008).

SKAP-HOM contains several modular interaction domains including a PH domain, a C-terminal SH3 domain, an N-terminal coiled-coil domain and multiple tyrosine phosphorylation sites (Y). The potential interaction partners are indicated below.

Binding Partners of SKAP-HOM

ADAP constitutively binds to SKAP55 or SKAP-HOM. The dominant binding occurs via the SKAP-HOM SH3 domain which binds to the proline rich-regions in ADAP (Liu et al. 1998; Marie-Cardine et al. 1998). A second interaction was described to occur between the ADAP helical SH3 domain and a non-canonical RKxxYxxY motif in SKAP55. Preliminary data indicated that this RKxxYxxY motif in SKAP-HOM also binds to the SH3 domain of ADAP (Wang and Rudd 2008).

PRAM-1 (PML-Retinoic acid receptor alpha regulated adaptor molecule-1) shares structural homologies with ADAP and is expressed early during myeloid differentiation and in mature granulocytes. Co-immunoprecipitation studies documented a constitutive association between PRAM-1 and SKAP-HOM. Although the interaction site was not characterized, PRAM-1 contains several proline-rich repeats corresponding to the SH3 recognition site (Moog-Lutz et al. 2001).

Another interaction partner of SKAP-HOM is the serine/threonine kinase ▶ *HPK1* (hematopoietic progenitor kinase 1) which constitutively associates with SKAP-HOM in mouse B cells. In addition, as shown for SKAP55 in T cells, SKAP-HOM interacts in B cells with *RIAM*, a Rap1 effector molecule involved in integrin activation (Königsberger et al. 2010).

In murine macrophages, SKAP-HOM has been reported to interact with *SHPS-1* (Src homology 2 domain-containing protein tyrosine phosphatase

substrate 1, also known as SIRP alpha), a transmembrane receptor of the immunoglobulin (Ig) superfamily. SHPS-1 functions as a scaffolding protein mediating the assembly of adhesion-regulated multi-protein complexes including SKAP-HOM, ADAP, Pyk2, and the tyrosine phosphatase SHP-1 (Timms et al. 1999).

Pathogenic species of *Yersinia* harbor a plasmid-encoded type III secretion system that translocates virulence-associated bacterial proteins into host cells. *YopH* (*Yersinia* outer protein H) is a highly active protein tyrosine kinase. When translocated into host cells, YopH disrupts focal adhesion complexes thereby causing detachment of infected cells and abrogation of the phagocytic process. It has been demonstrated that YopH dephosphorylates SKAP-HOM, ADAP, and Cas (Crk-associated substrate) in murine macrophages, thus interfering with adhesion-regulated signal transduction pathways (Fällman et al. 2002).The short peptide sequence of SKAP-HOM (DEYDDPF), located in the linker region between the dimerization domain and the PH domain, is recognized by the N-terminal substrate-binding domain of YopH. Based on this SKAP-HOM sequence, cyclic peptides have been designed to inhibit *Yersinia* infections (Leone et al. 2012).

Function of SKAP-HOM

SKAP-HOM seems to play a role during myelopoesis. When overexpressed in myeloid cells, SKAP-HOM inhibited proliferation without affecting differentiation (Bourette et al. 2005; Curtis et al. 2000). Furthermore, there is clear evidence that SKAP-HOM plays a role in adhesion events. In murine macrophages, the scaffolding protein SHPS-1 assembles an adhesion-regulated multi-protein complex including SKAP-HOM, ADAP, Pyk2, and the tyrosine phosphatase SHP-1. Timms et al. reported that SKAP-HOM is tyrosine phosphorylated in response to macrophage adhesion to fibronectin (Timms et al. 1999). Macrophages that lack SHP-1, display hyperphosphorylation and show increased adhesivness. In addition, Bourette et al. showed that macrophage colony-stimulating factor (M-CSF) induces tyrosine phosphorylation of SKAP-HOM in myeloid cells and its association with other proteins including actin (Bourette et al. 2005). Collectively, these data support the hypothesis that SKAP-HOM is involved in the regulation of actin dynamics in myeloid cells.

In T cells, ADAP and SKAP55 play an essential role in TCR-mediated inside-out signaling that is needed for LFA-1 clustering and conjugate formation between T cells and antigen-presenting cells (APC). Given the high homology, the question arises whether SKAP-HOM plays a redundant role in the same events. According to Wang and coworkers, SKAP-HOM failed to compensate for the loss of SKAP55 in LFA-1 clustering in T cells (Wang and Rudd 2008).

SKAP-HOM-deficient B cells show strongly reduced adhesion to fibronectin and ICAM-1 after BCR-stimulation, whereas B lymphocyte development and differentiation was normal (Togni et al. 2005). The signaling module regulating activation of B-cell integrins involves HPK1, SKAP-HOM, and RIAM. The loss of HPK1 induces enhanced integrin activity suggesting a negative regulatory role for this signaling complex. In conclusion, these data indicate that SKAP-HOM and SKAP55 may have similar but specialized functions in T and B cells (Königsberger et al. 2010).

In SKAP-HOM-deficient bone marrow-derived dendritic cells (BMDCs) the spontaneous motility is enhanced. By contrast, adhesion of mature BMDCs to fibronectin is reduced and antigen-dependent conjugate formation between SKAP-HOM-deficient DCs and T cells is delayed (Reinhold et al. 2009). Bone marrow-derived macrophages from SKAP-HOM-deficient mice exhibit abortive actin-rich ruffle formation (Swanson et al. 2008).

Overall, the results obtained from the characterization of the knockout mice (Table 1) confirm the role of SKAP-HOM in the regulation of integrin activation and adhesion in immune cells.

Only recently, new functions of SKAP-HOM outside the immune system were described. For example, heat shock factor *HSF4b* belongs to the transcriptional regulator family of heat shock proteins (Hsp). HSF4b is mainly expressed in lens epithelial cells and plays a major role in lens development. SKAP-HOM was identified as a novel target of HSF4b that associates with Nck2 and F-actin at membrane ruffles thereby regulating actin remodeling in lens cells (Zhou et al. 2011).

In an attempt to search for novel genes that are involved in pancreatic carcinogenesis, SKAP-HOM was the most frequently detected overexpressed gene in microdissections of pancreatic ductal adenocarcinoma specimen compared to reference samples. There was a significant correlation between DNA copy

SKAP-HOM, Table 1 Summary of the characterization of SKAP-HOM-deficient mice

Cell type	Phenotype	References
T cells	Normal	Togni et al. (2005)
B cells	Reduced proliferation	Togni et al. (2005)
	Reduced BCR-mediated adhesion to fibronectin and ICAM-1	
	Reduced serum immunoglobulins	
Platelets	Normal spreading and aggregation	Togni et al. (2005)
	Normal platelet activation	
BMDC Bone marrow dendritic cells	Increased migration into the lymph nodes in vivo	Reinhold et al. (2009)
	Higher motility in vitro	
	Reduced adhesion to fibronectin	
	Delayed conjugate formation with antigen-specific T cells	
	Reduced proliferation of antigen-specific T cells	
BMM Bone marrow macrophages	Normal proliferation	Togni et al. (2005), Swanson et al. (2008)
	Normal phagocytosis	
	Impaired ruffle formation	
Disease model	Reduced severity of experimental autoimmune encephalomyelitis (EAE)	Togni et al. (2005)

number and mRNA expression level. This dysregulation of SKAP-HOM expression might be associated with the development of pancreatic cancer (Harada et al. 2008). Another study was performed to identify progression-associated metastasis genes in malignant melanoma. Functional assays were performed on a subset of 30 candidate genes including SKAP-HOM. siRNA-mediated knockdown of SKAP-HOM significantly inhibited invasion of melanoma cells in the Boyden Chamber assay (Kabbarah et al. 2010).

Summary

SKAP-HOM is a ubiquitously expressed cytosolic adaptor molecule. In immune cells, the molecular complex formed between SKAP-HOM, ADAP, and RIAM is involved in the regulation of inside-out signaling from immunoreceptors to integrins. To dissect the specialized function of SKAP-HOM and SKAP55 in T cells, an analysis of the respective knockout mice in parallel with a SKAP-HOM/SKAP55 double knockout mouse would be helpful. Further studies are needed to investigate the function of SKAP-HOM outside the immune system. The potential role of SKAP-HOM as a cancer-related gene seems to be of special interest. The knowledge of SKAP-HOM interaction partners and their protein-protein interaction domains might provide useful information for the design of synthetic inhibitors to modulate the invasive phenotype of tumor cells.

References

Bourette RP, Therier J, Mouchiroud G. Macrophage colony-stimulating factor receptor induces tyrosine phosphorylation of SKAP55R adaptor and its association with actin. Cell Signal. 2005;17:941–9.

Curtis DJ, Jane SM, Hilton DJ, Dougherty L, Bodine DM, Begley CG. Adaptor protein SKAP55R is associated with myeloid differentiation and growth arrest. Exp Hematol. 2000;28:1250–9.

Fällman M, Deleuil F, McGee K. Resistance to phagocytosis by Yersinia. Int J Med Microbiol. 2002;291:501–9.

Harada T, Chelala C, Bhakta V, Chaplin T, Caulee K, Baril P, Young BD, Lemoine NR. Genome-wide DNA copy number analysis in pancreatic cancer using high-density single nucleotide polymorphism arrays. Oncogene. 2008;27:1951–60.

Huang Y, Norton DD, Precht P, Martindale JL, Burkhardt JK, Wange RL. Deficiency of ADAP/Fyb/SLAP-130 destabilizes SKAP55 in Jurkat T cells. J Biol Chem. 2005;280:23576–83.

Kabbarah O, Nogueira C, Feng B, Nazarian RM, Bosenberg M, Wu M, Scott KL, Kwong LN, Xiao Y, Cordon-Cardo C, Granter SR, Ramaswamy S, Golub T, Duncan LM, Wagner SN, Brennan C, Chin L. Integrative genome comparison of primary and metastatic melanomas. PLoS One. 2010;5:e10770.

Kliche S, Breitling D, Togni M, Pusch R, Heuer K, Wang X, Freund C, Kasirer-Friede A, Menasche G, Koretzky GA, Schraven B. The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. Mol Cell Biol. 2006;26:7130–44.

Königsberger S, Peckl-Schmid D, Zaborsky N, Patzak I, Kiefer F, Achatz G. HPK1 associates with SKAP-HOM to negatively regulate Rap1-mediated B-lymphocyte adhesion. PLoS One. 2010;5:e12468.

Kouroku Y, Soyama A, Fujita E, Urase K, Tsukahara T, Momoi T. RA70 is a src kinase-associated protein expressed ubiquitously. Biochem Biophys Res Commun. 1998;252:738–42.

Leone M, Barile E, Dahl R, Pellecchia M. Design and NMR studies of cyclic peptides targeting the N-terminal domain of the protein tyrosine phosphatase YopH. Chem Biol Drug Des. 2011;77:12–9.
Liu J, Kang H, Raab M, da Silva AJ, Kraeft SK, Rudd CE. FYB (FYN binding protein) serves as a binding partner for lymphoid protein and FYN kinase substrate SKAP55 and a SKAP55-related protein in T cells. Proc Natl Acad Sci USA. 1998;95:8779–84.
Marie-Cardine A, Verhagen AM, Eckerskorn C, Schraven B. SKAP-HOM, a novel adaptor protein homologous to the FYN-associated protein SKAP55. FEBS Lett. 1998;435:55–60.
Moog-Lutz C, Peterson EJ, Lutz PG, Eliason S, Cave-Riant F, Singer A, Di Gioia Y, Dmowski S, Kamens J, Cayre YE, Koretzky G. PRAM-1 is a novel adaptor protein regulated by retinoic acid (RA) and promyelocytic leukemia (PML)-RA receptor alpha in acute promyelocytic leukemia cells. J Biol Chem. 2001;276:22375–81.
Reinhold A, Reimann S, Reinhold D, Schraven B, Togni M. Expression of SKAP-HOM in DCs is required for an optimal immune response in vivo. J Leukoc Biol. 2009;86:61–71.
Swanson KD, Tang Y, Ceccarelli DF, Poy F, Sliwa JP, Neel BG, Eck MJ. The Skap-hom dimerization and PH domains comprise a 3′-phosphoinositide-gated molecular switch. Mol Cell. 2008;32:564–75.
Takahashi T, Yamashita H, Nagano Y, Nakamura T, Ohmori H, Avraham H, Avraham S, Yasuda M, Matsumoto M. Identification and characterization of a novel Pyk2/related adhesion focal tyrosine kinase-associated protein that inhibits alpha-synuclein phosphorylation. J Biol Chem. 2003;278:42225–33.
Timms JF, Swanson KD, Marie-Cardine A, Raab M, Rudd CE, Schraven B, Neel BG. SHPS-1 is a scaffold for assembling distinct adhesion-regulated multi-protein complexes in macrophages. Curr Biol. 1999;9:927–30.
Togni M, Swanson KD, Reimann S, Kliche S, Pearce AC, Simeoni L, Reinhold D, Wienands J, Neel BG, Schraven B, Gerber A. Regulation of in vitro and in vivo immune functions by the cytosolic adaptor protein SKAP-HOM. Mol Cell Biol. 2005;25:8052–63.
Wang H, Rudd CE. SKAP-55, SKAP-55-related and ADAP adaptors modulate integrin-mediated immune-cell adhesion. Trends Cell Biol. 2008;18:486–93.
Zhou L, Zhang Z, Zheng Y, Zhu Y, Wei Z, Xu H, Tang Q, Kong X, Hu L. SKAP2, a novel target of HSF4b, associates with NCK2/F-actin at membrane ruffles and regulates actin reorganization in lens cell. J Cell Mol Med. 2011;15:783–95.

SKIP

► Tribbles

SLC14A1

► UT (Urea Transporter)

SLC14A2

► UT (Urea Transporter)

SLC30A

► Zinc Transport in the Pancreatic β-Cell: Roles of ZnT (SLC30A) and ZiP (SLC39A) Family Members

SLC39A

► Zinc Transport in the Pancreatic β-Cell: Roles of ZnT (SLC30A) and ZiP (SLC39A) Family Members

SLC9A3R1 (Solute Carrier Family 9 Member 3 Regulator 1)

► NHERF

Slp (Synaptotagmin-Like Protein)

Mitsunori Fukuda
Laboratory of Membrane Trafficking Mechanisms, Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi, Japan

Synonyms

Exophilin; Sytl (Syanptotagmin-Like)

Historical Background

Synaptotagmin-like proteins (Slps) were originally identified as synaptotagmin-related molecules containing two C2 calcium/phospholipid-binding motifs (named the C2A domain and the C2B domain) at their C terminus and no transmembrane domain at

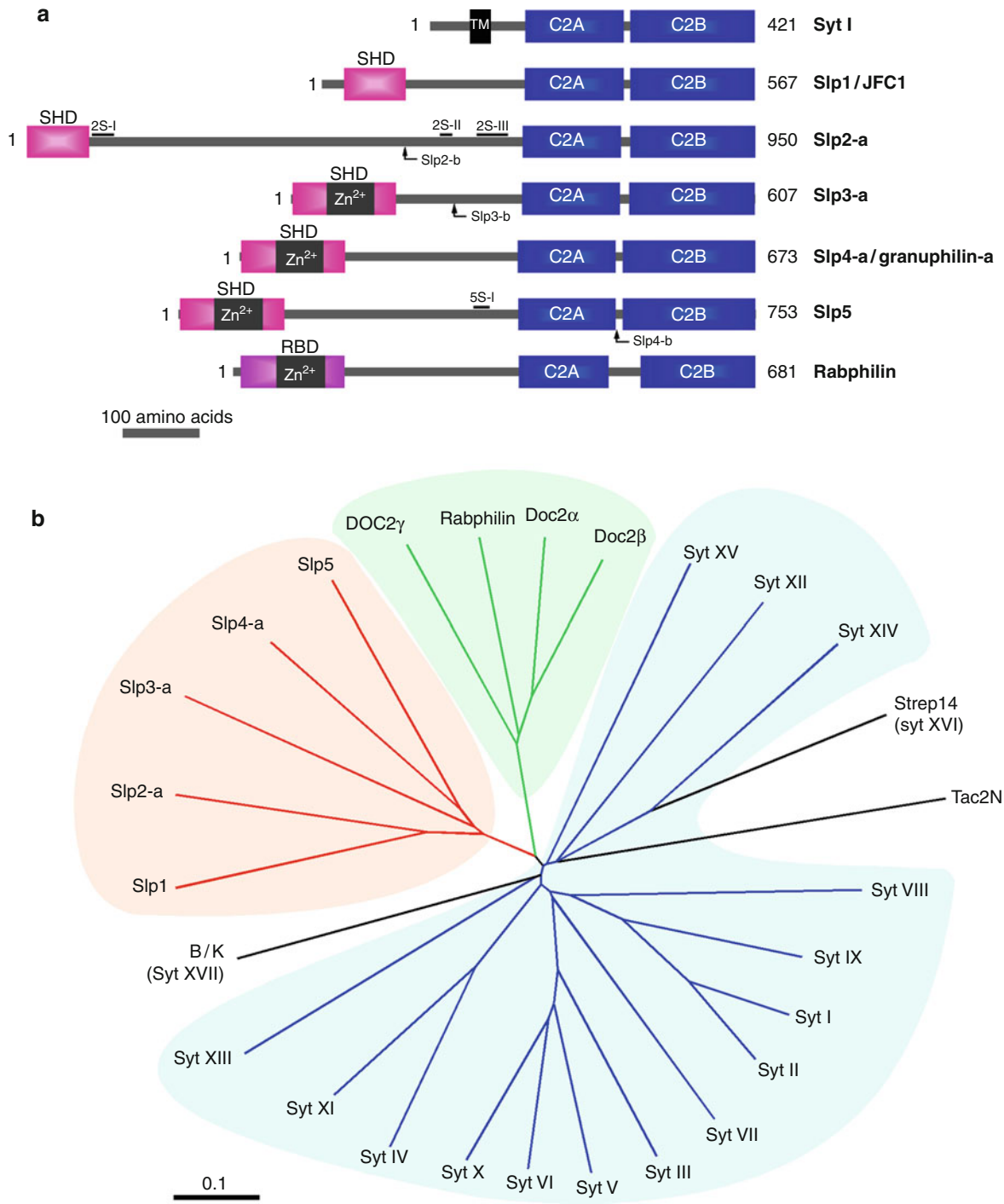
their N terminus (Fig. 1a), which suggested that they regulate certain intracellular membrane traffic events, the same as synaptotagmin I (Syt I) does in synaptic vesicle traffic (Fukuda and Mikoshiba 2001). The Slp family of proteins is now defined as a family of proteins, each of which contains an N-terminal conserved motif named the Slp homology domain (SHD) (Fig. 1a, magenta boxes) and C-terminal tandem C2 domains, although some members of the Slp family have several isoforms (e.g., Slp2-b, Slp3-b, and Slp4-b) that lack one of these domains because of alternative splicing (Fig. 1a, bars and arrows) (Fukuda et al. 2001). The Slp family is also recognized as the third group of C-terminal-type tandem C2 proteins that form a branch distinct from the synaptotagmin family and the rabphilin/Doc2 family in the phylogenetic tree (Fig. 1b). Slp family members appear to be conserved in all vertebrates, and five members of the Slp family (Slp1-5) have been reported in humans and mice (Wang et al. 1999; Fukuda and Mikoshiba 2001; McAdara Berkowitz et al. 2001; Kuroda et al. 2002b). Since mouse Slp mRNAs are differentially distributed in different tissues and at different developmental stages, Slp family members have been suggested to play a tissue-specific or cell-type-specific role in membrane traffic.

The common function of Slp family proteins was first revealed by biochemical analysis of the SHD, which has relatively weak amino acid sequence similarity to the Rab-binding domain of rabphilin, another type of C-terminal-type tandem C2 protein (Fig. 1a, bottom) (Fukuda et al. 2001). The results of an investigation of the ability of the SHD to bind all Rab isoforms clearly indicated that it serves as a specific effector domain for Rab27A and Rab27B (Kuroda et al. 2002a). The Rab27-binding SHD is also found in the N-terminal domain of another protein family, the Slac2 (Slp homologue lacking C2 domains) family, which consists of Slac2-a/melanophilin, Slac2-b, and Slac2-c/MyRIP (Fukuda 2008). Rab27 belongs to the Rab-type small GTPase family and functions as a molecular switch by cycling between two nucleotide-bound states, a GTP-bound active state and a GDP-bound inactive state. The GTP-bound form of Rab27 is recruited to specific membrane compartments, e.g., to melanosomes in melanocytes and to secretory vesicles in secretory cells, and interacts with its specific effector, namely, one of the members of the Slp family. The Rab27 effector function of the Slp family members in melanosome transport and secretory granule exocytosis has been revealed during the past decade, and the proposed function of each Slp is described below.

Slp1/JFC1

Slp1 was independently identified as JFC1, an NADPH oxidase- and phosphatidylinositol 3,4,5-trisphosphate (PIP_3)-binding protein (McAdara Berkowitz et al. 2001). In contrast to other SHDs, the SHD of Slp1 and Slp2-a lacks zinc finger motifs (Fig. 1a), but it still specifically recognizes Rab27 isoforms (Kuroda et al. 2002a). Mouse Slp1 protein is most abundantly expressed in the pancreas, especially in pancreatic acinar cells (Saegusa et al. 2008), and it is also expressed in other cell types, including neutrophils (Brzezinska et al. 2008), neurons (Arimura et al. 2009), platelets (Neumüller et al. 2009), and cytotoxic T lymphocytes (Holt et al. 2008). In neutrophils, Slp1 is co-localized with Rab27A on myeloperoxidase (MPO)-containing azurophilic granules, and knockdown of Slp1 by short hairpin RNA has been found to impair MPO secretion (Brzezinska et al. 2008). The Rab27A·Slp1 complex has been proposed to regulate the tethering and/or docking step of azurophilic granule exocytosis, possibly through interaction with PIP_3 in the plasma membrane. Similarly, Slp1 has been suggested to control other secretion events, including prostate-specific antigen secretion by human prostate carcinoma cells, dense granule exocytosis by platelets, and lytic granule exocytosis by cytotoxic T lymphocytes. Notably, Slp1 has also been found to form a complex with Rab27B·CRMP-2·TrkB·kinesin-1 and to mediate anterograde transport in axons (Arimura et al. 2009).

Despite the important roles of Slp1 at the cellular level, Slp1 knockout (KO) mice are viable, and no obvious abnormalities have been detected in their neurons or immune cells (Saegusa et al. 2008; Holt et al. 2008). The Slp1-deficiency in Slp1 KO mice may be compensated by a Slp1-related protein, Slp2-a. The only difference between wild-type and Slp1 KO mice that has been reported is a higher number of zymogen granules in the pancreatic acinar cells of fasted Slp1 KO mice (Saegusa et al. 2008).



Slp (Synaptotagmin-Like Protein), Fig. 1 (continued)

Slp2

Slp2-a is the largest member of the Slp family. A variety of Slp2 isoforms that have resulted from tissue/cell type-specific alternative splicing events have been reported in the literature (Fukuda et al. 2001; Holt et al. 2008) (Fig. 1a), but the physiological significance of the presence of multiple Slp2 isoforms remains unknown. The Slp2-a isoform contains an N-terminal SHD, whereas the Slp2-b and Slp2-c isoforms lack this domain. Mouse Slp2-a protein is most abundantly expressed in the stomach, especially in gastric surface mucous cells (Saegusa et al. 2006), and it is also expressed in other cell types, including melanocytes (Kuroda and Fukuda 2004), pancreatic α cells, and cytotoxic T lymphocytes (Holt et al. 2008). The well-known function of Slp2-a is to anchor melanosomes to the plasma membrane by simultaneously interacting with Rab27A on the melanosome via its SHD and with phosphatidylserine (PS) in the plasma membrane via its C2A domain (Kuroda and Fukuda 2004). Slp2-a also regulates the elongated cell shape of melanocytes by an unknown mechanism that is independent of Rab27A (Kuroda and Fukuda 2004). Slp2-a is also involved in the docking of mucus granules to the apical plasma membrane of the gastric surface mucous cells, because a smaller number of mucus granules and docking-defect have been observed in the gastric surface mucous cells of Slp2-a KO mice (Saegusa et al. 2006). An inhibitory role of Slp2-a in glucagon secretion has been proposed based on the results of an overexpression study, but the function of endogenous Slp2-a in pancreatic α cells is currently unknown.

Two possible links between human disease and Slp2-a have been reported. The first link is based on the fact that the Slp2-a protein level is dramatically reduced in the cytotoxic T lymphocytes of type II Griscelli syndrome patients (i.e., Rab27A-deficient) (Holt et al. 2008). Its reduction is presumably attributable to proteolysis, because Slp2-a contains multiple PEST sequences, which are potential signals for rapid protein degradation (Holt et al. 2008). However, cytotoxic T lymphocytes from Slp2-a KO mice exhibit normal killing activity (i.e., normal lytic granule exocytosis), suggesting that the Slp2-a-deficiency may be compensated by a Slp2-a-related protein, Slp1. The second link is based on the fact that dysferlin-deficiency has been shown to cause compensatory induction of Slp2-a (and also Rab27A) in limb girdle muscular dystrophy 2B (LGMD2B) muscles, and its induction may contribute to the onset of inflammation in patient muscles.

Slp3

Although Slp3 mRNA is expressed in mouse spleen, lung, kidney, and testis (Fukuda et al. 2001), almost nothing is known about the protein expression of Slp3. Two alternative splicing isoforms, Slp3-a and Slp3-b (Fig. 1a), have been reported, and the former form contains an N-terminal SHD with zinc finger domains. The results of an in vitro binding study indicated that the C2A domain of Slp3 and Slp5 functions as an atypical Ca^{2+}-dependent phospholipid-binding domain (Fukuda 2002), although key Glu/Asp residues responsible for Ca^{2+} binding of the C2 domain of Syt I and protein kinase C (PKC) are missing in the C2A domain of Slp3 and Slp5. Interestingly, expression of these Ca^{2+}-dependent-type Slps (i.e., Slp3-a and Slp5) in neuroendocrine PC12 cells strongly induced hormone secretion without changing the number of hormone granules docked at the plasma membrane (Fukuda 2003; Tsuboi and Fukuda 2006). By contrast, Ca^{2+}-independent-type Slps (i.e., Slp1, Slp2-a, and Slp4-a) had no effect or else had an inhibitory effect on hormone secretion by PC12 cells.

Slp (Synaptotagmin-Like Protein), Fig. 1 Structure of Slp family members. (**a**) Comparison of Slp family members with Syt I and rabphilin. Syt I, the Slp family members, and rapbphilin share the tandem C2 domains (i.e., the C2A domain and the C2B domain; *blue* boxes) at their C terminus, but their N-terminal structures differ. Syt I contains a single transmembrane domain (TM, black box), whereas the Slp family members contain a unique Slp homology domain (SHD, magenta boxes), also known as RBD27 (Rab-binding domain specific for Rab27 isoforms) (Fukuda et al. 2001; Kuroda et al. 2002a). The SHD of Slp3-5 is divided by zinc finger motifs (Zn^{2+}). Short bars (2 S-I, 2 S-II, 2 S-III, 5 S-I) and arrows (Slp2-b, Slp3-b, and Slp4-b) indicate the positions of alternative splicing sites. Amino acid numbers are given on both sides. (**b**) Molecular dendrogram of the mouse C-terminal-type tandem C2 proteins, including members of the synaptotagmin family (*blue* branch), the Slp family (*red* branch), and the rabphilin/Doc2 family (*green* branch). The dendrogram was drawn by using the ClustalW program set at the default parameters (available at http://clustalw.ddbj.nig.ac.jp/top-e.html)

Slp (Synaptotagmin-Like Protein), Table 1 Proposed functions and distribution of Slp family members

Name (gene ID)	Binding partners (binding site)	Proposed functions, distribution, and other features	KO mouse phenotypes
Slp1/JFC1 (mouse: 269589) (human: 84958)	CRMP-2 (N terminus) Rab27 (SHD) Rab8[a] PIP_3 (C2A) TrkB (C2A) Rap1GAP2 (C2A) NADPH oxidase	Control of prostate-specific antigen secretion by human prostate carcinoma cells Control of azurophilic granule exocytosis by neutrophils Control of TrkB transport in axons Control of dense granule secretion by platelets Control of amylase secretion by pancreatic acinar cells Possible involvement of lytic granule exocytosis by cytotoxic T lymphocytes Phosphorylation of Slp1 at serine 241 by Akt	Viable No apparent abnormalities in general appearance or behavior Increased zymogen granules in pancreatic acinar cells of the fasted Slp1 KO mice
Slp2 (mouse: 83671) (human: 54843)	Rab27 (SHD) PS (C2A)	Melanosome anchoring to the plasma membrane Control of basal mucus secretion by gastric surface mucous cells Control of glucagon secretion by pancreatic α cells Compensatory induction of Slp2-a in limb girdle muscular dystrophy 2B (LGMD2B) muscles Deficiency of Slp2-a protein in cytotoxic T lymphocytes from type II Griscelli syndrome patients	Viable No apparent abnormalities in general appearance or behavior Decreased basal mucus secretion by gastric surface mucous cells of Slp2-a KO mice
Slp3 (mouse: 83672) (human: 94120)	Rab27 (SHD) PS (C2A)[b]	Enhancement of hormone secretion by PC12 cells, when Slp3-a is overexpressed	None produced yet
Slp4/granuphilin (mouse: 27359) (human: 94121)	Rab3/8/27 (SHD) Munc18-1·syntaxin-1a (linker domain) Munc18-2·sytanxin-2/3 (linker domain)	Control of insulin secretion by pancreatic β cells Control of dense-core vesicle exocytosis by PC12 cells Control of amylase secretion by parotid acinar cells Control of pituitary hormone secretion by pituitary cells Control of exosome secretion by HeLa cells	Viable No apparent abnormalities in general appearance or behavior Very mild growth defect (~10% weight reduction) Increased insulin secretion
Slp5 (mouse: 236643) (human: 94122)	Rab27 (SHD) PS (C2A/C2B)[b]	Enhancement of hormone secretion by PC12 cells, when Slp5 is overexpressed Expression of Slp5 protein in pancreatic β cell lines	None produced yet

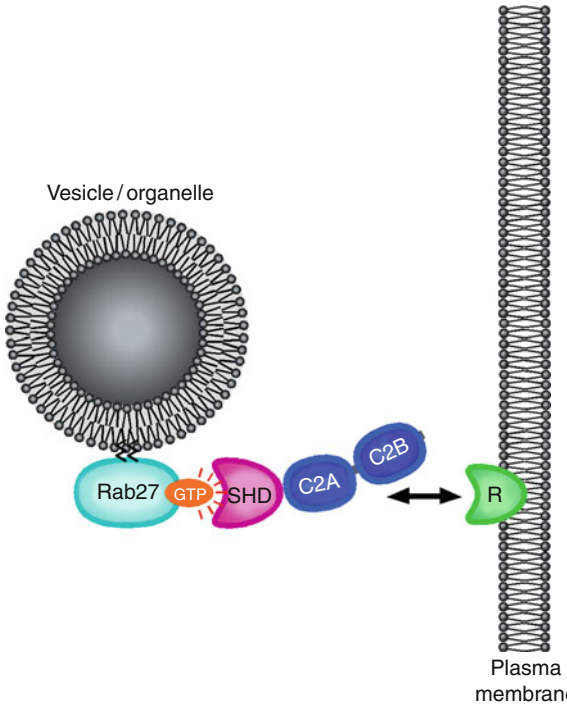
[a]Binding of Rab8 to Slp1 is a matter of controversy in the literature.
[b]The C2A domain of Slp3 and Slp5 exhibits Ca^{2+}-dependent phospholipid-binding activity.

Slp4/Granuphilin

Slp4/granuphilin was originally identified as a rabphilin-like protein associated with insulin granules in pancreatic β cells (Wang et al. 1999). Two alternative splicing isoforms, Slp4-a/granuphilin-a and Slp4-b/granuphilin-b, have been reported, and both of them contain an N-terminal SHD (Fig. 1a). However, no differences in the function of the two isoforms are unknown. Mouse Slp4-a protein is most abundantly expressed in the pancreas, especially in pancreatic β cells (Wang et al. 1999), and it is also expressed in other neuroendocrine cells (Tsuboi and Fukuda 2006), parotid acinar cells, and HeLa cells (Ostrowski et al. 2010). In contrast to other Slp family members, Slp4-a functions as a negative regulator of hormone secretion by neuroendocrine cells, including pancreatic β cells and PC12 cells (Wang et al. 1999; Fukuda 2003), although expression of Slp4-a promotes docking of hormone granules to the plasma membrane, possibly through interaction with Munc18-1·syntaxin-1a complex (Gomi et al. 2005; Tsuboi and Fukuda 2006; Tomas et al. 2008). Small interfering RNA-mediated

knockdown of endogenous Slp4-a or targeted disruption of *Slp4-a* gene actually caused increased hormone secretion despite decreasing the number of plasma membrane-docked hormone granules (Gomi et al. 2005; Tsuboi and Fukuda 2006). Therefore, granuphilin may be a potential therapeutic target for the treatment of type 2 diabetes.

In contrast to the negative role of Slp4-a in hormone secretion, positive roles of Slp4-a have been reported in other cell types. For example, Slp4-a is present on amylase-containing granules in parotid acinar cells together with Rab27A/B and forms a complex with Munc18-2·syntaxin-2/3. Disruption of the Slp4-a·Munc18-2·syntaxin-2/3 complex either by a GST-Slp4-a linker, which contains a Munc18-2-binding region, or antibody against the Slp4-a linker domain, attenuates isoproterenol-stimulated amylase release from streptolysin O-permeabilized parotid acinar cells. Slp4-a is also expressed in HeLa cells and contributes to exosome secretion through interaction with Rab27A.



Slp (Synaptotagmin-Like Protein), Fig. 2 Proposed function of Rab27·Slp complex in the transport of a Rab27-bearing vesicle/organelle. The Slp is recruited to a certain vesicle/organelle by direct binding of the SHD to GTP-Rab27 via an active GTP-Rab27 specifically localized there. The Slp also interacts with a certain receptor (R) in the plasma membrane, and the resulting Rab27·Slp complex mediates tethering, docking, and/or fusion of the transport vesicle/organelle. Thus, Slp family members function as a linker protein between Rab27 on a vesicle/organelle and protein/lipid in the plasma membrane (Fukuda 2008). Examples of "R" are syntaxin-1a/Munc18-1 for Slp4-a/granuphilin-a (Gomi et al. 2005; Tsuboi and Fukuda 2006; Tomas et al. 2008), PS for Slp2-a (Kuroda and Fukuda 2004), and PIP_3 for Slp1/JFC1

Slp5

Slp5 was identified as a protein closely related to Slp4-a, and the genes encoding both are located on the same X chromosome in humans, rats, and mice (Kuroda et al. 2002b). However, the biochemical properties of Slp4-a and Slp5 are clearly different in terms of Ca^{2+}-dependent phospholipid-binding activity and Munc18-1·syntaxin-1a–binding activity (Wang et al. 1999; Kuroda et al. 2002b; Fukuda 2003; Tsuboi and Fukuda 2006). Abundant expression of human Slp5 mRNA has been found in the placenta and liver, but its precise protein expression patterns remain to be determined. Although expression of Slp5 protein has been reported in some pancreatic β cell lines, its involvement in insulin secretion has never been investigated. As noted above in the section on Slp3, however, expression of Slp5 in neuroendocrine PC12 cells strongly induces hormone secretion without changing the number of hormone granules docked at the plasma membrane (Fukuda 2003; Tsuboi and Fukuda 2006).

Summary

The members of the Slp family of proteins are well-known Rab27 effectors that function in specific membrane traffic events (reviewed in Fukuda 2008) (summarized in Table 1). Although each member participates in a different type of membrane traffic event, we found a common molecular mechanism that underlies the Slp-mediated membrane traffic (Fig. 2). Since Slp family proteins are first recruited to the specific vesicle/organelle where the GTP-bound active form of Rab27 is present, and their C-terminal domain interacts with their specific receptor protein (R in Fig. 2) in the plasma membrane, members of the Slp family of proteins function as linkers between cargo (i.e., Rab27-containing vesicle/organelle) and target membrane and promote tethering/docking/fusion of the Rab27-containing vesicle/organelle to the target

membrane. Future determination of the detailed expression pattern of each Slp member and identification of more tissue/cell-type-specific binding partners of Slps, especially Slp3-a and Slp5, will be necessary to fully understand the molecular mechanism of Slp-mediated membrane traffic.

References

Arimura N, Kimura T, Nakamuta S, Taya S, Funahashi Y, Hattori A, et al. Anterograde transport of TrkB in axons is mediated by direct interaction with Slp1 and Rab27. Dev Cell. 2009;16:675–86. doi:10.1016/j.devcel.2009.03.005.

Brzezinska AA, Johnson JL, Munafo DB, Crozat K, Beutler B, Kiosses WB, et al. The Rab27a effectors JFC1/Slp1 and Munc13-4 regulate exocytosis of neutrophil granules. Traffic. 2008;9:2151–64. doi:10.1111/j.1600-0854.2008.00838.x.

Fukuda M. The C2A domain of synaptotagmin-like protein 3 (Slp3) is an atypical calcium-dependent phospholipid-binding machine: comparison with the C2A domain of synaptotagmin I. Biochem J. 2002;366:681–7. doi:10.1042/BJ20020484.

Fukuda M. Slp4-a/granuphilin-a inhibits dense-core vesicle exocytosis through interaction with the GDP-bound form of Rab27A in PC12 cells. J Biol Chem. 2003;278:15390–6. doi:10.1074/jbc.M213090200.

Fukuda M. Regulation of secretory vesicle traffic by Rab small GTPases. Cell Mol Life Sci. 2008;65:2801–13. doi:10.1007/s00018-008-8351-4.

Fukuda M, Mikoshiba K. Synaptotagmin-like protein 1-3: a novel family of C-terminal-type tandem C2 proteins. Biochem Biophys Res Commun. 2001;281:1226–33. doi:10.1006/bbrc.2001.4512.

Fukuda M, Saegusa C, Mikoshiba K. Novel splicing isoforms of synaptotagmin-like proteins 2 and 3: identification of the Slp homology domain. Biochem Biophys Res Commun. 2001;283:513–9. doi:10.1006/bbrc.2001.4803.

Gomi H, Mizutani S, Kasai K, Itohara S, Izumi T. Granuphilin molecularly docks insulin granules to the fusion machinery. J Cell Biol. 2005;171:99–109. doi:10.1083/jcb.200505179.

Holt O, Kanno E, Bossi G, Booth S, Daniele T, Santoro A, et al. Slp1 and Slp2-a localize to the plasma membrane of CTL and contribute to secretion from the immunological synapse. Traffic. 2008;9:446–57. doi:10.1111/j.1600-0854.2008.00714.x.

Kuroda TS, Fukuda M. Rab27A-binding protein Slp2-a is required for peripheral melanosome distribution and elongated cell shape in melanocytes. Nat Cell Biol. 2004;6:1195–203. doi:10.1038/ncb1197.

Kuroda TS, Fukuda M, Ariga H, Mikoshiba K. The Slp homology domain of synaptotagmin-like proteins 1-4 and Slac2 functions as a novel Rab27A binding domain. J Biol Chem. 2002a;277:9212–8. doi:10.1074/jbc.M112414200.

Kuroda TS, Fukuda M, Ariga H, Mikoshiba K. Synaptotagmin-like protein 5: a novel Rab27A effector with C-terminal tandem C2 domains. Biochem Biophys Res Commun. 2002b;293:899–906. doi:10.1016/S0006-291X(02)00320-0.

McAdara Berkowitz JK, Catz SD, Johnson JL, Ruedi JM, Thon V, Babior BM. JFC1, a novel tandem C2 domain-containing protein associated with the leukocyte NADPH oxidase. J Biol Chem. 2001;276:18855–62. doi:10.1074/jbc.M011167200.

Neumüller O, Hoffmeister M, Babica J, Prelle C, Gegenbauer K, Smolenski AP. Synaptotagmin-like protein 1 interacts with the GTPase-activating protein Rap1GAP2 and regulates dense granule secretion in platelets. Blood. 2009;114:1396–404. doi:10.1182/blood-2008-05-155234.

Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol. 2010;12:19–30. doi:10.1038/ncb2000.

Saegusa C, Tanaka T, Tani S, Itohara S, Mikoshiba K, Fukuda M. Decreased basal mucus secretion by Slp2-a-deficient gastric surface mucous cells. Genes Cells. 2006;11:623–31. doi:10.1111/j.1365-2443.2006.00964.x.

Saegusa C, Kanno E, Itohara S, Fukuda M. Expression of Rab27B-binding protein Slp1 in pancreatic acinar cells and its involvement in amylase secretion. Arch Biochem Biophys. 2008;475:87–92. doi:10.1016/j.abb.2008.04.031.

Tomas A, Meda P, Regazzi R, Pessin JE, Halban PA. Munc 18-1 and granuphilin collaborate during insulin granule exocytosis. Traffic. 2008;9:813–32. doi:10.1111/j.1600-0854.2008.00709.x.

Tsuboi T, Fukuda M. The Slp4-a linker domain controls exocytosis through interaction with Munc18-1·syntaxin-1a complex. Mol Biol Cell. 2006;17:2101–12. doi:10.1091/mbc.E05-11-1047.

Wang J, Takeuchi T, Yokota H, Izumi T. Novel rabphilin-3-like protein associates with insulin-containing granules in pancreatic beta cells. J Biol Chem. 1999;274:28542–8. doi:10.1074/jbc.274.40.28542.

SLP-76

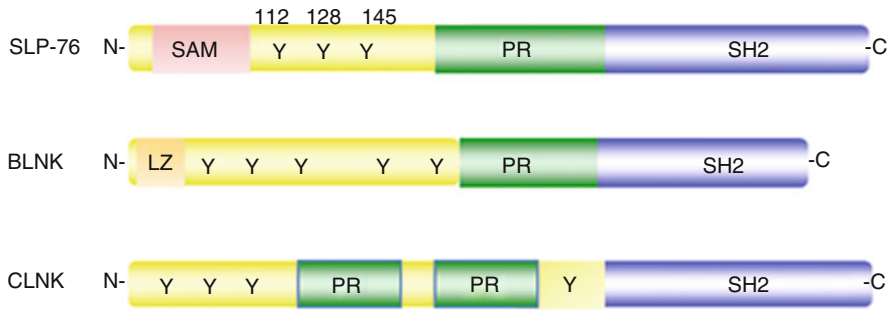
Jong Ran Lee
Department of Life Science, College of Natural Sciences, Ewha Womans University, Seoul, South Korea

Synonyms

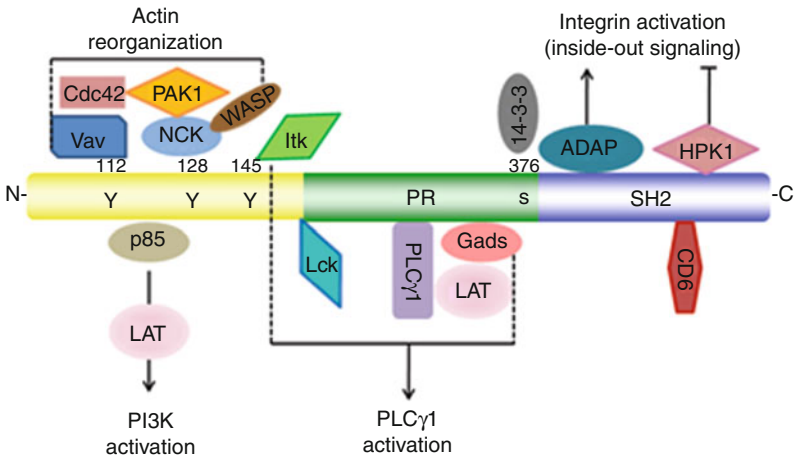
LCP2; Lymphocyte cytosolic protein 2; Src homology 2 (SH2) domain–containing leukocyte protein of 76 kDa

Historical Background

SLP-76 was first identified as a substrate of the protein tyrosine kinases that are activated by T-cell antigen



SLP-76, Fig. 1 Domain structures of SLP-76 adaptor family members. Domains found in the SLP-76 adaptor family members are schematically shown. *LZ* leucine-zipper motif domain, *SAM* sterile alpha motif domain, *SH2* Src homology 2 domain, *PR* proline-rich region. The amino acid position numbers are indicated in SLP-76



SLP-76, Fig. 2 Binding partners for functional domains of SLP-76. Binding partners for each domain structure of SLP-76 and the consequent functional outcomes are shown. *ADAP* adhesion- and degranulation-promoting adaptor protein, *Gads* Grb2-related adaptor proteins, *HPK1* hematopoietic progenitor kinase 1, *Itk* interleukin 2-inducible T-cell kinase, *LAT* linker for activation of T cells, *NCK* non-catalytic region of tyrosine kinase, *PAK1* p21-activated kinase 1, *PI3K* phosphatidylinositol 3-kinase, *PLC* phospholipase C, *WASP* Wiskott-Aldrich syndrome protein

receptors (TCRs) (Jackman et al. 1995). SLP-76, which functions as a signal transducer downstream of TCRs, is an adaptor protein that has three modular domains: an amino (N)-terminal acidic domain with three tyrosine (Y) phosphorylation motifs, a central proline-rich region, and a carboxy (C)-terminal SH2 domain (Fig. 1). Two other proteins, B-cell linker protein (BLNK/BASH/BCA/SLP-65) and cytokine-dependent hematopoietic cell linker (CLNK/MIST), constitute a family of SLP-76 adaptors, each expressed exclusively in hematopoietic cells. The three family members have only a moderate degree of sequence homology, but share a characteristic domain structure (Fig. 1). These SLP-76 family members are critical for nucleation of large signaling complexes in diverse hematopoietic cell types.

SLP-76 as a Scaffold for Signaling Complexes

Investigation into the three functional domains of SLP-76 has revealed how SLP-76 operates in TCR signaling. SLP-76 regulates T-cell function in various ways through its binding partners (Fig. 2).

The amino-terminal tyrosines of SLP-76 are essential for SLP-76 function. Three tyrosines at amino acids 112 (113 in humans), 128, and 145 become phosphorylated after TCR stimulation. Mutation of

tyrosine 112 and/or 128 abrogates the inducible association of SLP-76 with the guanine nucleotide exchange factor (GEF) ▶ Vav1 (Wu et al. 1996), the adaptor non-catalytic region of tyrosine kinase (NCK) (Wunderlich et al. 1999), and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Shim et al. 2004). Mutation of tyrosine 145 results in the loss of binding to Tec family tyrosine kinase interleukin 2-inducible T-cell kinase (Itk) (Su et al. 1999). Recently, a sterile-α motif (SAM) was identified at amino acids 12–78 just proximal to the three tyrosines, but it is not currently known what interaction(s) the SAM domain of SLP-76 mediates.

The central proline-rich region of SLP-76 contains a characterized SH3-domain-binding motif (RxxK) that mediates constitutive interaction with Grb2-related adaptor proteins (Gads) (Berry et al. 2002). In addition to the Gads-binding site, the P1 domain at amino acids 157–223 was defined as a region necessary for the basal association of SLP-76 with phospholipase C-γ1 (PLCγ1) (Yablonski et al. 2001). However, the critical aspect of the P1 domain probably lies more in its role as a molecular spacer, preventing the premature recruitment of PLCγ1 to the cell membrane by associating with the transmembrane adaptor linker for activation of T cells (▶ LAT), rather than as a mediator of an essential protein/protein interaction (Gonen et al. 2005; Jung et al. 2010). The Src family kinase Lck also binds the proline-rich domain of SLP-76 (Sanzenbacher et al. 1999). Recently, serine 376 within the proline-rich region was shown to be phosphorylated by the serine-threonine kinase hematopoietic progenitor kinase 1 (HPK1), one of the binding partners of the SLP-76 SH2 domain. Phosphorylation at this site results in decreased TCR signal transduction (Shui et al. 2007), but the mechanism has yet to be defined.

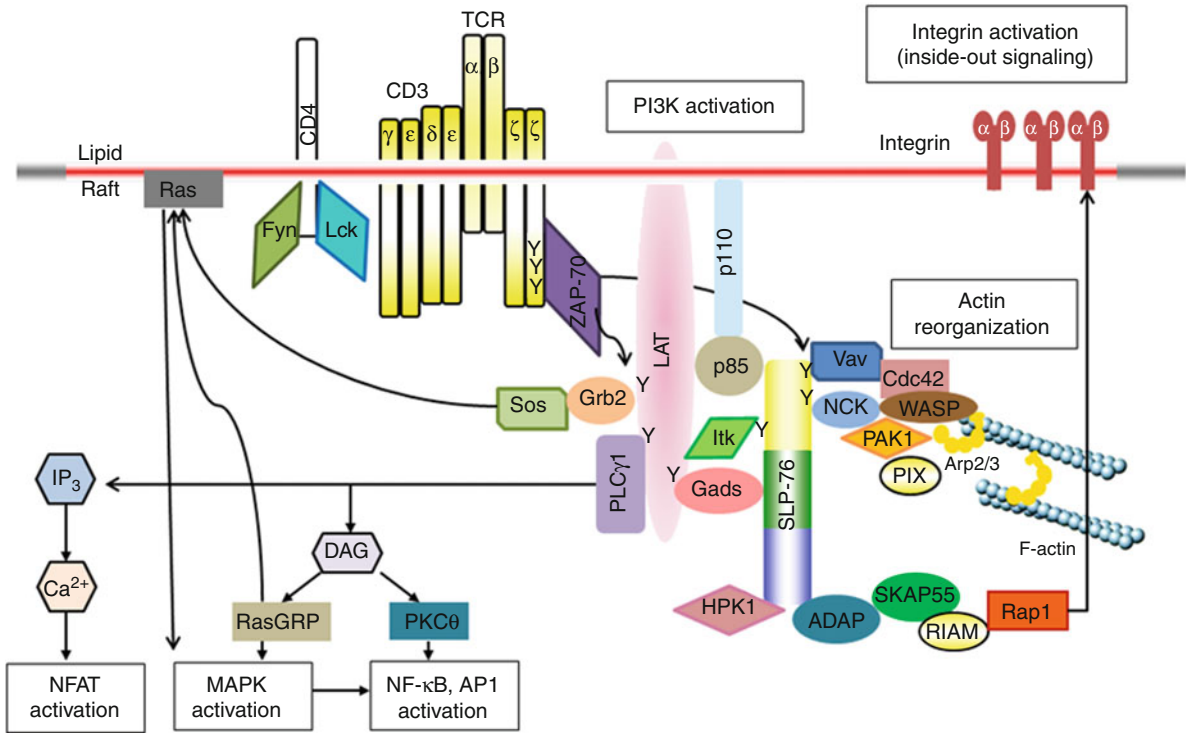
The carboxy-terminal SH2 domain of SLP-76 inducibly associates with adhesion- and degranulation-promoting adaptor protein (▶ ADAP) (Musci et al. 1997) and HPK1 (Sauer et al. 2001). The SLP-76 SH2 domain can also associate with the cytoplasmic tail of the T-cell surface receptor CD6 (Hassan et al. 2006).

Role of SLP-76 in TCR Signaling

TCR ligation results in activation of the Src family kinase Lck, which phosphorylates tyrosines in the immunoreceptor tyrosine–based activation motifs (ITAMs) present within the ▶ CD3 chains of the TCR complex. Phosphorylated ITAMs recruit the Syk family kinase ζ-chain-associated protein kinase of 70 kDa (▶ ZAP-70), leading to phosphorylation of SLP-76 and LAT, which is a critical step in the assembly of the signaling complex by the two adaptors. Phosphorylation of LAT indirectly recruits SLP-76 via Gads binding, and the two adaptors nucleate a multimolecular complex of several signaling proteins and transport this complex to the cell membrane, where LAT is constitutively localized within microdomains known as lipid rafts or glycolipid-enriched membrane domains (GEMs). For PLCγ1 enzyme activation, recruitment to the GEM and tyrosine phosphorylation is induced by binding to the phosphotyrosine of LAT via its SH2 domain, and SLP-76 recruitment to LAT via Gads is also required for this process (Yablonski et al. 1998). Thus, LAT, SLP-76, and Gads appear to constitute an integrated signaling unit that couples TCR-mediated activation of cytoplasmic protein kinases to PLCγ1 enzyme activation (Fig. 3).

The role of SLP-76 in TCR signaling was determined from overexpression studies in the Jurkat human leukemic T-cell line, demonstrating that SLP-76 is a positive regulator of TCR signaling. Complementary loss-of-function studies in SLP-76-deficient Jurkat cells known as J14 cells (Yablonski et al. 1998) revealed considerably reduced phosphorylation of PLCγ1, but not of ZAP-70, LAT, and Itk, showing that SLP-76 links proximal signaling molecules and downstream effectors. Thus, signaling events due to PLCγ1 activation, such as the generation of inositol-1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG) and resulting intracellular calcium flux, the activation of protein kinase C (PKC) family members, and indirect activation of Ras through Ras guanyl-releasing protein (▶ RasGRP), are all reduced. Upregulation of a T-cell activation marker CD69, transcriptional activity of nuclear factor of activated T cells (▶ NFAT), and activator protein 1 (AP1) is significantly reduced with SLP-76 deficiency (Yablonski et al. 1998).

The SLP-76-binding partners Vav1 and NCK, which inducibly associate with the amino-terminal tyrosines of SLP-76 after TCR stimulation, regulate cytoskeletal organization via Cdc42 recruitment through p21-activated kinase 1 (PAK1) and Wiskott-Aldrich syndrome protein (WASP). This model of TCR-mediated cytoskeletal rearrangement implies that SLP-76 regulates actin polymerization in response to TCR stimulation by regulating colocalization of Vav1 with NCK (Zeng et al. 2003) (Fig. 3).



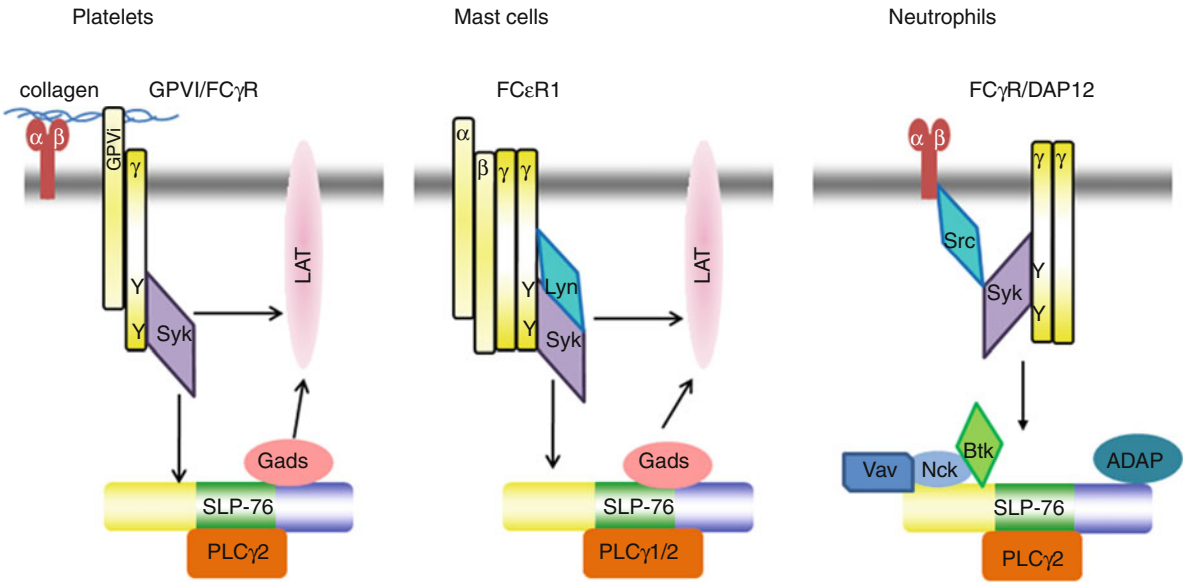
SLP-76, Fig. 3 Role of SLP-76 in T-cell function. SLP-76-mediated signaling pathways downstream of the T-cell receptor: cellular activation and inside-out signaling are shown. *AP1* activator protein 1, *DAG* diacylglycerol, *IP3* inositol-1, 4, 5-trisphosphate, *MAPK* mitogen-activated protein kinase, *NFAT* nuclear factor of activated T cells, *NF-κB* nuclear factor-κB, *PKC* protein kinase C, *Rap1* Ras-proximity-1, *RasGRP* Ras guanyl-releasing protein, *RIAM* Rap1-GTP-interacting adaptor molecule, *SKAP* Src kinase–associated phosphoprotein, *Sos* son of sevenless, *TCR* T-cell receptor

SLP-76 has been implicated in TCR-induced integrin activation, so-called "inside-out signaling" because of its association with ADAP through the SH2 domain. Mutational analysis of ADAP at sites of interaction with SLP-76 diminishes the ability of T cells to adhere to integrin-coated surfaces. The molecular mechanism of SLP-76-mediated inside-out signaling to integrins is thus likely ADAP dependent. Recently, direct involvement was shown for SLP-76 in membrane targeting and activation of the small GTPase Ras-proximity-1 (Rap1), which promotes clustering and affinity modulation of integrins for their full activation, although the domain requirements have not been identified (Horn et al. 2009) (Fig. 3).

SLP-76 Signaling in Other Receptor Systems

The amino-terminal tyrosine motifs of SLP-76 are the earliest phosphorylation substrates of Src and Syk family kinases and are activated by ligation of tyrosine kinase–associated receptors on a variety of hematopoietic cell types. This tyrosine phosphorylation of SLP-76 induces associations with SH2 domain–containing proteins. The central proline-rich sequences mediate constitutive interactions with SH3 domain–containing proteins, and the carboxy-terminal SH2 domain of SLP-76 induces phosphotyrosine-dependent interactions with additional binding partners. Consequently, the SLP-76 adaptor nucleates large signaling complexes and plays critical roles in a variety of tyrosine kinase–associated receptor signaling pathways in various hematopoietic cell types (Fig. 4).

SLP-76 expressed in platelets is phosphorylated after stimulation of the ITAM-bearing collagen receptor, glycoprotein (GP) VI. As in T cells, SLP-76 lies downstream of the Src and Syk family kinases but upstream of PLCγ activation. SLP-76 is also inducibly phosphorylated upon ligation of the platelet integrin αIIβB3, which is required for normal platelet



SLP-76, Fig. 4 Role of SLP-76 in signaling triggered by other surface receptors in hematopoietic cell types. SLP-76 signaling in other receptor systems that are associated with a variety of tyrosine kinases is shown

spreading on its ligand fibrinogen. Unlike TCR signaling, which depends critically on SLP-76 and LAT as well as the inducible interaction between these two adaptor proteins, collagen receptor and integrin signaling in platelets rely more on SLP-76 than on LAT for their function (Judd et al. 2002).

In addition to T cells and platelets, SLP-76 is expressed in multiple hematopoietic lineages including neutrophils, mast cells, macrophages, and natural killer (NK) cells (Jackman et al. 1995). The predominant ITAM-bearing receptors on these cells are Fc receptors (FcRs) including FcεRI in mast cells and FcγRs in other cell types. The signaling molecules that are both upstream and downstream of SLP-76 are similar among these receptors and across cell types. However, important differences exist in how SLP-76 coordinates signal transduction across different cell and receptor types.

Cross-linking of FcεRI, which constitutively interacts with the Src family kinase Lyn, with antigen-bound IgE induces activation and recruitment of Lyn and Syk. Consequently, tyrosine phosphorylation of SLP-76 and nucleation of a signaling complex similar to the one formed in T cells upon TCR activation occur. This complex includes LAT, Vav1, Btk, Gads, PLCγ1/2, and ADAP. SLP-76-deficient bone marrow–derived mast cells degranulate poorly and produce negligible amounts of the cytokine interleukin-6 and also show biochemical defects such as diminished calcium flux and phosphorylation of PLCγ2 (Pivniouk et al. 1999).

Upon FcγR stimulation in neutrophils, signals and effector functions are induced by activation of the Src family kinases Hck and Fgr, followed by Syk activation. These responses in neutrophils are synergistically augmented by cell adhesion. SLP-76-deficient neutrophils show reduced calcium flux and production of reactive oxygen species in response to FcγR stimulation and also fail to spread upon stimulation with integrins (Newbrough et al. 2003). These data suggest that SLP-76 is involved in both FcγR and integrin signaling in neutrophils.

In contrast to neutrophils, SLP-76-deficient macrophages respond normally to FcγR signals, leading to intact phagocytosis function and production of reactive oxygen species (Nichols et al. 2004). Similarly, SLP-76 appears dispensable for the FcγRIIIA-mediated killing function in NK cells (Peterson et al. 1999).

SLP-76-Deficient Mice and Defects in Multiple Lineages

The importance of SLP-76 as a regulator of signaling pathways for cellular function was highlighted by SLP-76-deficient mice. SLP-76-deficient mice present in Mendelian ratios in utero suffer perinatal mortality in more than 60% of the mice. SLP-76-deficient fetuses show

varying degrees of subcutaneous hemorrhage, and surviving mice have blood in the peritoneum (Clements et al. 1998). Adult SLP-76-deficient mice have very small thymi and lack peripheral T cells and lymph nodes. By contrast, normal numbers of macrophages and NK cells are seen in these mice (Clements et al. 1998).

SLP-76-deficient thymocytes are unable to induce allelic exclusion and β-selection due to the failure to transduce pre-TCR signals, resulting in a complete block of thymocyte development at the double-negative 3 stage (Clements et al. 1998). A possible role for SLP-76 in pre-B-cell receptor signaling at the pre-B-cell stage has been shown. Despite hemorrhage in the peritoneum in SLP-76-deficient mice, normal platelet development and normal bleeding times are observed (Clements et al. 1998). Loss of SLP-76 in platelets results in decreased PLCγ2 activation, aggregation, and degranulation in response to collagen as well as decreased spreading on fibrinogen. SLP-76-deficient neutrophils show defective responses to integrin stimulation, with a loss of reactive oxygen species production and failure to spread, as well as a reduced response to FcγR stimulation (Newbrough et al. 2003). SLP-76-deficient mice have a normal number and distribution of mast cells, but SLP-76 deficiency results in loss of mast cell–dependent responses: reduced degranulation, cytokine production, and consequent loss of passive systemic anaphylaxis (Pivniouk et al. 1999).

The abnormal subcutaneous hemorrhagic phenotype in SLP-76-deficient mice is due to a failure of separation of the lymphatic and blood vascular networks during development (Abtahian et al. 2003). SLP-76-deficient mice have chimeric vessels composed of lymphatic and blood endothelial cells, with blood-filled lymphatics sometimes observed. The exact role of SLP-76 for vascular development has yet to be determined.

Summary

SLP-76 was first cloned in 1995 as a substrate of the TCR-activated protein tyrosine kinases and a binding protein for Grb2. Since then, initial characterization of the role of SLP-76 in TCR signaling has documented SLP-76 as an adaptor protein containing multiple protein interaction domains, creating a molecular scaffold on which key signaling complexes are built. The adaptor protein SLP-76 is expressed in multiple lineages of hematopoietic cells including T cells, platelets, and neutrophils. The details of how SLP-76 functions were elucidated both in T cells and other hematopoietic lineages using cell lines and SLP-76-deficient mice. Besides having critical roles for signaling downstream of both immunoreceptors and integrins, SLP-76 may function as a regulator of additional cell surface receptor signals such as those in the vasculature. Insights from studies of how SLP-76 regulates immune cell development and coordinates signal transduction across different cell and receptor types will provide important information about how the immune system uses various strategies for optimal host defense. Based on these observations, potential therapeutic targets can be explored.

References

Abtahian F, Guerriero A, Sebzda E, Lu MM, Zhou R, Mocsai A, Myers EE, Huang B, Jackson DG, Ferrari VA, Tybulewicz V, Lowell CA, Lepore JJ, Koretzky GA, Kahn ML. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science. 2003;299:247–51.

Berry DM, Nash P, Liu SK, Pawson T, McGlade CJ. A high-affinity Arg-X-X-Lys SH3 binding motif confers specificity for the interaction between Gads and SLP-76 in T cell signaling. Curr Biol. 2002;12:1336–41.

Clements JL, Yang B, Ross-Barta SE, Eliason SL, Hrstka RF, Williamson RA, Koretzky GA. Requirement for the leukocyte-specific adaptor protein SLP-76 for normal T cell development. Science. 1998;281:416–9.

Gonen R, Beach D, Ainey C, Yablonski D. T cell receptor-induced activation of phospholipase C-gamma 1 depends on a sequence-independent function of the P-I region of SLP-76. J Biol Chem. 2005;280:8364–70.

Hassan NJ, Simmonds SJ, Clarkson NG, Hanrahan S, Puklavec MJ, Bomb M, Barclay AN, Brown MH. CD6 regulates T-cell responses through activation-dependent recruitment of the positive regulator SLP76. Mol Cell Biol. 2006;26:6727–38.

Horn J, Wang X, Reichardt P, Stradal TE, Warnecke N, Simeoni L, Gunzer M, Yablonski D, Schraven B, Kliche S. Src homology 2-domain containing leukocyte-specific phosphoprotein of 76 kDa is mandatory for TCR-mediated inside-out signaling, but dispensable for CXCR4-mediated LFA-1 activation, adhesion, and migration of T cells. J Immunol. 2009;183:5756–67.

Jackman JK, Motto DG, Sun Q, Tanemoto M, Turck CW, Peltz GA, Koretzky GA, Findell PR. Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. J Biol Chem. 1995;270:7029–32.

Judd BA, Myung PS, Obergfell A, Myers EE, Cheng AM, Watson SP, Pear WS, Allman D, Shattil SJ, Koretzky GA. Differential requirement for LAT and SLP-76 in GPVI versus T cell receptor signaling. J Exp Med. 2002;195:705–17.

Jung SH, Jeong JH, Seul HJ, Lee JR. Competition between SLP76 and LAT for PLCγ1 binding in resting T cells. Eur J Immunol. 2010;40:2330–9.

Musci MA, Hendricks-Taylor LR, Motto DG, Paskind M, Kamens J, Turck CW, Koretzky GA. Molecular cloning of SLAP-130, an SLP-76-associated substrate of the T cell antigen receptor-stimulated protein tyrosine kinases. J Biol Chem. 1997;272:11674–7.

Newbrough SA, Mocsai A, Clemens RA, Wu JN, Silverman MA, Singer AL, Lowell CA, Koretzky GA. SLP-76 regulates Fcgamma receptor and integrin signaling in neutrophils. Immunity. 2003;19:761–9.

Nichols KE, Haines K, Myung PS, Newbrough S, Myers E, Jumaa H, Shedlock DJ, Shen H, Koretzky GA. Macrophage activation and Fcgamma receptor-mediated signaling do not require expression of the SLP-76 and SLP-65 adaptors. J Leukoc Biol. 2004;75:541–52.

Peterson EJ, Clements JL, Ballas ZK, Koretzky GA. NK cytokine secretion and cytotoxicity occur independently of the SLP-76 adaptor protein. Eur J Immunol. 1999;29:2223–32.

Pivniouk VI, Martin TR, Lu-Kuo JM, Katz HR, Oettgen HC, Geha RS. SLP-76 deficiency impairs signaling via the high-affinity IgE receptor in mast cells. J Clin Invest. 1999;103:1737–43.

Sanzenbacher R, Kabelitz D, Janssen O. SLP-76 binding to p56lck: a role for SLP-76 in CD4-induced desensitization of the TCR/CD3 signaling complex. J Immunol. 1999;163:3143–52.

Sauer K, Liou J, Singh SB, Yablonski D, Weiss A, Perlmutter RM. Hematopoietic progenitor kinase 1 associates physically and functionally with the adaptor proteins B cell linker protein and SLP-76 in lymphocytes. J Biol Chem. 2001;276:45207–16.

Shim EK, Moon CS, Lee GY, Ha YJ, Chae SK, Lee JR. Association of the Src homology 2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) with the p85 subunit of phosphoinositide 3-kinase. FEBS Lett. 2004;575:35–40.

Shui JW, Boomer JS, Han J, Xu J, Dement GA, Zhou G, Tan TH. Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. Nat Immunol. 2007;8:84–91.

Su YW, Zhang Y, Schweikert J, Koretzky GA, Reth M, Wienands J. Interaction of SLP adaptors with the SH2 domain of Tec family kinases. Eur J Immunol. 1999;29:3702–11.

Wu J, Motto DG, Koretzky GA, Weiss A. Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity. 1996;4:593–602.

Wunderlich L, Farago A, Downward J, Buday L. Association of Nck with tyrosine-phosphorylated SLP76 in activated T lymphocytes. Eur J Immunol. 1999;29:1068–75.

Yablonski D, Kuhne MR, Kadlecek T, Weiss A. Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76-deficient T cell. Science. 1998;281:413–6.

Yablonski D, Kadlecek T, Weiss A. Identification of a phospholipase C-γ1 (PLC-γ1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-γ1 and NFAT. Mol Cell Biol. 2001;21:4208–18.

Zeng R, Cannon JL, Abraham RT, Way M, Billadeau DD, Bubeck-Wardenberg J, Burkhardt JK. SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. J Immunol. 2003;171:1360–8.

SLP-76 Adapter-Binding Protein of 130 kDa (SLAP-130)

► ADAP

SMADIP, Smad Interacting Protein

► SARA

Small G Protein Indispensable for Equal Chromosome Segregation 1

► Arl8b

Small G-Protein

► Rin (Ras-Like Protein in Neurons)

Small GTPase

► Rin (Ras-Like Protein in Neurons)

Small GTP-Binding Protein

► Rin (Ras-Like Protein in Neurons)

SMARCA

► SWI/SNF Chromatin Remodeling Complex

smMLCK

► MYLK (Myosin Light Chain Kinase)

S-Modulin

▶ Recoverin

Smooth Muscle Myosin Light Chain Kinase

▶ MYLK (Myosin Light Chain Kinase)

SOCS

Julia Strebovsky and Alexander H. Dalpke
Department of Infectious Diseases, Medical Microbiology and Hygiene, University of Heidelberg, Heidelberg, Germany

Synonyms

CIS (cytokine-inducible SH2-containing protein); JAB (Janus kinase binding protein); SSI (STAT-induced STAT inhibitor); TIP3 (Tec interacting protein-3 = SOCS1); (Nck-associated protein-4 = SOCS7)

Historical Background

Cytokines play an important role in the regulation of innate and adaptive immunity. In general, cytokines exert rapidly inducible but transient cellular responses. Whereas signal transduction by cytokines is nowadays known in molecular details, counteraction and termination by negative regulation has only recently begun to be understood. However, the integration of positive and negative signals is mandatory to ensure immunological homeostasis. Type I and II cytokine receptors (including receptors for interferons, interleukins, and hematopoietic growth factors) typically lack intrinsic kinase activity. Instead, cytokines that trigger those receptors activate receptor-associated cytoplasmic Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) factors. The JAK/STAT signaling pathway regulates a variety of responses in immune cells, including development, activation, and differentiation. Negative regulation which terminates JAK/STAT signaling comprises internalization of cytokine receptors, secretion of decoy receptors that capture secreted cytokines, expression of constitutively activated regulators including PIAS (protein inhibitors of activated STAT) proteins, protein tyrosine phosphatases (PTP) such as SHP-1 (SH2-containing phosphatase 1), ▶ SHIP (SH2-containing inositol phosphatase), and protein modifications (ubiquitination and ISGylation) of signaling components. In 1995, CIS (cytokine-inducible SH2-containing protein) was discovered as the first member of the SOCS family during studies on the expression of immediate-early cytokine-induced genes (Yoshimura et al. 1995). In 1997, Suppressor of Cytokine Signaling (SOCS) proteins were subsequently identified independently by three groups as a protein family that regulates JAK/STAT signaling (Endo et al. 1997; Naka et al. 1997; Starr et al. 1997). Importantly, expression of SOCS proteins was induced by JAK/STAT signaling itself (explaining the synonyms STAT-induced STAT inhibitor, SSI and cytokine-inducible SH2-containing protein, CIS) and in turn SOCS proteins limited further signal transduction of this signaling pathway. Thus, SOCS proteins were identified and characterized as the first intracellular feedback inhibitors of type I and II cytokine receptors. They build up a negative regulatory pathway thereby limiting duration of JAK/STAT signaling. Since then, it has been discovered that the eight SOCS family members can be induced by a variety of cytokines that in turn are regulated in a (1) feedback inhibition mode. Moreover, it has turned out that beside this feedback loop SOCS proteins can also be induced by other immune receptors and signals, including Toll-like receptors, TNF receptor, T-cell receptors, cAMP, thereby regulating a cell's sensitivity towards subsequent cytokine stimulation, referred to as (2) crosstalk inhibition.

The SOCS Family

Based upon structural similarities, the SOCS family consists of eight members: SOCS1-7 and CIS (Alexander 2002; Alexander and Hilton 2003; Yoshimura et al. 2007). Since SOCS proteins are negative feedback inhibitors that are induced through JAK/STAT signaling, they contain STAT binding regions within their promoters. Hence, SOCS

expression is induced by activated STATs. However, SOCS proteins are pleiotropic, thus individual family members can be induced by and regulate different STAT signaling modules. SOCS genes have only few introns thus identifying them as rapidly inducible, immediate early genes. SOCS expression is regulated at the transcriptional level: Promoters bear STAT factor binding elements but also additional transcription factors drive SOCS expression (e.g., ▶ NF-κB, egr-1, IRF1). Moreover, SOCS protein expression is also controlled by translational mechanisms. Thus, SOCS proteins feature a short half-life time (approximately 1–4 h) ensuring timely and rapid regulation of transient cytokine responses. SOCS1 and SOCS3 abundance is determined by protein stability: ▶ Pim-1-induced phosphorylation stabilizes SOCS1 which in turn increases half-life time of the latter. Protein stability of SOCS3 is regulated through a specific 35-residue PEST sequence (Pro, Glu, Ser, Thr) that enhances its degradation. Additionally, the SOCS box is discussed to influence the stability of SOCS proteins. Structures for CIS in conjunction with Elongin B/C and SOCS3 together with a tyrosine-phosphorylated peptide from the IL-6 receptor have been solved.

Structure and Mode of Action

All SOCS proteins share the same principal domain structure (Fig. 1) with a SOCS box, a Src homology 2 (SH2) domain, and an optional kinase inhibitory region (KIR). The SOCS box domain is a 40 amino acid-comprising domain that can be found in the carboxy terminus of all SOCS family members. Additional proteins containing a SOCS box motif but not sharing the basic structure of SOCS1-7 and CIS (as defined by the SH2 domain) have also been assigned to the SOCS superfamily which includes more than 40 proteins united to four subfamilies depending on the motif located N-terminally of the SOCS box. These subfamilies contain ankyrin repeats, WD-40 repeats, SPRY domains, and small GTPases.

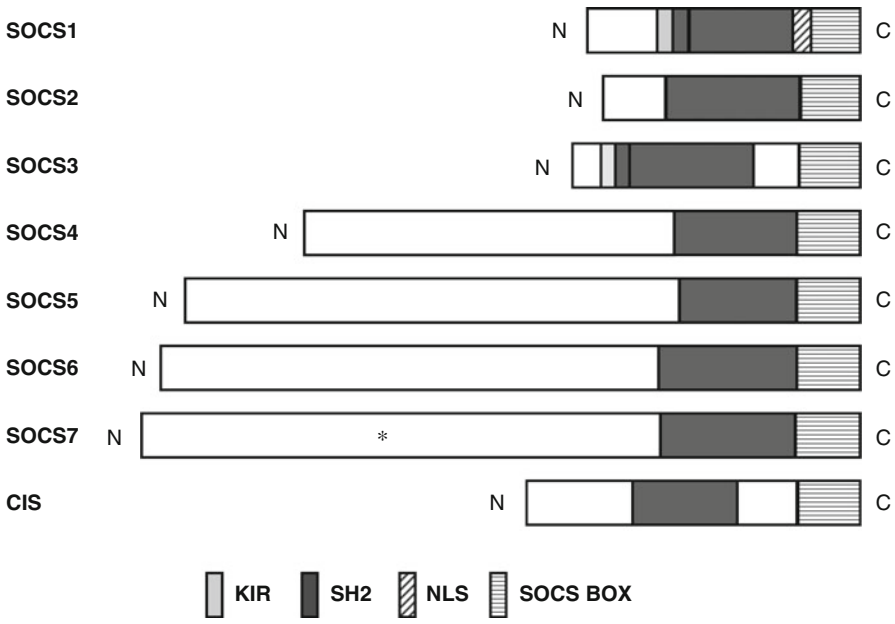
The SOCS box has been shown to operate as E3 ubiquitin ligase by interacting with Elongin B and C, cullin-5, and RING box 2 (Kile et al. 2002). In concert with two other enzymes of the ubiquitin system, E1 (ubiquitin-activating) and E2 (ubiquitin-conjugating), the E3 ubiquitin ligase induces ubiquitination of a specifically bound protein thus leading to the proteasomal degradation of the latter. SOCS proteins thus operate by inducing proteasomal decay of substrates which they bind by an additional N-terminal protein interaction domain, the SH2 domain. Inhibition of proteasomal degradation dampens the inhibitory effects of SOCS on cytokine signaling.

To access their substrates, SOCS1-7 and CIS bear a central SH2 domain which mediates binding to phosphorylated tyrosine residues within target proteins. SOCS1 binds to activated, phosphorylated Y1007 in JAK2 thereby inhibiting JAK2 signaling. It also interferes with JAK1-3 and Tyk2 activation. SOCS1 also binds to the type I and II IFN receptor chains IFNAR1 and IFNGR1. SOCS3 is also capable of binding to JAK2-pY1007 but with lower affinity as compared to SOCS1. Moreover, SOCS3, SOCS2, and CIS bind to phosphorylated residues exposed at cytoplasmic sites of various cytokine receptors. SOCS3 has a specific affinity to bind to gp130, thus inhibiting IL-6 and other gp130-dependent cytokines. In turn, STAT3 activity is controlled. Importantly, IL-10-induced STAT3 activity is not limited by SOCS3. SOCS3 inhibits JAK activity whereas CIS and SOCS2 do not interfere with JAKs but rather compete with STAT recruitment to the receptor. A consensus sequence to which the SH2 domain of SOCS3 binds has been identified as (S/A/V/Y/F)-hydrophopbic-(V/I/L)-hydrophobic-(H/V/I/Y).

Interestingly, the SH2 domain is also involved in binding proteins distinct from JAK/STAT, e.g., ▶ NF-κB p65, c-kit, insulin receptor, and tumor suppressor p53. Moreover, SOCS3 interferes also with ▶ MAP kinase activation by blocking the SHP2 binding site in gp130.

Additional small extended SH2 subdomains (ESS) N-terminal (SOCS1 and 3) of the SH2 domain essentially contribute to the binding of phosphorylated tyrosine residues. In case of SOCS1 and SOCS3 the SH2 domain mediates binding to substrates; however, inhibition of JAKs is achieved by a kinase inhibitory region (KIR) which is N-terminally attached to the SH2 domain and can only be found in SOCS1 and SOCS3. The KIR domain blocks the catalytic cleft of JAKs thereby contributing to termination of JAK/STAT signaling.

The further N-terminus is quite diverse among the SOCS family members and varies in length and sequence. In contrast to the specific function of SOCS proteins located at the cytoplasmic site of the



SOCS, Fig. 1 Domain structure of the eight SOCS family members. SOCS1 and SOCS3 bear an extended SH2 domain, as well as a KIR domain. SOCS1 contains a bipartite nuclear localization signal and SOCS7 possesses an ill-defined, central monopartite nuclear localization signal (*)

cytokine receptor, SOCS1 revealed an unexpected subcellular localization pattern with mainly nuclear occurrence. This was attributed to a specific sequence composed of two clusters of basic amino acid residues which function as classical bipartite nuclear localization signal (NLS) (Baetz et al. 2008). Furthermore, SOCS3 and 6 are found ubiquitously expressed throughout the whole cell by immunofluorescence and SOCS7 also has the capacity to translocate to the nucleus where it contributes in regulating the DNA damage response. The N-terminus of SOCS7 was shown to bear a basic region with homology to a classical monopartite NLS. The findings indicate that SOCS proteins might exert additional functions beyond inhibiting JAK/STAT signaling at the receptor level.

Physiological Functions of SOCS Proteins

SOCS proteins can be induced by a variety of cytokines and in turn show inhibitory effects on many different cytokines, hematopoietic growth factors, and further immunological stimuli within in vitro assays. However, generation of knockout or transgenic mice and subsequent analysis of the resulting phenotype have revealed important insights into the physiological importance of individual SOCS family members (Table 1) (Dalpke 2004).

$SOCS1^{-/-}$ mice succumb to perinatal death due to multiorgan inflammation. These mice reveal enhanced peripheral T cell (TC) and natural killer (NK) cell activation, macrophage organ infiltration, and fatty liver degeneration. Furthermore, thymus size is decreased and B lymphocytes undergo increased apoptosis. A major underlying dysregulation is undampened IFNγ signaling. Backcrossing to $Rag2^{-/-}$ or $IFN\gamma^{-/-}$ mice rescues $SOCS1^{-/-}$ mice from early death and the disease can be reinduced in $Rag2^{-/-}$ mice by transfer of hematopoietic $SOCS1^{-/-}$ cells confirming the importance of hyperactivated lymphocytes. Natural killer cell depletion reduces hepatotoxicity in $SOCS1^{-/-}$ mice. SOCS1/IFNγ double knockout mice show a delayed phenotype with polycystic kidneys, pneumonia, chronic skin ulcers, and granulomas in various organs. Moreover, the lethal phenotype can also be ameliorated by crossing $SOCS1^{-/-}$ mice with $STAT4^{-/-}$ or $STAT6^{-/-}$ mice, emphasizing that SOCS1 action is not limited to IFNγ signaling. Additionally, $SOCS1^{-/-}$ macrophages show higher proinflammatory protein production in response to Toll-like receptor (TLR) stimulation by lipopolysaccharide (LPS) indicating a role for SOCS1 in termination of TLR signal transduction. In dendritic cells, SOCS1 was required to suppress induction of autoimmune responses and to restrict breakdown of tolerance. Mice with isolated knockout of the SOCS box in

SOCS, Table 1 Functions of SOCS proteins as determined from genetic models

	Knockout phenotype	Transgene phenotype
SOCS1	Neonatal lethality, multiorgan inflammation, fatty liver degeneration, lymphocyte apoptosis, aberrant T-cell activation, increased IFNγ responsiveness, aberrant DC and B-cell activation, autoimmunity	Spontaneous T-cell activation, increase in $CD4^+$ T cells, decrease in γδ T cells, disturbed T-cell development
SOCS2	Gigantism, dysregulated growth hormone, and IGF-I signaling	Gigantism, dysregulated growth hormone signaling
SOCS3	Embryonic lethality with placental defects and aberrant erythropoiesis, increased hepatocarcinogenesis, prolonged and aberrant IL-6/STAT-3 signaling in macrophages	Embryonic lethality with anemia, increased T_H2 differentiation
SOCS4		
SOCS5	No abnormalities	Impaired T_H2 development, disturbed IL-4 signaling
SOCS6	Mild growth retardation	
SOCS7	Growth retardation, increased lethality postnatal due to hydrocephalus	
CIS	No obvious phenotype	IL-2/STAT-5 signaling defects, lactation failure, reduced numbers of NK, NKT cells, reduced body weight

SOCS1 have been generated and also show an amelioration of the acute lethal phenotype with a delayed onset of the multi-inflammatory disease of SOCS1 knockouts.

$SOCS2^{-/-}$ mice exhibit augmented growth which is supposed to occur due to altered growth hormone and insulin-like growth factor I signaling. Interestingly, SOCS2 transgenic mice also show increased growth. SOCS2 is also necessary to mediate the anti-inflammatory effects of lipoxin A4 in dendritic cells.

$SOCS3^{-/-}$ mice are embryonic lethal, presumably due to placenta defects and increased LIF receptor sensitivity. They also show defects in erythropoiesis, but $SOCS3^{-/-}$ fetal liver stem cells can reconstitute erythropoiesis when transferred into lethally irradiated adults. SOCS3 deficiency in macrophages shows altered IL-6 signaling: On the one hand, IL-6 signal transduction is not limited anymore resulting in prolonged STAT3 activity with IL-10-like effects. On the other hand, in SOCS3 deficient macrophages IL-6 adopts IFN-like activities due to unchecked STAT1 activity. SOCS3 is also crucially involved in the regulation leptin and G-CSF signaling.

SOCS5 transgenic mice show diminished T_H2 differentiation due to constrained IL4 signaling. $SOCS6^{-/-}$ and $SOCS7^{-/-}$ mice are smaller than wild-type littermates, the latter showing increased lethality due to hydrocephalus development. Both SOCS6 and SOCS7 regulate insulin receptor signaling. However, SOCS4-7 mice are less studied so far. $CIS^{-/-}$ mice do not show a distinct phenotype, but CIS transgenic mice have defects in lactation, a disturbed IL2/STAT5 signaling and reduced numbers of γδ T cells, natural killer cells, and natural killer T cells.

SOCS proteins also act as tumor suppressors (Rottapel et al. 2002): SOCS1 is involved in the early stage of tumor development by limiting inflammation and tissue damage whereas SOCS3 terminates STAT3-mediated proliferation and tissue remodeling. SOCS1 expression is lost in many tumors. Especially in liver fibrosis and hepatocellular carcinomas, the SOCS1 promoter is hypermethylated resulting in decreased SOCS1 expression, enhanced inflammation, and tumorigenesis. Likewise, SOCS1 was downregulated by hepatitis C virus core protein. SOCS1 has also been discovered as essential activator of the p53 tumor suppressor thus impeding with tumor development. Besides, SOCS1 has been identified to degrade HPV (human papillomavirus) E7 oncogene thus blocking the proliferation of infected cells. The SOCS3 gene was hypermethylated in lung cancer with restoration of SOCS3 expression resulting in STAT3 inhibition and concomitant growth inhibition.

Finally, SOCS proteins are also involved in chronic inflammatory diseases. SOCS3 is elevated in human ulcerative colitis and regulates STAT3 activity in an animal model of inflammatory bowel disease. SOCS1 is also involved in suppression of experimental colitis. Patients with allergic contact dermatitis and psoriasis have dysregulated SOCS1-3 expression and SOCS3 can also be found elevated in rheumatoid arthritis. Also hematopoietic malignancies and loss of

therapeutic IFN responsiveness might be associated with SOCS expression. The question remains to be solved whether disturbed SOCS expression is a consequence of altered cytokine networks or, at least in some conditions, might cause and contribute directly to pathogenesis of those diseases. Nevertheless, those observations implicate that SOCS manipulation is a promising approach to alter cytokine signaling.

Further Functions of SOCS within the Immune System

To address the role of SOCS within the immune system, regulation of both, innate and adaptive immunity, has been studied experimentally (Dalpke et al. 2008; Yoshimura 2009). SOCS1 has been shown to affect TLR responses, with increased secretion of proinflammatory cytokines once SOCS1 is missing. SOCS1 and 3 are also strongly induced by TLR triggering thus limiting the action of subsequent cytokine receptor triggering. Thus, SOCS1 limited GM-CSF (granulocyte–monocyte-colony-stimulating factor) and IL-4-mediated differentiation of $CD14^+$ monocytes to dendritic cells. SOCS1 also is important for STAT6 to STAT1 switching during dendritic cell maturation. Further experiments show that SOCS1 encounters activated NF-κB within the nucleus and inhibits its action as transcription factor via ubiquitination and subsequent degradation of the NF-κB subunit p65. Also, TLR-induced SOCS1 inhibits paracrine signal amplification by type I IFN and this circuit is mainly responsible for increased LPS sensitivity of $SOCS1^{-/-}$ mice because $SOCS1^{-/-}/Tyk2^{-/-}$ mice which lack type I IFN signaling had no increased LPS sensitivity anymore. Furthermore, single reports claim interaction of SOCS1 with the TLR2/4 adaptor protein ▶ Mal through interaction with Bruton's tyrosine kinase and with IRAKs as well as inhibition of TLR-induced JAK activity. In turn, $SOCS1^{-/-}$ mice exhibit lack of endotoxin tolerance. SOCS1 is therefore involved in regulating innate immune responses; however, the distinct outcome of its action seems to be dependent on the respective gene analyzed. SOCS1 deficiency also leads to hyperactivated dendritic cells that supported autoimmune responses.

Concerning adaptive immunity, SOCS proteins play an important role in the differentiation of T-cell subsets. On the one hand, SOCS1 is upregulated during T-cell development in the thymus and $SOCS1^{-/-}$ mice show a defect in the development of single positive thymocytes with a bias toward $CD8^+$ T cells. On the other hand, SOCS proteins are induced during cytokine-mediated T helper (T_H) development which in turn is regulated by SOCS proteins.

T_H1 cells preferentially develop in an IL-12 environment: They have high levels of SOCS1 and SOCS5 which suppresses IL-4 induced STAT6 activation directing T cells into T_H2 development. In contrast, T_H2 cells mainly develop in a "non-IL-12" environment and express GATA-3 as transcription factor due to IL-4-mediated STAT6 activation. T_H2 cells possess high levels of SOCS3 suppressing IL-12 signaling and thus inhibiting T-bet induction and T_H1 differentiation. Indeed, patients with T_H2-shifted diseases like asthma and atopic dermatitis show a correlation between SOCS3 levels and T cell-mediated disease activity. T_H17 cells for which IL-6, IL-21, and IL-23 with subsequent activation of STAT3 are important are also affected by SOCS3. Since SOCS3 blocks STAT3 action it has been identified as negative regulator of T_H17 differentiation. SOCS1 and 3 have also been implicated to play a role in development of regulatory T cells (Treg). Treg cells are $CD4^+$ cells defined by high expression of CD25 and the transcription factor FOXP3. Expression of FOXP3 induces microRNA (miRNA) 155 which targets SOCS1. This miRNA-mediated SOCS1 downregulation enhances IL-2/STAT5 signaling which in turn increases the number of Treg cells. Furthermore, miRNA-155 has also been shown to target SOCS1 which is used by the host's immune system upon viral infection to positively regulate type I IFN signaling.

The role of SOCS proteins as negative regulators of the immune system is confirmed by pathogenic microbes that misuse cell intrinsic negative regulation circuits to evade immune recognition. Murid herpesvirus-4 (MuHV-4) encodes for a SOCS box motif containing protein (ORF73) that targets NF-κB for ubiquitination and proteasomal degradation thereby inhibiting NF-κB activity. Other viruses have evolved SOCS-dependent mechanisms to escape host immune system: HIV-1 Tat protein is a key regulator of viral replication; however, it additionally induces SOCS2 which in turn interferes with JAK/STAT signaling thus supporting virus replication. Likewise, RSV surface proteins G and F induce

SOCS1 expression. For HSV-1 direct effects on the SOCS1 promoter, followed by increased SOCS1, have been shown. Not only viruses but also parasites take advantage of the SOCS negative regulatory system: *Toxoplasma gondii* replicates in macrophages and induces SOCS1, 3 and CIS which deactivate JAK/STAT signaling. *Leishmania major* infection was associated with lower pathogenicity in SOCS1-deficient cells, though at the cost of detrimental effects due to uncontrolled cytokine signaling. Also, intracellular mycobacteria are reported to interfere with macrophage activation by IFNγ through SOCS1 and −3 induction. Furthermore, SOCS3 induction by hepatitis C virus might be a mean to circumvent antiviral activities of type I interferons.

Besides JAK/STAT regulation, SOCS proteins show further effects in a variety of additional signaling pathways: Thus SOCS1 has been reported to regulate the insulin receptor, TNF receptor, and Toll-like receptors; SOCS3 in vitro also inhibits insulin receptor, IL1R, and CXCR4 signaling to name a few. Although the molecular details are not known it seems probable that the function of SOCS proteins is not restricted to the inhibition of classical type I and II cytokine receptors.

Future Directions

SOCS proteins have been shown to be involved in a variety of cellular processes and could therefore present a potential target for the treatment of inflammatory diseases. To confirm the function of specific domains of SOCS and to imitate SOCS expression, SOCS mimetics have been produced. SOCS mimetics comprise peptides corresponding to functional domains coupled to protein translocation domains facilitating cell penetration. A peptide that resembles the KIR region has been described to mimic the inhibitory effects of SOCS on JAK activity. Treatment of mice with SOCS mimetics prevented vaccinia virus transcription and replication. Regarding the role of SOCS1 in regulating NF-κB signaling, there is evidence that SOCS1 administration limits the TNF production in synovial fluid macrophages isolated from patients suffering from rheumatoid arthritis. Similarly, delivery of recombinant whole proteins has been proposed. Other techniques to control SOCS expression include siRNA or miRNA approaches which will lead to a downregulation of the respective protein.

Summary

SOCS proteins comprise a family of eight proteins (CIS, SOCS1-7) that have first been identified as intracellular negative regulators of JAK/STAT signaling upon type I and II cytokine receptor stimulation. Therefore, SOCS proteins contain an extended SH2 domain which allows them to bind to tyrosine-phosphorylated cytokine receptors or Janus kinases. Through a kinase inhibitory region (SOCS1 and 3) as well as a SOCS box domain which targets substrates for proteasomal degradation SOCS proteins limit signal transduction. However, the activity of SOCS exceeds the control of JAK/STAT signal transduction: SOCS proteins are also involved in the termination of NF-κB responses, negatively influence insulin signaling and function as tumor suppressors. Depending on the cytokine milieu, SOCS proteins shape the immune system by influencing macrophages, dendritic cells, and lymphocyte differentiation. Evidence for crucial roles of individual SOCS proteins within the immune system comes from mice with targeted disruption of SOCS genes that show major deregulated cytokine pathways. Since SOCS proteins are essential modulators of immunity, precise understanding of their defined mode of action could be beneficial to develop new therapeutic strategies to combat inflammatory and autoimmune diseases as well as cancer.

References

Alexander WS. Suppressors of cytokine signalling (SOCS) in the immune system. Nat Rev Immunol. 2002;2(6):410–6.

Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu Rev Immunol. 2003;22:503–29.

Baetz A, Koelsche C, Strebovsky J, Heeg K, Dalpke AH. Identification of a nuclear localization signal in suppressor of cytokine signaling 1. FASEB J. 2008;22(12):4296–305.

Dalpke AH. Suppressor of cytokine signaling (SOCS) proteins in innate immunity. In: Pandalai SG, editor. Recent res develop. Immunology, vol. 6. Trivandrum: Research Signpost; 2004. p. 317–39.

Dalpke A, Heeg K, Bartz H, Baetz A. Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. Immunobiology. 2008;213(3–4):225–35.

Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, et al. A new protein containing an SH2 domain that inhibits JAK kinases. Nature. 1997;387(6636):921–4.
Kile BT, Schulman BA, Alexander WS, Nicola NA, Martin HM, Hilton DJ. The SOCS box: a tale of destruction and degradation. Trends Biochem Sci. 2002;27(5):235–41.
Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, et al. Structure and function of a new STAT-induced STAT inhibitor. Nature. 1997;387(6636):924–9.
Rottapel R, Ilangumaran S, Neale C, La Rose J, Ho JM, Nguyen MH, et al. The tumor suppressor activity of SOCS-1. Oncogene. 2002;21(28):4351–62.
Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, et al. A family of cytokine-inducible inhibitors of signalling. Nature. 1997;387(6636):917–21.
Yoshimura A. Regulation of cytokine signaling by the SOCS and Spred family proteins. Keio J Med. 2009;58(2):73–83.
Yoshimura A, Ohkubo T, Kiguchi T, Jenkins NA, Gilbert DJ, Copeland NG, et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoetin receptors. EMBO J. 1995;14(12):2816–26.
Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol. 2007;7(6):454–65.

Somatostatin Receptor

Barbara Mariniello
Endocrinology Unit, Department of Medical and Surgical Sciences, University of Padua, Padua, Italy

Synonyms

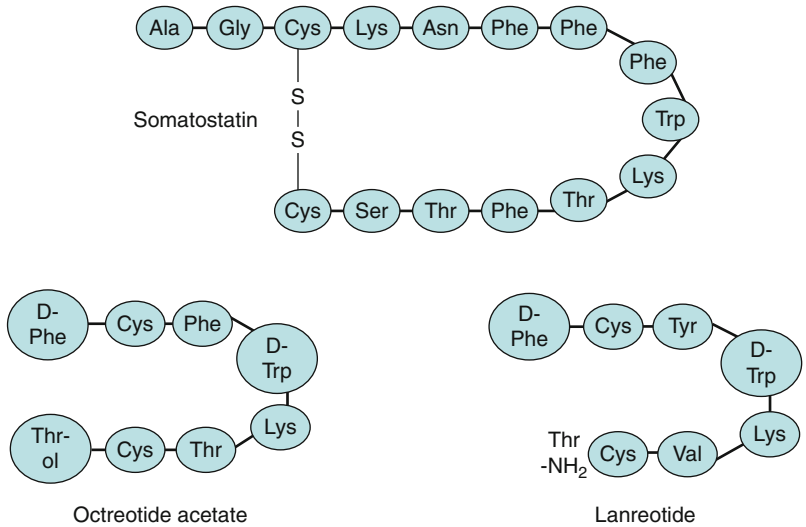
SSTR

Historical Background

Somatostatin (SST) is a cyclopeptide that was first identified in the hypothalamus inhibiting GH secretion and immediately after in pancreatic islets inhibiting the insulin secretion in vitro, but it is produced also throughout the central and peripheral nervous system and in most major peripheral organs as endocrine pancreas, thyroid, adrenals, spleen, submandibular glands, gut, liver, kidneys, prostate, and placenta (Reisine and Bell 1995). SST is initially synthesized as a large precursor molecule, preprosomatostatin that undergoes tissue-specific enzymatic degradation generating two bioactive products: SST-14, identified in hypothalamus, and SST-28 that corresponds to somatostatin with a NH_2-terminal extension of 14 amino acids. Nutrients (glucose, amino acids, lipids), neurotransmitters, neuropeptides (glucagons, growth hormone releasing hormone, bombesin), hormones (insulin, glucocorticoids) and cytokines (interleukin-1, interleukin-6, transforming growth factor-β, tumor necrosis factor-α, insulin-like growth factor, leptin, interferon-γ, etc.), and several intracellular mediators including cyclic AMP, cyclic GMP, Ca2+, and nitric oxide, all influence the transcription and/or secretion of SST. The two peptides act through the inhibition of endocrine and exocrine secretion, inhibition of intestinal motility, absorption of nutrients and ions, vascular contractility, and immune cell functions. Moreover, they have a role in neurotransmission and cognitive functions, and they have antiproliferative effects acting on cell secretion, proliferation, and differentiation. SST can also inhibit angiogenesis in vitro and in vivo inhibiting the production of vascular endothelial growth factor and platelet-derived growth factor (Öberg et al. 2010).

Somatostatin Analogues

SST, which takes effect through high-affinity plasma membrane five SST receptors ($SSTR_{1–5}$) (see below), has limited clinical use due to its short half-life (<3 min) but the identification of stable SST analogues with a longer half-life has raised new hopes for the treatment of tumors (Fig. 1). Octreotide, the first SST analogue available commercially, contains three substituted amino acids (D-Phe, LThr [ol], D-Trp) that make it resistant to metabolic degradation with a longer half-life (2 h). It is an SSTR2-preferring agonist with a moderate affinity for SSTR3 and SSTR5. Lanreotide is a second analogue with a similar binding profile of octreotide. Furthermore, radiolabeled SST analogues are used as diagnostic tools for the detection of endocrine tumors, as well as for the therapy. A novel multiligand SST analogue, a cyclohexapeptide named pasireotide, has a 20–30, 5, and 40–100 times higher binding affinity than octreotide and lanreotide for the receptors SSTR1, SSTR3, and SSTR5, respectively (Bruns et al. 2002). The very favorable 24 h elimination half-life of pasireotide makes this novel compound suitable for clinical application. Interestingly, pasireotide modulates SST receptor trafficking and leads to the formation of unstable SSTR2 complex with beta-arrestin that dissociate at or near the plasma membrane.



Somatostatin Receptor, Fig. 1 Chemical structure of SST-14 and SST analogues

Consequently, SSTR2 are recycled faster to the plasma membrane after endocytosis in cells treated with pasireotide than in those treated with octreotide. This finding suggests that a longer-lasting functional response could be achieved with pasireotide (Cescato et al. 2010).

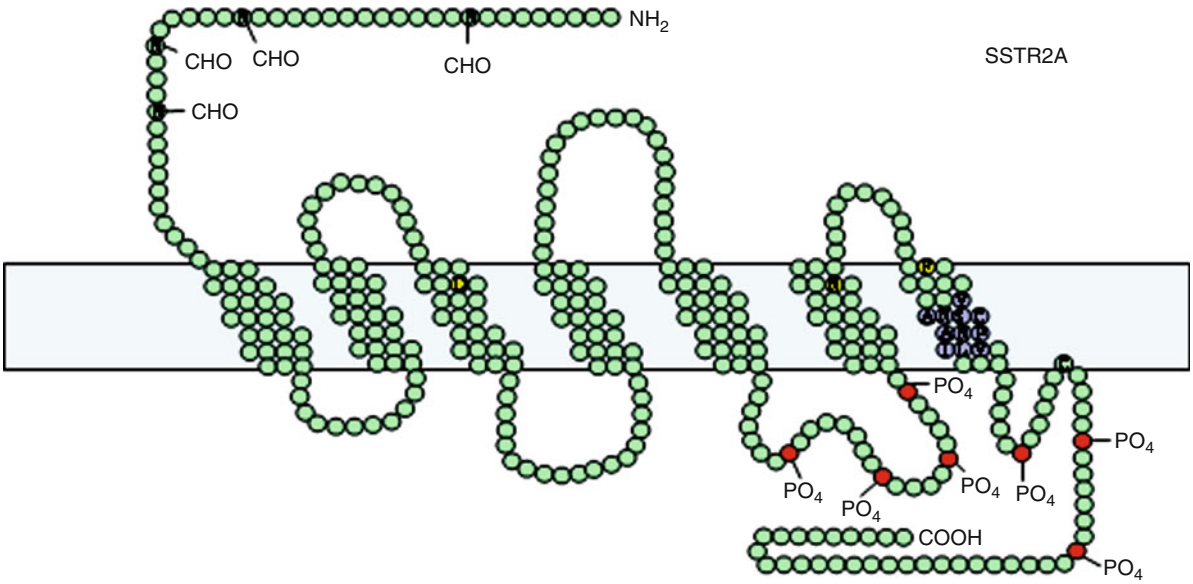
Somatostatin Receptors

SSTRs were first described in the pituitary GH4C1 cell line. Studies using a variety of techniques such as membrane binding analyses, in vivo and in vitro autoradiography, covalent cross-linking, and purification of a solubilized receptor showed SSTR expression in various densities in brain, gut, pituitary, endocrine and exocrine pancreas, adrenals, thyroid, kidneys, and immune cells. SSTRs have also been identified in a large variety of human cancers including pituitary adenomas, islet tumors, carcinoids, adenocarcinomas of the breast, prostate, ovary, kidney and colon origin, lymphomas, as well as astrocytomas, neuroblastomas, and medulloblastomas. Analyses of SSTR mRNAs demonstrate that various human tumors from neuroendocrine and gastroenteropancreatic origin, brain tumors, pheochromocytomas, medullary thyroid carcinomas, prostate, lung, and breast cancers express various SSTR mRNA, each tumor expressing more than one subtype and SSTR2 being the most frequently expressed (Benali et al. 2000).

Photoaffinity labeling and purification studies have provided evidence for the existence of several SSTR proteins of 32–85 kDa that are expressed in a tissue-specific manner and some that exhibit selective agonism for SST-14 or SST-28 (Patel 1999). The $SSTR_{1\text{-}5}$, identified in the early 1990s (Yamada et al. 1992), are codified by five genes that segregate on five different chromosomes: 14q13, 17q24, 22q13.1, 20p11.2, and 16p13.3. Genes for SSTR1, 3, 4, and 5 lack classical introns, the SSTR2 gene displays a cryptic intron at the 3′ end of the coding segment, which determine two spliced variants, a long (SSTR2A) form and a short (SSTR2B) form. The 2A and 2B variants differ only in the length of the cytoplasmic tail. There are thus six putative SSTR subtypes of closely related size that present a high degree of sequence identity (39–57%), with seven α helical transmembrane domains (TM) typical of G-protein-coupled receptors (GPCR) (Fig. 2). The sequence differences reside in the extracellular and intracellular domains and are probably responsible for their signaling specificity. SST binding induces a conformational change in the receptor that results in activation of an associated heterotrimeric G protein complex leading to exchange of GTP for GDP on the α-subunit. The GTP bound form of the free α-subunit and β/γ complex interact with the specific cellular effector pathways (Patel 1999). These receptors differ in their signaling pathways and cellular and tissue distribution, which are crucial factors determining tissue response to SST and SSTR agonists.

Intracellular Pathways Coupled to SSTRs

Using recombinant SSTR expressed in various eukaryote cells, the intracellular signaling pathways coupled to SSTRs have been extensively studied; they are complex and vary among receptor, cell, and organ types.



Somatostatin Receptor, Fig. 2 Model for the orientation of the SST receptors in the plasma membrane. The four potential sites for N-linked glycosylation, indicated by "CHO," in the extracellular NH_2-terminal domain are noted. PO_4 are the putative sites for phosphorylation by protein kinase A, protein kinase C, and casein kinase in the extracellular COOH-terminal domain, shown by the closed red circles. The YANSCANPI/VLY sequence, shown by the closed *blue circles*, in the VIIth TM is highly conserved in all SSTRs. The cysteine residue 12 amino acids downstream from the VIIth TM is conserved in SSTR1,2,4,5 and may be the site of a potential palmitoyl membrane anchor. Residues Asp^{122}, Asn^{276}, and Phe^{294}, shown by the closed *yellow circles*, in TMs III, VI, and VII, respectively, have been proposed to form part of a ligand-binding pocket for octreotide

The complexity arises not only from the large number of SSTR subtypes and range of cell types that express them, but also because each receptor subtype is coupled to multiple intracellular transduction pathways (Table 1 and Fig. 3).

All five SSTRs are functionally coupled to inhibition of adenylate cyclase and cAMP production that is the first pathway implicated. In neuronal and neuroendocrine cells, SST and analogues also regulate several subsets of K+ channels causing hyperpolarization of the plasma membrane and leading to decreased Ca2+ influx through voltage-gated Ca2+ channels and consequently to a reduction in intracellular Ca2+. Moreover, a third pathway affected by SST and analogues is represented by the activation of a number of protein phosphatases including serine/threonine phosphatases, tyrosine phosphatases, and a Ca2 + -dependent phosphatase. Somatostatins inhibit proliferation directly by regulating tyrosine kinase, tyrosine phosphatase, nitric oxide synthase, cyclic guanosine 3′, 5′ cyclic monophosphate-dependent protein kinase, and RAS/extracellular signal-regulated kinase signaling pathways. The regulation of these pathways varies according to SSTR subtype and the cell environment. All five receptors stimulate phospholipase C and increase Ca2+ mobilization via both pertussis toxin–sensitive and pertussis toxin–insensitive G protein.

In vitro data and biodistribution studies in animals have shown that SST and agonists induce internalization of SSTR2, SSTR3, and also SSTR5 with a consequent desensitization and attenuated receptor relating signaling. This is a crucial feedback mechanism that prevents persistent stimulation by the agonist. Agonist-induced internalization is rapid; almost all SSTR2 moved from the cell membrane to endosome-like cellular structures within the cytoplasm. Agonist binding to SSTR5 induces mobilization of intracellular stores of the receptor to the cell membrane and then internalization of membrane SSTR5 following ligand binding (Öberg et al. 2010).

Biological Effects of Somatostatin Receptors

Because the same cell expresses multiple receptor subtypes and the coupling of SSTRs to similar transduction pathways, it can be assumed that the SSTR subtypes may act in concert (Table 2). In the cell, the

Somatostatin Receptor, Table 1 G-protein-coupled signal transduction: characteristics of SSTR subtypes

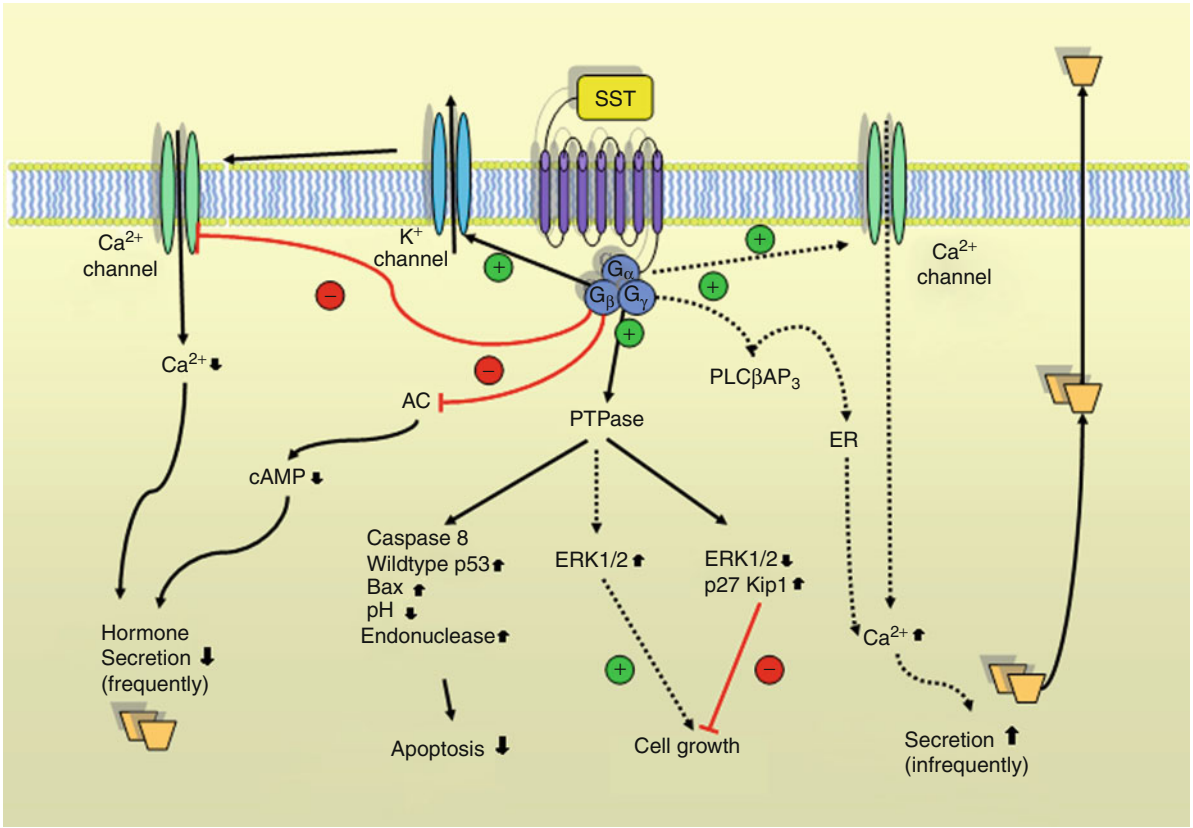
	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Molecular size (kDa)	53–72	71–95	65–85	45	52–66
Amino acids	391	369	418	388	363
mRNA (Kb)	4.8	8.5	5.0	4.0	4.0
Adenylate cyclase	↓	↓	↓	↓	↓
Thyrosine phosphatase	↑	↑	↑	↑↓	↑
MAP kinase (ERK)	↑	↑↓	↑↓	↑	↓
K+ channels		↑	↑	↑	↑
Ca2+ channels	↓	↓			
Na+/H+ exchanger	↑				
AMPA kainite glutamate channels	↑	↓			
Phospholipase C/IP3	↑	↑	↑	↑	↑↓
Phospholipase A2	↓	↓		↑	
Tissue distribution	Brain, pituitary, islet, stomach, liver, kidneys, hypothalamus	Brain, pituitary, islet, stomach, kidneys, lymphocytes, adrenals, hypothalamus	Brain, pituitary, islet, stomach, T cell, hypothalamus	Brain, stomach, islet, lungs, placenta, heart, hypothalamus, pituitary	Brain, pituitary, islet, stomach, hypothalamus, lymphoid cells

blockade of secretion by SST is mediated through inhibition of Ca2+ and to a reduction of cAMP production. In the pituitary, SSTR2, SSTR5, and SSTR1 are involved in inhibiting growth hormone release. In the pancreas, SSTR2 mediates inhibition of glucagon release, whereas SSTR5 is a negative regulator of insulin secretion. SSTR2 also mediates insulin secretion in human pancreas. The high expression of SSTR1 in human pancreatic islet cells suggests that it may also be involved in regulating insulin release. SSTR5 is involved in the inhibition of pancreatic exocrine secretion. In the stomach, SSTR2 contributes to the inhibition of histamine and gastrin release and the inhibition of gastric acid secretion. SSTR1 and SSTR2 mediate the inhibition of intestinal ionic secretion. In human neuroendocrine gut tumor cells, SST and octreotide inhibit L-type voltage-dependent calcium channels with the same amplitude suggesting that at least SSTR2 and SST5R may be involved in the inhibition of Ca2+ influx and thereby inhibition of tumor-produced neurotransmitters and hormone. SSTR3 could be involved in the stimulation of gastric and intestinal smooth muscle cell contraction and SSTR5 in the inhibition of colonic muscle cell contraction (Benali et al. 2000).

SST analogues are also thought to inhibit cell proliferation by inducing apoptosis, which may be mediated through the SSTR2 and SSTR3. When SSTR3 is transfected into previously SST-free cell lines, addition of octreotide causes the upregulation of the tumor suppressor protein ▶ p53, which subsequently induces apoptosis. Furthermore, in athymic mouse and in hamster models, SSTR2 re-expression caused dramatic decrease in tumor growth and inhibition of primary tumor growth without metastatic progression, respectively.

Homo- and Heterodimerization of SST and Dopamine (DA) Receptors

Membrane SSTRs exist in the basal state as a monomeric species, but activation by ligand induces SSTR dimerization (Rocheville et al. 2000a), both homo- and heterodimerization with other members of the SSTR family, and dimerization alters the functional properties of the receptor such as ligand binding affinity and agonist-induced receptor internalization and upregulation. Both homo- and heterooligomeric receptors are occupied by two ligand molecules. Evidences show that monomeric, homooligomeric, and heterooligomeric receptor species occur in the same cell cotransfected with two SSTRs, and that oligomerization of SSTRs is regulated by ligand binding by a selective process that is restricted to some (SSTR5) but not other (SSTR1) subtypes. SSTR5 formed heterodimers with SSTR1 but not with SSTR4 suggesting that heterodimerization is



Somatostatin Receptor, Fig. 3 Intracellular signaling pathways coupled to SSTRs, leading to changes in hormone secretion, apoptosis, and cell growth. *AC* adenylyl cyclase, *ER* endoplasmic reticulum, *ERK* extracellular signal-regulated kinase, *Gα*, *Gβ*, *Gγ* G protein subunit, *IP3* inositol triphosphate, *PLC* phospholipase C, *PTPase* phosphotyrosine phosphatase

a specific process that is restricted to some but not all receptor subtype combinations (Patel et al. 2002).

SST and DA are two major neurotransmitter systems that share a number of structural and functional characteristics. SSTR and DA receptor (DR) families share about 30% sequence homology, are colocalized in neuronal subgroups, and SST is involved in modulating DA-mediated control of motor activity. DA, like SST, acts through its own family of five GPCRs (D1R to D5R). D2R and SSTR5 associate on the plasma membrane and the SSTR5-D2R heterooligomer displays enhanced signaling when simultaneously activated by both SST and DA ligands (Rocheville et al. 2000b). Hetero-oligomerization defines a new level of functional diversity in endogenous GPCR signaling, and should constitute novel unrecognized drug targets for combinations of agonists or antagonists.

Effect of SST Analogues on Various Tumors

SSTR2 and SSTR5 are the most important subtypes in inhibiting hormonal secretions in functioning gastrointestinal neuroendocrine tumors (GI-NETs) due to their wide-ranging effects; it is thought the dual inhibition of both subtypes may have an increased inhibitory effect (Öberg et al. 2010). The two subtypes may also mediate antiproliferative effects. Patients with NETs receiving SSTR2-preferring analogues such as octreotide and lanreotide may experience a loss of response. It has led to interest in new, multi-receptor ligand SST analogues that could be as effective and well tolerated in patients who experience an escape from response. Pasireotide may fulfill this role due to its high affinity for SSTR1–3 and SSTR5. Preliminary data are promising, with effective control of diarrhea and flushing observed in NET patients refractory or resistant to octreotide.

Somatostatin Receptor, Table 2 Biological effect of each SSTR

Inhibitory effect on	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Hormone and mediator secretion					
GH	+	+			+
TSH		+			+
Gastrin		+			
Insulin		+			+
Glucagon		+			
Cytokine (IL6, IFNγ), histamine		+			
Exocrine secretion					
Gastric acid		+			
Amylase					+
Intestinal Cl- secretion	+	+			
Motility					
Gastric and intestinal relaxation			+		
Colonic contraction					+
Cell proliferation					
Induction of G1 cell cycle arrest	+	+		+	+
Induction of apoptosis		+	+		

The SSTRs are also demonstrated expressed in neuroblastoma, therefore the treatment with SST analogues or radiolabeled SST could represent a new therapeutic possibility in patients with neuroblastoma where high-dose chemotherapy, irradiation, and autologous stem cell transplantation have failed (Georgantzi et al. 2010).

The finding of SSTRs ubiquitously distributed in normal kidney and IgA nephropathy suggests that the increased expression of these receptors might be the potential pathogenesis of inflammatory renal disease (Bhandari et al. 2008).

SST system, through SSTR1 and SSTR2, exerts mainly an immunosuppressive effect in human macrophages and may, therefore, represent a therapeutic window that can be exploited for the development of new strategies in pharmacological therapy of inflammation (Armani et al. 2007).

One of the organs in which SST has an important inhibitory effect on hormone secretion is the anterior pituitary gland (Hofland et al. 2010). SSTRs have a predominant inhibitory role of growth hormone secretion, although the secretion of other pituitary hormones, e.g., prolactin, thyroid stimulating hormone, and adrenocorticotropic hormone is regulated by SST as well. Also DRs, in particular the D2R, have an important regulatory role in the PRL secretion. Pituitary tumors express both SST and/or D2 receptors. The SSTR subtype, as well as the co-expression of SSTR and D2R, has significant impact on the possibility to treat patients with pituitary tumors with SST analogues and DA agonists to control hormonal hypersecretion and tumor growth. Pasireotide is also a promising pituitary-directed medical therapy for patients with persistent or recurrent Cushing's disease after unsuccessful surgery, in patients awaiting the effects of pituitary radiation or in inoperable patients (Arnaldi and Boscaro 2010).

Moreover, pasireotide affects cell growth, apoptosis, and catecholamine levels in pheochromocytoma (Pasquali et al. 2008). Finally, a markedly reduced cortisol and aldosterone secretion by pasireotide was also evident in human adrenocortical cell line (H295R) and in primary adrenocortical cultures that strongly expressed SSTR5 and SSTR2 (Mariniello et al. 2010).

However, with the availability of newer SST analogues such as pasireotide, the determination of differential expression of various SSTR subtypes and an assessment of heterogeneity of such expression in larger series of primary and metastatic tumors is clinically relevant. Furthermore, SSTR subtype expression should be correlated with the pattern of clinical response of such patients to SST analogue therapies.

Summary

SST and all five receptor subtypes were ubiquitously distributed in many organs and in a large variety of

human cancers. Synthetic derivatives of SST have similar activity to native SST but with a longer half-life, they lack the key enzyme cleavage sites and are more stable than native SST. The analogues, such as octreotide, lanreotide, and the multiple SSTR ligand, pasireotide, are currently in preclinical evaluation or in early clinical trials. There is evidence for a number of direct and indirect mechanisms by which SST analogues can exert antitumor effects. Direct antitumor activities, mediated through SSTRs expressed in tumor cells, include blockade of autocrine/paracrine growth-promoting hormone and growth factor production, inhibition of growth factor-mediated mitogenic signals, and induction of apoptosis. Indirect antitumor effects, that do not require the presence of SSTRs in tumor cells, include inhibition of growth-promoting hormone and growth factor secretion, and antiangiogenic actions. Therefore, not only the receptor profiles but also the cell types, as well as the dimerization, are responsible for the final effect of a given ligand. These elements open new perspectives for the use of the new chimeric compounds in human tumors.

References

Armani C, Catalani E, Balbarini A, Bagnoli P, Cervia D. Expression, pharmacology, and functional role of somatostatin receptor subtypes 1 and 2 in human macrophages. J Leukocyte Biol. 2007;81:845–55.

Arnaldi G, Boscaro M. Pasireotide for the treatment of Cushing's disease. Expert Opin Investig Drugs. 2010;19:1–10.

Benali N, Ferjoux G, Puente E, Buscail L, Susini C. Somatostatin receptors. Digestion. 2000;62(Suppl 1):27–32.

Bhandari S, Watson N, Long E, Scarpe S, Zhong W, Xu SZ, Atkin SL. Expression of somatostatin and somatostatin receptor subtypes1–5 in human normal and diseased kidney. J Histochem Cytochem. 2008;56:733–43.

Bruns C, Lewis I, Briner U, et al. SOM230: a novel somatostatin peptidomimetic with broad somatotropin release inhibiting factor (SRIF) receptor binding and a unique antisecretory profile. Eur J Endocrinol. 2002;146:707–16.

Cescato R, Loesch KA, Waser B, et al. Agonist-biased signaling at the sst2A receptor: the multi-somatostatin analogs KE108 and SOM230 activate and antagonize distinct signaling pathways. Mol Endocrinol. 2010;24:240–9.

Georgantzi K, Tsolakis AV, Stridsberg M, Jakobson A, Christofferson R, Tiensuu Janson E. Differentiated expression of somatostatin receptor subtypes in experimental models and clinical neuroblastoma. Pediatr Blood Cancer. 2010. doi:10.1002/pbc22913.

Hofland LJ, Feelders RA, de Herder WW, Lamberts SWJ. Pituitary tumors: the sst/D2 receptors as molecular targets. Mol Cell Endocrinol. 2010;326:89–98.

Mariniello B, Finco I, Sartorato P, Patalano A, Iacobone M, Guzzardo V, Fassina A, Mantero F. Somatostatin receptor expression in adrenocortical tumors and effect of a new somatostatin analog SOM230 on hormone secretion in vitro and in ex vivo adrenal cells. J Endocrinol Invest. 2010. doi:10.3275/7324.

Öberg KE, Reubi JC, Kwekkeboom DJ, Krenning EP. Role of somatostatins in gastroenteropancreatic neuroendocrine tumor development and therapy. Gastroenterology. 2010;139:742–53.

Pasquali D, Rossi V, Conzo G, et al. Effects of somatostatin analog SOM230 on cell proliferation, apoptosis, and catecholamine levels in cultured pheochromocytoma cells. J Mol Endocrinol. 2008;40:263–71.

Patel YC. Somatostatin and its receptor family. Front Neuroendocrinol. 1999;20:157–98.

Patel RC, Kumar U, Lamb DC, Eid JS, Rocheville M, Grant M, Rani A, Hazlett T, Patel SC, Gratton E, Patel YC. Ligand binding to somatostatin receptors induces receptor-specific oligomer formation in live cells. Proc Natl Acad Sci USA. 2002;99:3294–9.

Reisine T, Bell GI. Molecular biology of somatostatin receptors. Endocr Rev. 1995;16:427–42.

Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC, Patel YC. Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. J Biol Chem. 2000a;275:7862–9.

Rocheville M, Lange DC, Kumar U, Patel SC, Patel RC, Patel YC. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. Science. 2000b;288:154–7.

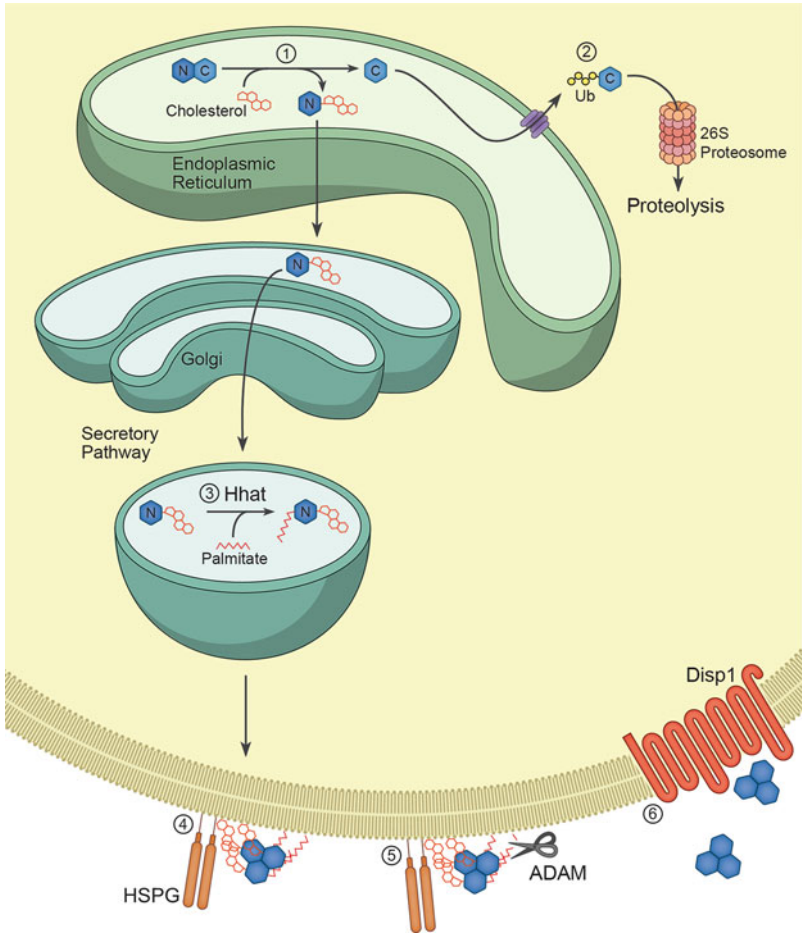
Yamada Y, Post SR, Wang K, Tager HS, Bell GI, Seino S. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. Proc Natl Acad Sci USA. 1992;89:251–5.

Sonic Hedgehog (Shh)

Kaitlyn Ryan and Chin Chiang
Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, USA

Historical Background

The Hedgehog (Hh) family of secreted proteins plays an essential role in metazoan development, and defects in the Hh pathway lead to congenital abnormalities such as holoprosencephaly and certain forms of cancer. The importance of *Hh* during embryogenesis was first discovered in a genetic screen for mutations disrupting larval patterning in *Drosophila melanogaster* by Nusslein-Volhard and Wieschaus in



Sonic Hedgehog (Shh), Fig. 1 Shh processing and release. Shh is synthesized as a 45 kDa precursor in the endoplasmic reticulum. (*1*) An autocatalytic cleavage event results in the formation of C- and N-terminal fragments. The cholesterol-modified N-terminal fragment enters the secretory pathway and ultimately serves as the mature signaling ligand, whereas (*2*) the C-terminal fragment is degraded via ER-associated degradation (ERAD). (*3*) Hh acyltransferase (Hhat) catalyzes the covalent attachment of palmitate to the N-terminus of the N-terminal fragment. Dually lipidated Shh is targeted to the cell membrane where (*4*) Heparan sulfate proteoglycans (HSPG) facilitate the formation of Shh multimers. Multimeric Shh is secreted via (*5*) A disintegrase and metalloproteases (ADAM) and the (*6*) 12-pass transmembrane protein Dispatched1 (Disp1)

1980 (Varjosalo and Taipale 2008). Unlike *Drosophila*, which harbors a single *Hh* gene, the mammalian genome encodes three *Hh* ligands: *Dessert Hedgehog* (*Dhh*), *Indian Hedgehog* (*Ihh*), and *Sonic Hedgehog* (*Shh*), all of which activate the vertebrate Hh pathway in a similar manner. *Shh* is the most widely expressed of these ligands, and much of what is known about vertebrate Hh signaling stems from studies on Shh. Though similar in many ways to the *Drosophila* Hh pathway, several features of vertebrate Hh signaling make it unique. Perhaps the most striking divergence is in the role that the primary cilium, a vestigial organelle that is largely absent in flies, plays in vertebrate Hh signaling. Over the past two decades considerable process has been made in understanding vertebrate Hh signaling and Shh in particular. Nonetheless, why the primary cilium plays such a central role in the vertebrate pathway is one of several unanswered questions requiring further inquiry and exploration. Thus, in addition to summarizing the current understanding of Shh signaling, this entry will also introduce unanswered questions and remaining areas of ambiguity.

Sonic Hedgehog Processing and Release

Hedgehog proteins undergo a unique series of post-translational processing steps that result in the covalent attachment of two lipid moieties: cholesterol at the C-terminus and palmitate at the N-terminus. Shown in Fig. 1, Shh is synthesized as a 45 kD precursor molecule in the endoplasmic reticulum. Following removal of its signal sequence, Shh undergoes a cholesterol-dependent autocatalytic cleavage, generating two peptides roughly equal in size (Varjosalo and Taipale 2008). The C-terminal fragment has no known signaling function and undergoes ER-associated degradation (ERAD), a process that requires the ubiquitin

ligase Hrd1 and its partner Sel1 as well as the ATPase p97 (Chen et al. 2011). The N-terminal fragment ultimately gives rise to the dually lipidated mature signaling molecule. A cholesterol moiety is added to its C-terminus during the aforementioned cleavage event, and Hh acyltransferase (Hhat) catalyzes the attachment of a palmitate moiety at the N-terminus (Varjosalo and Taipale 2008). Dually lipidated Shh is targeted to the cell surface through the secretory pathway.

While the process of cholesterol and palmitate attachment to Shh is relatively well-defined, the functional significance of these modifications is more complex. One of the key features of Shh is its ability to signal over long distances, and cholesterol was originally thought to promote such long-range signaling (Lewis et al. 2001). However, initial studies relied upon a mouse model in which Shh expression was severely impaired, and additional studies showed that cholesterol restricts, rather than promotes, the spread of Shh (Li et al. 2006). By contrast, palmitoylation appears to primarily affect the potency of Shh, as mouse mutants producing a non-palmitoylated form of Shh have significantly reduced levels of Shh pathway activity (Chen et al. 2004). Recent studies in vitro indicate that the palmitate moiety promotes the formation of active Shh oligomers by facilitating the cleavage of N-terminal peptides that otherwise obstruct important receptor binding sites in multimeric Shh (Ohlig et al. 2011). This cleavage is mediated by A disintegrase and metalloproteases (ADAMs), which remove the palmitate moiety and adjacent amino acids before Shh is released (Ohlig et al. 2011). In the absence of palmitoylation, either due to loss of Hhat or to mutations in the N-terminal cysteine residue where palmitate attaches, this cleavage cannot occur and the resulting multimer is far less potent. Cholesterol also plays an essential role in multimer formation, as Shh lacking cholesterol (ShhN) is secreted solely as monomers (Guerrero and Chiang 2007). Cholesterol may promote multimer formation by tethering Shh to the plasma membrane, allowing negatively charged heparan sulfate proteoglycans (HSPG) to bind positively charged residues in the Cardin Wientraub (CW) motif and cluster Shh on the membrane (Dierker et al. 2009). Taken together, these data provide insight into why Shh receives such unusual posttranslational modifications in order to properly function.

The covalent attachment of cholesterol and palmitate render Shh membrane-tethered, and the release of lipid-modified Shh into the extracellular environment requires the activity of the 12-pass transmembrane protein Dispatched1. Mouse *Disp1* mutants die around embryonic day 9.5 with severe defects in the Shh pathway, indicating that *Disp1* plays an essential role in vertebrate Hh signaling (Caspary et al. 2002; Kawakami et al. 2002; Ma et al. 2002). Cells lacking *Disp1* synthesize and process Shh normally, but are unable to secrete the mature ligand. Instead, Shh accumulates on the plasma membrane of *Disp1*$^{-/-}$ cells, able to signal to neighboring cells but unable to signal distally (Ingham et al. 2011). In addition to Disp1, recent in vitro work suggests that ADAMs may also facilitate Shh secretion by cleaving both lipid moieties, rendering the secreted form of Shh unlipidated (Dierker et al. 2009; Ohlig et al. 2011). Nonetheless, the importance of ADAM-mediated cleavage in vivo has not been shown, nor has whether ADAMs interact and cooperate with Disp1 to facilitate Shh secretion. Addressing these two questions will help complete the story of how Shh is released from producing cells.

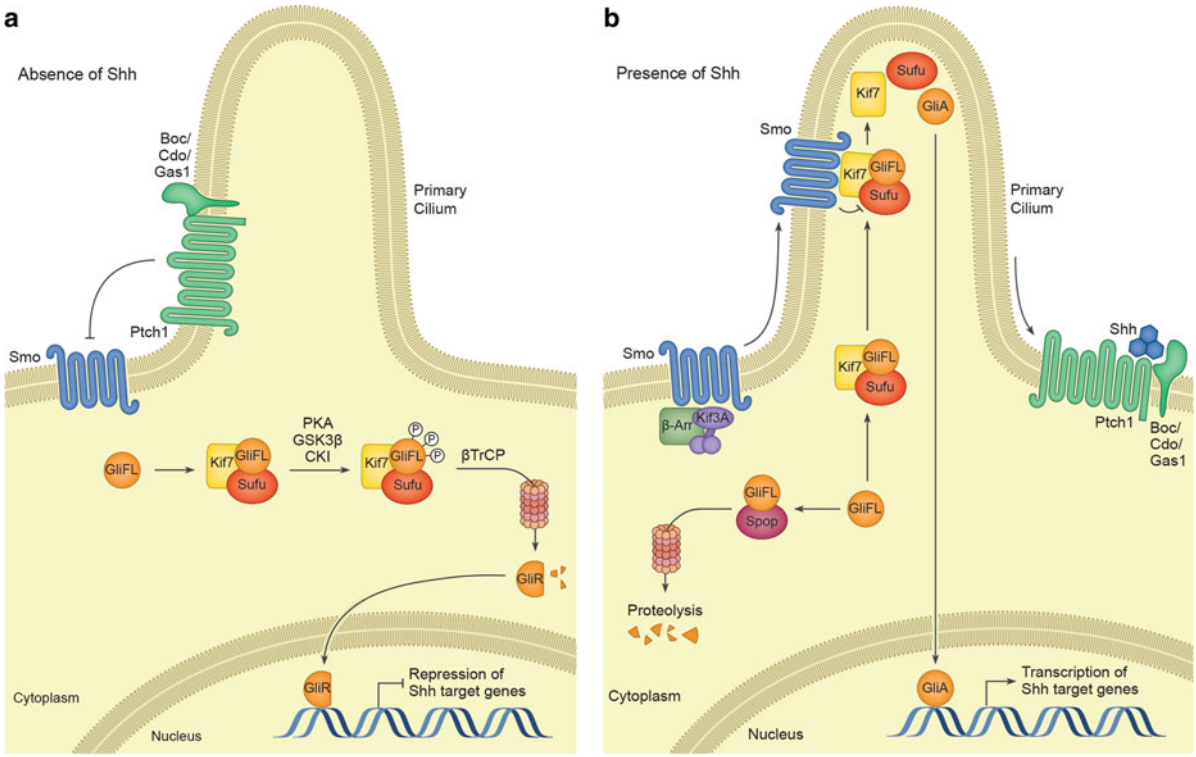
Cytosolic Sonic Hedgehog Signaling

Overview

The core components of *Drosophila* Hh signaling are conserved in vertebrates. Shh-producing cells secrete Shh, which binds Patched1 (Ptch1), a 12-pass transmembrane protein that serves as the Shh receptor. Binding of Shh to Ptch1 relieves Ptch1-mediated inhibition of the signal transducer Smoothened (Smo), and Smo activates the zinc finger transcription factors Gli2 and Gli3, which translocate into the nucleus and promote the transcription of Shh target genes. In the absence of Shh, these transcription factors are partially processed into N-terminal transcriptional repressors that suppress the transcription of Shh targets. Thus, the relative abundance of transcriptional activators and inhibitors ultimately regulates the transcription of Shh target genes.

The Primary Cilium

Although many aspects of *Drosophila* Hh signaling are conserved in vertebrates, vertebrate Hh signaling depends upon the primary cilium. Primary cilia are slim, microtubule-based nonmotile structures projecting



Sonic Hedgehog (Shh), Fig. 2 Cytosolic Shh signaling. (**a**) In the absence of ligand, Ptch1 localizes to the cilium and inhibits Smo. Sufu-Gli-Kif7 complexes are phosphorylated by PKA, GSK3β and CKI, enabling their recognition by the E3 Ubiquitin ligase β-TrCP and resulting in the proteolytic processing of C-terminal peptides. The resulting of N-terminal transcriptional repressors (GliR) repress the transcription of Shh target genes. (**b**) In the presence of Shh ligand, Shh binds Ptch1-co-receptor complexes, causing them to exit the cilium. No longer inhibited by Ptch1, Smo enters the cilium by lateral transport. Activated Smo recruits and dissociates Sufu-Gli-Kif7 complexes, and additional modifications lead to the formation of transcriptional activators (GliA), which enter the nucleus and promote the transcription of Shh target genes. Spop competes with Sufu for binding to full-length Gli molecules and promotes the proteolytic degradation of Gli-FL

from the cell surface of nearly all vertebrate cell types. The assembly and maintenance of primary cilia requires intraflagellar transport (IFT) proteins, and several members of the IFT family are essential for proper Shh signaling (Goetz and Anderson 2010). Mutations in components of the IFT-B complex, which mediates the anterograde transport of molecules from the base of the cilium to the tip, lead to a complete loss of Shh signaling. By contrast, mutations in members of the IFT-A complex, which controls retrograde transport, lead to unkempt Shh pathway activation (Goetz and Anderson 2010). Nonetheless, it not currently known whether IFT-A and -B complexes interact directly with Shh pathway components to control their localization and activity or if, instead, these complexes facilitate Shh signaling simply by maintaining proper cilia architecture. Indeed, recent genetic studies suggest that the primary cilium may function primarily as a scaffold for Shh signaling, arguing against a direct role for IFT proteins in the movement of Shh pathway components (Ocbina et al. 2011).

Cytosolic Shh Signaling in the Absence of Ligand

Shown in Fig. 2a, in the absence of Shh ligand, Ptch1 localizes to the cilium and inhibits the seven-pass transmembrane protein Smoothened (Wilson and Chuang 2010). The precise details of this inhibition are not well understood, but may involve the transport of small molecules that maintain Smo in an inactive conformation, a possibility that is supported by the fact that Smo is susceptible to inhibition and activation by small molecules (Ingham et al. 2011). In its inactive conformation, Smo is unable to inhibit the partial proteolytic processing of the zinc finger

transcription factors Gli2 and Gli3 from their full-length form into N-terminal repressors. Gli2 and Gli3 processing is facilitated by Suppressor of Fused (Sufu), which promotes the phosphorylation of Gli2 and Gli3 by Protein Kinase A (PKA), Casein Kinase I (CKI), and Glycogen Synthase Kinase β (GSK3β) (Humke et al. 2010; Tukachinsky et al. 2010). The Kinesin 4 family member Kif7 may also facilitate this phosphorylation. Phosphorylated Gli2 and Gli3 are recognized by the E3 ubiquitin ligase β-TrCP, leading to the proteolytic degradation of C-terminal peptides and the formation of Gli2 and Gli3 transcriptional repressors (Gli2R and Gli3R) (Wilson and Chuang 2010). Because Gli3 is more efficiently processed than Gli2, Gli3R serves as the principle repressor of Shh signaling. These transcriptional repressors translocate into the nucleus where they suppress the transcription of Shh target genes.

While Sufu has a relatively minor role in *Drosophila*, it is indispensible for proper Shh signaling in vertebrates (Ingham et al. 2011). *Sufu*$^{-/-}$ mice die around embryonic day 9.5, displaying aberrant Shh pathway activation and dramatically reduced levels of both full length and repressor forms of Gli, supporting a model wherein Sufu stabilizes full-length Gli and assists in GliR formation (Tukachinsky et al. 2010). Recent studies indicate that the Cul3 adaptor Speckle-type POZ protein (Spop) promotes the degradation of full-length Gli2 and Gli3 and competes with Sufu for binding of these molecules (Wang et al. 2010). Accordingly, genetic ablation of *Spop* from *Sufu*$^{-/-}$ cells partially rescues full-length Gli2 and Gli3 protein levels.

In addition to Sufu, the kinesin Kif7 also promotes the processing of full-length Gli and may form a complex with Gli and Sufu (Endoh-Yamagami et al. 2009; Wilson and Chuang 2010). Mice lacking functional *Kif7* have reduced levels of Gli3R, increased levels of full-length Gli2, and exhibit features of aberrant Shh pathway activation such as polydactyl (Cheung et al. 2009; Endoh-Yamagami et al. 2009; Liem et al. 2009). Thus, much as its *Drosophila* homolog Cos2, Kif7 may serve as a scaffolding molecule for GliR production in the vertebrate Hh pathway (Wilson and Chuang 2010). However, unlike Cos2, Kif7 has a functional motor domain, and mutations in this domain impair the ability of Kif7 to promote GliR formation (Liem et al. 2009). Nonetheless, whether Kif7's motor function influences the trafficking of Shh pathway components remains unclear, as does the precise role of Kif7 in Shh signaling.

Cytosolic Shh Signaling in the Presence of Ligand

Shown in Fig. 2b, activation of the Shh pathway results in both inhibition of Gli repressor formation and induction of Gli activator formation, ultimately leading to the transcription of Shh target genes. In the presence of Shh ligand, Shh binds Ptch1, relieving Ptch1-mediated inhibition of Smo. Smo levels increase on the plasma membrane, and Smo moves into the cilium where it promotes the disassembly of Sufu-Gli-Kif7 complexes and inhibits the proteolytic processing of Gli2 and Gli3 into their repressor forms (Milenkovic et al. 2009; Tukachinsky et al. 2010; Humke et al. 2010). Full-length Gli2 and Gil3 acquire additional poorly characterized modifications that convert them to their activator forms (Gli2A and Gli3A). Whereas Gli3 acts primarily as a transcriptional repressor, Gli2 acts primarily as a transcriptional activator. In the presence of Shh, Kif7, Smo, and Gli are enriched in the cilium tip, which may serve the location of GliA formation. Activated Gli2 induces the expression of *Gli1*, which acts solely as an activator of Shh signaling. In addition to inducing the transcription of genes involved in proliferation and differentiation, Gli1 and Gli2 promote the transcription of *Ptch1*, which inhibits further pathway activation in the absence of additional ligand.

Co-receptors

Three membrane-associated co-receptors – Growth arrest-specific 1 (Gas1), CAM-related/downregulated by oncogenes (Cdo), and Brother of Cdo (Boc) – facilitate Shh reception and positively regulate the pathway (Beachy et al. 2010). While removal of any one of these molecules leads to a modest, tissue-specific reduction in Shh pathway activity, loss of all three co-receptors completely abolishes Shh signaling, indicating that *Cdo*, *Boc*, and *Gas1* are indispensible for vertebrate Hh signaling (Allen et al. 2011; Izzi et al. 2011). These molecules likely form distinct receptor complexes with Ptch1 and enable proper Shh binding (Izzi et al. 2011). In contrast to these positive regulators of Shh signaling, Hedgehog interacting protein (Hhip) functions as a decoy receptor and serves as a negative regulator of the pathway (Beachy et al. 2010). Whereas the expression of *Cdo*, *Boc*, and *Gas1*

are downregulated in the presence of Shh, the expression of *Hhip* is upregulated, thus providing a means of tempering pathway activity.

Shh Signaling in Development and Disease

Shh functions as a morphogen, mitogen, and survival factor to regulate the development and patterning of many tissues in the vertebrate embryo. In the embryonic neural tube, for instance, floor plate-derived Shh promotes the proliferation, survival, and specification of neural progenitors. Similarly, Shh arising from the zone of proliferation (ZPA), a Shh-producing center in the posterior region of the limb bud, regulates digit specification in the embryonic limb (Jiang and Hui 2008). In the developing foregut, endoderm-derived Shh provides instructional cues to the surrounding mesoderm, resulting in proper development of both the gastrointestinal tract and lung (McMahon et al. 2003). In some tissues, the importance of Shh persists even after birth. For example, during the late embryonic and early postnatal stages, Purkinje cell-derived Shh is essential for granule cell precursor proliferation in the developing cerebellum (Jiang and Hui 2008). In the adult, Shh is required for the maintenance of stem cell populations both in the brain and the epithelium (Jiang and Hui 2008). Defects in Shh signaling have been linked to numerous congenital abnormalities, including holoprosencephaly, polydactyl, and tracheal-esophageal fistula, underscoring the importance of the pathway for normal development (McMahon et al. 2003).

Whereas loss of Shh signaling leads to congenital abnormalities, increased pathway activity has been linked to certain forms of cancer. Somatic mutations in *Ptch1*, the Shh receptor and a potent inhibitor of the pathway, are observed in nearly all cases of basal cell carcinoma (BCC), a common but rarely metastatic form of skin cancer (Barakat et al. 2010). Similarly, inactivating mutations in *Ptch1* or activating mutations in *Smo* are observed in a subset of medulloblastomas (MB), a malignant tumor of the cerebellum and the most common pediatric brain tumor (Jiang and Hui 2008). Additionally, aberrant Shh pathway activity may contribute to rhabdomyosarcoma (RMS), a pediatric cancer thought to arise from skeletal muscle progenitors (Barakat et al. 2010). While aberrant pathway activation underlies BCC, MB, and RMS, other cancers such as pancreatic ductal adenocarcinoma exhibit paracrine Shh signaling, whereby tumor cells secrete Shh into the surrounding stroma and influence the tumor microenvironment (Yauch et al. 2008). Given the role of Shh signaling in oncogenesis, it is unsurprisingly that efforts have been made to target the pathway pharmacologically. Many of these agents have recently entered phase II and III clinical trials, and the efficacy of Shh inhibitors will become apparent in years to come (Ng and Curran 2011).

Summary

The most widely expressed of the three Hh ligands in mammals, Sonic Hedgehog is essential for proper embryonic development and patterning. Over the past 20 years, significant progress has been made in understanding the production, release, and downstream signaling of Shh ligands. Nonetheless, several fundamental questions regarding the pathway remain unanswered. First, a detailed understanding of how Disp1 and ADAMs mediate the secretion of multimeric Shh remains elusive. Second, the process by which Ptch1 inhibits and regulates the subcellular localization of Smo is not yet known, nor is the process by which Sufu-Gli complexes are dissociated by activated Smo. Finally, and perhaps most perplexing, is the question of how, and why, the primary cilium plays such an essential role in vertebrate Hh signal transduction. Answering these questions will not only provide insight into vertebrate development, but may inform the treatment of Hedgehog-driven tumors.

References

Allen BL, Song JY, Izzi L, Althaus IW, Kang JS, Charron F, et al. Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. Dev Cell. 2011;20(6):775–87.

Barakat MT, Humke EW, Scott MP. Learning from Jekyll to control Hyde: hedgehog signaling in development and cancer. Trends Mol Med. 2010;16(8):337–48.

Beachy PA, Hymowitz SG, Lazarus RA, Leahy DJ, Siebold C. Interactions between hedgehog proteins and their binding partners come into view. Genes Dev. 2010;24(18):2001–12.

Caspary T, Garcia-Garcia MJ, Huangfu D, Eggenschwiler JT, Wyler MR, Rakeman AS, et al. Mouse Dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling. Curr Biol. 2002;12(18):1628–32.

Chen MH, Li YJ, Kawakami T, Xu SM, Chuang PT. Palmitoylation is required for the production of a soluble

multimeric Hedgehog protein complex and long-range signaling in vertebrates. Genes Dev. 2004;18(6):641–59.
Chen X, Tukachinsky H, Huang CH, Jao C, Chu YR, Tang HY, et al. Processing and turnover of the hedgehog protein in the endoplasmic reticulum. J Cell Biol. 2011;192(5):825–38.
Cheung HO, Zhang X, Ribeiro A, Mo R, Makino S, Puviindran V, et al. The kinesin protein Kif7 is a critical regulator of Gli transcription factors in mammalian hedgehog signaling. Sci Signal. 2009;2(76):ra29.
Dierker T, Dreier R, Petersen A, Bordych C, Grobe K. Heparan sulfate-modulated, metalloprotease-mediated sonic hedgehog release from producing cells. J Biol Chem. 2009;284(12):8013–22.
Endoh-Yamagami S, Evangelista M, Wilson D, Wen X, Theunissen JW, Phamluong K, et al. The mammalian Cos2 homolog Kif7 plays an essential role in modulating Hh signal transduction during development. Curr Biol. 2009;19(15):1320–6.
Goetz SC, Anderson KV. The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet. 2010;11(5):331–44.
Guerrero I, Chiang C. A conserved mechanism of Hedgehog gradient formation by lipid modifications. Trends Cell Biol. 2007;17(1):1–5.
Humke EW, Dorn KV, Milenkovic L, Scott MP, Rohatgi R. The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes Dev. 2010;24(7):670–82.
Ingham PW, Nakano Y, Seger C. Mechanisms and functions of Hedgehog signalling across the metazoa. Nat Rev Genet. 2011;12(6):393–406.
Izzi L, Levesque M, Morin S, Laniel D, Wilkes BC, Mille F, et al. Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation. Dev Cell. 2011;20(6):788–801.
Jiang J, Hui CC. Hedgehog signaling in development and cancer. Dev Cell. 2008;15(6):801–12.
Kawakami T, Kawcak T, Li YJ, Zhang W, Hu Y, Chuang PT. Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling. Development. 2002;129(24):5753–65.
Lewis PM, Dunn MP, McMahon JA, Logan M, Martin JF, St-Jacques B, et al. Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. Cell. 2001;105(5):599–612.
Li Y, Zhang H, Litingtung Y, Chiang C. Cholesterol modification restricts the spread of Shh gradient in the limb bud. Proc Natl Acad Sci U S A. 2006;103(17):6548–53.
Liem Jr KF, He M, Ocbina PJ, Anderson KV. Mouse Kif7/Costal2 is a cilia-associated protein that regulates sonic hedgehog signaling. Proc Natl Acad Sci U S A. 2009;106(32):13377–82.
Ma Y, Erkner A, Gong R, Yao S, Taipale J, Basler K, et al. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. Cell. 2002;111(1):63–75.
McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol. 2003;53:1–114.
Milenkovic L, Scott MP, Rohatgi R. Lateral transport of smoothened from the plasma membrane to the membrane of the cilium. J Cell Biol. 2009;187(3):365–74.
Ng JM, Curran T. The Hedgehog's tale: developing strategies for targeting cancer. Nat Rev Cancer. 2011;11(7):493–501.
Ocbina PJ, Eggenschwiler JT, Moskowitz I, Anderson KV. Complex interactions between genes controlling trafficking in primary cilia. Nat Genet. 2011;43(6):547–53.
Ohlig S, Farshi P, Pickhinke U, van den Boom J, Hoing S, Jakuschev S, et al. Sonic hedgehog shedding results in functional activation of the solubilized protein. Dev Cell. 2011;20(6):764–74.
Tukachinsky H, Lopez LV, Salic A. A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. J Cell Biol. 2010;191(2):415–28.
Varjosalo M, Taipale J. Hedgehog: functions and mechanisms. Genes Dev. 2008;22(18):2454–72.
Wang C, Pan Y, Wang B. Suppressor of fused and Spop regulate the stability, processing and function of Gli2 and Gli3 full-length activators but not their repressors. Development. 2010;137(12):2001–9.
Wilson CW, Chuang PT. Mechanism and evolution of cytosolic Hedgehog signal transduction. Development. 2010;137(13):2079–94.
Yauch RL, Gould SE, Scales SJ, Tang T, Tian H, Ahn CP, et al. A paracrine requirement for hedgehog signalling in cancer. Nature. 2008;455(7211):406–10.

Sphingenine-1-Phosphate

► Sphingosine-1-Phosphate

Sphingosine-1-Phosphate

Ashok Kumar and Julie D. Saba
Center for Cancer Research, Children's Hospital Oakland Research Institute, Oakland, CA, USA

Synonyms

D-*erythro*-sphingosine-1-phosphate; Long-chain base phosphate; S1P; Sphingenine-1-phosphate; Sphingosine-1-phosphoric acid

Historical Background

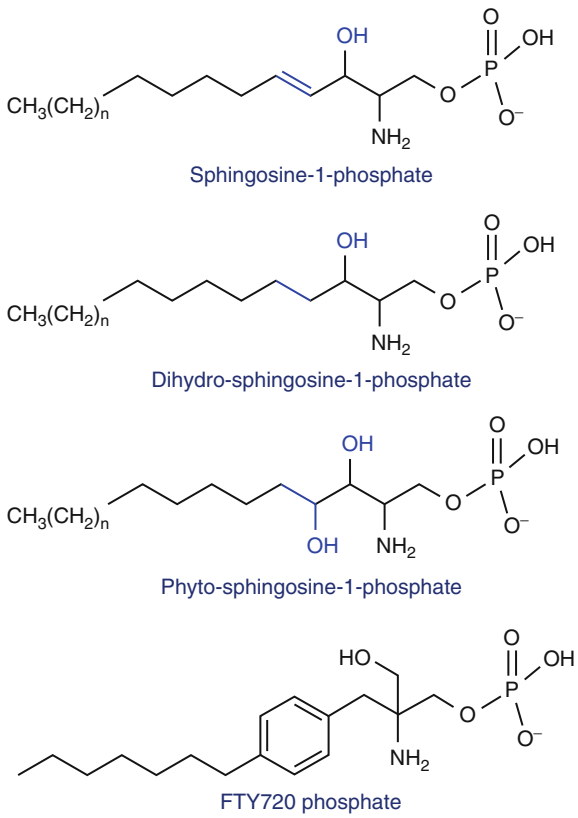
Sphingosine-1-phosphate (S1P) is a naturally occurring bioactive sphingolipid metabolite. Sphingolipids

are essential structural constituents of the plasma membrane of all eukaryotic organisms and are also produced by some bacteria. Sphingolipids are highly enriched along with cholesterol in microdomains or lipid rafts. In response to a wide range of stressful stimuli, membrane sphingomyelin and to lesser extent other complex sphingolipids are rapidly metabolized to the bioactive sphingolipid intermediate, *ceramide* and subsequently to sphingosine (Hannun and Obeid 2008). Phosphorylation of sphingosine by two *sphingosine kinases* results in the formation of S1P. S1P has emerged as a potent signaling molecule that regulates diverse cellular processes including cell proliferation, survival, differentiation, and migration (Spiegel and Milstien 2003). S1P plays a critical role in several physiological processes, including lymphocyte trafficking, vascular development, and inflammation. S1P exerts its biological actions both in cell-extrinsic and cell-intrinsic manner. S1P acts as ligand for five G-protein coupled receptors (GPCRs) known as S1P receptors 1-5 ($S1P_{1\text{-}5}$). S1P also acts as an intracellular second messenger. In 2002, it was discovered that the phosphorylated form of FTY720, an immunosuppressive compound, is an analog of S1P and binds to four out of five S1P receptors (Mandala et al. 2002). Subsequent to this discovery, intense research by many groups has provided a comprehensive understanding the role of S1P signaling in lymphocyte development, immune cell migration, and inflammation (Rivera et al. 2008; Spiegel and Milstien 2011). In addition to its role in immune cell trafficking and signaling, S1P functions as an oncogenic lipid that promotes tumor growth and progression. S1P plays a critical role in the regulation of many essential biological and developmental processes (Fyrst and Saba 2010). Not surprisingly, both the deficiency of S1P as well as its excessive accumulation are involved in pathological conditions including cancer, vascular abnormalities, inflammation, and autoimmune diseases and are likely to be linked to human congenital defects. Conversely, targeting the S1P signaling pathways and S1P metabolism appears promising as a therapeutic strategy for a number of human diseases.

Sphingosine, which comprises the backbone of most mammalian sphingolipids, was named in 1884 by JLW Thudichum after the Greek mythological creature, the sphinx, because of its enigmatic nature. One century later, the biological effects of sphingosine and its metabolite S1P are beginning to be elucidated. In the early 1990s, Sarah Spiegel's research group discovered that S1P regulates cell growth and stimulates DNA synthesis in response to platelet-derived growth factor (PDGF) (Olivera and Spiegel 1993). Further, they demonstrated that S1P suppresses ceramide-mediated apoptosis and thus proposed the concept of a sphingolipid rheostat (Cuvillier et al. 1996; Spiegel and Milstien 2003). In this model, the balance between intracellular ceramide/sphingosine and S1P levels influences whether cells exposed to stressful conditions will undergo growth arrest/apoptosis or proliferate and survive. Initially, it was proposed that S1P, like ceramide, mediates all its functions by acting as an intracellular second messenger. In the late 1990s, Timothy Hla and Sarah Spiegel discovered that S1P is a natural agonist for the *Endothelial Differentiation Gene-1* (EDG1), renamed S1P receptor 1 ($S1P_1$) (Lee et al. 1998). It is now well established that in higher eukaryotes, S1P exerts many of its actions by binding to its five cognate GPCRs (S1P receptors; $S1P_{1\text{-}5}$), thereby triggering intracellular signaling pathways through which it regulates a diverse array of biological processes such as angiogenesis, vascular integrity, cardiac development, and lymphocyte trafficking (Hla and Brinkmann 2011).

Structure, Biosynthesis, and Metabolism

Structure: S1P is synthesized by phosphorylation of its precursor sphingolipid, sphingosine. All sphingolipids contain a sphingoid or long-chain base (LCB) backbone. The term sphingoid base refers to a group of related compounds that contain two hydroxyl groups at the C1 and C3 positions, and an amino group at the C2 position (Chen et al. 2010). The most prevalent of sphingoid bases are sphinganine [(2 S,3R)-2-aminooctadece-1,3-diol] and sphingenine [(2 S,3R,4E)-2-aminooctadec-4-ene- 1,3-diol]. In mammalian cells, the most common sphingoid base is (2 S,3R,4E)-2-aminooctadec-4-ene-1,3-diol, which is also called *D-erythro*-sphingosine or just sphingosine. Other sphingoid bases vary in alkyl chain length and branching, the number and position of double bonds, the presence of additional hydroxyl groups, and other features (Chen et al. 2010). In fungi and plants, *D-ribo-phyto-sphingosine* (phyto-sphingosine) is predominant, and it carries an additional hydroxyl group at C4. *D-erythro-dihydrosphingosine* (dihydrosphingosine or sphinganine) is also detectable



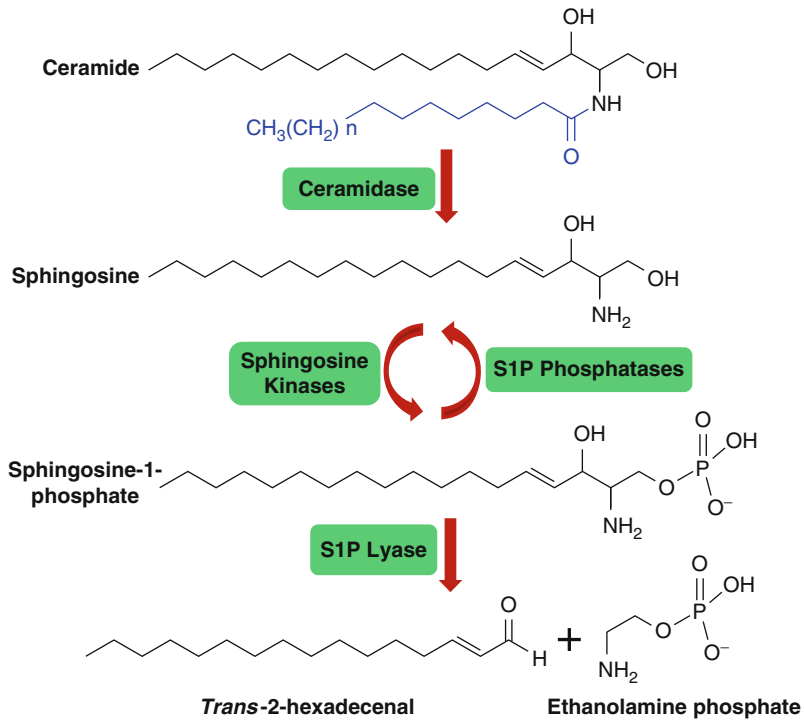
Sphingosine-1-Phosphate, Fig. 1 *Structure of natural and synthetic long-chain base* (*LCB*) *phosphates*. Sphingosine-1-phosphate and dihydro-sphingosine-1-phosphate are major long-chain base phosphates found in yeast, nematodes, flies, and mammals, whereas phyto-sphingosine-1-phosphate is abundant in fungi and plants. LCB phosphates vary in chain length among the species, with mammals carrying C18 chain length. (n = 3–7). FTY720 phosphate is a synthetic analog of S1P

in most organisms. Sphingoid bases vary in chain length among species, with mammals carrying primarily C18; yeast, C16, C18, and C20; fly, C14 and C16; and nematode, C17. Some species, such as nematodes, contain unusual branch chain sphingoid bases. Unless otherwise stated, S1P biosynthesis, metabolism, and functions have been referred to the mammalian S1P.

Biosynthesis and Metabolism: Two highly homologous sphingosine kinases, known as sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2) catalyze the phosphorylation of the hydroxyl group (1-OH) of sphingosine to form S1P (Fig. 1) (Spiegel and Milstien 2003). Both SphK1 and SphK2 phosphorylate sphingosine and dihydrosphingosine, whereas SphK2 exhibits broader substrate specificity and can also phosphorylate phyto-sphingosine and FTY720 (a synthetic drug structurally related to sphingosine). Sphingosine and other LCBs are produced mainly by two pathways: *de novo* sphingolipid biosynthesis or by breakdown of ceramide. In *de novo* biosynthesis, the first step is the condensation of L-serine and palmitoyl-CoA through the action of serine palmitoyltransferase to form 3-ketodihydrosphingosine, which is then reduced to dihydrosphingosine. In yeast, hydroxylation at the C4 position of dihydrosphingosine by the hydroxylase Sur2p yields the predominant yeast LCB, phyto-sphingosine. However, sphingosine cannot be synthesized by the *de novo* pathway and is generated instead via the deacylation of ceramide catalyzed by ceramidase (salvage pathway).

S1P levels in the tissues and plasma are tightly regulated. SphK1 is the primary enzyme responsible for S1P generation and is mainly localized in the cytosol. Activation of SphK1 is regulated by many factors such as its intracellular localization, epigenetic or post-translational modification. SphK1 can be activated by wide variety of growth factors including PDGF, vascular endothelial growth factor, epidermal growth factor, hepatocyte growth factor and tumor necrosis factor-α (TNF-α) (Pitson 2011). Upon stimulation by growth factors, cytokines, and lipopolysaccharide, SphK1 translocates to the plasma membrane. SphK1 generated S1P has been implicated in many pathophysiological conditions including vascular permeability, angiogenesis and inflammation. Compared to SphK1, much less is known about the regulation and function of SphK2. SphK2 is localized in mitochondria, nucleus, and endoplasmic reticulum and regulates apoptosis and gene expression.

Circulating and tissue S1P levels are also regulated by its catabolism. There are six enzymes known to catabolize S1P and which play a role in maintaining S1P levels in the tissues (Fig. 2). The first enzyme is S1P lyase, which degrades S1P irreversibly to ethanolamine phosphate and a long-chain aldehyde (*trans*-2-hexadecenal) (Kumar and Saba 2009). S1P phosphatase-1 and -2 remove the phosphate group reversibly from S1P and convert it back to sphingosine. These three enzymes are localized in the endoplasmic reticulum and are highly selective for S1P and LCB phosphate substrates. Additionally, there are three plasma membrane-bound phosphatases known as lipid phosphate phosphatases 1, 2, and 3 (LPP1-3) that can dephosphorylate a broad range of lipid



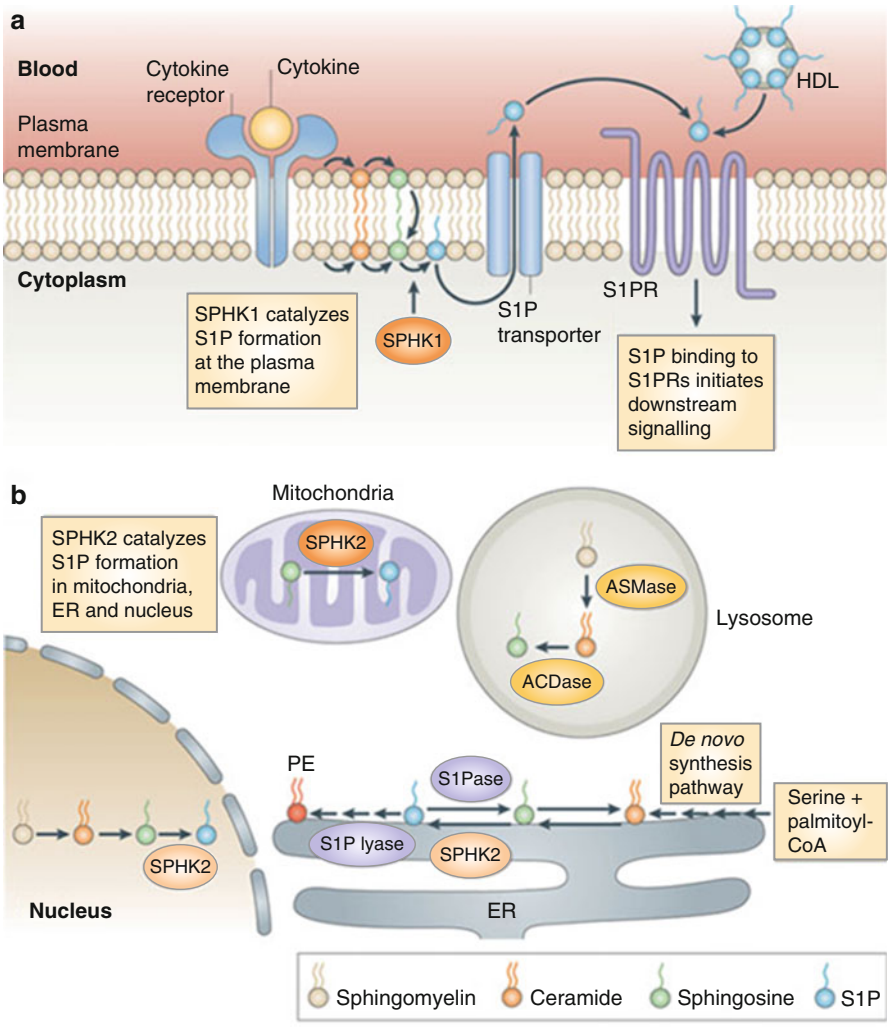
Sphingosine-1-Phosphate, Fig. 2 *Sphingosine-1-Phosphate* (*S1P*) *metabolic pathway in mammalian cells.* S1P is generated by the conversion of ceramide to sphingosine by the enzyme ceramidase and the subsequent phosphorylation of sphingosine to S1P, which is catalyzed by two sphingosine kinases. S1P can be dephosphorylated back to sphingosine in a reversible reaction catalyzed by S1P phosphatases or lipid phosphate phosphates. This sphingosine can be reused in the salvage pathway. S1P can also be irreversible degraded by the enzyme S1P lyase forming a long-chain aldehyde (*trans*-2-hexadecenal) and ethanolamine phosphate. Several molecular species of ceramide are found in mammals based on fatty acid chain length (n = 4–16)

phosphate substrates including S1P, ceramide-1-phosphate, lysophosphatidic acid, and phosphatidic acid (Pyne and Pyne 2010).

Occurrence, Source, and Transport

S1P is generated by most cell types in mammals. In blood, S1P concentrations vary between 0.2 and 1.0 μM. In lymph, S1P is present in the hundred-nanomolar range and is produced by radiation-resistant cells of non-hematopoietic origin, presumably lymphatic endothelial cells. Erythrocytes and platelets are rich sources of S1P, as both of these cell types are exposed to circulating sphingosine, contain sphingosine kinase activity, and lack most of the S1P degrading enzymes. Vascular endothelium and mast cells also secrete S1P. In blood, S1P remains bound to albumin and lipoproteins, most notably high-density lipoproteins (HDL). Albumin-bound S1P and free S1P are susceptible to degradation, whereas HDL-bound S1P is more stable. HDL likely facilitates the delivery of S1P to its receptors (Rivera et al. 2008).

Many cells that produce S1P also export it, thereby allowing S1P to bind and activate the S1P receptors present on neighboring cells (paracrine action) or the same cell (autocrine action). The latter mechanism is also known as "Inside-Out Signaling" (Fig. 3). S1P is an amphiphilic molecule that requires facilitated export from cells. The ATP-binding cassette (ABC) family of transporters has been shown to be involved in the export of S1P from the cells. Mast cells and platelets secrete S1P in a stimulus-dependent manner and require the activity of ABCC1 and ABCA-like transporters, respectively. On the contrary, erythrocytes and endothelial cells export S1P constitutively in a stimulus-independent manner. Erythrocytes mediate S1P export through the ABCA1 transporter protein.



Sphingosine-1-Phosphate, Fig. 3 *Mechanism of action of S1P*. In response to cytokine or other stimuli, S1P is synthesized by phosphorylation of sphingosine by sphingosine kinase 1 (SPHK1) at the plasma membrane (**a**) and by SPHK2 at the endoplasmic reticulum (ER), mitochondria and nucleus (**b**). At the ER, S1P is irreversibly degraded by S1P lyase or dephosphorylated to sphingosine by an S1P phosphatase (S1Pase). S1P produced at the plasma membrane in response to stimuli is released by specific transporters and regulates cellular functions by binding to specific S1P receptors (S1PRs) and initiating downstream signaling pathways. S1P produced in the mitochondria and nucleus by SPHK2 has direct intracellular targets, and S1P generated by SPHK1 at the plasma membrane can also function intracellularly. In the blood, S1P is produced mainly by erythrocytes (although platelets, endothelium, and other cellular sources also contribute to S1P levels under various conditions), is bound to albumin and high-density lipoprotein (HDL), and can activate S1PRs. ACDase, acid ceramidase; ASMase, acid sphingomyelinase (Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology, Spiegel and Milstien 11:403, copyright (2011))

Recently, a novel S1P transporter *Spns2* was discovered in zebrafish. Mutations in *Spns2* caused the cardiac bifida phenotype that resembled that of the S1P receptor zebrafish mutant, thus revealing the two genes to be operating in the same pathway (Kawahara et al. 2009). The human orthologue of *Spns2* has also been shown to export S1P and other phosphorylated LCBs including FTY720 from the cells.

Mechanism of Action

With the discovery of EDG1 as a high-affinity receptor for S1P, it became evident that an important biological function of S1P is to act as a natural ligand for the EDG GPCR family of receptors, now referred to as S1P receptors (Lee et al. 1998). To date, five S1P receptors EDG1/$S1P_1$, EDG5/$S1P_2$, EDG3/$S1P_3$, EDG6/$S1P_4$, and EDG8/$S1P_5$ that bind to S1P and dihydro-S1P have been identified in vertebrates (Hla and Brinkmann 2011). In mammalian cells, S1P receptors are ubiquitous, but their expression patterns vary among the different tissues. Each S1P receptor couples to different heterotrimeric G-proteins. For example, $S1P_1$ and $S1P_4$ couple mainly to G_i; $S1P_2$ and $S1P_3$ activate G_i, G_q, and $G_{12/13}$; and $S1P_5$ binds to G_i and $G_{12/13}$. After coupling to G-proteins, these receptors either activate or inhibit downstream signaling pathways including extracellular signal-regulated kinase, c-Jun N-terminal Kinase, phosphatidylinositol 3-kinase, phospholipase C, Rac, Rho, cyclic AMP, and phospholipase D (Spiegel and Milstien 2003). The functional response of each cell to S1P varies depending on S1P receptor expression and intracellular signaling configuration. By activating these receptors, S1P regulates diverse biological processes including vascular development, angiogenesis, and immunity. To date, S1P receptors have not been identified in invertebrates, leading to the current opinion that these receptors evolved with the coelomates.

S1P regulates many biological processes including cell survival, DNA damage repair, calcium mobilization, and stress response by acting as an intracellular second messenger. Many studies conducted in lower organisms such as yeast, flies, slime molds, nematodes, and plants that lack identifiable S1P receptors revealed that S1P regulates important developmental processes through direct intracellular signaling. In fact, S1P has a universal signaling role in calcium mobilization in yeast, plants, and mammals. However, the question of how S1P mediates its intracellular signaling effects remained unanswered, and specific intracellular targets of S1P remained elusive. Recently, several direct intracellular S1P targets have been identified. For examples, S1P directly binds to histone deacetylase (HDAC)-1 and -2, TNF receptor-associated factor 2 (TRAF2), p21-activated protein kinase 1 and prohibitin 2. Whether these or other intracellular interactions are responsible for the function of S1P in simple metazoans remains to be determined.

S1P in Health and Disease

S1P in immunity: In a landmark study by Mandala and colleagues, it was shown that FTY720, an immunosuppressive agent is an analog of sphingosine and after phosphorylation in vivo it (phosphorylated compound of FTY720) binds to four of five S1P receptors and induces lymphopenia (Mandala et al. 2002). A plethora of immunological investigations have now revealed the major impact of S1P signaling on the mammalian immune system. S1P signaling through its receptors was found to regulate the trafficking and migration of many bone marrow–derived immune cells including T and B lymphocytes, natural killer (NK) T cells, dendritic cells, macrophages, neutrophils, mast cells, and hematopoietic progenitors cells (Rivera et al. 2008; Spiegel and Milstien 2011). Pharmacological and genetic approaches combined with sophisticated in vivo imaging of immune cells have provided novel insights regarding the role of $S1P_1$ signaling in mature lymphocyte egress from the thymus and secondary lymph nodes in to the circulation. Due to high levels of plasma and lymph S1P and low S1P levels in lymphoid tissues, an S1P gradient exists between plasma and lymphoid organs. Mature thymocytes expressing high levels of $S1P_1$ sense the gradient and migrate toward the blood stream. Inhibition or genetic deficiency of S1P catabolizing enzymes, such as S1P lyase or lipid phosphate phosphatase 2 raises thymic S1P levels, disrupting the S1P gradient and resulting in blockade of exit of mature thymocytes, thereby producing lymphopenia (Schwab et al. 2005). In addition to its role in leukocyte migration, S1P signaling contributes to T lymphocyte differentiation, allergy, inflammation, endothelial barrier integrity in anaphylactic shock, and other immune functions.

S1P in cell survival and cell death: The metabolic balance between ceramide, sphingosine, and S1P plays an important role in determining cell fate. In general, ceramide and sphingosine accumulation result in growth arrest and/or induce cell death, whereas S1P has a mitogenic function and inhibits apoptosis. S1P inhibits ceramide-induced apoptosis in many cell types (Cuvillier et al. 1996; Spiegel and Milstien 2011). Moreover, overexpression of SphK1 inhibits apoptosis and induces chemoresistance, whereas overexpression of S1P lyase which depletes intracellular S1P promotes apoptosis in response to DNA damaging agents and ionizing radiation (Min et al. 2004; Kumar et al. 2011).

S1P increases the expression of anti-apoptotic proteins Bcl-2 and Mcl1 and downregulates pro-apoptotic Bcl-2 family proteins Bad and Bax. In vivo, S1P protects small intestine from radiation-induced endothelial apoptosis and it also prevents radiation-induced cell death in germ cells (Morita et al. 2000; Bonnaud et al. 2010). However, the rheostat model has recently been challenged due to some interesting findings. For example, under certain conditions, S1P can induce autophagy, similar to ceramide. However, the cellular outcome of S1P-induced autophagy is different than the ceramide-induced autophagy, because S1P-induced autophagy promotes cell survival whereas ceramide-induced autophagy leads to cell death (Pyne and Pyne 2010). There is also evidence that S1P pools generated by SphK1 versus SphK2 have opposite effects on cellular growth and apoptosis. While SphK1 generates S1P, which promotes cell growth and rescues cells from ceramide-induced apoptosis, overexpression of SphK2 suppresses growth and enhances cell death (Pyne and Pyne 2010; Pitson 2011).

S1P in cardiovascular system: S1P signaling through endothelial $S1P_1$ is required for vascular maturation during development, as dramatically illustrated by the embryonic lethality of $S1P_1$ knockout mice (Lee et al. 1998; Hla and Brinkmann 2011). S1P signaling also contributes to cardiac development, angiogenesis, HDL-mediated effects, and regulation of vascular tone and permeability. S1P enhances survival in cardiomyocytes during hypoxia, and both SphK1 and SphK2 appear to contribute to ischemic preconditioning, a mechanism by which brief exposure to minor ischemic episodes affords cardioprotection against larger ischemic events. Deficiency of SphK1 or S1P receptors $S1P_2$/$S1P_3$ exacerbates infarction size in cardiac ischemia-reperfusion model in mice. In contrast, S1P lyase deficiency in mice or inhibition of its enzymatic activity by a pharmacological agent enhances recovery from cardiac ischemia-reperfusion. In addition to the direct effects of S1P in the heart, there is increasing evidence that some of the protective effects of HDL against atherosclerosis and heart disease are mediated through S1P (Fyrst and Saba 2010).

S1P in cancer: Through its roles in regulating cell death pathways, proliferation, migration, and angiogenesis, S1P signaling has a significant impact on the processes of carcinogenesis, cancer progression, and drug and radiation resistance patterns. Many components of S1P signaling pathways including sphingosine kinases, S1P lyase, and S1P receptors have been mechanistically linked with cancer cell aggressiveness, invasion, metastasis, and chemoresistance. SphK1 is overexpressed in various types of human cancers including prostate, gastric, breast, lung, colon cancer, glioma, and non-Hodgkin's lymphoma (Pyne and Pyne 2010). Further, overexpression of SphK1 in mouse fibroblast induces transformation through a Ras-dependent pathway, demonstrating that SphK 1 is an oncogene. Elevation of SphK1 expression in cancers is often associated with clinical grade of tumors and chemoresistance. Inhibition of SphK1 enzyme activity or genetic knockdown blocks tumor progression in many types of cancers and induces apoptosis and autophagy (Pyne and Pyne 2010). On the contrary, S1P lyase expression is downregulated in melanomas, colon and ovarian cancer and may act as a tumor suppressor gene (Kumar and Saba 2009). S1P promotes tumor invasion, neovascularization, and metastasis by activating its cognate receptors. However, no consistent pattern of S1P receptor gene expression has been associated with clinical tumor grade. Notably, mice lacking $S1P_2$ develop diffuse large B-cell lymphoma, and several mutations have been reported in $S1P_2$ gene in diffuse large B-cell lymphoma patients.

Therapeutic targeting of S1P signaling pathways: Due to the central involvement of S1P signaling in immunity and tumorigenesis, substantial activity in the pharmaceutical industry has developed around the goals of targeting S1P (the monoclonal antibodies developed by LPath), S1P receptors (S1PR-specific agonist and antagonist compounds generated by many research groups), sphingosine kinase (inhibitors), and S1P lyase (inhibitors) for therapeutic purposes. Notably, FTY720, a synthetic analogue of sphingosine, has been approved by the US Federal Drug Administration in relapsing multiple sclerosis patients. S1P-specific monoclonal antibody (ASONEP; LPath, Inc.) which reduces tumor progression in various mouse models of cancer has been approved for Phase 1 clinical trial in cancer. LX2931 (Lexicon Inc.), an oral S1P lyase inhibitor is in Phase II clinical trial for use in rheumatoid arthritis. Several sphingosine kinase inhibitors are being tested in mouse models for cancer, inflammation and sepsis. As more potent and selective agents become available, their evaluation in pre-clinical models and clinical trials will reveal the true potential of targeting S1P signaling for therapeutic purposes.

Summary

S1P signaling regulates myriad biological functions, and each year new targets are being identified. In fact, over the last two decades, S1P has become one of the most highly investigated signaling lipids. Although the S1P signaling field has advanced tremendously, some important questions remained unanswered. For example, the exact role of S1P in regulating diverse physiological functions in lower eukaryotes in the absence of identifiable S1P receptors is still an enigma. Direct intracellular binding of S1P to TRAF2 and HDAC has been shown recently. However, it is highly likely that additional intracellular targets remain to be discovered. FTY720 has been approved for clinical use in relapsing multiple sclerosis patients, providing the first major impact of the S1P pathway on medical care. Whether FTY720 will be efficacious and safe in other conditions such as chronic inflammatory demyelinating neuropathy, which is the peripheral counterpart to multiple sclerosis, in other autoimmune diseases, or in other conditions such as regenerative diseases, radioprotection, and cancer are still outstanding questions. While it seems that FTY720's main function is by receptor downregulation, there is evidence that agonism of S1P receptors contributes to some of its beneficial effects. It is hoped that other S1P receptor agonists/antagonists will be useful in various clinical contexts. As biological and physiological research continues to reveal the functions of S1P signaling, the clinical contexts in which S1P signaling can be targeted for patient benefit will also likely expand.

References

Bonnaud S, Niaudet C, Legoux F, Corre I, Delpon G, Saulquin X, et al. Sphingosine-1-phosphate activates the AKT pathway to protect small intestines from radiation-induced endothelial apoptosis. Cancer Res. 2010;70:9905–15.

Chen Y, Liu Y, Sullards MC, Merrill Jr AH. An introduction to sphingolipid metabolism and analysis by new technologies. Neuromolecular Med. 2010;12:306–19.

Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind S, et al. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature. 1996;381:800–3.

Fyrst H, Saba J. An update on sphingosine-1-phosphate and other sphingolipid mediators. Nat Chem Biol. 2010;6:489–97.

Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol. 2008;9:139–50.

Hla T, Brinkmann V. Sphingosine 1-phosphate (S1P): physiology and the effects of S1P receptor modulation. Neurology. 2011;76:S3–8.

Kawahara A, Nishi T, Hisano Y, Fukui H, Yamaguchi A, Mochizuki N. The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. Science. 2009;323:524–7.

Kumar A, Saba JD. Lyase to live by: sphingosine phosphate lyase as a therapeutic target. Expert Opin Ther Targets. 2009;13:1013–25.

Kumar A, Oskouian B, Fyrst H, Zhang M, Paris F, Saba JD. S1P lyase regulates DNA damage responses through a novel sphingolipid feedback mechanism. Cell Death Dis. 2011;2:e119.

Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, et al. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. Science. 1998;279:1552–5.

Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science. 2002;296:346–9.

Min J, Stegner A, Alexander H, Alexander S. Overexpression of sphingosine-1-phosphate lyase or inhibition of sphingosine kinase in *Dictyostelium discoideum* results in a selective increase in sensitivity to platinum-based chemotherapy drugs. Eukaryot Cell. 2004;3(3):795–805.

Morita Y, Perez GI, Paris F, Miranda SR, Ehleiter D, Haimovitz-Friedman A, et al. Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. Nat Med. 2000;6:1109–14.

Olivera A, Spiegel S. Sphingosine 1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. Nature. 1993;365:557–9.

Pitson SM. Regulation of sphingosine kinase and sphingolipid signaling. Trends Biochem Sci. 2011;36:97–107.

Pyne NJ, Pyne S. Sphingosine 1-phosphate and cancer. Nat Rev Cancer. 2010;10:489–503.

Rivera J, Proia RL, Olivera A. The alliance of sphingosine-1-phosphate and its receptors in immunity. Nat Rev Immunol. 2008;8:753–63.

Schwab S, Pereira J, Matloubian M, Xu Y, Huang Y, Cyster J. Lymphocyte sequestration through S1P lyase inhibition an disruption of S1P gradients. Science. 2005;309:1735–9.

Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol. 2003;4:397–407.

Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. Nat Rev Immunol. 2011;11:403–15.

Sphingosine-1-Phosphoric Acid

► Sphingosine-1-Phosphate

Spi1

▶ Ran

Spindle Assembly Checkpoint, SAC

▶ Monopolar Spindle 1 (Mps1)

Spindle Pole Body, SPB

▶ Monopolar Spindle 1 (Mps1)

Spk1 (Yeast *Saccaromyces pombe*)

▶ ERK1/ERK2

sPLA2

▶ Phospholipase A_2

Spo4 (Second Cdc7 Homologue in *S. pombe*)

▶ Cdc7

SPRK

▶ MLK3

SRC

▶ c-Src Family of Tyrosine Kinases

Src Homology 2 (SH2) Domain–Containing Leukocyte Protein of 76 kDa

▶ SLP-76

Src Homology Region 2 (SH2)-Domain Phosphatase or Src Homology Region 2 Domain-Containing PTP-1 (SHP-1 or SH-PTP1)

▶ PTPN6

SRC1

▶ c-Src Family of Tyrosine Kinases

SRC-1

▶ Steroid Receptor Coactivator Family

SRC-2

▶ Steroid Receptor Coactivator Family

SRC-3

▶ Steroid Receptor Coactivator Family

SRK

▶ ZAP-70

Srx

▶ Sulfiredoxin

Srx1

▶ Sulfiredoxin

Srxn1

▶ Sulfiredoxin

Sry

David A. Jans and Gurpreet Kaur
Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

Synonyms

Sex determining region on the Y chromosome; Testis determining factor; TDF

Historical Background

Mammalian sex determination occurs during a short period in embryonic development, with SRY (Sex determining Region on the Y chromosome) playing a key role. In the 1940s, Jost et al. demonstrated that a testis is necessary for development of the male phenotype in mammals, as implied by the fact that castrated rabbits of either chromosomal sex developed as females (Jost et al. 1973). Karyotypes of patients with Turner Syndrome (45, X) and Klinefelter syndrome (47, XXY) demonstrated that sex is chromosomally controlled, and that the Y chromosome, in particular, determines male development regardless of X chromosome number (Vilain and McCabe 1998). Based on these observations, it was hypothesized that a dominant inducer of testis formation, the so-called testis determination factor (TDF), was located on the Y chromosome and encoded a gene or genes specifying the male phenotype; in the absence of this TDF, a female phenotype ensued (Vilain and McCabe 1998).

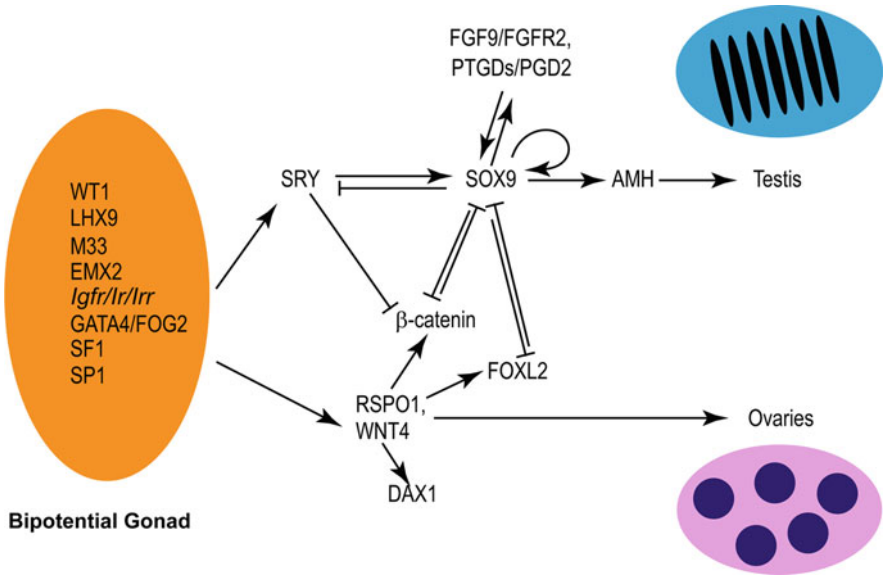
In 1990, the master gene on the Y chromosome, *Sry*, was isolated by positional cloning and identified as the TDF (Polanco and Koopman 2007). In the genome of true hermaphrodites and XX males, the small fragment of the Y chromosome that had translocated to the X chromosome was found to encode *Sry*, where XX mice transgenic for *Sry* developed as males with normal testes and testicular cords, implying that *Sry* was the only Y-encoded gene necessary for testis formation and the resulting male phenotype (Harley et al. 2003).

In mammalian males, SRY acts on the undifferentiated genital ridge as a switch to initiate the development of a testis, instead of an ovary, from the genital primordium; driving cells to a Sertoli (i.e., male) rather than a granulosa (female) fate (Sekido and Lovell-Badge 2009). *Sry* transcripts are expressed for a brief period during gonadal development in mice, consistent with SRY's role being that of initiating, rather than maintaining testis differentiation through triggering a complex cascade of events leading to testicular development (see Fig. 1) (Sekido and Lovell-Badge 2009).

Expression of SRY During Development

In mammals, the gonad initially develops in a nonspecific manner, and is morphogenically identical in XX and XY mouse embryos until *Sry* expression, which occurs between 10.5 and 12.5 days (d) post coitum (pc) (Sekido and Lovell-Badge 2009). At 11.5 dpc, sex-specific differences become apparent, with the differentiation of the supporting lineage into Sertoli cells and alignment into testis cord and presumptive seminiferous tubules. In the absence of SRY, the ovary forms via a default pathway 2–3 days later in female embryos forming granulosa or theca cells. After 12.5 dpc, SRY is detectable only at very low levels in the mouse (Polanco and Koopman 2007).

In humans, the gonadal ridge forms around day 33 of gestation, with *Sry* detectable in XY embryos at day 41, peaking at day 44 when the testis cords are visible. By 52 days gestation, the germ cells are surrounded by Sertoli cells, which continue to express *Sry* at low levels (Harley et al. 2003). In contrast to in the mouse, the *Sry* transcript is expressed widely in the human embryo, including in the brain and continues to be transcribed even in human adult testis, consistent



Sry, Fig. 1 *Signaling pathways in sex determination*. Development of the gonads is highly regulated through complex signaling and antagonistic interactions between the two sex determining pathways, with SRY as the key initiator of male sex determination and *Sox9* expression to favor testis development. *Arrow-headed lines* indicate positive upregulation and *bar-headed lines* indicated repressive influences. Key: *DAX1* Dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on gene 1 of the X chromosome, *AMH* Anti-Mullerian hormone, *EMX2* Empty spiracles homeobox 2, *FGF9* Fibroblast growth factor 9, *FGFR2* Fibroblast growth factor receptor 2, *FOG2* Zinc finger protein, multitype 2, *FOXL2* Forkhead box protein L2, *GATA4* GATA binding protein 4, *Igfr* Insulin-like growth factor receptor 1, *Ir* Insulin receptor, *Irr* Insulin receptor-related receptor, *LHX9* LIM homeobox gene 9, *M33* M33 polycomb-like protein, chromobox homologue 2, *PGD2* Prostaglandin D 2, *PTGD* Prostaglandin, *RSPO1* R-spondin 1, *SF1* Steroidogenic factor 1, *SP1* Specificity protein 1, *SOX9* Sry-related HMG box 9, *SRY* Sex determining Region on the Y chromosome, *WNT4* Wingless-type MMTV integration site family member 4, *WT1* Wilm's tumor suppressor gene 1

with roles additional to those in sex determination, such as spermatogenesis (Harley et al. 2003).

Little is known of how *Sry* expression is regulated, with the transcription factors WT1 (Wilm's tumor suppressor gene 1) and SF1 (Steroidogenic factor 1) playing key roles (see Fig. 1) (Polanco and Koopman 2007). Mutations in either can result in sex reversal and gonadal dysgenesis, with SF1 able to bind and transactivate the human and porcine *Sry* promoter in vitro. Two isoforms of WT1 have been found to play key roles in testis development, −KTS and +KTS, which exclude or include the amino acid sequence lysine-threonine-serine in the zinc finger nucleic acid binding domain, respectively. The −KTS isoform regulates *Sf1* expression, with evidence that it can also bind and transactivate the human and porcine *Sry* promoter. Intriguingly, the +KTS isoform shows higher affinity for RNA than DNA, and has been proposed to be involved in enhancing *Sry* translation and/or stabilizing the *Sry* mRNA (Polanco and Koopman 2007). Lhx9 (LIM-homeobox gene 9) and a polycomb group protein M33 are also involved in regulating *Sry* expression, with both being able to upregulate *Sf1* expression (Piprek 2009). CITED1 [CBP(CREB-binding protein)/p300-interacting transactivator with ED-rich tail 2], a non-DNA binding transcriptional cofactor, may also act with SF1 and WT1 to increase *Sry* expression (Buaas et al. 2009).

The transcription factor GATA-4, along with its cofactor FOG2 can also upregulate *Sry* expression, with synergism between GATA-4 and the +KTS isoform of WT1 resulting in strong transactivation of the porcine and mouse *Sry* promoter (Piprek 2009). The transcription factor SP1 (specificity protein 1) may also activate *Sry* expression, with mutations affecting its binding to the *Sry* promoter associated with human XY sex reversal (Polanco and Koopman 2007). The insulin receptor, insulin-related receptor, and the insulin-like growth factors are also implicated in testis development, with sex reversal resulting from a triple

null mutation in mice (Sekido and Lovell-Badge 2009). A transcription factor, EMX2 (empty spiracles homeobox 2), is also believed to play a role in testis development, although little is known of the mechanism (Piprek 2009).

The expression of *Sry* in the embryonic gonad appears to occur as a dynamic wave, originating from the center of the gonads and diffusing out toward the anterior and posterior poles between 10.5 and 12.5 dpc in mice (Polanco and Koopman 2007). SRY positive cells are detectable at 12 tail somites (ts; ~11.0 dpc), by 18 ts (~11.5 dpc) one third of cells express SOX9 [SRY-related HMG (high mobility group)-box 9], half of which are SRY positive, and by 25 ts (~12.0 dpc) 50% of cells show SOX9 expression, with 90% of all cells being SRY negative (Oh and Lau 2006). Recent studies indicate that SRY has a critical time window of 6 h to trigger *Sox9* upregulation and induce testis differentiation (Hiramatsu et al. 2009). Once *Sox9* is expressed, *Sry* expression is switched off through a negative feedback mechanism either through the actions of SOX9, since *Sry* expression is higher in gonads with low levels of *Sox9*, or through *Sry* itself, where mice carrying weak *Sry* alleles have prolonged *Sry* expression, presumably as a result of delayed *Sox9* expression (Sekido and Lovell-Badge 2009).

Genetic studies of the variants of Y chromosome present on the C57BL/6 J (B6) mouse strain, as well as transgenic mice, indicate that threshold levels of *Sry* are required to be expressed at critical times during male sex development, with reduced levels and delayed expression of *Sry* resulting in aberrant testis development (Sekido and Lovell-Badge 2009; Polanco and Koopman 2007). These results indicate that *Sry* must act during a critical time window to appropriately activate *Sox9* and must be expressed before the ovarian pathway is engaged for testis development to proceed (Polanco and Koopman 2007).

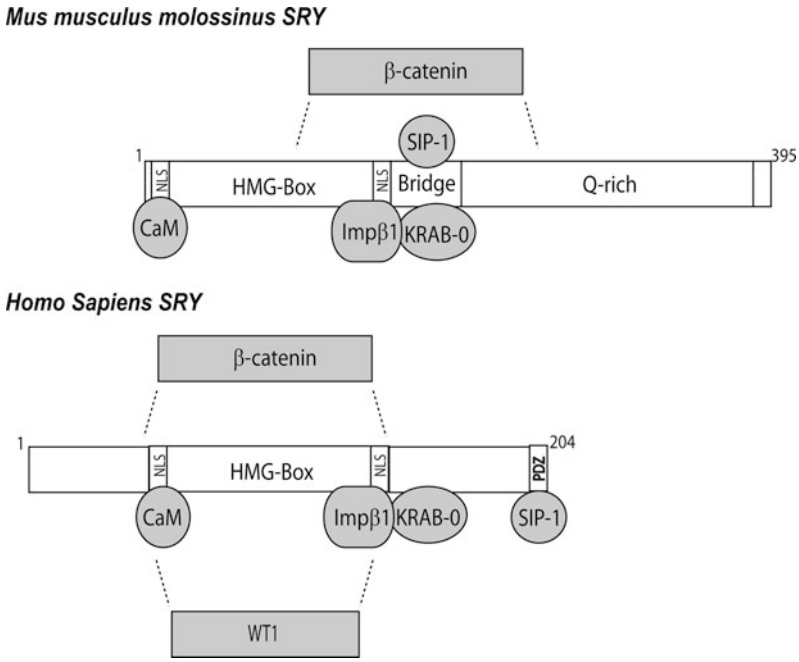
Targets of SRY in the Cell

A property of the family of SOX DNA-binding proteins is the ability to bind to the consensus DNA sequence AACAAT, with SRY having the highest affinity for the (A/T)AACAA(T/A) sequence, being able to bind DNA specifically and inducing strong bending of specific target DNAs (Polanco and Koopman 2007). The 204 amino acid human SRY is encoded as a single exon, which can be divided into three regions; a central 79 amino acid HMG-box domain that confers DNA binding, flanked by the N- and C-terminal domains (Polanco and Koopman 2007). Significantly for SRY's role in the nucleus, the HMG-box domain is flanked by two basic sequences that have been shown to be functional as nuclear localization signals (NLSs) (see Fig. 2) (Kaur and Jans 2011). In mouse (*Mus musculus molossinus*), SRY contains a large C-terminal glutamine-rich region, separated from the HMG-box by a "bridge domain" (see Fig. 2) (Sekido and Lovell-Badge 2009). This C-terminal region is absent in the subspecies *M.m. domesticus*, however, consistent with the idea that the HMG region is the key domain for SRY function (Harley et al. 2003).

There is essentially no sequence conservation outside of the HMG domain of SRY between different mammalian and reptile species, signifying its rapid divergence through evolution (Harley et al. 2003). The SRY HMG domain shows >70% amino acid sequence similarity for the SRY sequences from human, mouse, rabbit, wallaby, marsupial mouse, and sheep (Harley et al. 2003). The nuclear magnetic resonance structure of the HMG domain of SRY in complex with DNA has been determined, showing three α-helices forming an L shape. Through binding the minor groove of DNA, SRY can induce a large conformational change involving DNA helix unwinding, minor groove expansion, and DNA bending. These events collectively represent a molecular switch, allowing interactions between proteins bound at distant sites on the DNA to modulate transcription (Harley et al. 2003).

The N-terminal domain of SRY has also been shown to play an important role in DNA binding, with phosphorylation, by cAMP-dependent protein kinase (PKA), of serine residues at the N-terminal enhancing binding in the case of hSRY to DNA. Intriguingly, impaired PKA phosphorylation due to the R30I mutation of the PKA recognition site ($RRSSS^{33}$) reduces DNA binding activity, resulting in XY sex reversal (Assumpcao et al. 2002). Recent data also indicates the importance of the C-terminal domain of SRY in contributing to DNA binding in vitro (Sanchez-Moreno et al. 2009).

Timing of *Sry* expression is clearly crucial for testis development, as is the appropriate cellular environment provided by the Sertoli cells through provision



Sry, Fig. 2 *Functional Domains of SRY*. Schematic diagram of the domain structures for the *Mus musculus molossinus* and *Homo sapiens* SRY proteins. *Dotted lines* denote regions within SRY mediating interactions with the indicated binding partners. Key: *CaM* calmodulin, *HMG* High mobility group, *Impβ1* Importin β1, *KRAB-O* Kruppel-associated box only, *NLS* nuclear localization signal, *PDZ* Protein–protein interacting domain, *Q-rich* Glutamine-rich, *SIP*-1, SRY interacting protein 1, *WT1* Wilm's tumor gene 1

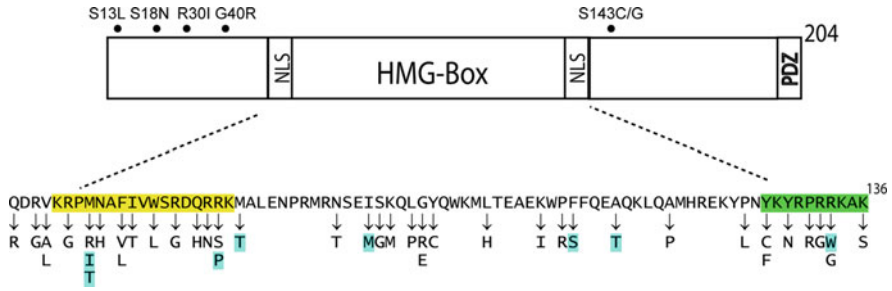
of cofactors, posttranslational modification, subcellular localization, and access to target DNA (Oh and Lau 2006). Although DNA binding, through the HMG domain, appears to be critical for sex determination, several proteins have also been identified to interact with SRY (see Fig. 2). The SRY-Interacting Protein 1 (SIP-1/NHERF-2), for example, may perhaps be involved in the modulation of SRY transcriptional activity, interacting through its protein–protein interacting (PDZ) domain, with the Thr-Lys-Leu (KTL) motif in hSRY's C-terminus, and the bridge domain in mSRY (Sekido and Lovell-Badge 2009).

KRAB-O (Kruppel-associated box only) has also been found to interact with SRY (see Fig. 2) through the bridge domain of mSRY and a domain adjacent to the hSRY HMG-box (aa 138–155) (Oh and Lau 2006). KRAB-O is believed to be involved in modulating SRY transcriptional activity, where it interacts directly with the KRAB-association protein 1 (KAP1) and indirectly with heterochromatin protein 1 (HP1), which acts as a transcriptional repressor (Oh and Lau 2006). Intriguingly, reduced expression of *Sox9* is observed in KRAB-O knockout mouse models, although no effects are evident in terms of testis development (Oh and Lau 2006).

Wilm's tumor gene (WT1; see also above) is also essential for testis development, with mutations such as R394W and D369N impairing the interaction of WT1 with SRY (see Fig. 2) and resulting in reduced transactivation, leading to Denys-Drash syndrome (DDS), which is characterized by Wilm's tumor, pseudohermaphroditism, and neuropathy (Matsuzawa-Watanabe et al. 2003). Other proteins that interact with SRY include PARP1 (poly(ADP-ribose) polymerase 1), which upon binding SRY's HMG domain is thought to interfere with SRY's DNA binding activity (Li et al. 2006).

SRY and Sex Reversal

That, as a general rule, XY embryos become males and XX embryos become females does not hold true for sex-reversed individuals where the normal process of sexual differentiation has been altered. Since gonadal differentiation in most mammals cannot be induced by environmental variations or external manipulations, these must arise from genetic abnormalities. XX female-to-male sex reversal is a rare event, observed in 1:20,000 newborns, with 80% of cases due to *Sry* translocation. However, XY male-to-female sex reversal occurs at a frequency of 1:3,000 in newborns (Sarafoglou and Ostrer 2000), although only 15% of all cases can be attributed to mutations within *Sry*,



Sry, Fig. 3 *Sex-reversing mutations in human SRY cluster in the HMG-box domain.* Sex reversing point mutations are denoted by *arrows*, with the amino acid substitution indicated. Few point mutations are located outside the HMG-box domain, indicated by the *circles* above the N- and C-terminal domains. The N-terminal "CaM-NLS" (*yellow*) and the C-terminal "β-NLS" (*green*) are indicated, with mutations highlighted in *blue* having no significant affects on SRY's abilities to bind/bend DNA

consistent with the idea that a number of other key genes are involved in sex determination, such as *Sox9*, *Sf1*, and *Wt1*.

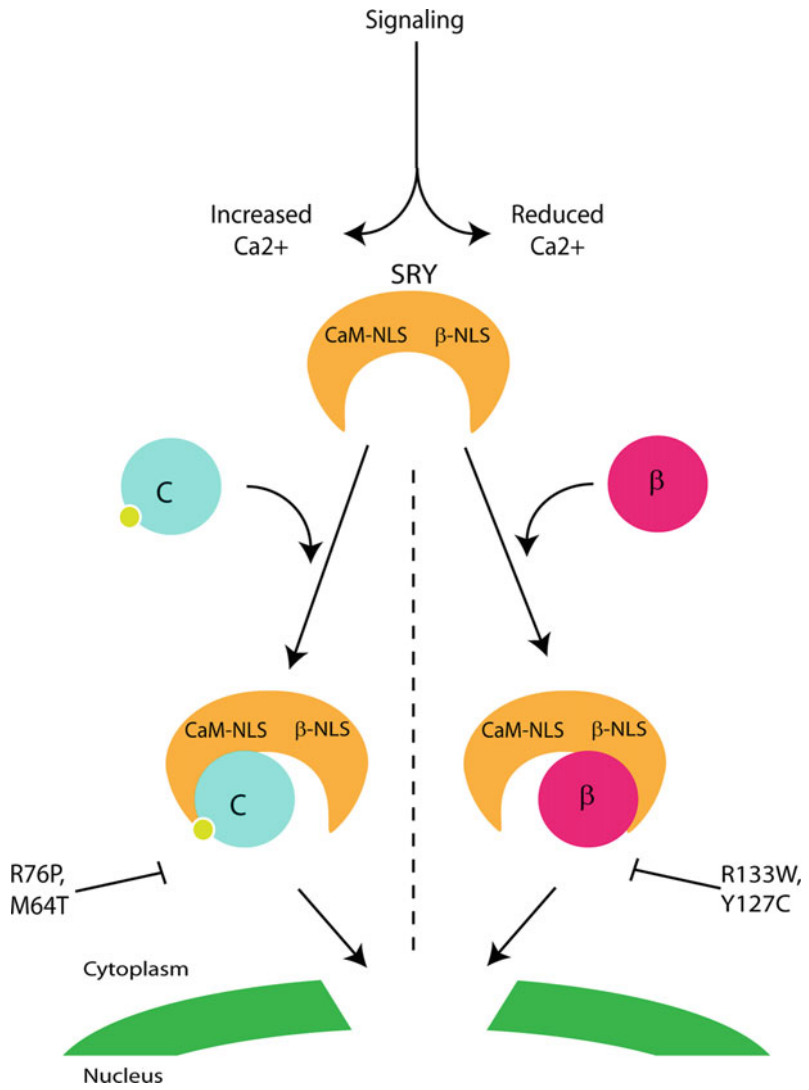
Almost all patients with SRY mutations show pure or complete gonadal dysgenesis (CGD), lacking testicular development with well-developed Mullerian structures and streak gonads in place of normal ovaries (Sarafoglou and Ostrer 2000). XY sex reversal patients develop as normal females with female internal and external genitalia, but are sterile, lack ovarian function, and present clinically with primary amenorrhea (Sarafoglou and Ostrer 2000). About 50% of XY female sex reversal cases show gonadoblastomas, additional to CGD, necessitating surgical removal of gonadal tissues (Mitchell and Harley 2002). The majority of SRY sex-reversing mutations occur de novo, thus affecting only one individual in the family (Mitchell and Harley 2002). However, mutations in the HMG domain have also been described in fathers of XY females. In these so-called familial cases, it is thought that genetic background compensates for the mutation in the father, but not in the affected individual (Mitchell and Harley 2002).

Sex reversing mutations in h*Sry*, including frameshift, nonsense or missense mutations, are largely clustered in the central region of the HMG domain, consistent with the idea that the DNA binding motif plays an essential role in vivo. Of the approximately 50 known *Sry* missense mutations (see Fig. 3), 4 mutations are found in the N-terminal domain (S3L, S18N, R30I, and G40R), with 2 more recently characterized mutations localized to the C-terminal region (S143C and S143G), highlighting the importance of these domains in sex determination (Assumpcao et al. 2002; Sanchez-Moreno et al. 2009). All other SRY missense mutations reside within the HMG-box, indicating that mutations that impair interaction between SRY and its DNA targets or protein-binding partners result in sex reversal. Importantly, a number of sex reversing mutations (e.g. R76P, R133W) do not impair DNA binding or bending significantly, but appear to affect nuclear accumulation specifically (Kaur et al. 2010).

Nuclear Entry of SRY

SRY gains access to the nucleus through the arginine-rich N-terminal (^{61}KRPMNAFIVWSRDQRRK) CaM- and C-terminal (^{126}KYRPRRKAK) β-NLSs at either end of the HMG domain (Kaur and Jans 2011). Nuclear import of SRY via its β-NLS is mediated by Impβ1/RanGTP transport factors of the importin (Imp) superfamily which mediate translocation of the cargo through the nuclear pore complex for subsequent release into the nucleus (see Fig. 4) (Kaur and Jans 2011). Interestingly, Impβ1 binding and nuclear accumulation has been reported to be enhanced by acetylation at residue K136, by the histone acetyl transferase p300, while deacetylation by histone deacetylase 3 (HDAC3) induces relocation of SRY from the nucleus to the cytoplasm (Thevenet et al. 2004).

Nuclear import via the CaM-NLS is mediated in an unconventional manner through direct interaction with the calcium-binding protein calmodulin (CaM), in a Ca^{2+}-dependent and Ran-independent manner (see Fig. 4) (Kaur and Jans 2011); precisely how CaM mediates nuclear import is not known but it seems



Sry, Fig. 4 *Schematic representation of SRY's dual nuclear import pathways; Ca^{2+} as a switch*. SRY possesses dual NLSs; the CaM-NLS mediates nuclear import through interaction with CaM (C) in Ca^{2+}-bound state (*green circle*) and the β-NLS, which mediates nuclear import through binding Impβ1 (β); both CaM and Impβ1 bind SRY in a mutually exclusive manner, presumably through a simple masking mechanism. Cellular signals that increase intracellular Ca^{2+} levels facilitate nuclear import through the CaM-NLS by enabling CaM to bind to SRY, as well as hindering transport through the β-NLS. Under non-stimulated conditions (low intracellular Ca^{2+}), CaM is unable to bind to the CaM-NLS, allowing Impβ1 to bind to the β-NLS to mediate nuclear import. Sex-reversing mutations that specifically inhibit CaM or Impβ1 binding, and hence nuclear import in the presence or absence of elevated intracellular Ca^{2+}, respectively, are delineated (single letter code); all evidence suggests that both NLS-dependent nuclear import pathways are required for male sex determination, so that optimal SRY nuclear import can occur under conditions of both high and low intracellular Ca^{2+}

reasonable to suggest that CaM is an adaptor binding SRY to another molecule that then transports SRY through the nuclear pore. Intriguingly, changes in intracellular Ca^{2+} concentrations may also play a role in modulating nuclear accumulation of SRY through switching between the nuclear import mechanisms, with impaired nuclear import via the β-NLS pathway observed in the presence of increasing Ca^{2+} concentrations (Kaur and Jans 2011). What is clear, however, is that both NLSs are essential for SRY's role in sex determination, since mutations in the β-NLS (R133W) and CaM-NLS (M64T and R76P) that do not affect

DNA binding/bending but result in reduced binding to Impβ1 and CaM, respectively, result in sex reversal (Kaur et al. 2010).

Targets of SRY Action

SRY clearly can act as a transcriptional activator, with *Sox9* being a strong candidate as a direct target of SRY. *Sox9* is strongly upregulated soon after *Sry* expression, with *Sry* positive cells becoming *Sox9* positive cells as well in mouse testis (Sekido and Lovell-Badge 2009). More recently, transgenic mice studies have demonstrated that SRY, acting synergistically with SF1, can induce the upregulation of *Sox9* transcription through a testicular specific enhancer of *Sox9* core (TESCO) situated 14 kb upstream of the *Sox9*'s start codon, with SF1 and SRY found to directly bind multiple elements within TES in vivo (Sekido and Lovell-Badge 2009). Consistent with the idea that *Sox9* is a key target of SRY action, a number of studies indicate that SOX9 can replace SRY function (Sekido and Lovell-Badge 2009). Sex reversal resulting from mutations in *Sox9* has also been described in XY campomelic dysplasia (CD) patients (Sekido and Lovell-Badge 2009).

Recent evidence suggests that SRY mediates testis development by repressing Wnt/β-catenin signaling (see Fig. 1), which is stimulated by a small secreted factor, R-spondin 1 (RSPO1; see Fig. 1). RSPO1 synergizes with Wnt4 to activate ovarian development in XX females, where mutations in *Rspo1* have been found to result in female-to-male sex reversal (Lau and Li 2009). SRY's role appears to be through binding to β-catenin via the HMG-box in the case of hSRY, and the HMG-box and Q-rich domains in the case of mSRY (see Fig. 2) (Sekido and Lovell-Badge 2009). SRY inhibits Wnt/β-catenin signaling in the nucleus at the level of β-catenin, possibly through inducing degradation of β-catenin upon SRY binding, or interfering with β-catenin's ability to transactivate target genes, thus, acting to repress ovarian differentiation and thereby promote male sex development (Lau and Li 2009).

Summary

SRY plays a key role in mammalian sex determination with mutations in *Sry* resulting in sex reversal. Since its discovery several decades ago, slow progress has been made in determining the mechanisms of SRY function. Lack of experimental systems, especially in humans, has limited the study of sex determination to the mouse, with further limitations due to the brief period of expression of *Sry* in a small number of embryonic cells. It is clear however, that upregulation of SOX9 by SRY is a key event in mediating male sex differentiation, with emerging evidence implicating a role for SRY in repressing ovarian development through Wnt/β-catenin. Significantly, the dual nuclear import mechanisms of SRY play a key role in male sex determination, with mutations therein resulting in impaired SRY targeting to the nucleus and sex reversal. Intriguingly, calcium plays a role in switching between the two import mechanisms of SRY to enable efficient SRY nuclear accumulation to be maintained under various physiological conditions. A key to understanding the development of the male sex will be in unraveling the regulation of *Sry* expression in early gonadal development, the dynamic movements of SRY within the cell during its brief period of expression, and the interplay of key sex determining proteins leading to repressed ovarian function and favored testis development.

References

Assumpcao JG, Benedetti CE, Maciel-Guerra AT, Guerra Jr G, Baptista MT, Scolfaro MR, et al. Novel mutations affecting SRY DNA-binding activity: the HMG box N65H associated with 46, XY pure gonadal dysgenesis and the familial non-HMG box R30I associated with variable phenotypes. J Mol Med. 2002;80(12):782–90.

Buaas FW, Val P, Swain A. The transcription co-factor CITED2 functions during sex determination and early gonad development. Hum Mol Genet. 2009;18(16):2989–3001.

Harley VR, Clarkson MJ, Argentaro A. The molecular action and regulation of the testis-determining factors, SRY (sex-determining region on the Y chromosome) and SOX9 [SRY-related high-mobility group (HMG) box 9]. Endocr Rev. 2003;24(4):466–87.

Hiramatsu R, Matoba S, Kanai-Azuma M, Tsunekawa N, Katoh-Fukui Y, Kurohmaru M, et al. A critical time window of Sry action in gonadal sex determination in mice. Development. 2009;136(1):129–38.

Jost A, Vigier B, Prepin J, Perchellet JP. Studies on sex differentiation in mammals. Recent Prog Horm Res. 1973;29:1–41.

Kaur G, Delluc-Clavieres A, Poon IK, Forwood JK, Glover DJ, Jans DA. Calmodulin-dependent nuclear import of HMG-box family nuclear factors; importance for the role of SRY in sex reversal. Biochem J. 2010;430(1):39–48.

Kaur G, Jans DA. Dual nuclear import mechanisms of sex determining factor SRY: intracellular Ca2+ as a switch. FASEB J. 2011;25(2):665–75.

Lau YF, Li Y. The human and mouse sex-determining SRY genes repress the Rspol/beta-catenin signaling. J Genet Genomics. 2009;36(4):193–202.
Li Y, Oh HJ, Lau YF. The poly(ADP-ribose) polymerase 1 interacts with Sry and modulates its biological functions. Mol Cell Endocrinol. 2006;257–258:35–46.
Matsuzawa-Watanabe Y, Inoue J, Semba K. Transcriptional activity of testis-determining factor SRY is modulated by the Wilms' tumor 1 gene product, WT1. Oncogene. 2003;22(39):7900–4.
Mitchell CL, Harley VR. Biochemical defects in eight SRY missense mutations causing XY gonadal dysgenesis. Mol Genet Metab. 2002;77(3):217–25.
Oh HJ, Lau YF. KRAB: a partner for SRY action on chromatin. Mol Cell Endocrinol. 2006;247(1–2):47–52.
Piprek RP. Genetic mechanisms underlying male sex determination in mammals. J Appl Genet. 2009;50(4):347–60.
Polanco JC, Koopman P. Sry and the hesitant beginnings of male development. Dev Biol. 2007;302(1):13–24.
Sanchez-Moreno I, Canto P, Munguia P, de Leon MB, Cisneros B, Vilchis F, et al. DNA binding activity studies and computational approach of mutant SRY in patients with 46, XY complete pure gonadal dysgenesis. Mol Cell Endocrinol. 2009;299(2):212–8.
Sarafoglou K, Ostrer H. Clinical review 111: familial sex reversal: a review. J Clin Endocrinol Metab. 2000;85(2):483–93.
Sekido R, Lovell-Badge R. Sex determination and SRY: down to a wink and a nudge? Trends Genet. 2009;25(1):19–29.
Thevenet L, Mejean C, Moniot B, Bonneaud N, Galeotti N, Aldrian-Herrada G, et al. Regulation of human SRY subcellular distribution by its acetylation/deacetylation. EMBO J. 2004;23(16):3336–45.
Vilain E, McCabe ER. Mammalian sex determination: from gonads to brain. Mol Genet Metab. 1998;65(2):74–84.

SSI (STAT-Induced STAT Inhibitor)

► SOCS

SSTR

► Somatostatin Receptor

STANK

► HIPK2

STAR

► Guanylyl Cyclase C

STAT1

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STAT2

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STAT3

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STAT4

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STAT5A

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STAT5B

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STAT6

► Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STAT92E

▶ Phospho- and Unphospho-STATs in Signal Transduction and Gene Regulation (STAT)

STD

▶ ZAP-70

Sterile Alpha and TIR Motif-Containing Protein

▶ Toll-like Receptor Adaptor Protein Family Members

Sterile-Alpha Motif and Leucine Zipper–Containing Kinase AZK (ZAK)

▶ MLTK

Steroid Receptor Coactivator Family

Marc J. Tetel[1] and Pui Man Rosalind Lai[1,2]
[1]Neuroscience Program, Wellesley College, Wellesley, MA, USA
[2]Harvard Medical School, Boston, MA, USA

Synonyms

ACTR; AIB1; GRIP1; NcoA-1; NCoA-2; pCIP; RAC3; SRC-1; SRC-2; SRC-3; TIF2; TRAM-1

Historical Background

Steroid hormones have profound effects on physiology and behavior. Most of these biological effects of steroid hormones are mediated through their respective receptors, which are members of the steroid/nuclear receptor superfamily of transcriptional activators. These receptors can act in a classic genomic mechanism by interacting directly with DNA to alter transcription or at the membrane to rapidly activate cytoplasmic signaling pathways (Tetel and Lange 2009). In the classic genomic mechanism of action, nuclear receptor coregulators act to enhance (coactivators) or repress (corepressors) the transcriptional activity of these receptors. While over 300 coactivators have been identified to function in receptor transcription, the role of these coactivators in a wide range of human diseases is becoming better understood (Lonard et al. 2010). This review will focus on the function of the steroid receptor coactivator family as signaling molecules in physiology and behavior and in human disease.

p160 Steroid Receptor Coactivator Family

The steroid receptor coactivator (SRC) family of p160 proteins consists of SRC-1 (NcoA-1), SRC-2 (GRIP1/TIF2/NCoA-2), and SRC-3 (AIB1/TRAM-1/ACTR/RAC3/pCIP). The SRC family and the other nuclear receptor coactivators share a common set of characteristics. The SRC family of coactivators physically interacts with steroid receptors, including receptors for androgens (AR), estrogens (ER), progestins (PR), and glucocorticoids (GR), in a ligand-dependent manner (Johnson and O'Malley 2011). The SRCs physically associate with agonist-bound receptors through centrally located multiple LXXLL motifs (L, leucine; X, any amino acid) that make up nuclear receptor (NR) boxes. The SRCs, as well as other coactivators, do not bind DNA and thus differentiate them from traditional transcription factors. The C-terminus of the SRCs contains two activation domains (AD-1 and AD-2). The N-terminus contains a third activation domain (AD-3) and a bHLH-PAS motif (basic helix loop helix-Per Arnt Sims), which is the most conserved domain within this family of proteins. The activation domains interact with secondary coactivators known as co-coactivators. These co-coactivators act as bridging molecules between the receptor and the general transcription machinery and modify chromatin within the promoter and enhancer regions by histone acetylation and methylation (Johnson and O'Malley 2011).

p160 SRC Family in Physiology

SRC-1

In addition to what is known about the molecular mechanisms of the SRCs from in vitro studies, more is being learned about their role in hormone action in vivo. SRC-1 knockout mice, while fertile, have decreased responsiveness in progestin target tissues (Xu et al. 1998), partial resistance to thyroid hormone (Weiss et al. 1999), and delayed development of cerebellar Purkinje cells (Nishihara et al. 2003). Interestingly, in these mice SRC-2 is up-regulated in steroid-sensitive tissues, including brain and testes, suggesting that increased expression of SRC-2 compensates for the loss of SRC-1 (Xu et al. 1998).

SRC-2

Studies of SRC-2 knockout mice reveal that this coactivator is important in fertility and ductal branching in mammary gland (Fernandez-Valdivia et al. 2007). Disruption of SRC-2 expression in uterine PR-positive cells of $PR^{Cre/+}SRC\text{-}2^{flox/flox}$ mice led to an early block in embryo implantation (Fernandez-Valdivia et al. 2007). Furthermore, removal of SRC-1 in $PR^{Cre/+}SRC\text{-}2^{flox/flox}$ uteri caused a block in decidualization, suggesting that both SRC-1 and SRC-2 are required for complete PR-dependent decidualization. In addition, SRC-2 is important for PR action in mammary gland as demonstrated by the lack of significant branching and alveolar morphogenesis in the $PR^{Cre/+}SRC\text{-}2^{flox/flox}$ mammary gland (Fernandez-Valdivia et al. 2007). Microarray analysis of uteri from SRC-2 null mice reveals that SRC-2 is involved in the ability of progesterone to repress specific genes involved in a variety of functions, including cell cycle and immunity (Jeong et al. 2007).

SRC-3

Female SRC-3 null mice, while fertile, have delayed puberty and longer estrous cycles, ovulate fewer eggs, and have impaired mammary gland development (Han et al. 2006). Studies in SRC-3 null mice reveal that this coactivator is critical for normal PR-dependent mammary gland development and function (Han et al. 2006). Interestingly, gonadotropin-releasing hormone (GnRH) can activate PR-dependent transcription of a reporter gene in a pituitary cell line (An et al. 2006). Knockdown of SRC-3 by siRNA abolishes this effect, suggesting that SRC-3 is required for this GnRH-induced activation of PR (An et al. 2006).

p160 SRC Family in Metabolism and Adipogenesis

SRC-1

All three members of the p160 family of coactivators are involved in metabolic homeostasis and adipogenesis, which increases risk for clinical conditions including cardiovascular disease and diabetes. SRC-1 is critical in maintaining energy balance by regulating both energy intake and expenditure (Louet and O'Malley 2007). In support, SRC-1 knockout mice have decreased energy expenditure and a reduced thermogenic capacity and are, thus, prone to obesity. One proposed mechanism for SRC-1 in metabolism is through its interactions with PPARγ coactivator-1α (PGC-1α), a protein important in the control of mitochondrial biogenesis and oxidative phosphorylation (Louet and O'Malley 2007).

SRC-2

In contrast to SRC-1, SRC-2 knockout mice are leaner compared with wild type mice and have an increase in adaptive thermogenesis (Chopra et al. 2008). It appears that the absence of SRC-2 increases the interaction of SRC-1 with PGC-1α, enhancing thermogenic activity. Thus, it has been proposed that the ratio of SRC-1 and SRC-2 plays a role in maintaining the balance of energy expenditure and adipogenesis through controlling the metabolic activity of PGC-1α. In the liver, SRC-2 acts as a coactivator for the nuclear receptor RORα in the regulation of hepatic G6Pase, an important regulator of glucose production. Ablation of SRC-2 in mice leads to phenotypes of Von Gierke's disease, an inherited glycogen storage disease (Chopra et al. 2008).

SRC-3

SRC-3 knockout mice have lower body fat content compared with wild type animals (Louet and O'Malley 2007). SRC-3 controls the transcription of PPARγ2, important for adipocyte differentiation through enhancing the CAAT enhancer binding protein-β (Chopra et al. 2008). In support, adipocyte

differentiation and adipogenesis were impaired in both mouse embryonic fibroblasts from cells isolated from SRC-3 knockout mice and a knockdown of SRC-3 in 3T3-L1 adipocyte cells (Chopra et al. 2008). However, a knockdown of SRC-1 and SRC-2 had little effect on adipocyte differentiation. Taken together, these studies suggest that all three members of the p160 family of coactivators play an important role in energy homeostasis and adipogenesis.

SRC Family Functions in Brain and Behavior

A variety of recent studies indicate that two of the p160 SRC family members, SRC-1 and SRC-2, are important for hormone action in brain and the regulation of behavior (Tetel et al. 2009).

SRC-1 is expressed at high levels in the hypothalamus, cortex, and hippocampus of rodents (Tetel et al. 2009). Moreover, SRC-1 is expressed in the majority of estradiol-induced PR cells in regions involved in metabolism and female reproduction, including the ventromedial nucleus of the hypothalamus (VMN), medial preoptic area, and arcuate nucleus in rodents (Tognoni et al. 2011). Furthermore, SRC-1 interacts with steroid receptors; recent studies reveal that SRC-1 from brain physically associates with ER and PR in a receptor subtype- and brain region-specific manner (Molenda-Figueira et al. 2008). SRC-1 is critical for normal development of hormone-dependent sexual differentiation of the brain and adult sexual behavior (Auger et al. 2000). In the adult brain, SRC-1 functions in the VMN to modulate ER-mediated transactivation of the behaviorally relevant PR gene (Molenda et al. 2002). In addition, SRC-1 functions in distinct ER- and PR-dependent aspects of female sexual behavior (Molenda-Figueira et al. 2006). Interestingly, reduction of SRC-1 expression in brain by antisense altered PR function and reduced PR-dependent proceptivity (behavior by the female to solicit interaction by the male), but not PR-dependent receptivity (Molenda-Figueira et al. 2006). These findings suggest that reduction of SRC-1 by antisense disrupted the activity of PR signaling pathway(s) that influence(s) proceptivity, while alternate PR signaling pathways, that regulate PR-dependent receptivity, remained intact and functional.

SRC-2 is coexpressed with PR and ERα in rodent hypothalamus and physically associates with these receptors in a hormone-dependent manner (Yore et al. 2010; Tognoni et al. 2011). In further support of a role for SRC-2 in hormone action in brain, SRC-2 functions in estradiol-induction of hypothalamic PR and female sexual behavior in mice and rats (Apostolakis et al. 2002). Interestingly, in contrast to the other members of the SRC family, *SRC-3* is only sparsely expressed in the hypothalamus and does not appear to modulate the expression of reproductive behavior (Apostolakis et al. 2002).

p160 SRC Family in Breast and Prostate Cancer

SRC-1

SRC-1 has been found to be elevated in breast tumors and a strong predictor of breast cancer recurrence and hormone-independent tumors. In vitro studies demonstrate that SRC-1 promotes cancer metastasis through the estrogenic pathway. MCF-7 breast cancer cells over-expressed with SRC-1 have increased expression of estrogen-induced genes and enhanced estrogen-induced cell growth. However, MCF-7 cells treated with antisense against SRC-1 show a decrease in cell proliferation and invasion, as well as a lower level of SDF-1α, a protein that controls cell proliferation through autocrine and paracrine mechanisms (Xu et al. 2009). These findings suggest that SRC-1 increases breast cancer cell proliferation through regulating the SDF-1α pathway.

SRC-1 promotes estrogen-independent breast cancer metastasis through the integrin α5 (ITGA5) signaling pathway. In ER-negative tumors, SRC-1 expression is positively correlated with ITGA5, an important molecule involved in mediating cell adhesion and migration (Qin et al. 2011). In further support of SRC-1 promoting cancer proliferation through an estrogen-independent pathway, ITGA5 promoter activity is enhanced by SRC-1.

In prostate cancer, SRC-1 is correlated with increased tumor aggressiveness and promotes cell proliferation by enhancing AR activation and function (Agoulnik et al. 2006). The phosphorylation of SRC-1 by mitogen-activated protein kinase (MAPK) leads to an increase in AR activity (Suzuki et al. 2003). Reduction of SRC-1 in AR-positive cell lines, but not in AR-negative cell lines, resulted in

decreased tumor proliferation, suggesting the major effect of SRC-1 is through the androgenic pathway.

SRC-2

The role of SRC-2 in oncogenesis remains controversial. While one study reported a correlation of SRC-2 with cyclin D1 in ERα-positive breast tumors, another study found no changes in SRC-2 levels (Xu et al. 2009). However, the over-expression of SRC-2 in MCF-7 breast cancer cells led to a decrease in cell proliferation and invasion and a reduction in the expression of ERα target genes. In prostate cancer, SRC-2 expression correlates with recurrence of the disease. The down-regulation of SRC-2 by antisense reduced expression of AR-induced genes and decreased cell proliferation in both AR-dependent and AR-independent prostate cancer cell lines (Agoulnik et al. 2006).

SRC-3

SRC-3 (aka amplified in breast cancer-1, AIB1) is highly over-expressed in breast cancer and is correlated with ER and PR expression and tumor size (Anzick et al. 1997; Bautista et al. 1998). SRC-3 expression is also correlated with poor clinical prognosis. Breast cancer cells transfected with SRC-3 had enhanced estrogen-dependent transcription, suggesting that SRC-3 promotes tumorigenesis through an estrogenic pathway (Lanz et al. 2010). Several mechanisms have been proposed on how SRC-3 acts to promote cancer growth. SRC-3 regulates E2F1-mediated cell cycle progression and GRB-2 associated binding protein2, which activates the AKT/▶ mTOR pathway and increases cancer growth. SRC-3 has also been shown to activate epidermal growth factor receptor EGFR and ERBB2, resulting in the hyperactivation of Akt and MAPK which contributes to cancer proliferation, growth, and migration (Yan et al. 2008). In further support, Akt signaling is down-regulated in SRC-3 knockout mice. In another study, SRC-3 increased PEA3 and AP-mediated matrix metalloproteinase expression which promote breast and prostate tumor cell metastasis (Yan et al. 2008).

In prostate cancer, SRC-3 is correlated with both tumor stage and poor clinical prognosis (Culig et al. 2004). SRC-3 is involved in the androgenic pathway by acting as a coactivator for AR. However, SRC-3 is also involved in androgen-independent cancer proliferation as the down-regulation of SRC-3 in androgen-insensitive prostate cancer cells also leads to a decrease in proliferation (Zou et al. 2006).

Summary

The p160 family of nuclear receptor coactivators has a critical role in modulating steroid receptor action and thus the appropriate cellular response to steroids. A variety of studies indicate that these coactivators are essential in physiology, including metabolism and adipogenesis. In addition, this family of coactivators functions in brain to regulate important aspects of steroid action and the regulation of behavior. Thus, it is no surprise that alteration of the function of these molecules can contribute to steroid-responsive cancers, such as breast and prostate cancer, in profound ways. The findings reviewed above indicate that each of these members of the p160 family of coactivators can mediate distinct signaling pathways of steroid receptors. Therefore, understanding the recruitment of different coactivator complexes to the promoter, which is likely to be cell- and tissue-specific, will be critical for understanding how hormones regulate complex physiological and behavioral events, as well as hormone-dependent diseases.

Acknowledgments Studies contributed by the author's laboratory were supported by grants from National Science Foundation IBN 0080818 and National Institutes of Health R01 DK61935 (MJT).

References

Agoulnik IU, Vaid A, Nakka M, Alvarado M, Bingman WE, Erdem H, Frolov A, Smith CL, Ayala GE, Ittmann MM, Weigel NL. Androgens modulate expression of transcription intermediary factor 2, an androgen receptor coactivator whose expression level correlates with early biochemical recurrence in prostate cancer. Cancer Res. 2006;66:10594–602.

An BS, Selva DM, Hammond GL, Rivero-Muller A, Rahman N, Leung PC. Steroid receptor coactivator-3 is required for progesterone receptor trans-activation of target genes in response to gonadotropin-releasing hormone treatment of pituitary cells. J Biol Chem. 2006;281:20817–24.

Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS.

AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science. 1997;277:965–8.
Apostolakis EM, Ramamurphy M, Zhou D, Onate S, O'Malley B. Acute disruption of select steroid receptor coactivators prevents reproductive behavior in rats and unmasks genetic adaptation in knockout mice. Mol Endocrinol. 2002;16:1511–23.
Auger AP, Tetel MJ, McCarthy MM. Steroid receptor coactivator-1 mediates the development of sex specific brain morphology and behavior. Proc Natl Acad Sci USA. 2000;97:7551–5.
Bautista S, Valles H, Walker RL, Anzick S, Zeillinger R, Meltzer P, Theillet C. In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. Clin Cancer Res. 1998;4:2925–9.
Chopra AR, Louet JF, Saha P, An J, Demayo F, Xu J, York B, Karpen S, Finegold M, Moore D, Chan L, Newgard CB, O'Malley BW. Absence of the SRC-2 coactivator results in a glycogenopathy resembling Von Gierke's disease. Science. 2008;322:1395–9.
Culig Z, Comuzzi B, Steiner H, Bartsch G, Hobisch A. Expression and function of androgen receptor coactivators in prostate cancer. J Steroid Biochem Mol Biol. 2004;92:265–71.
Fernandez-Valdivia R, Mukherjee A, Amato P, Allred DC, Nguyen J, DeMayo FJ, Lydon JP. Progesterone-action in the murine uterus and mammary gland requires steroid receptor coactivator 2: relevance to the human. Front Biosci. 2007;12:3640–7.
Han SJ, Demayo FJ, Xu J, Tsai SY, Tsai MJ, O'Malley BW. Steroid receptor coactivator (SRC)-1 and SRC-3 differentially modulate tissue-specific activation functions of the progesterone receptor. Mol Endocrinol. 2006;20:45–55.
Jeong JW, Lee KY, Han SJ, Aronow BJ, Lydon JP, O'Malley BW, Demayo FJ. The p160 steroid receptor coactivator-2, SRC-2, regulates murine endometrial function and regulates progesterone-independent and -dependent gene expression. Endocrinology. 2007;148:4238–50.
Johnson AB, O'Malley BW. Steroid receptor coactivators 1, 2 and 3: critical regulators of nuclear receptor activity and steroid receptor modulator (SRM)-based cancer therapy. Mol Cell Endocrinol. In press 2011.
Lanz RB, Bulynko Y, Malovannaya A, Labhart P, Wang L, Li W, Qin J, Harper M, O'Malley BW. Global characterization of transcriptional impact of the SRC-3 coregulator. Mol Endocrinol (Baltim). 2010;24:859–72.
Lonard DM, Kumar R, O'Malley BW. Minireview: the SRC family of coactivators: an entree to understanding a subset of polygenic diseases? Mol Endocrinol (Baltim). 2010;24:279–85.
Louet JF, O'Malley BW. Coregulators in adipogenesis: what could we learn from the SRC (p160) coactivator family? Cell Cycle. 2007;6:2448–52.
Molenda HA, Griffin AL, Auger AP, McCarthy MM, Tetel MJ. Nuclear receptor coactivators modulate hormone-dependent gene expression in brain and female reproductive behavior in rats. Endocrinology. 2002;143:436–44.
Molenda-Figueira HA, Williams CA, Griffin AL, Rutledge EM, Blaustein JD, Tetel MJ. Nuclear receptor coactivators function in estrogen receptor- and progestin receptor-dependent aspects of sexual behavior in female rats. Horm Behav. 2006;50:383–92.
Molenda-Figueira HA, Murphy SD, Shea KL, Siegal NK, Zhao Y, Chadwick JG, Denner LA, Tetel MJ. Steroid receptor coactivator-1 from brain physically interacts differentially with steroid receptor subtypes. Endocrinology. 2008;149:5272–9.
Nishihara E, Yoshida-Kimoya H, Chan C, Liao L, Davis RL, O'Malley BW, Xu J. SRC-1 null mice exhibit moderate motor dysfunction and delayed development of cerebellar Purkinje cells. J Neurosci. 2003;23:213–22.
Qin L, Chen X, Wu Y, Feng Z, He T, Wang L, Liao L, Xu J. Steroid receptor coactivator-1 upregulates integrin $\alpha 5$ expression to promote breast cancer cell adhesion and migration. Cancer Res. 2011;71:1742–51.
Suzuki H, Ueda T, Ichikawa T, Ito H. Androgen receptor involvement in the progression of prostate cancer. Endocr Relat Cancer. 2003;10:209–16.
Tetel MJ, Lange CA. Molecular genomics of progestin actions. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, editors. Hormones, brain and behavior. San Diego: Academic; 2009. p. 1439–65.
Tetel MJ, Auger AP, Charlier TD. Who's in charge? Nuclear receptor coactivator and corepressor function in brain and behavior. Front Neuroendocrinol. 2009;30:328–42.
Tognoni CM, Chadwick Jr JG, Ackeifi CA, Tetel MJ. Nuclear receptor coactivators are coexpressed with steroid receptors and regulated by estradiol in mouse brain. Neuroendocrinology. 2011;94(1):49–57.
Weiss RE, Xu J, Ning G, Pohlenz J, O'Malley BW, Refetoff S. Mice deficient in the steroid receptor co-activator 1 (SRC-1) are resistant to thyroid hormone. EMBO J. 1999;18:1900–4.
Xu J, Qiu Y, Demayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science. 1998;279:1922–5.
Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer. 2009;9:615–30.
Yan J, Erdem H, Li R, Cai Y, Ayala G, Ittmann M, Yu-Lee LY, Tsai SY, Tsai MJ. Steroid receptor coactivator-3/AIB1 promotes cell migration and invasiveness through focal adhesion turnover and matrix metalloproteinase expression. Cancer Res. 2008;68:5460–8.
Yore MA, Im D, Webb LK, Zhao Y, Chadwick JG, Molenda-Figueira HA, Haidacher SJ, Denner L, Tetel MJ. Steroid receptor coactivator-2 expression in brain and physical associations with steroid receptors. Neuroscience. 2010;169:1017–28.
Zou JX, Zhong Z, Shi XB, Tepper CG, deVere White RW, Kung HJ, Chen H. ACTR/AIB1/SRC-3 and androgen receptor control prostate cancer cell proliferation and tumor growth through direct control of cell cycle genes. Prostate. 2006;66:1474–86.

STK17B

► DRAK2

Striatal-Enriched Protein Tyrosine Phosphatase (STEP)

Pradeep Kurup[1], Jian Xu[1], Susan Goebel-Goody[1], Surojit Paul[2] and Paul Lombroso[1]
[1]Child Study Center, School of Medicine, Yale University, New Haven, CT, USA
[2]Neurology, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Synonyms

PTPN5

Historical Background

Protein tyrosine phosphatases (PTPs) play a significant role in diverse signaling mechanisms ranging from cellular differentiation to synaptic plasticity (Paul and Lombroso 2003; Tonks 2006). *ST*riatal-*E*nriched protein tyrosine *P*hosphatase (STEP) is a brain-specific protein tyrosine phosphatase belonging to the non-receptor tyrosine phosphatase family. Although enriched in the striatum, it is also localized to neurons in the cortex, hippocampus, and related brain regions (Boulanger et al. 1995). Interestingly, it is absent in cerebellum, where a highly related PTP, PTP-STEP-like is present (Watanabe et al. 1998). STEP exists as two major isoforms, named after their mobility in SDS-PAGE and designated $STEP_{61}$ and $STEP_{46}$. These isoforms are produced by alternative splicing of a single *STEP* (*Ptpn5*) gene (Bult et al. 1997). Since its discovery 20 years ago (Lombroso et al. 1991), a series of studies have established a role for STEP in opposing the development of synaptic strengthening in neurons. This occurs through STEP-mediated tyrosine (Tyr) dephosphorylation and inactivation of a number of key signaling molecules and receptors that are required for normal synaptic function (Braithwaite et al. 2006). As will be discussed at length below, disruption of STEP function is implicated in several neuropsychiatric disorders due to this critical regulatory function at synapses.

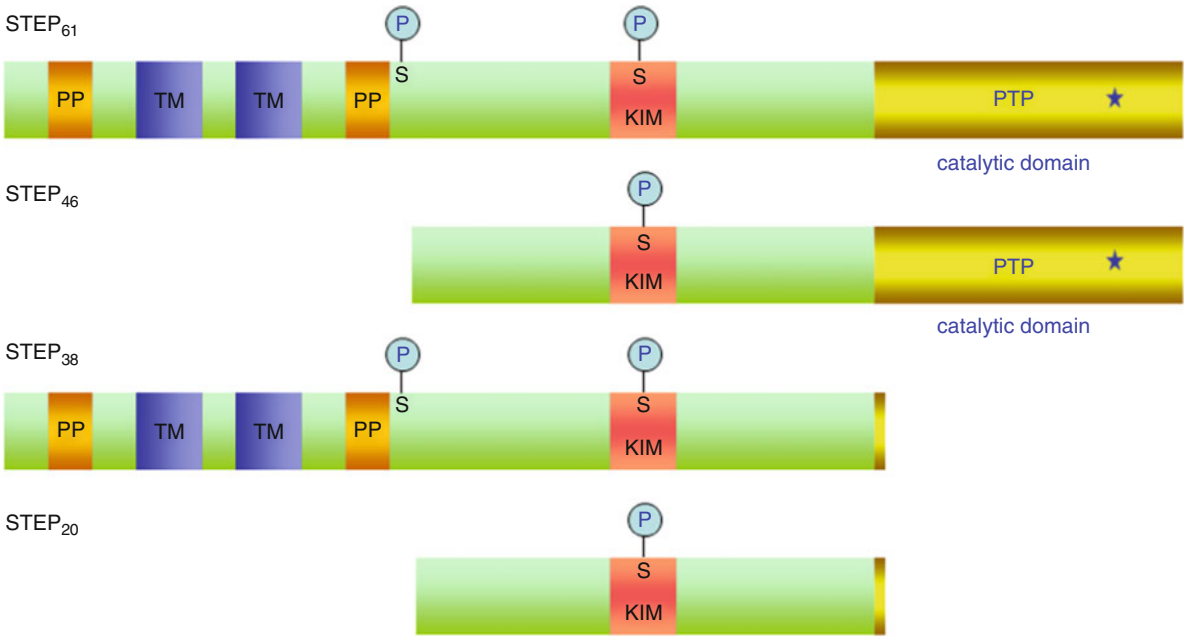
Domain Structure

Like other PTPs, STEP has a highly conserved catalytic domain ([I/V]HCxAGxxR[S/T]G) within the ~280 amino acid phosphatase domain that is present at its C-terminal region. In addition to the catalytic domain, various amino acid motifs exist that are responsible for the interaction of STEP isoforms with diverse signaling proteins (Fig. 1). Both $STEP_{61}$ and $STEP_{46}$ contain a regulatory kinase-interacting motif (KIM) that is required for the interaction of STEP with its substrates that include Fyn tyrosine kinase and two members of the mitogen-activated protein kinase family (MAPK), ERK (extracellular-regulated kinase) and p38 MAPKs (Pulido et al. 1998; Nguyen et al. 2002; Poddar et al. 2010). The KIM domain in STEP has a serine phosphorylation site for protein kinase A (PKA), and phosphorylation of this site sterically prevents the interaction of STEP with its substrates (Paul et al. 2000).

$STEP_{61}$ has an additional 172 amino acids at its N-terminus that consists of two hydrophobic domains, two polyproline-rich regions and PEST sequences. The hydrophobic domains in $STEP_{61}$ are responsible for targeting it to membrane compartments, including the endoplasmic reticulum and postsynaptic density (PSD) (Lombroso et al. 1993; Boulanger et al. 1995; Oyama et al. 1995). One of the polyproline-rich domains is necessary for the interaction of $STEP_{61}$ with its substrate Fyn (Nguyen et al. 2002). The PEST sequences in $STEP_{61}$ serve as potential signals for proteolytic cleavage sites under certain physiological conditions (Nguyen et al. 1999). Two additional isoforms of STEP are reported ($STEP_{38}$ and $STEP_{20}$), which are produced by alternative splicing and lack a catalytic domain (Sharma et al. 1995). The exact functions of these isoforms are not known, but they may act as dominant-negative isoforms to protect STEP substrates from dephosphorylation while trafficking within neurons and future studies are needed to address the role of these family members.

Regulation of STEP

STEP activity is modulated by phosphorylation, local translation, proteolytic cleavage, ubiquitination, and oligomerization (Fig. 2). Alterations in STEP activity



Striatal-Enriched Protein Tyrosine Phosphatase (STEP), Fig. 1 *Domain structure of STEP*. Alternative splicing of STEP gene produces four STEP isoforms, $STEP_{61}$, $STEP_{46}$, $STEP_{38}$, and $STEP_{20}$. $STEP_{61}$ contains two transmembrane (TM) domains and two polyproline-rich sequences (PP) in the N-terminal 172 region, whereas $STEP_{46}$ lacks this region. Both $STEP_{46}$ and $STEP_{61}$ share the binding site for ERK, the kinase-interacting motif (KIM), and the PTP catalytic domain, which contains an 11-amino-acid catalytic site (*asterisks*). $STEP_{61}$ has two serine residues that are phosphorylated by PKA (S), whereas $STEP_{46}$ contains one PKA site, within the KIM domain. $STEP_{38}$ and $STEP_{20}$ are catalytically inactive, due to the absence of PTP domain and possibly function as dominant-negative variants toward active STEP

by these mechanisms have a significant impact on normal synaptic function as well as various disease states, and these are discussed next.

Phosphorylation

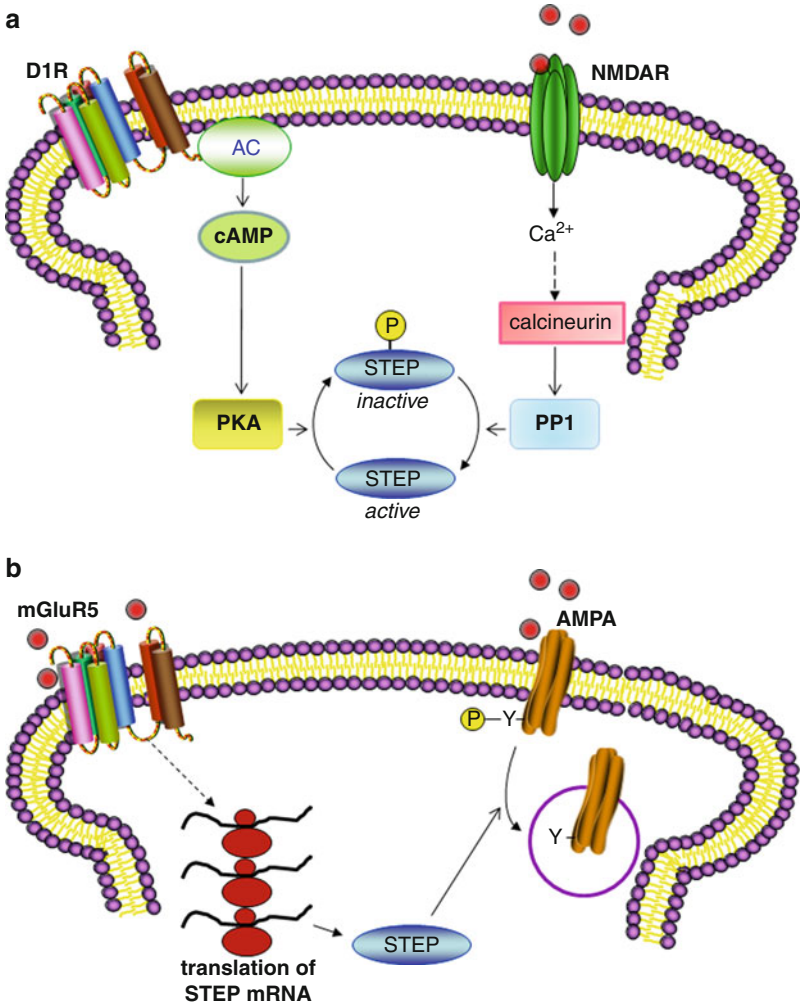
Phosphorylation is an important posttranslational modification that regulates numerous intracellular signaling pathways. Both $STEP_{61}$ and $STEP_{46}$ are phosphorylated by protein kinase A (PKA). Dopamine (DA)-mediated D1 receptor (D1R) stimulation leads to activation of adenyl cyclase and increases in intracellular cAMP levels and PKA activity. PKA phosphorylates both $STEP_{61}$ and $STEP_{46}$ at a conserved serine residue within the KIM domain (ser^{49} in $STEP_{46}$ equivalent to ser^{221} in $STEP_{61}$). $STEP_{61}$ is also phosphorylated by PKA at an additional site (ser^{160}) in its unique N-terminal domain (Paul et al. 2000). Phosphorylation of the serine residue within the KIM domain renders STEP inactive in terms of its ability to bind to its substrates. Additional studies have also shown that psychostimulants such as amphetamine inactivate STEP by promoting a DA/D1R/cAMP/PKA-mediated phosphorylation of this residue, as well as inhibiting dephosphorylation of STEP by PP1 through the PKA activation of ▶ DARPP-32 (Valjent et al. 2005).

In contrast, glutamate-mediated stimulation of GluN2B-containing *N*-methyl-D-aspartate receptors (NMDARs), subsequent influx of Ca^{2+}, and activation of the calcium-dependent phosphatase calcineurin (PP2B) lead to dephosphorylation of the serine residue within the KIM domain. Dephosphorylation of this residue allows STEP to bind to its substrate (Paul et al. 2003; Paul and Connor 2010). Beta-amyloid (Aβ), the peptide implicated in Alzheimer's disease (AD), has also been shown to modulate the activity of STEP through calcineurin/PP1-mediated dephosphorylation of this residue. Active STEP, in turn, leads to tyrosine dephosphorylation and subsequent internalization of functional NMDARs (Snyder et al. 2005). Impairment of NMDA receptor trafficking plays a role in the pathogenesis of AD and suggests

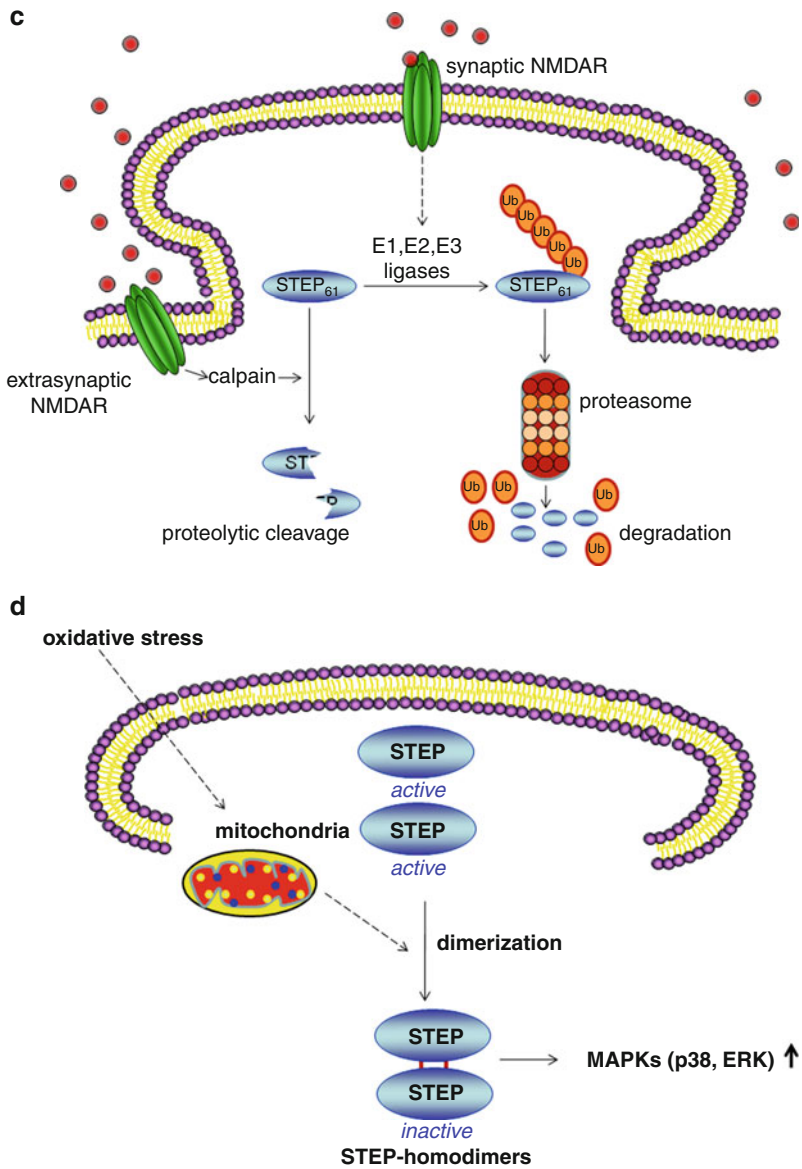
a role of STEP in AD-related dementia (Kurup et al. 2010a). The role of phosphorylation of ser^{160} in $STEP_{61}$ remains unknown, although its proximity to a PEST sequence suggests that it may play a role in the degradation of $STEP_{61}$ through either ubiquitination or proteolysis, as will be discussed further below.

Local Translation

Local translation is a process by which specific mRNA transcripts are translated at distant dendritic sites upon synaptic activity (Wang et al. 2010). This mechanism helps to remodel spines to either strengthen or weaken synaptic connections, depending on incoming synaptic stimuli. For local translation to occur, mRNA transcripts are transported along dendrites and kept in a repressed state by inhibitory RNA-binding proteins. Upon synaptic stimuli, the repression is removed and the message is translated. STEP mRNA is present in synaptoneurosomal fractions and is translated upon group I metabotropic glutamate receptor 5 (mGluR5) stimulation (Zhang et al. 2008). mGluR signaling is involved in long-term depression (LTD) and mGluR-dependent translation has been implicated in several disorders including fragile X syndrome (Waung and Huber 2009). STEP translation regulates AMPAR trafficking during mGluR-dependent LTD. In STEP knock out (KO) cultures, mGluR stimulation with the agonist DHPG failed to induce AMPAR endocytosis. Moreover, at baseline, STEP KO mice show increased surface AMPARs subunits (GluA1 and GluA2)



Striatal-Enriched Protein Tyrosine Phosphatase (STEP), Fig. 2 (continued)



Striatal-Enriched Protein Tyrosine Phosphatase (STEP), Fig. 2 *Regulation of STEP at synapse*. (**a**) *Phosphorylation*: Dopamine receptor (D1) stimulation leads to activation of PKA by adenyl cyclase/cAMP pathway, which results in phosphorylation of a regulatory serine within the KIM domain, resulting in inactivation of STEP. Glutamate stimulation reverses the KIM domain phosphorylation of STEP by activating the phosphatase calcineurin/PP1 pathway through NMDAR calcium influx. Thus, STEP is activated by a PKA/PP2B/PP1-mediated mechanism. (**b**) *Local translation*: mGluR5 activation by the agonist (DHPG) leads to local translation of STEP mRNA. STEP dephosphorylates a regulatory tyrosine residue (s) of the GluA2 subunit of AMPA receptor that results in increased internalization of AMPA receptor subunits from the neuronal surface. (**c**) *Ubiquitin-proteasome system (UPS) and proteolytic cleavage*: Synaptic NMDAR activation recruits ubiquitin enzymes and addition of ubiquitin molecules to $STEP_{61}$, which eventually leads to degradation of $STEP_{61}$ by the proteasome. Stimulation of extrasynaptic NMDARs activate calpain, which proteolytically cleaves STEP within the KIM domain to form a lower molecular weight $STEP_{33}$ isoform that is unable to bind to STEP substrates. (**d**) *Oligomerization*: Oxidative stress induced by H_2O_2 inactivates $STEP_{61}$ catalytic activity by homodimerization. This results in increased activation of MAPK signaling molecules, such as p38 and ERK

(Zhang et al. 2008). These studies suggest that local translation of STEP is required for AMPAR internalization during mGluR-dependent LTD. There are several potential Tyr residues at the carboxy-terminus of GluA2, but whether STEP directly dephosphorylates one or more of these residues to induce endocytosis of GluA1/GluA2 receptor complexes is under investigation.

Ubiquitination

Ubiquitination and subsequent degradation of cellular proteins by the ubiquitin-proteasome system (UPS) plays a role in the development of synaptic plasticity (Yi and Ehlers 2007). Local degradation of proteins at the synapse increases the efficacy of synaptic signaling in specific regions of the spine and permits spine remodeling (Haas and Broadie 2008). The degradation of STEP is differentially regulated by synaptic and extrasynaptic NMDARs (Xu et al. 2009). Synaptic and extrasynaptic NMDARs are differentially localized in the neuron (Goebel-Goody et al. 2009), and activate distinct signaling pathways (Hardingham and Bading 2003). Synaptic NMDAR stimulation preferentially activates pERK signaling and promotes neuronal survival pathways, whereas stimulation of extrasynaptic NMDARs activates p-p38 signaling and initiates the cell-death pathways (Hardingham et al. 2002). The UPS degrades STEP after synaptic NMDAR stimulation, whereas STEP is proteolytically cleaved by calpain after extrasynaptic stimulation. In both cases, STEP is removed from synaptic sites and is no longer able to dephosphorylate its substrates (Xu et al. 2009).

In primary neuronal cultures, proteasome inhibitor treatment results in an increase of $STEP_{61}$ levels (Kurup et al. 2010a). The increase in $STEP_{61}$ is insensitive to transcription or translation inhibitors. Moreover, accumulation of STEP-ubiquitin conjugates is observed in $STEP_{61}$-transfected HEK cells, as well as in mouse brain tissues. In several different AD mouse models, $STEP_{61}$ levels are elevated due to inhibition of the proteasome system by beta-amyloid oligomers. This results in the accumulation of active $STEP_{61}$, which subsequently reduces surface NMDAR levels (Kurup et al. 2010a). More details on the role of STEP in Alzheimer's disease are presented in a separate section below. In summary, UPS-mediated degradation regulates many proteins involved in synaptic plasticity, including STEP, and this process is dysregulated in various CNS disorders.

Proteolytic Cleavage

Proteolytic cleavage is another mechanism by which signaling proteins are either activated or inactivated during synaptic signaling (Bingol and Sheng 2011). Exposure to high levels of glutamate leads to overactivation of NMDARs primarily at extrasynaptic sites and influx of Ca^{2+}, which activates a number of proteases including calpain (Doshi and Lynch 2009). Calpain is a Ca^{2+}-dependent cysteine protease that cleaves $STEP_{61}$ to release a lower MW isoform $STEP_{33}$ during excitotoxic conditions. Cleavage occurs within the KIM domain to produce $STEP_{33}$, which no longer has an intact substrate-binding site, and can no longer associate with STEP substrates (Xu et al. 2009).

Recent studies have shed light on how extrasynaptic NMDAR stimulation can couple to neuronal cell-death pathways through a STEP-regulated activation of the MAPK family member, p38. STEP dephosphorylates a tyrosine residue in the activation loop of p38 and thereby blocks p38-mediated signaling (Poddar et al. 2010). Calpain cleavage of $STEP_{61}$ produces the truncated isoform $STEP_{33}$ that no longer inactivates p38, and results in sustained activation of p38 and cell-death pathways. Blocking STEP cleavage by a competition peptide corresponding to the calpain cleavage site protected cortical cultures from glutamate-mediated excitotoxicity, as well as cortical slices from neuronal death after oxygen-glucose deprivation (Xu et al. 2009). In summary, this study addresses the mechanisms by which STEP exerts a neuroprotective role or promotes cell-death pathways depending on the type of NMDAR stimulation that occurs.

Oligomerization

Dimerization is a well-known regulatory mechanism for transmembrane proteins that includes tyrosine phosphatases (den Hertog et al. 2008). A recent study showed that homo dimerization of $STEP_{61}$ is readily detectable under basal condition in hippocampus, cortex, as well as cultured neurons (Deb et al. 2011). Dimerization of $STEP_{61}$ involves intermolecular disulfide bond formation between two cysteine residues

present in the hydrophobic region at the N-terminus and involves a domain that is not conserved among tyrosine phosphatases. Oxidative stress leads to increase in dimerization and higher order oligomer formation of $STEP_{61}$ resulting in reduced phosphatase activity. $STEP_{46}$ does not form dimers under basal condition, but does following oxidative stress, resulting in subsequent loss of activity. The precise implication of oxidative stress–induced oligomerization of STEP in neurons is not well understood. However, a concomitant increase in the phosphorylation of ERK MAPK, a physiological substrate of STEP, was observed in neurons treated with hydrogen peroxide, suggesting the inability of oligomerized STEP to inactivate it. Consistent with these findings, several studies have suggested a role of ERK and p38 MAPKs in oxidative stress–induced neurodegeneration (Choi et al. 2004; Stanciu et al. 2000). Taken together, these findings suggest that inactivation of STEP through oligomerization could play a role in the initiation of neuronal degeneration by promoting chronic activation of ERK and p38 MAPK that eventually leads to activation of pro-apoptotic pathways.

STEP Substrates

Mitogen-Activated Protein Kinases (MAPKs)

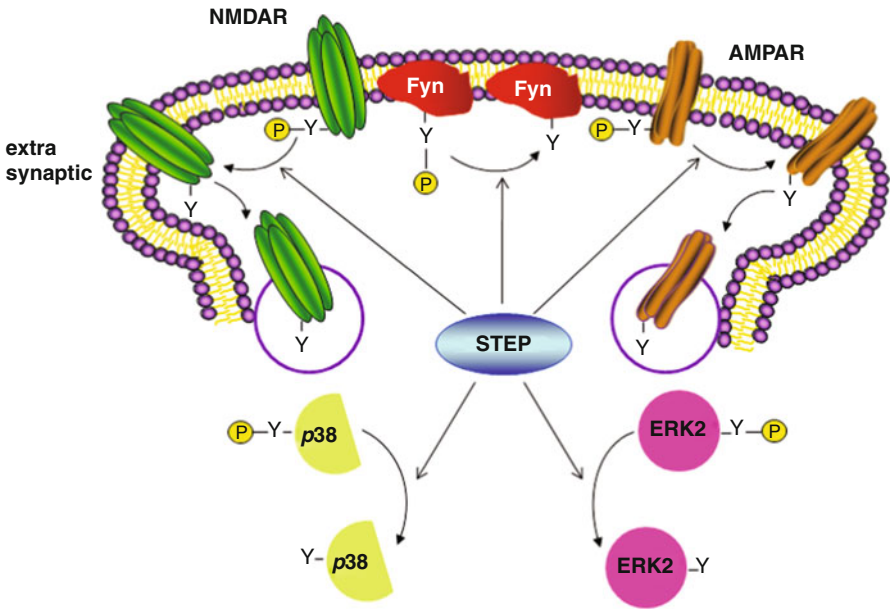
MAPKs play an important role in transducing extracellular signals to intracellular targets (Keshet and Seger 2010). ERK1/2 and p38 are members of the MAPK family and are phosphorylated and activated by their respective MEKs in cells. MEK is a dual specificity kinase that phosphorylates at tyrosine and threonine/serine residues. ERK1/2 are widely expressed protein kinases whose functions include the development of synaptic plasticity, regulation of transcription and translation, and the consolidation of long-term memory (Sweatt 2001). Many different stimuli, including growth factors and ligands for heterotrimeric G protein–coupled receptors, activate the ERK1/2 pro-survival pathways, while p38 MAPK responds to stress stimuli and its activation initiates cell-death pathways.

ERK1/2: ERK1 and ERK2 are substrates of STEP (Paul et al. 2003; Munoz et al. 2003). Most of the studies on STEP and ERK were carried out with ERK2 isoform, so the contribution focuses on this isoform. Activated ERK2 strengthens synaptic plasticity by promoting local translation of proteins, initiating gene transcription, neurotransmitter release, and spine reorganization (Sweatt 2001). Both $STEP_{61}$ and $STEP_{46}$ dephosphorylate a regulatory tyrosine residue (Tyr^{204}) in ERK2, thereby inactivating it. Thus, STEP regulates the duration of ERK2 signaling in neurons (Paul et al. 2003). STEP interacts with ERK through its KIM domain, and PKA phosphorylation of STEP prevents this interaction and prolongs ERK activity in neurons. In STEP KO brain tissues (e.g., striatum, hippocampus, amygdala), activated pERK1/2 levels are significantly increased. In comparison to wild-type neuronal cultures, STEP KO neuronal cultures show exaggerated activation of active ERK after group I metabotrophic glutamate receptor (mGluR) stimulation with DHPG (Venkitaramani et al. 2009).

Activated ERK2 is required for memory consolidation. The inactivation of ERK2 opposes this process and a role for STEP was demonstrated in Pavlovian fear conditioning learning in rats (Paul et al. 2007). Infusion of a substrate-trapping mutant of STEP, which binds to ERK2 and does not release it, blocked translocation of ERK2 to the nucleus and prevented consolidation of fear conditioning learning. These results support a role of STEP in normally opposing long-term memory formation.

Recent studies have indicated that subunits of NMDARs can differentially regulate ERK2 activation. The activation of NMDARs leads to a rapid influx of calcium and transient activation of ERK initially, which is dependent on GluN2A-containing receptors. In contrast, delayed calcium influx through GluN2B-containing receptors results in inactivation of ERK2 through activation of the calcineurin/PP1 pathway and dephosphorylation of STEP (Paul and Connor 2010).

p38: Both $STEP_{46}$ and $STEP_{61}$ isoforms bind p38 and inactivate it. The contribution of STEP in p38-mediated cell death was discussed above. In summary, glutamate stress or physiological stress induced by oxygen-glucose deprivation induces p38 activation. This is due in part to calpain-mediated cleavage of STEP to its lower MW inactive isoform $STEP_{33}$, which no longer dephosphorylates p38 and results in activation of cell-death pathways (Xu et al. 2009). Taken together with the earlier ERK studies, these findings point to the important role of STEP in regulating MAPKs in the development of both synaptic plasticity as well as activation of neuronal cell-death pathways.



Striatal-Enriched Protein Tyrosine Phosphatase (STEP), Fig. 3 *STEP substrates*. Active STEP dephosphorylates critical tyrosine residues in its substrates (Tyr^{182} of p38, Tyr^{204} of ERK) to inactivate them. Active Fyn phosphorylates Tyr^{1472} residue in GluN2B and facilitates the exocytosis of NMDARs to the neuronal surfaces. STEP dephosphorylates Tyr^{420} of Fyn and inactivates it, thereby opposing surface expression of NMDARs. STEP also directly dephosphorylates Tyr^{1472} residue of the GluN2B subunit to internalize surface GluA1/GluA2B. STEP also dephosphorylates unidentified tyrosine residue(s) in the GluA2 subunit of AMPARs that promotes internalization of surface AMPAR

NMDARs

Glutamate receptors are required for the development of synaptic plasticity and consolidation of long-term memories (Peng et al. 2011). NMDARs are ligand-gated ionotrophic channels, and are named for the selective agonist that binds to NMDARs but not to other glutamate receptors. A unique property of the NMDAR is its voltage-dependent activation, a result of ion channel block by extracellular Mg^{2+} ions. The NMDARs forms a heterotetramer between two GluN1 and two GluN2 subunits (Paoletti and Neyton 2007). The surface expression of NMDARs is regulated by various kinases and phosphatases during long-term potentiation (LTP). LTP-inducing stimuli promote trafficking of GluN1/GluN2B complexes to neuronal surfaces (Grosshans et al. 2002) and activate a number of proteins required for synaptic strengthening.

Dysregulation of NMDAR trafficking is found in several neuropsychiatric diseases, including Alzheimer's disease and schizophrenia (Lau and Zukin 2007). STEP directly binds to GluN1 subunit of the NMDAR complex and inhibits high-frequency stimulation (HFS)-LTP (Pelkey et al. 2002). Moreover, trafficking of GluN1/GluN2B complex to neuronal surfaces is regulated by STEP (Snyder et al. 2005; Braithwaite et al. 2006; Zhang et al. 2010). STEP directly dephosphorylates Tyr^{1472} (Y^{1472}) in the GluN2B subunit, leading eventually to clathrin-mediated internalization of GluN1/GluN2B (Roche et al. 2001; Snyder et al. 2005; Braithwaite et al. 2006).

Fyn

Fyn is a member of the Src family of non-receptor tyrosine kinases. Fyn phosphorylates tyrosine residues on multiple targets to initiate several signaling pathways. Fyn is activated by auto-phosphorylation of its tyrosine residue (Y^{420}) to generate a binding site that recruits other signaling molecules. Conversely, $STEP_{61}$ inactivates Fyn by binding to it via the proline-rich domain present in $STEP_{61}$ and by dephosphorylating Tyr^{420} (Nguyen et al. 2002). Fyn regulates NMDAR trafficking by directly phosphorylating Tyr (Y^{1472}) on the GluN2B subunit (Nakazawa et al. 2001; Dunah et al. 2004). STEP thereby opposes the surface expression of GluN2B-containing NMDARs in two ways: by decreasing Fyn-mediated phosphorylation of Tyr^{1472} and by

directly dephosphorylating the GluN2B subunit at Y^{1472} (Braithwaite et al. 2006; Baum et al. 2010). See Fig. 3 for list of STEP substrates.

STEP Knockout Mice

The current model of STEP function is that it opposes synaptic strengthening and memory consolidation by inactivating a number of synaptic proteins. A prediction of this hypothesis would therefore be that loss of STEP might facilitate learning and memory in certain cognitive tasks. STEP KO mice were generated using a target vector to replace 1.3 kb genomic region containing the phosphatase domain with the neomycin gene in ES cells by homologous recombination (Venkitaramani et al. 2009). STEP KO mice are viable, fertile, and with no obvious phenotypic abnormalities, suggesting that STEP is not essential for embryonic or postnatal survival. Biochemically, STEP KO progeny show no expression of STEP, but have increased tyrosine phosphorylation of STEP substrates. STEP KOs have increased synaptosomal membrane expression of glutamate receptors, including both NMDARs (Zhang et al. 2010) and AMPARs (Zhang et al. 2008), confirming the critical role STEP plays in the regulation of glutamate receptor trafficking.

Recent studies with STEP KO mice have clarified the role of STEP on hippocampal-dependent learning and memory (Venkitaramani et al. 2011). When subjected to the Morris water maze and radial arm maze, STEP KO mice show enhanced cognitive flexibility and fewer working memory errors than wild-type (WT) littermates. STEP KO mice show improved performance in these tasks, correlating with enhanced tyrosine phosphorylation of ERK1/2, the GluN2B subunit of the NMDAR, as well as increased phosphorylation of the transcription factors CREB and Elk-1 downstream of ERK1/2 activation (Venkitaramani et al. 2011). These findings suggest a role for STEP in negatively regulating hippocampal-dependent memory.

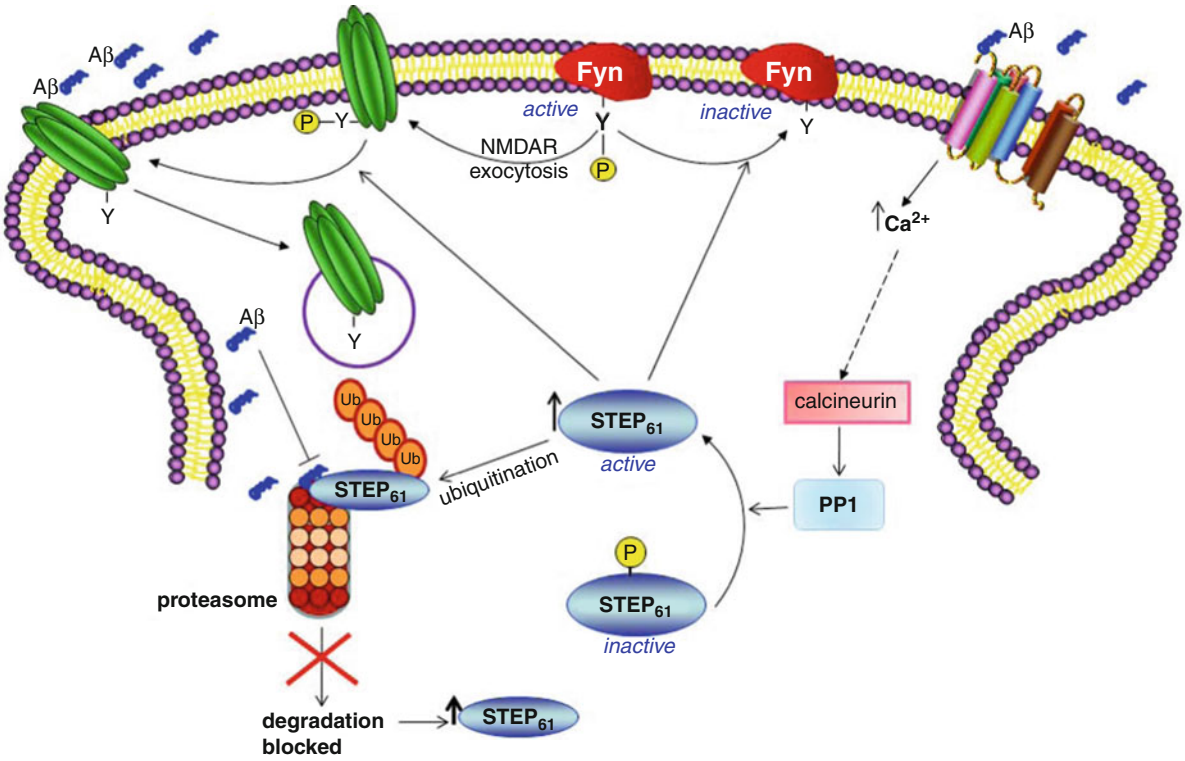
Diseases

Alzheimer's Disease

Alzheimer's is the most common neurodegenerative disorder associated with loss of memory and other cognitive symptoms. A hallmark of AD is the accumulation of beta-amyloid (Aβ) peptide in brains, a process that has been implicated in the progression of the disease (Selkoe 2002). Aβ peptides are formed from amyloid precursor protein (APP) by the sequential cleavage of β- and γ-secreteases (Turner et al. 2003). APP overexpressing transgenic mouse models mimic some, but not all, of the biochemical and behavioral defects observed in AD (Elder et al. 2010). These models have been used in efforts to uncover the molecular basis and pathophysiology of AD.

STEP has recently been shown to play a role in the etiology of AD. STEP levels are elevated in the brains of human AD as well as in several mouse AD models (Chin et al. 2005; Kurup et al. 2010a). Elevated STEP levels were associated with increased internalization of surface NMDARs (Snyder et al. 2005; Kurup et al. 2010a) and AMPARs (Zhang et al., submitted). An earlier study found that Aβ leads to activation of STEP through a calcineurin/PP1-mediated dephosphorylation of STEP. Aβ directly bound to α7 nicotinic acetylcholine receptor (α7nAChR) on neuronal surface, leading to calcium influx and activation of calcineurin and PP1. This pathway dephosphorylates STEP at the regulatory KIM domain serine residue and activates STEP. STEP is now able to dephosphorylate its substrates. STEP dephosphorylates Y^{1472} of GluN2B and causes internalization of GluN1/GluN2B receptor complexes. This study also showed the effect of Aβ was blocked by transducing a dominant-negative STEP, which competes for endogenous STEP binding to GluN2B. This model was confirmed using STEP KO neuronal cultures in which Aβ failed to induce NMDAR endocytosis, and internalization was rescued after the addition of membrane-permeable active STEP protein back into the cultures prior to Aβ treatment (Kurup et al. 2010b).

A recent study established a second mechanism by which STEP levels are elevated in AD (Kurup et al. 2010a). STEP is normally degraded by the ubiquitin proteasomal system (UPS). In human AD brains, proteasomal activity is decreased and associates with an accumulation of ubiquitin-immunoreactive inclusion bodies (Lam et al. 2000; Mori et al. 1987). Aβ peptide inhibits proteasome activity, which leads to the accumulation of numerous proteins normally degraded by the UPS. Treatment of cortical cultures with Aβ-enriched medium (7PA2-conditioned medium) results in an increase in $STEP_{61}$ levels, which is insensitive to translational or transcriptional inhibitors. $STEP_{61}$ and $STEP_{61}$ ubiquitin conjugates are increased upon Aβ



Striatal-Enriched Protein Tyrosine Phosphatase (STEP), Fig. 4 *Regulation of STEP in Alzheimer's disease*. STEP levels and activity are regulated by amyloid beta (Aβ) peptide. Aβ binds to the α7 nicotinic acetylcholine receptor (α7nAChR), which results in calcium influx and activation of calcineurin and PP1. This pathway dephosphorylates STEP at the regulatory KIM domain serine residue and activates STEP. Active STEP then dephosphorylates GluN2B at Tyr^{1472} and causes the internalization of GluN1/GluN2B receptor complexes from neuronal surfaces. In addition, STEP inactivates Fyn and blocks exocytosis of NMDARs to the neuronal surface. Aβ peptide also inhibits proteasome activity to block normal degradation of STEP. This leads to an increase in active STEP levels in the presence of Aβ and internalization of NMDA receptors. Thus, Aβ surface expression of NMDARs by two parallel pathways that involve STEP

treatment, suggesting that there is a blockade in proteasomal degradation and clearance of ubiquitinated proteins. These observations were confirmed in an AD mouse model (Tg2576 mouse), which show progressive increases in $STEP_{61}$ levels and $STEP_{61}$-ubiquitin conjugates in the cortex over the first year of life that correlates with an increase in Aβ levels and a decrease in surface glutamate receptor levels. Taken together, these studies indicate that Aβ regulates STEP through two pathways (Fig. 4): (1) by favoring calcineurin/PP1-mediated dephosphorylation and activation of STEP, and (2) by blocking the proteasome-mediated degradation of $STEP_{61}$, thereby accumulating active $STEP_{61}$.

The aforementioned studies demonstrate a role for $STEP_{61}$ in AD and suggest that genetic reduction of STEP might rescue the cognitive defects in AD mouse models. This hypothesis was tested by crossing a well-established AD model (triple transgenic mice; 3xTg-AD) with STEP KO mice to obtain progeny with elevated Aβ levels but null for STEP (double mutant; DM). Characterization of DM mice using several behavioral tests indicates significantly improved cognitive performance in spatial and nonspatial hippocampal-dependent memory tasks compared to 3xTg-AD. These behavioral improvements in DM mice are associated with increase in surface levels of NMDAR subunits (GluN1/GluN2B), increase in active ERK (pERK) and active Fyn (pY^{420} Fyn) (Zhang et al. 2010). As discussed earlier, both ERK and Fyn are STEP substrates. ERK mediates synaptic strengthening in part via regulating gene expression and local translation, while Fyn directly phosphorylates GluN2B at Y^{1472} and promotes increased surface expression of NMDARs. Thus, loss of STEP restores cognitive function in the 3xTg-AD mouse model by increasing tyrosine phosphorylation of its substrates and by favoring glutamate receptor surface expression.

Status Epileptics

Status epilepticus (SE) is a severe condition consisting of a prolonged seizure. SE often leads to long-lasting changes in hippocampal synaptic physiology that results in the development of temporal lobe epilepsy. A prominent feature of SE is the reduction of GABAergic hippocampal interneurons. A recent study demonstrates that STEP is highly expressed in hilar somatostatin-sensitive interneurons. Moreover, the abundance of STEP in these neurons renders them more vulnerable to SE-induced cell death by opposing ERK/MAPK activation. Activation of the ERK/MAPK cascade leads to expression of neuroprotective factors, whereas high expression of STEP in these interneurons suppresses ERK/MAPK-mediated neuroprotective responses. Accordingly, blocking calcineurin/PP1-mediated activation of STEP using the calcineurin inhibitor (FK506) rescues cell death in the pilicarpine-induced SE model (Choi et al. 2007). These findings suggest that a reduction of STEP in these interneurons might increase their resistance to pilicarpine-induced SE. A follow-up study extended these findings in the STEP KO model. STEP KO mice display a higher seizure threshold for the development of pilicarpine-induced SE compared to WT littermates (Briggs et al. 2011). This inherent property is due to increased excitation of inhibitory hilar neurons and reduced excitation of dentate gyrus granular cells. Enhanced inhibitory input to granule cell neurons in STEP KOs compared to WT increases their resistance to the development of seizures. These data suggest that targeted inhibition of STEP in hilar interneurons region may prove beneficial for the treatment of seizure disorders.

Summary

STEP is a non-receptor tyrosine phosphatase uniquely expressed in the brain. It plays a significant role in synaptic plasticity by regulating MAPK signaling and glutamate receptor trafficking. Several STEP substrates have been identified that include ERK1/2, p38, Fyn, and GluN2B. STEP dephosphorylates these substrates and thereby opposes the development of synaptic plasticity and the consolidation of long-term memories. STEP activity is regulated by PKA-mediated KIM domain phosphorylation, local translation, degradation by the ubiquitin-proteasome system, and by proteolytic cleavage. Excessive STEP levels and activity is implicated in a number of disorders, including Alzheimer's disease. STEP levels are increased in AD mouse models and human AD patients. Genetically reducing STEP levels in an AD mouse model rescues the biochemical and cognitive deficits associated with AD. In addition, recent studies on STEP show a potential role in epilepsy. Overall, these studies suggest that small molecule inhibitors of STEP might have therapeutic potential for the treatment of these disorders.

References

Baum ML, Kurup P, Xu J, Lombroso PJ. A STEP forward in neural function and degeneration. Commun Integr Biol. 2010;3:419–22.

Bingol B, Sheng M. Deconstruction for reconstruction: the role of proteolysis in neural plasticity and disease. Neuron. 2011;69:22–32.

Boulanger LM, Lombroso PJ, Raghunathan A, During MJ, Wahle P, Naegele JR. Cellular and molecular characterization of a brain-enriched protein tyrosine phosphatase. J Neurosci. 1995;15:1532–44.

Braithwaite SP, Adkisson M, Leung J, et al. Regulation of NMDA receptor trafficking and function by striatal-enriched tyrosine phosphatase (STEP). Eur J Neurosci. 2006;23:2847–56.

Briggs SW, Walker J, Asik K, Lombroso P, Naegele J, Aaron G. STEP regulation of seizure thresholds in the hippocampus. Epilepsia. 2011;52:497–506.

Bult A, Zhao F, Dirkx Jr R, et al. STEP61: a member of a family of brain-enriched PTPs is localized to the endoplasmic reticulum. Eur J Cell Biol. 1997;72:337–44.

Chin J, Palop JJ, Puolivali J, Massaro C, Bien-Ly N, Gerstein H, Scearce-Levie H, Masliah E, Mucke L. Fyn kinase induces synaptic and cognitive impairments in a transgenic mouse model of Alzheimer's disease. J Neurosci. 2005;25:9694–703.

Choi WS, Eom DS, Han BS, Kim WK, Han BH, Choi EJ, Oh TH, Markelonis GJ, Cho JW, Oh YJ. Phosphorylation of p38 MAPK induced by oxidative stress is linked to activation of both caspase-8- and -9-mediated apoptotic pathways in dopaminergic neurons. J Biol Chem. 2004;279:20451–60.

Choi YS, Lin SL, Lee B, et al. Status epilepticus-induced somatostatinergic hilar interneuron degeneration is regulated by striatal enriched protein tyrosine phosphatase. J Neurosci. 2007;27:2999–3009.

Deb I, Poddar R, Paul S. Oxidative stress-induced oligomerization inhibits the activity of the non-receptor tyrosine phosphatase STEP61. J Neurochem. 2011;116:1097–111.

den Hertog J, Ostman A, Böhmer FD. Protein tyrosine phosphatases: regulatory mechanisms. FEBS J. 2008;275:831–47.

Doshi S, Lynch DR. Calpain and the glutamatergic synapse. Front Biosci (Schol Ed). 2009;1:466–76.

Dunah AW, Sirianni AC, Fienberg AA, Bastia E, Schwarzschild MA, Standaert DG. Dopamine D1-dependent trafficking of

striatal N-methyl-D-aspartate glutamate receptors requires Fyn protein tyrosine kinase but not DARPP-32. Mol Pharmacol. 2004;65:121–9.

Elder GA, Gama Sosa MA, De Gasperi R. Transgenic mouse models of Alzheimer's disease. Mt Sinai J Med. 2010;77: 69–81.

Goebel-Goody SM, Davies KD, Alvestad Linger RM, Freund RK, Browning MD. Phospho-regulation of synaptic and extrasynaptic N-methyl-d-aspartate receptors in adult hippocampal slices. Neuroscience. 2009;158:1446–59.

Grosshans DR, Clayton DA, Coultrap SJ, Browning MD. LTP leads to rapid surface expression of NMDA but not AMPA receptors in adult rat CA1. Nat Neurosci. 2002;5:27–33.

Haas KF, Broadie K. Roles of ubiquitination at the synapse. Biochim Biophys Acta. 2008;1779:495–506.

Hardingham GE, Bading H. The Yin and Yang of NMDA receptor signalling. Trends Neurosci. 2003;26:81–9.

Hardingham GE, Fukunaga Y, Bading H. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci. 2002;5:405–14.

Keshet Y, Seger R. The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. Methods Mol Biol. 2010;661:3–38.

Kurup P, Zhang Y, Xu J, et al. A beta-mediated NMDA receptor endocytosis in Alzheimer's disease involves ubiquitination of the tyrosine phosphatase STEP61. J Neurosci. 2010a;30:5948–57.

Kurup P, Zhang YF, Venkitaramani DV, Xu J, Lombroso PJ. The role of STEP in Alzheimer's disease. Channels. 2010b;4:347–50.

Lam YA, Pickart CM, Alban A, et al. Inhibition of the ubiquitin-proteasome system in Alzheimer's disease. Proc Natl Acad Sci USA. 2000;97:9902–6.

Lau CG, Zukin RS. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat Rev Neurosci. 2007;8:413–26.

Lombroso PJ, Murdoch G, Lerner M. Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. Proc Natl Acad Sci USA. 1991;88:7242–69.

Lombroso PJ, Naegele JR, Sharma E, Lerner M. A protein tyrosine phosphatase expressed within dopaminoceptive neurons of the basal ganglia and related structures. J Neurosci. 1993;13:3064–74.

Mori H, Kondo J, Ihara Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. Science. 1987;235:1641–4.

Munoz JJ, Tarrega C, Blanco-Aparicio C, Pulido R. Differential interaction of the tyrosine phosphatases PTP-SL, STEP and HePTP with the mitogen-activated protein kinases ERK1/2 and p38alpha is determined by a kinase specificity sequence and influenced by reducing agents. Biochem J. 2003; 372:193–201.

Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K, Mishina M, Manabe T, Yamamoto T. Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the N-methyl-D-aspartate receptor. J Biol Chem. 2001;276:693–9.

Nguyen TH, Paul S, Xu Y, Gurd JW, Lombroso PJ. Calcium-dependent cleavage of striatal enriched tyrosine phosphatase (STEP). J Neurochem. 1999;73:1995–2001.

Nguyen TH, Liu J, Lombroso PJ. Striatal enriched phosphatase 61 dephosphorylates Fyn at phosphotyrosine 420. J Biol Chem. 2002;277:24274–9.

Oyama T, Goto S, Nishi T, Sato K, Yamada K, Yoshikawa M, Ushio Y. Immunocytochemical localization of the striatal enriched protein tyrosine phosphatase in the rat striatum: a light and electron microscopic study with a complementary DNA-generated polyclonal antibody. Neuroscience. 1995;69:869–80.

Paoletti P, Neyton J. NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol. 2007;7:39–47.

Paul S, Connor JA. NR2B-NMDA receptor-mediated increases in intracellular Ca^{2+} concentration regulate the tyrosine phosphatase, STEP, and ERK MAP kinase signaling. J Neurochem. 2010;114:1107–18.

Paul S, Lombroso PJ. Receptor and nonreceptor protein tyrosine phosphatases in the nervous system. Cell Mol Life Sci. 2003;60:2465–82.

Paul S, Snyder GL, Yokakura H, Picciotto MR, Nairn AC, Lombroso PJ. The Dopamine/D1 receptor mediates the phosphorylation and inactivation of the protein tyrosine phosphatase STEP via a PKA-dependent pathway. J Neurosci. 2000;20:5630–8.

Paul S, Nairn AC, Wang P, Lombroso PJ. NMDA-mediated activation of the tyrosine phosphatase STEP regulates the duration of ERK signaling. Nat Neurosci. 2003;6:34–42.

Paul S, Olausson P, Venkitaramani DV, et al. The striatal-enriched protein tyrosine phosphatase gates long-term potentiation and fear memory in the lateral amygdala. Biol Psychiatry. 2007;61:1049–61.

Pelkey KA, Askalan R, Paul S, et al. Tyrosine phosphatase STEP is a tonic brake on induction of long-term potentiation. Neuron. 2002;34:127–38.

Peng S, Zhang Y, Zhang J, Wang H, Ren B. Glutamate receptors and signal transduction in learning and memory. Mol Biol Rep. 2011;38:453–60.

Poddar R, Deb I, Mukherjee S, Paul S. NR2B-NMDA receptor mediated modulation of the tyrosine phosphatase STEP regulates glutamate induced neuronal cell death. J Neurochem. 2010;115:1350–62.

Pulido R, Zúñiga A, Ullrich A. PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. EMBO J. 1998;17:7337–50.

Roche KW, Standley S, McCallum J, Dune LC, Ehlers MD, Wenthold RJ. Molecular determinants of NMDA receptor internalization. Nat Neurosci. 2001;4:794–802.

Selkoe DJ. Alzheimer's disease is a synaptic failure. Science. 2002;298:789–91.

Sharma E, Zhao F, Bult A, Lombroso PJ. Identification of two alternatively spliced transcripts of STEP: a subfamily of brain-enriched protein tyrosine phosphatases. Brain Res Mol Brain Res. 1995;32:87–93.

Snyder EM, Nong Y, Almeida CG, et al. Regulation of NMDA receptor trafficking by amyloid-beta. Nat Neurosci. 2005;8:1051–8.

Stanciu M, Wang Y, Kentor R, et al. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem. 2000;275:12200–6.

Sweatt JD. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J Neurochem. 2001;76:1–10.
Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. Nat Rev Mol Cell Biol. 2006;7:833–46.
Turner PR, O'Connor K, Tate WP, Abraham WC. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog Neurobiol. 2003;70:1–32.
Valjent E, Pascoli V, Svenningsson P, et al. Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. Proc Natl Acad Sci USA. 2005;102:491–6.
Venkitaramani DV, Paul S, Zhang Y, et al. Knockout of striatal enriched protein tyrosine phosphatase in mice results in increased ERK1/2 phosphorylation. Synapse. 2009;63:69–81.
Venkitaramani DV, Moura PJ, Picciotto MR, Lombroso PJ. Striatal-enriched protein tyrosine phosphatase (STEP) knockout mice have enhanced hippocampal memory. Eur J Neurosci. 2011;33:2288–98.
Wang DO, Martin KC, Zukin RS. Spatially restricting gene expression by local translation at synapses. Trends Neurosci. 2010;33:173–82.
Watanabe Y, Shiozuka K, Ikeda T, et al. Cloning of PCPTP1-Ce encoding protein tyrosine phosphatase from the rat cerebellum and its restricted expression in Purkinje cells. Brain Res Mol Brain Res. 1998;15:83–94.
Waung MW, Huber KM. Protein translation in synaptic plasticity: mGluR-LTD, Fragile X. Curr Opin Neurobiol. 2009;19:319–26.
Xu J, Kurup P, Zhang Y, et al. Extrasynaptic NMDA receptors couple preferentially to excitotoxicity via calpain-mediated cleavage of STEP. J Neurosci. 2009;29:9330–43.
Yi JJ, Ehlers MD. Emerging roles for ubiquitin and protein degradation in neuronal function. Pharmacol Rev. 2007;59:14–39.
Zhang Y, Venkitaramani DV, Gladding CM, et al. The tyrosine phosphatase STEP mediates AMPA receptor endocytosis after metabotropic glutamate receptor stimulation. J Neurosci. 2008;28:10561–6.
Zhang Y, Kurup P, Xu J, et al. Genetic reduction of striatal-enriched tyrosine phosphatase (STEP) reverses cognitive and cellular deficits in an Alzheimer's disease mouse model. Proc Natl Acad Sci USA. 2010;107:19014–9.

Structure and Functions of the Urokinase Receptor

Pia Ragno
Department of Chemistry and Biology,
University of Salerno, Fisciano, Salerno, Italy

Synonyms

CD87; PLAUR; uPA Receptor; uPAR; Urokinase-type plasminogen activator receptor

Historical Background

The receptor (uPAR) for the urokinase-type plasminogen activator (uPA) was firstly identified in 1985 on the surface of monocyte-like cells but only in 1990 uPAR protein was purified and cDNA cloned and sequenced. The first role proposed for uPAR was the focusing of the uPA proteolytic activity on the cell membrane, thus allowing cell migration through the extracellular matrix (ECM), without affecting the general architecture of the tissue. Since then, a large body of evidence clearly showed various roles for uPAR, independent of uPA enzymatic activity. In fact, uPAR is able to transduce proliferation, differentiation, adhesion, and migration signals into the cells, despite the absence of a transmembrane region and a cytosolic tail. However, the ability of uPAR to focus uPA activity on the cell membrane attributed to this specific receptor a key role in the plasminogen activation system.

Plasminogen is a plasma zymogen; the cleavage at the Arg_{561}-Val_{562} bond converts the single polypeptide chain plasminogen into plasmin, that consists of two polypeptide chains held together by a disulfide bond. Plasminogen can associate to fibrin via lysine-binding sites located in the noncatalytic region. In fact, the primary in vivo function of plasminogen/plasmin is to regulate fibrinolysis, i.e., the process wherein the fibrin clot, end product of the coagulation process, is degraded. However, plasminogen/plasmin ability to bind low-affinity cellular receptors and to degrade extracellular matrix (ECM) proteins indicated a crucial role for plasmin in other processes involving protein degradation, in particular in cell migration, an important event occurring in various physiologic and pathologic processes, such as embryogenesis, wound healing, angiogenesis, and tumor cell dissemination (Castellino and Ploplis 2005).

Plasminogen can be activated by different proteases, but its specific physiologic activators are the tissue-type (tPA) and the urokinase-type (uPA) plasminogen activators. Both serine-proteases are secreted as single chains and are activated by a single cleavage, that yields two chains held together by a disulfide bond.

tPA is mainly involved in fibrinolysis since it binds fibrin with high affinity. The assembly of fibrinolytic components, plasminogen and tPA, at the surface of fibrin results in efficient fibrin degradation (Rijken and Lijnen 2009). uPA, which lacks fibrin-binding sites but

is able to bind cellular receptors, is instead mainly involved in processes including cell migration and tissue invasion, such as inflammation, angiogenesis, and tumor invasion (Irigoyen et al. 1999).

The plasminogen activation system represents a very efficient proteolytic machinery, thus its activity must be strictly regulated. The primary plasmin inhibitor is the alfa2-antiplasmin; the most efficient inhibitor of plasminogen activators (PAs) is the type-1 inhibitor (PAI-1) (Balsara and Ploplis 2008). The other specific PA inhibitor is the type-2 inhibitor (PAI-2); its lower inhibitory efficiency, as compared to PAI-1, and the intracellular detection of PAI-2 nonglycosylated forms suggested a regulatory role for PAI-2 in the activity of enzymes other than uPA (Medcalf and Stasinopoulos 2005).

Plasminogen, tPA, and uPA can bind cellular receptors. Binding to cellular receptors represents another crucial regulatory step, since focusing enzymes and substrates on the cell surface strongly enhances the proteolytic cascade. Plasminogen binds in a lysine- and/or carbohydrate-dependent manner to the cell surface, with low affinity but high capacity. Cell-bound plasmin is protected from its natural inhibitors, since both receptors and inhibitors utilize plasminogen/plasmin C-terminal lysines. Different cellular binding sites for tPA have been described.

uPA binds a specific high-affinity cell-anchored receptor, the urokinase-type plasminogen activator receptor (uPAR), which, in the last decades, has been largely characterized in its expression, structure, and function.

uPAR Expression

The human uPAR gene is located on the long arm of chromosome 19 (19q13) and contains seven exons and six introns extending over 23 kb of genomic DNA. This region lacks TATA and CAAT boxes and contains GC-rich sequences and consensus elements for SP1, AP1, AP2, NF-k B, and the Kruppel-like transcription factor, which have been reported to drive uPAR transcription.

uPAR expression can be regulated also at a posttranscriptional level. In fact, several sequence elements regulating uPAR mRNA decay have been identified throughout the transcript. These sequences are present both in the coding and in the 3′ untranslated region (UTR) of the uPAR transcript and are able to bind proteins that promote stability or degradation of uPAR mRNA. Interestingly, uPA regulates the expression of its receptor both at transcriptional and posttranscriptional levels (Nagamine et al. 2005).

uPAR is expressed constitutively in many cell lines; however, its expression in culture is inducible by several factors as phorbol esters or various growth factors. In vivo, uPAR is moderately expressed in various tissues including lungs, kidneys, spleen, vessels, uterus, bladder, thymus, heart, liver, and testis. uPAR expression can be strongly up-regulated in organs undergoing extensive tissue remodeling, as in gestational tissues during embryo implantation and placental development or in keratinocytes during epidermal wound healing. Stress, injury, and inflammation also induce uPAR expression. In blood, the expression of uPAR is strongly increased upon activation of neutrophils, monocytes, and T cells (Smith and Marshall 2010). Up-regulation of uPAR expression has been also reported in peripheral CD33+ myeloid and CD14 + monocytic cells, following granulocyte-colony-stimulating factor (G-CSF)-induced mobilization of CD34+ hematopoietic stem cells (Ragno and Blasi 2008).

A wide variety of human and mouse cancers overexpress uPAR. uPAR-deficient mice show phenotypes consistent with these observations and not always coincident with phenotypes of uPA-deficient mice, strongly suggesting additional proteolysis-independent in vivo role of uPAR.

uPAR Structure

uPAR is synthesized as a single polypeptide chain of 313 amino acid residues, with a 21-residues signal peptide. Posttranslational cleavage and removal of the last 30 C-terminal residues allow the attachment of a glycosyl-phosphatidyl-inositol (GPI) tail to Gly 283 that anchors the receptor to the cell surface. Mature uPAR consists of three homologous domains of approximately 90 amino acids (D1, D2 and D3, from the N-terminus), belonging to the Ly-6/uPAR/alfa-neurotoxin protein domain family. By virtue of its GPI-tail, uPAR partitions preferentially to lipid rafts, cholesterol-rich microdomains of cell membrane, which represent anchoring platforms for specific cell-signaling mediators.

The crystal structure of a uPAR soluble form bound to an antagonist peptide has been solved, thus confirming that uPAR consists of three domains with a typical three-finger fold, each domain containing three adjacent loops rich in beta-pleated sheets and a small C-terminal loop. The three domains of uPAR form an almost globular receptor with a breach between D1 and D3, thus generating a central cavity where the ligand peptide is located. The top of the cavity is quite large and progressively narrows toward the bottom. The peptide establishes multiple contacts with the walls of the cavity; D1 plays a predominant role in this ligand interaction by providing half of the binding interface. uPAR exhibits a large outer surface that harbors the interdomain linker regions and the five possible N-linked glycosylation sites. This model suggests that uPA is embedded in the central cavity and that the large outer receptor surface is available to bind additional ligands (Kjaergaard et al. 2008).

The three domains of the GPI-anchored uPAR are joined by linker sequences. The D1-D2 linker region is particularly exposed to proteolysis and can be cleaved by several enzymes, including plasmin and uPA itself. The cleavage can occur at different sites in the D1-D2 linker region and may disrupt or not a specific sequence (SRSRY), corresponding to amino acids 88–92, involved in cell migration. In both cases, the cleavage generates truncated forms of GPI-uPAR, lacking D1 (D2-D3 uPAR), which have been detected on the surface of different cell lines and in normal and cancer tissues.

Both full-length and cleaved uPAR can be shed, thus generating soluble uPAR forms (suPAR and D2-D3 suPAR, respectively) identified in biological fluids, both in vitro and in vivo. The shedding can be due to the activity of glycosyl-phosphatidyl-inositol specific phospholipase C or D, or to a juxtamembrane proteolytic cleavage of uPAR. D2-D3 suPAR can also be generated by proteolytic cleavage of full-length suPAR mediated by metalloproteases, cathepsin G or elastase (Montuori et al. 2005).

uPAR Interactors

uPAR Extracellular Ligands

uPAR was firstly identified as the cellular receptor for the urokinase-type plasminogen activator (uPA). uPA is secreted as a 54 kDa single-chain pro-enzyme that can be converted, likely following binding to uPAR, into the two-chain active form by a single cleavage at Lys_{158}-Ile_{159}. The N-terminal A chain or its amino terminal fragment (ATF, residues 1–135) binds uPAR with high affinity and specificity. The C-terminal B chain contains the serine-protease domain and is unable to bind the receptor (Carriero et al. 2009).

uPA binds the N-terminal uPAR domain 1 (D1), even though the full-length uPAR is required for an efficient uPA binding. uPA binding to GPI-anchored uPAR allows the cell to focus uPA proteolytic activity, which activates cell-bound or free plasminogen into plasmin. Plasmin promotes ECM degradation directly, by degrading ECM components, including fibrinogen, fibronectin, and vitronectin, and indirectly, by activating several metalloproteases.

In spite of its name, uPAR acts also as a receptor for other extracellular molecules, such as vitronectin (VN), an ECM component, and the cleaved form of high molecular mass kininogen (HKa). The functional epitope on uPAR that is responsible for its interaction with the full-length, extended form of vitronectin is formed by three residues in domain I (Trp(32), Arg (58), and Ile(63)) and two residues located in the flexible linker peptide connecting uPAR domains I and II (Arg(91) and Tyr(92)). uPA positively regulates VN binding to uPAR, likely by inducing formation of uPAR dimers that exhibit a higher affinity for VN as compared to the monomers. HKa, unlike uPA, inhibits uPAR-mediated cell adhesion to VN, likely because it binds and blocks the VN-binding site in uPAR. Furthermore, the type-1 plasminogen activator inhibitor (PAI-1) competes with uPAR in the binding to VN because they bind overlapping regions on VN located in the N-terminal somatomedin B (SMB) domain, close to the integrin-binding site. uPAR expression confers to the cell the ability to adhere to VN, thus it is a non-integrinic VN receptor (Madsen and Sidenius 2008).

uPA and VN can be bound only by full-length uPAR, both in the cell-anchored and in the soluble form, even if, in the latter case, with less efficiency; the cleaved forms of uPAR are unable to bind both uPA and VN (Montuori et al. 2005).

Cell Surface uPAR Interactors

Cell surface uPAR interactors encompass a long list of proteins. The structural basis of these interactions is still poorly understood; however, much evidence

demonstrates that the physical/functional interaction of uPAR with these proteins is essential for its non-proteolytic functions. uPAR functionally interacts with receptor tyrosine kinases, as the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR)-beta, various integrins, caveolin, the receptors for bacterial formylated peptides (fMLP receptors, fMLP-Rs), the collagen receptor uPARAP/Endo180, and receptors of the low-density lipoprotein receptor family including the LDL receptor-related protein (LRP) and LRP1B. In addition, uPAR has been shown to associate with the cation-independent Mannose 6-phosphate/insulin-like growth factor-II receptor that has been implicated in the targeting of uPAR to lysosomes. As a consequence of some of these interactions, uPAR activates intracellular signaling molecules involved in cell proliferation, survival, differentiation, adhesion, and migration. In fact, uPAR lacks a transmembrane region and a cytosolic tail, thus it could not transduce signals inside the cell unless it associates to signaling partners; the best candidates to this role seem to be integrins and fMLP receptors (Blasi and Sidenius 2010).

uPAR and Integrins

uPAR co-capping with the β2 integrin Mac-1 was firstly demonstrated in resting neutrophils; subsequently, the presence of uPAR and β2 integrins was identified in large receptor complexes that included signaling molecules. Fluorescence resonance energy transfer analysis (FRET), immunolocalization, and co-immunoprecipitation have identified uPAR in complex with several integrin families, such as β1, β2, β3, and β5. uPAR-binding sites have been identified on α- and β1-integrin chains. α3β1 integrin associates with uPAR via a surface loop within the α3 β-propeller, outside the ligand-binding region; cell treatment with a 17 mer α3β1 integrin peptide (peptide α 325) or Ala mutations within the uPAR-interacting loop of α3 (H245A) chain abolishes uPAR co-immunoprecipitation with α3β1 integrin and impairs uPA-uPAR-dependent signals. A uPAR-binding site has also been identified in a β1-chain loop (residues 224–232) of α5β1 integrin; this loop is very close to the β-propeller of α5 chain in the energy-minimized model of the integrin structure. The synthetic peptide corresponding to the uPAR-binding site of β1 or a β1 chain Ser227Ala point mutation abrogates functional uPAR effects on α5β1 integrin.

Subsequently, also putative integrin-binding sites on uPAR have been identified in the uPAR domains 2 and 3. The peptide covering the interaction site in D2 abolishes uPAR co-immunoprecipitation with αvβ3 and α5β1 integrins, activates αvβ3 integrin-dependent signaling pathways, and stimulates cell migration, whereas peptide D2A-Ala, generated by mutating two glutamic acids into two alanines, lacks chemotactic activity, and, in addition, inhibits VN-, FN-, and CG-dependent cell migration. The peptide covering D3 interaction site binds purified α5β1 integrin; substituting a single amino acid in this peptide or in full-length soluble uPAR impairs binding of the purified integrin. uPAR binding to integrins requires the full-length receptor, as in the case of uPAR binding to uPA and VN (Tang and Wei 2008).

Interestingly, uPA may bind simultaneously both uPAR and integrins. In fact, uPA interacts with uPAR through its growth factor-like domain (GFD) in the amino terminal fragment (ATF), whereas it recognizes Mac-1 and β1 integrins through the kringle domain and the αvβ5 integrin through the "connecting peptide" (residues 132–158). Mac-1 also is able to bind at the same time uPA and uPAR, through its I and non-I domains, respectively (Carriero et al. 2009; Tang and Wei 2008).

uPAR–integrin interactions seem to exert opposite effects, depending on the cell type examined. In fact, the first evidence of uPAR interaction with β1 integrins was reported in uPAR-transfected HEK-293 cells, in which uPAR and integrins form stable complexes that inhibit cell adhesion to fibronectin (FN) and, in parallel, increase RGD-independent uPAR-mediated cell adhesion to VN. By contrast, uPAR activates α5β1 integrin in human epidermoid carcinoma HEp-3 cells.

uPAR and fMLP Receptors

fMLP (fMet-Leu-Phe) is a formylated peptide of bacterial origin that stimulates chemotaxis by activating seven transmembrane domain receptors coupled to G-proteins. Three fMLP receptors (fMLP-R) have been identified and cloned: the high-affinity *N*-formyl-peptide receptor (FPR) and its homologues FPR-like 1 (FPRL1) and FPR-like2 (FPRL2). FPR is a high-affinity receptor for fMLP. FPRL1 has a much lower affinity for fMLP, whereas it is efficiently activated by several different molecules, such as lipoxin A_4, serum amyloid A, the prion peptide $PrP_{106\text{-}126}$, HIV-1 envelope peptides, the Helicobacter Pylori Hp(2–20)

peptide, and various synthetic peptides. FPRL2 shows a high homology with the other two fMLP receptors but does not bind fMLP and shares some ligands with FPRL1, as Hp(2–20) and synthetic peptides. fMLP-Rs were firstly identified in leukocytes, and subsequently in several different cell types, including epithelial cells (Le et al. 2002).

Full-length GPI-anchored uPAR functionally interacts with fMLP-Rs through a specific SRSRY sequence (residues 88–92) in the D1-D2 linker region; in fact, uPAR expression is required for fMLP-dependent migration in monocytes and HEK-293 epithelial cells and, conversely, fMLP-R expression is required in cell migration induced by uPA or its amino terminal fragment (ATF).

Interaction of uPAR with fMLP-Rs was originally demonstrated by showing that the soluble cleaved form of uPAR (D2-D3 suPAR), or its derived peptides containing the SRSRY sequence (residues 88–92), bind and activate FPRL1 in monocyte-like cells, thus inducing their migration. Interestingly, D2-D3 suPAR does not induce calcium mobilization, unlike fMLP and other FPRL1 ligands. Subsequently, D2-D3 suPAR capability to induce cell migration through activation of all members of the fMLP-R family has been shown in various cell types. Unlike full-length GPI-anchored uPAR, full-length soluble uPAR does not bind fMLP receptors, even though it is able to interact with integrins and VN (Ragno 2006).

uPAR as Signal Transducer

A large body of evidence clearly shows that uPAR is able to activate intracellular signaling leading to cellular responses such as cell adhesion, migration, differentiation, proliferation, and survival. uPAR-dependent signaling can be activated by uPA (independently of its proteolytic activity), VN, and by uPAR overexpression itself. Many signaling partners have been proposed for uPAR, which lacks a transmembrane region and a cytosolic tail; however, the most important transmembrane receptors associated with uPAR signaling seem to belong to the integrin and fMLP-R families.

Even if a direct and physical uPAR interaction with integrins has been questioned, many studies show that uPAR signaling requires integrin cooperation. uPAR-β1 and uPAR-αv integrin interactions have been often associated with the activation of FAK/src signaling, leading to increased activities of Rac1 and/or ERK1/2.

The first uPAR–integrin interaction to be described involved the β2 integrin Mac-1, which is expressed primarily in leukocytes, where it regulates migration, differentiation, and phagocytosis. uPAR association with Mac-1 improves Mac-1 binding to fibrinogen; up-regulation and/or engagement of Mac-1 enhance uPAR-dependent adhesion to VN in monocytes. Signaling promoted by the uPAR-Mac-1 interaction seems to involve Src family kinases. A crucial role for uPAR in regulating β2 integrin activity has also been suggested in vivo; in fact, the β2 integrin-dependent recruitment of leukocytes to inflamed peritoneum and of neutrophils to the lung in response to *P. aeruginosa* pneumonia infection is significantly reduced in uPAR-deficient mice (Smith and Marshall 2010).

In normal microvascular endothelial cells (MVECs), full-size uPAR is connected with the actin cytoskeleton via the alphaM- and alphaX-subunits of β2 integrins. In systemic sclerosis (SSc), MVECs angiogenesis is blocked by uPAR cleavage; in fact, uPAR cleavage induces uPAR uncoupling from β2 integrins, impairing the activation of Rac and Cdc42 and the integrin-delivered signals to the actin cytoskeleton (Margheri et al. 2006).

One of the first reports on uPAR involvement in cell-signaling described a uPAR-dependent mechanism in which, in tumorigenic cells, high uPAR levels activated α5β1 integrin, thus promoting its binding to fibronectin and the assembly of integrin-bound fibronectin into insoluble structures during fibronectin fibrillogenesis. α5β1-dependent signaling induced a strong and persistent ERK1/2 activation, which promoted cell proliferation. By contrast, cells expressing low uPAR levels showed p38 MAPK activation, which inhibited ERK1/2 activation. In vivo, tumor cells with low ERKs/p38 activity ratio rapidly arrested in G_0–G_1 and remained viable but dormant for a prolonged period of time (Aguirre-Ghiso et al. 2003). Subsequently, several reports have shown that signaling events downstream of uPAR-β1 integrin interactions promote FAK phosphorylation and ERK1/2 activation. Src also co-immunoprecipitates with uPAR-β1 integrin complexes and is activated by uPAR signaling through both α5β1 and α3β1 integrins (fibronectin and laminin receptors, respectively). ERK1/2 and Src activation induced by uPAR-β1 integrin mediates cell adhesion, migration, invasion of ECM, proliferation,

epithelial-mesenchymal transition (EMT), and, in addition, increase in the expression of uPA and metalloproteases, suggesting that uPAR signaling through β1 integrins can contribute to invasion also by increasing pericellular proteolysis (Smith and Marshall 2010).

αv- integrins have also been strongly implicated in uPAR signaling. Both uPAR and αv integrins bind vitronectin, uPAR recognizing the SMB domain and αv integrins the Arg-Gly-Asp sequence of vitronectin. The uPAR-av integrin interaction has an important role in signaling for cell migration. The functional effects of uPAR-αv integrin association were studied in fibrosarcoma and breast carcinoma cell lines, both of which exhibit uPA-dependent physical association between uPAR and αvβ5. uPA promoted cellular migration via a uPAR/αvβ5-dependent signaling cascade in which Ras, ERKs, and myosin light chain kinase (MLCK) serve as essential downstream effectors. In uPAR-transfected cells, uPAR signaling through αv-β3 integrin may activate Src, leading to phosphorylation of ▶ p130Cas and subsequent binding of CRK; the p130Cas–CRK complex recruits DOCK1 for Rac activation. Further, uPAR is required to activate αvβ3 integrin in podocytes, promoting cell motility and activation of the small GTPases Cdc42 and Rac1. Blocking αvβ3 integrin reduces podocyte motility in vitro and lowers proteinuria in mice; thus uPAR seems to play a physiologic role in the regulation of kidney permeability. Also, in some cells, uPAR signaling promotes the cell surface expression of αvβ3 integrin (Smith and Marshall 2010).

Different forms of uPAR are present on the cell surface: full-length uPAR and cleaved uPAR forms (D2-D3 uPAR), lacking the D1 domain and exposing or not a specific SRSRY sequence (residues 88–92), which is able to functionally interact with fMLP-Rs. uPAR and D2-D3 uPAR appear to mediate different signaling pathways. A report on fibroblast-to-myofibroblast differentiation showed that fibroblasts express increased amounts of full-length cell surface uPAR as compared to myofibroblasts, which have increased expression of D2-D3 uPAR. uPAR cleavage seems to be required to induce fibroblast transition to myofibroblast, since inhibition of uPAR cleavage prevents myofibroblast differentiation (Ragno 2006).

uPAR can be shed from the cell surface. However, the soluble receptor is able to activate cell-signaling pathways in uPAR negative cells and can still activate β2 and β1 integrins, thus inducing leukocyte adhesion to endothelium or ERK1/2 activation in Hep3 carcinoma cells (Montuori et al. 2005).

The soluble form of D2-D3 uPAR (D2-D3 suPAR), or its derived peptides containing the SRSRY sequence, bind and activate fMLP-Rs, stimulating cell migration. Thus, D2-D3 suPAR can be considered as a ligand for fMLP receptors and is also able to activate cell-signaling pathways. Indeed, the SRSRY peptide increases uPAR-αvβ5 association and stimulates PKC activity and ERK phosphorylation (Gargiulo et al. 2005).

uPAR can also cross-talk with growth factor receptors, such as EGFR and PDGFR-beta. Indeed, uPA binding to uPAR can initiate a cell-signaling pathway that is mediated by EGFR, and, conversely, uPAR-dependent cell-signaling may prime cells to proliferate in response to EGF.

uPAR cross-talk with the platelet-derived growth factor receptor (PDGFR)-beta has been reported in human vascular smooth muscle cells (VSMC), in which uPA induces uPAR association with PDGFR-beta, thus stimulating its phosphorylation. uPAR-PDGFR-beta association is required for uPA-induced migratory and proliferative downstream signals. However, unlike the association to EGFR, uPA strongly inhibits PDGF-induced migration (Ragno 2006).

Recently, the cross-talk between uPAR and CXCR4, the receptor for the stromal-derived factor 1 chemokine, has been reported; uPAR expression regulates CXCR4 activity on specific extracellular matrix components by a mechanism involving fMLP-Rs and αv integrins (Montuori et al. 2010).

Summary

The urokinase receptor (uPAR) was firstly identified in 1985 on the surface of monocyte-like cells. It is moderately expressed in various tissues and its expression can be strongly up-regulated in organs undergoing extensive tissue remodeling. Stress, injury, and inflammation induce uPAR expression; a wide variety of human and mouse cancers also overexpress uPAR.

uPAR is a GPI-anchored protein that binds with high affinity the serine-protease urokinase-type plasminogen activator (uPA), thus regulating cell membrane–associated proteolytic activity and, hence, increasing cell ability to move and migrate through barriers.

uPAR mobility along the cell membrane, due to its GPI-tail, and its structure, recently solved, strongly suggest that uPAR is a molecule prone to interact with other cell surface molecules. In fact, over the years, a large body of evidence clearly showed that uPAR is capable of multiple interactions and, likely by virtue of these interactions, it is able to activate intracellular signaling, leading to cellular responses such as cell adhesion, migration, differentiation, proliferation, and survival. The majority of reports is focused on its likely interaction with integrins, which has been questioned in the last years, even though a crucial role of integrins in uPAR signaling activities is generally accepted. Other possible uPAR signaling partners could be the receptors for the fMLP formylated peptide (fMLP-Rs). In this case also, there are no reports on a direct interaction of cell membrane uPAR with fMLP-R; however, a direct binding between the cleaved form of soluble uPAR has been demonstrated in the past on monocyte-like cells. Thus, at present, the mechanism enabling uPAR to signal is still unclear and represents a very intriguing area of research; elucidation of these mechanisms could shed light on the real meaning and importance of uPAR in the various biologic and pathologic processes in which it is overexpressed and involved.

References

Aguirre-Ghiso JA, Estrada Y, Liu D, Ossowski L. ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). Cancer Res. 2003;63:1684–95.

Balsara RD, Ploplis VA. Plasminogen activator inhibitor-1: the double-edged sword in apoptosis. Thromb Haemost. 2008;100:1029–36.

Blasi F, Sidenius N. The urokinase receptor: focused cell surface proteolysis, cell adhesion and signaling. FEBS Lett. 2010;584:1923–30.

Carriero MV, Franco P, Vocca I, Alfano D, Longanesi-Cattani I, Bifulco K, Mancini A, Caputi M, Stoppelli MP. Structure, function and antagonists of urokinase-type plasminogen activator. Front Biosci. 2009;14:3782–94.

Castellino FJ, Ploplis VA. Structure and function of the plasminogen/plasmin system. Thromb Haemost. 2005;93:647–54.

Gargiulo L, Longanesi-Cattani I, Bifulco K, Franco P, Raiola R, Campiglia P, Grieco P, Peluso G, Stoppelli MP, Carriero MV. Cross-talk between fMLP and vitronectin receptors triggered by urokinase receptor-derived SRSRY peptide. J Biol Chem. 2005;280:25225–32.

Irigoyen JP, Muñoz-Cánoves P, Montero L, Koziczak M, Nagamine Y. The plasminogen activator system: biology and regulation. Cell Mol Life Sci. 1999;56:104–32.

Kjaergaard M, Hansen LV, Jacobsen B, Gardsvoll H, Ploug M. Structure and ligand interactions of the urokinase receptor (uPAR). Front Biosci. 2008;13:5441–61.

Le Y, Murphy PM, Wang JM. Formyl-peptide receptors revisited. Trends Immunol. 2002;23:541–8.

Madsen CD, Sidenius N. The interaction between urokinase receptor and vitronectin in cell adhesion and signalling. Eur J Cell Biol. 2008;87:617–29.

Margheri F, Manetti M, Serratì S, Nosi D, Pucci M, Matucci-Cerinic M, Kahaleh B, Bazzichi L, Fibbi G, Ibba-Manneschi L, Del Rosso M. Domain 1 of the urokinase-type plasminogen activator receptor is required for its morphologic and functional, beta2 integrin-mediated connection with actin cytoskeleton in human microvascular endothelial cells: failure of association in systemic sclerosis endothelial cells. Arthritis Rheum. 2006;54:3926–38.

Medcalf RL, Stasinopoulos SJ. The undecided serpin. The ins and outs of plasminogen activator inhibitor type 2. FEBS J. 2005;272:4858–67.

Montuori N, Visconte V, Rossi G, Ragno P. Soluble and cleaved forms of the urokinase-receptor: degradation products or active molecules? Thromb Haemost. 2005;93:192–8.

Montuori N, Bifulco K, Carriero MV, La Penna C, Visconte V, Alfano D, Pesapane A, Rossi FW, Salzano S, Rossi G, Ragno P. The cross-talk between the urokinase receptor and fMLP receptors regulates the activity of the CXCR4 chemokine receptor. Cell Mol Life Sci. 2010; doi:10.1007/s00018-010-0564-7.

Nagamine Y, Medcalf RL, Muñoz-Cánoves P. Transcriptional and potsttranscriptional regulation of the plasminogen activator system. Thromb Haemost. 2005;93:661–75.

Ragno P. The urokinase receptor: a ligand or a receptor? Story of a sociable molecule. Cell Mol Life Sci. 2006;63:1028–37.

Ragno P, Blasi F. uPAR and proteases in mobilization of hematopoietic stem cells. In: Edwards D, Hoyer-Hansen G, Blasi F, Sloane BF, editors. The Cancerdegradome. New York: Springer; 2008. p. 433–50.

Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost. 2009;7:4–13.

Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. Nat Rev Mol Cell Biol. 2010;11:23–36.

Tang CH, Wei Y. The urokinase receptor and integrins in cancer progression. Cell Mol Life Sci. 2008;65:1916–32.

STY (CLK1)

► CLK

Styxl1

► MK-STYX

Suc 1-Associated Neurotrophic Factor Target (SNT-1)

▶ FRS2

Suc 1-Associated Neurotrophic Factor Target (SNT-2)

▶ FRS2

Sulfiredoxin

Robert R. Bowers[1], Danyelle M. Townsend[2] and Kenneth D. Tew[1]
[1]Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA
[2]Dept of Pharmaceutical and Biomedical Sciences, Medical University of South Carolina, Charleston, SC, USA

Synonyms

Cysteine sulfinic acid reductase; Npn3; Srx; Srx1; Srxn1

Historical Background

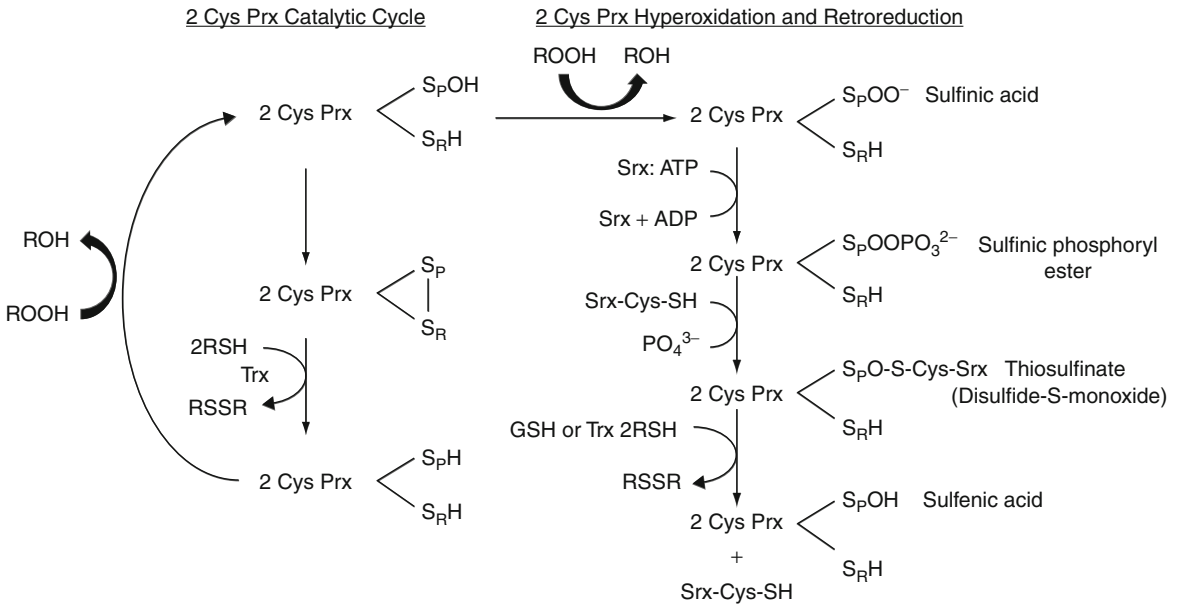
Redox homeostasis is critical for normal cellular function, and cellular redox imbalance is associated with a number of human pathologies including cardiovascular disease and cancer (Circu and Aw 2010). Reactive oxygen species (ROS) include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). ROS can oxidatively damage cellular constituents such as DNA, protein, and unsaturated lipids. Traditionally ROS were considered to be solely deleterious, but it has recently become apparent that evolution harnessed the labile nature of ROS for beneficial purposes (Circu and Aw 2010). Cells are exposed to ROS arising from both exogenous and endogenous sources. Environmental sources of ROS include ionizing radiation and certain drugs and toxins. The first endogenous source of ROS to be recognized is electrons leaking prematurely from mitochondrial electron transport chain complexes I and III that reduce molecular oxygen to superoxide anion. In addition, NADPH oxidases present in macrophages and neutrophils produce large bursts of superoxide anions in order to kill bacteria. Although NADPH oxidases were originally thought to be specific to immune cells, more recent work demonstrates NADPH oxidase isoforms are ubiquitously expressed (Brown and Griendling 2009). These NADPH oxidases are transiently activated in response to ligand binding to some peptide growth factor receptors, cytokine receptors, and G protein-coupled receptors (GPCRs). The superoxide produced is rapidly converted to hydrogen peroxide by spontaneous dismutation or by the activity of superoxide dismutase enzymes, and the hydrogen peroxide thus produced acts as a second messenger in cellular signaling pathways. Although low levels of hydrogen peroxide promote cell growth, high levels cause cellular damage and result in cell death. Consequently, controlling the cellular level of ROS is of critical importance.

Oxidative stress ensues when the level of ROS exceeds the antioxidant capacity of the cell, and cells possess a host of antioxidant enzymes and small molecule antioxidants that function to maintain redox homeostasis (Circu and Aw 2010). The enzymatic antioxidant defense system is composed of several enzyme families including superoxide dismutase, which catalyzes the dismutation of superoxide to hydrogen peroxide, and glutathione peroxidase, catalase, and peroxiredoxins, which reduce hydrogen peroxide to water. The sulfur of cysteine residues can exist in a number of chemical forms including thiol (S-H), thiolate anion (S^-), disulfide (S-S), sulfenic acid (S-OH), sulfinic acid ($S\text{-}O_2H$), and sulfonic acid (SO_3H). This versatility of sulfur chemistry is of paramount importance for maintaining redox homeostasis and redox signaling. The tripeptide glutathione (GSH) comprising glutamate-glycine-cysteine is present at millimolar concentrations in cells, and GSH is the primary cellular small molecule antioxidant (Townsend et al. 2003). GSH is in equilibrium with GSH disulfide (GSSG) in cells, and the ratio of reduced (GSH) to oxidized (GSSG) glutathione is indicative of the level of oxidative

stress. The intracellular environment is reducing and the GSH to GSSG ratio is typically 100:1, but during oxidative stress this ratio can be reduced to 10:1 or even less. In addition to acting as the primary small molecule antioxidant, the formation of mixed disulfides between proteins and GSH (P-S-S-G) – i.e., protein glutathionylation – is a mechanism to prevent the oxidation of cysteine residues to sulfinic and sulfonic states that may inactivate proteins or target them for degradation. The posttranslational glutathionylation modification of proteins is also involved in cell signaling (Townsend et al. 2003). The pKa of a typical cysteine residue is ~8.5 so most cysteine residues exist as sulfhydryls in the reducing environment of the cytosol. However, some cysteine residues have much lower pKa values, due to the presence of neighboring positively charged residues such as lysine and arginine that stabilize the thiolate anion. The thiolate anion is much more readily oxidized by ROS than sulfhydryls. Thus, when cells are exposed to oxidative stress, low pKa cysteine residues in proteins are readily oxidized to cysteine sulfenic acids. These cysteine sulfenic acids can condense with the free sulfhydryls of other cysteine residues to form disulfides including mixed disulfides with glutathione or can further be oxidized to sulfinic or sulfonic acids. Thus, through their ability to modify cysteine residues, ROS act as signaling molecules by altering protein structure and function. For example, one mechanism whereby hydrogen peroxide can act as a second messenger involves the oxidative inactivation of phosphatases, the catalytic cysteine of which exists as a thiolate anion. Redox regulation of protein cysteine residues is known to regulate the structure and function of a number of proteins including peroxiredoxins, actin, and some protein tyrosine phosphatases (Circu and Aw 2010).

Peroxiredoxins and the Identification of Sulfiredoxin

Peroxiredoxins (Prxs) are a ubiquitous and abundantly expressed family of enzymes found in both prokaryotes and eukaryotes that catalyze the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohol, respectively. In addition to their role as antioxidant enzymes, some eukaryotic Prxs are involved in cell signaling (reviewed in Hall et al. 2009). Mammals have six Prxs that are divided into three subgroups based on the presence of conserved cysteine residues and reaction mechanisms. All mammalian Prx isoforms have a conserved N-terminal cysteine residue designated the peroxidatic cysteine, and the most abundant subgroup of Prxs, the typical 2-Cys Prxs (PrxI-IV), also has a conserved C-terminal cysteine residue designated the resolving cysteine. Atypical 2-Cys Prxs (Prx V) have a second non-conserved cysteine whereas 1-Cys Prxs (Prx VI) only have the conserved N-terminal cysteine residue. Typical 2-Cys Prxs function as homodimers, and during enzymatic reduction of hydrogen peroxide the peroxidatic cysteine of Prx is oxidized to sulfenic acid (C–S–OH; Fig. 1). This peroxidatic cysteine sulfenic acid residue then condenses with the free sulfhydryl of the resolving cysteine of the other subunit of the dimer to form an intermolecular disulfide bond. The thiols are reconstituted by thioredoxin (Trx) using reducing equivalents derived from NADPH, thus completing the catalytic cycle. Before disulfide formation between the peroxidatic and resolving cysteines can occur, the peroxidatic cysteine sulfenic acid can be hyperoxidized to cysteine sulfinic acid (C–S–O_2H) by a second H_2O_2 molecule. Cysteine sulfinic acids cannot be reduced by cellular thiols such as Trx or GSH and their presence results in inactivation of the enzyme (Yang et al. 2002). Interestingly, eukaryotic 2-Cys Prxs are much more susceptible to hyperoxidative inactivation than their prokaryotic homologs. The crystal structure of human Prx II revealed that the peroxidatic cysteine sulfenic acid is located more than 12 Å away from the free sulfhydryl of the resolving cysteine requiring a conformational change for disulfide bond formation to occur. In addition, sequence analysis revealed that eukaryotic Prxs have two unique structural features, an additional C-terminal α helix (α7) with a conserved YF motif and a separate GGLG motif located near the peroxidatic cysteine. These structural features render the peroxidatic cysteine of eukaryotic Prxs susceptible to hyperoxidation to cysteine sulfinic acid (Jonsson and Lowther 2007; Hall et al. 2009). The sulfinic acid modification of the peroxidatic cysteine was initially thought to result in irreversible inactivation of the enzyme, but careful analyses of the fate of hyperoxidized Prxs subsequently revealed that the sulfinic acid modification of Prx is actually reversible (Woo et al. 2003), begging the question of which enzyme is responsible for this sulfinic acid reductase activity.



Sulfiredoxin, Fig. 1 The 2 Cys Prx catalytic cycle and reaction scheme for the retroreduction of sulfinic 2-Cys Prx by Srx. In the 2 Cys Prx catalytic cycle, the peroxidatic cysteine (S_P) is oxidized by peroxide (ROOH) forming a sulfenic acid (S_POH). The peroxidatic cysteine sulfenic acid condenses with the resolving cysteine (S_R) to form an intermolecular disulfide. Reduced 2 Cys Prx is reconstituted by thioredoxin (Trx). The peroxidatic cysteine sulfenic acid can be hyperoxidized by a second peroxide molecule to form a peroxidatic cysteine sulfinic acid. Srx with ATP bound catalyzes the formation of the sulfinic phosphoryl ester intermediate in the first step of the retroreduction reaction. In the second step, the catalytic cysteine of Srx attacks the Prx phosphoryl ester forming a thiosulfinate intermediate. Subsequent reduction occurs by glutathione (GSH) thiol-disulfide exchange reactions or is catalyzed by Trx

In 2003, Toledano and coworkers identified sulfiredoxin (Srx) in the yeast *S. cerevisiae* based on the observations that its expression was markedly induced by hydrogen peroxide exposure and that deletion of Srx reduced tolerance to hydrogen peroxide (Biteau et al. 2003). Srx was shown to associate with Tsa1 (yeast Prx) in cells by co-immunoprecipitation, and in vitro experiments demonstrated Mg^{2+} and ATP-dependent reduction of the sulfinic acid form of yeast Prx by purified Srx. Soon thereafter, Rhee and colleagues identified mammalian orthologs of yeast Srx in mice, rats, and humans (Chang et al. 2004). Using antibodies specific to the sulfinic form of 2-Cys Prxs, mammalian Srx was also demonstrated to have sulfinic acid reductase activity toward Prxs. The reduction of hyperoxidized 2-Cys Prxs by Srx was found to be a slow process (k_{cat} = 0.18/min) that required ATP hydrolysis, and both GSH and Trx were identified as potential electron donors as their K_m values were in the physiological range and similar V_{max} values were obtained with both reductants (Chang et al. 2004).

Mechanism of Action of Sulfiredoxin

Based on the observations that the reaction requires ATP hydrolysis, Mg^{2+} and the conserved cysteine of sulfiredoxin forming a transient disulfide with yeast Prx, the mechanism of sulfinic acid reductase activity by Srx was originally proposed to involve activation of Prx sulfinic acid by phosphorylation followed by a thiol-mediated reduction step involving the conserved Cys residue of Srx (Biteau et al. 2003). An alternate reaction scheme has been proposed whereby the initial step is attack of Srx Cys^{99} on the γ phosphate of ATP generating an Srx thiophosphate intermediate that is then attacked by the Prx sulfinic acid generating the same Prx sulfinic phosphoryl ester intermediate proposed in the original scheme (Jeong et al. 2006). In addition, this alternate scheme suggests that the thioltransferase is provided not by Srx but by GSH. Although some experimental evidence has been interpreted to support the alternate scheme (for review see Jonsson and Lowther 2007), the preponderance of evidence suggests that the original scheme whereby Srx acts as both

a phosphotransferase and a thioltransferase is correct. Thus, ATP bound Srx catalyzes the phosphorylation of Prx sulfinic acid forming a sulfinic phosphoryl ester intermediate (Fig. 1). This enables the sulfhydryl group of the catalytic cysteine of Srx to attack the peroxidatic cysteine phosphoryl ester forming a thiosulfinate (disulfide mono-oxide) intermediate that is reduced by thiols such as GSH to Prx sulfenic acid and Srx mixed disulfide. Several lines of evidence support this model. The crystal structure of human Srx in complex with ADP and a model of Srx:ATP based on this structure shows that the γ phosphate of ATP is near Srx Cys^{99} but does not directly interact with the Srx Cys^{99} residue (Jonsson et al. 2005). Superimposition of this Srx:ATP model onto the crystal structure of human Srx in complex with PrxI revealed that unfolding of the PrxI peroxidatic cysteine sulfinic acid would place the peroxidatic cysteine sulfinic acid near ATP and that the oxygen atom of the peroxidatic cysteine sulfinic acid is positioned for inline attack on the γ phosphate of ATP while Srx Cys^{99} is not positioned properly (Jonsson et al. 2008a). Studies with mutant Prx that cannot complete the reaction because the peroxidatic Cys is mutated to Asp also support sulfinic acid phosphorylation as the first step in the reaction (Jonsson et al. 2009). Finally, using chemical quenching and mass spectrometry a thiosulfinate intermediate between Srx and Prx was observed (Jonsson et al. 2008b). In sum, it appears the reaction mechanism proceeds as originally proposed whereby Srx acts first to properly position ATP for attack by the Prx peroxidatic cysteine sulfinic acid and then the Srx catalytic cysteine attacks the sulfinic acid phosphoryl ester to create a Prx-Srx thiosulfinate intermediate that can be reduced by GSH or Trx. At the present time it is unclear if the physiological reductant is GSH or Trx. If GSH is the reductant of the Prx-Srx thiosulfinate, a Prx cysteine sulfenic acid and an Srx mixed disulfide (glutathionylated Srx) would result. Reduced Srx could be regenerated after further reduction of glutathionylated Srx with additional GSH or other cellular thiols.

Sulfiredoxin as a Deglutathionylating Enzyme

In addition to its activity as a cysteine sulfinic acid reductase specific to 2-Cys Prxs, two recent studies demonstrate that Srx is capable of reversing glutathionylation of proteins (Findlay et al. 2006; Park et al. 2009). This observation has important implications for cell signaling because, as noted above, glutathionylation of protein cysteine residues is a posttranslational modification that affects protein structure and function. The anticancer prodrug PABA/NO is a potent inducer of glutathionylation of a number of proteins. Human embryonic kidney (HEK293) cells transfected with Srx exhibit a decrease in protein glutathionylation following PABA/NO treatment compared with control cells (Findlay et al. 2006). Further, actin and protein tyrosine phosphate 1B (PTP1B) were identified in vitro as specific targets of Srx deglutathionylation activity and these interactions were confirmed with in vivo studies (Findlay et al. 2006). Importantly, it was shown that glutathionylation of PTP1B inhibits its enzymatic activity and that deglutathionylation of PTP1B by Srx restored the phosphatase activity of PTP1B (Findlay et al. 2006). Another recent study demonstrates that 2-Cys Prxs are glutathionylated and that Srx is capable of reversing this modification (Park et al. 2009). Specifically, human Prx I has four cysteine residues – Cys^{52}, Cys^{71}, Cys^{83}, and Cys^{173} – and in vitro studies show Cys^{52}, Cys^{83}, and Cys^{173} can be glutathionylated. Using Cys mutants it was demonstrated that deglutathionylation of Cys^{83} and Cys^{173} is preferentially catalyzed by Srx whereas deglutathionylation of Cys^{52} is preferentially catalyzed by glutaredoxin. Thus, Srx impacts redox signaling pathways by both regulating hydrogen peroxide levels through reactivation of hyperoxidized Prxs and by reversing glutathionylation.

Regulation of Sulfiredoxin Expression

As mentioned Srx was originally identified, in part, through the observation that its expression is induced by hydrogen peroxide in yeast. More recent work shows that Srx transcription is induced by a variety of signaling pathways in mammals (Soriano et al. 2009). Several studies demonstrate mammalian Srx is a target of the activator protein-1 (AP-1) complex. The AP-1 complex is formed by transcription factors of the Jun and Fos families that bind as homo- and hetero-dimers to AP-1 response elements in promoters of target genes to activate transcription. An investigation of rodent pancreatic β cells stimulated with elevated glucose and cAMP first identified Srx as a target of the AP-1 complex (Glauser et al. 2007). AP-1 induction of Srx expression was also observed in studies of rat neurons (Papadia et al. 2008).

In this study (Papadia et al. 2008), synaptic activity acting through NMDA receptor signaling activated transcription of genes involved in intrinsic antioxidant defenses, and two active AP-1 response elements were identified in the rat Srx promoter. Although both of these AP-1 sites are conserved in most mammals, the distal site is not present in the primate lineage. Knockdown of Srx expression increased the sensitivity of neurons to oxidative stress (Papadia et al. 2008). Another group utilized the tumor promoter TPA (12-*O*-tetradecanoylphorbol-13-acetate) that activates AP-1 and TAM67, a dominant negative form of c-Jun, to show that Srx is an AP-1 target in mouse epidermal JB6 cells (Wei et al. 2008). Srx expression was found to be necessary for anchorage-independent growth of JB6 cells, and several human skin malignancies demonstrated elevated Srx protein levels (Wei et al. 2008).

Srx expression is also regulated by Nuclear factor erythroid 2–related factor (▶ Nrf2). Nrf2 is a transcription factor that binds as hetero-dimers with members of the Maf protein family to Antioxidant Response Elements (AREs) in target genes whose products are involved in protecting cells from oxidative stress. In the basal state, Nrf2 is targeted for degradation by Kelch-like ECH associated protein (Keap1). Oxidative stress and chemopreventive agents such as D3T (3 H 1,2 dithiole-3-thione) inhibit Keap1 and lead to the accumulation of Nrf2 resulting in increased expression of ARE-containing Nrf2 target genes. In studies of neurons over-expressing Nrf2 and by using the Nrf2 activator D3T it was demonstrated that Srx expression in rat neurons is induced by Nrf2, and an ARE was identified in the Srx promoter (Soriano et al. 2008). Another study showed that exposure of lung epithelial cells to cigarette smoke induced Srx through Nrf2 and an ARE in the Srx promoter (Singh et al. 2009). The ARE in the Srx promoter identified in this latter study (Singh et al. 2009) is in a different orientation and slightly different position relative to the first identified ARE (Papadia et al. 2008). In addition, the proximal AP-1 response element described above is located within the first identified ARE (reviewed in Soriano et al. 2009). Interestingly, both chemopreventative agents such as D3T and sulforaphanes and tumor-promoting phorbol esters can induce Srx expression. While Srx is demonstrated to protect against oxidative stress in neurons and in the lung, Srx expression is associated with transformation in skin cells. Moreover, Srx over-expression triggers altered expression and phosphorylation of cell cycle regulators p21, p27, and ▶ p53 and stabilizes the phosphatase ▶ PTEN (Lei et al. 2008). Perhaps equally importantly, Srx interacted directly with, and enhanced the activity of, phosphatase PTP1B. In turn, this promoted Src kinase activity by dephosphorylating its inhibitory tyrosine residue (Tyr^{530}). Also, Srx expression was stimulated by cell exposure to certain growth factors. Together, these data suggest a role for Srx in controlling the phosphorylation status of key regulatory kinases through effects upon phosphatase activity with an ultimate impact on pathways that influence cell proliferation (Lei et al. 2008).

Summary

Due to their involvement in cell signaling and their ability to damage cellular components, ROS levels must be tightly regulated. Cells have evolved a number of enzyme systems to guard against oxidative stress, and the Prx family of antioxidant enzymes is especially abundant and ubiquitously expressed. Hyperoxidized Prxs form higher order structures that function as chaperones to protect client proteins from oxidative damage during oxidative stress. Current understanding of the protective role played by GSH, Prxs, and other enzyme systems in protecting cells against oxidative insults is relatively advanced. Current understanding of the role of Prxs in hydrogen peroxide-mediated cell signaling in normal physiology and development, however, is somewhat limited. Much current research is focused on extending our knowledge of ROS-mediated signaling in normal physiology and on the role dysregulated ROS plays in the etiology and progression of diseases. Due to its ability to reactivate Prxs that have been hyperoxidized, Srx plays an important role in both the protective aspects and signaling aspects of Prx function. In addition, the recent realization that Srx possesses deglutathionylating activity is an interesting development that expands the functional implications of the homeostatic redox properties of this protein.

References

Biteau B, Labarre J, Toledano MB. ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. Nature. 2003;425(6961):980–4.

Brown DI, Griendling KK. Nox proteins in signal transduction. Free Radic Biol Med. 2009;47(9):1239–53.

Chang TS, Jeong W, Woo HA, Lee SM, Park S, Rhee SG. Characterization of mammalian sulfiredoxin and its reactivation of hyperoxidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site to cysteine. J Biol Chem. 2004;279(49):50994–1001.
Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med. 2010;48(6):749–62.
Findlay VJ, Townsend DM, Morris TE, Fraser JP, He L, Tew KD. A novel role for human sulfiredoxin in the reversal of glutathionylation. Cancer Res. 2006;66(13):6800–6.
Glauser DA, Brun T, Gauthier BR, Schlegel W. Transcriptional response of pancreatic beta cells to metabolic stimulation: large scale identification of immediate-early and secondary response genes. BMC Mol Biol. 2007;8:54.
Hall AP, Karplus PA, Poole LB. Typical 2-Cys peroxiredoxins–structures, mechanisms and functions. FEBS J. 2009;276(9):2469–77.
Jeong W, Park SJ, Chang TS, Lee DY, Rhee SG. Molecular mechanism of the reduction of cysteine sulfinic acid of peroxiredoxin to cysteine by mammalian sulfiredoxin. J Biol Chem. 2006;281(20):14400–7.
Jonsson TJ, Lowther WT. The peroxiredoxin repair proteins. Subcell Biochem. 2007;44:115–41.
Jonsson TJ, Murray MS, Johnson LC, Poole LB, Lowther WT. Structural basis for the retroreduction of inactivated peroxiredoxins by human sulfiredoxin. Biochemistry. 2005;44(24):8634–42.
Jonsson TJ, Johnson LC, Lowther WT. Structure of the sulphiredoxin-peroxiredoxin complex reveals an essential repair embrace. Nature. 2008a;451(7174):98–101.
Jonsson TJ, Tsang AW, Lowther WT, Furdui CM. Identification of intact protein thiosulfinate intermediate in the reduction of cysteine sulfinic acid in peroxiredoxin by human sulfiredoxin. J Biol Chem. 2008b;283(34):22890–4.
Jonsson TJ, Johnson LC, Lowther WT. Protein engineering of the quaternary sulfiredoxin.peroxiredoxin enzyme.substrate complex reveals the molecular basis for cysteine sulfinic acid phosphorylation. J Biol Chem. 2009;284(48):33305–10.
Lei K, Townsend DM, Tew KD. Protein cysteine sulfinic acid reductase (sulfiredoxin) as a regulator of cell proliferation and drug response. Oncogene. 2008;27:4877–87.
Papadia S, Soriano FX, Leveille F, Martel MA, Dakin KA, Hansen HH, et al. Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses. Nat Neurosci. 2008;11(4):476–87.
Park JW, Mieyal JJ, Rhee SG, Chock PB. Deglutathionylation of 2-Cys peroxiredoxin is specifically catalyzed by sulfiredoxin. J Biol Chem. 2009;284(35):23364–74.
Singh A, Ling G, Suhasini AN, Zhang P, Yamamoto M, Navas-Acien A, et al. Nrf2-dependent sulfiredoxin-1 expression protects against cigarette smoke-induced oxidative stress in lungs. Free Radic Biol Med. 2009;46(3):376–86.
Soriano FX, Leveille F, Papadia S, Higgins LG, Varley J, Baxter P, et al. Induction of sulfiredoxin expression and reduction of peroxiredoxin hyperoxidation by the neuroprotective Nrf2 activator 3 H-1,2-dithiole-3-thione. J Neurochem. 2008;107(2):533–43.
Soriano FX, Baxter P, Murray LM, Sporn MB, Gillingwater TH, Hardingham GE. Transcriptional regulation of the AP-1 and Nrf2 target gene sulfiredoxin. Mol Cells. 2009;27(3):279–82.
Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomed Pharmacother. 2003;57(3–4):145–55.
Wei Q, Jiang H, Mathews CP, Colburn NH. Sulfiredoxin is an AP-1 target gene that is required for transformation and shows elevated expression in human skin malignancies. Proc Natl Acad Sci USA. 2008;105(50):19738–43.
Woo HA, Chae HZ, Hwang SC, Yang KS, Kang SW, Kim K, et al. Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. Science. 2003;300(5619):653–6.
Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K, et al. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. J Biol Chem. 2002;277(41):38029–36.

SWI/SNF

► SWI/SNF Chromatin Remodeling Complex

SWI/SNF Chromatin Remodeling Complex

Payel Sen, Nilanjana Chatterjee and Blaine Bartholomew
Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, IL, USA

Synonyms

BAF; BRG1; BRM; hBRM; PBAF RSC; SMARCA; SWI/SNF

Historical Background

There are several large multi-subunit complexes that couple ATP hydrolysis with regulation of the chromatin landscape and are referred to as ATP-dependent chromatin remodelers. These complexes are primarily divided into four major classes based on the domain organization of their catalytic subunit. In this entry we focus on one of those major classes called the SWI/SNF subfamily. The SWI/SNF subfamily of chromatin remodelers are well conserved throughout all eukaryotes and typically the catalytic subunit has at least four signature motifs that are the ATPase, bromo,

AT-hook, and HSA domains. The ATPase domain has sequence homology with the two lobes of such ATP-dependent DNA translocases like RecA and Rad54. The DNA translocation activity of SWI/SNF is essential for these complexes to move nucleosomes along DNA. The bromo domain binds to acetylated lysines in histone tails which may modulate the activity of the remodeler. While the A/T hook domain in other complexes is used to bind to DNA, the role of the A/T hook in SWI/SNF is not well understood. The HSA domain is not unique to SWI/SNF complexes, but is found in other chromatin remodelers and is generally used to recruit actin or actin-related proteins (Arp) to the catalytic subunit. The Arps have been shown to stimulate the catalytic activity and to be an important part of a core complex. The subunit composition of SWI/SNF subfamily members ranges from 12 to 17 subunits and can be quite varied in humans. In yeast and Drosophila there are two SWI/SNF complexes present, while in humans the number is significantly higher. The two yeast complexes called SWI/SNF and RSC have different catalytic subunits, but share the same two Arps (Arp7 and 9). In Drosophila there is only one catalytic subunit called Brahma or Brm which is assembled into two different complexes that differ primarily by a subunit which contains multiple bromo domains and is called polybromo. In yeast the RSC complex also differs from SWI/SNF in that besides the catalytic subunit there are seven bromodomains spread throughout three different subunits. Human SWI/SNF in some ways is a combination of both yeast and Drosophila. There are two catalytic subunits in humans called BRG1 and hBRM, like yeast SWI/SNF, which are assembled into distinct complexes. However, like Drosophila, in humans there is also an ortholog of the polybromo subunit called BAF180 that is assembled with BRG1 or hBRM. In addition there are different forms of several of the other subunits which are respectively assembled into different SWI/SNF complexes which add to the diversity of human SWI/SNF. Many of the human SWI/SNF complexes are tissue or developmentally specific which lends itself well to the many different combinations of SWI/SNF that can be formed using different subunits. In this entry we focus on the various roles of SWI/SNF in regulating processes that involve DNA. Much of this work has come from studies in yeast where the functional role of SWI/SNF can more readily be studied.

Involvement in Transcription

Chromatin dynamics for transcription regulation involve the intricate interplay of ATP-dependent chromatin remodelers, ATP-independent histone modifiers, and trans-acting activator/coactivator or repressor/corepressor proteins. However, the temporal control of these factors presents a chicken-and-egg situation. Some promoters may be initially remodeled by chromatin remodelers to create accessible sites for activator binding which in turn can recruit other factors promoting transcription. In other promoters, activator proteins can first bind to promoter regions and recruit chromatin remodelers. Alternatively, activators may recruit histone modifiers like histone acetyl transferases (HATs) that put on active acetyl marks that are in turn recognized by remodeler subunits bearing special "modification-identifying" domains. In some other cases, activators may initially bind to exposed low-affinity sites, recruit remodelers which in turn expose more high-affinity sites. Repressors may function similarly, except that they interact with remodelers that evenly space nucleosomes to form a repressive chromatin structure. The repressed state of chromatin can also be achieved by recruitment of histone deacetylases (HDACs). Besides a normal recruitment establishment and maintenance function, activator/repressor proteins might directly modify the catalytic activities of the remodeler complexes.

Genome-wide gene expression studies with budding yeast SWI/SNF mutants Δ*swi2* and Δ*swi1* showed that they affected ~6% of genes in *Saccharomyces cerevisiae* (Sudarsanam et al. 2000). In *Schizosaccharomyces Pombe*, however, Δ*snf2* and Δ*snf5* mutants showed that they affected 2.6% of the genome but a different set of genes compared to *S. cerevisiae* (Monahan et al. 2008). In budding yeast, about 50% of the affected genes were downregulated in the mutants suggesting that SWI/SNF is involved equally in transcription activation and repression. Δ*swi2* and Δ*swi1* affected an overlapping set of genes suggesting they function as a complex. Given the spatial location of genes that were affected, it was evident that SWI/SNF affects individual promoters rather than chromosomal domains. More direct evidence of SWI/SNF's role in transcription activation was provided by biochemical studies on individual promoters like *SUC2, PHO5, PHO8, ARG1, RNR3, SNZ1* and *INO1* among others (Ford et al. 2007; Ransom et al. 2009; Tomar et al. 2009; Kim et al. 2005).

Chromatin immunoprecipitation (ChIP) studies provided evidence of the direct binding of SWI/SNF to these promoters. Swi2, Snf5, and Swi1 were shown to directly interact with acidic activators like Gcn4, Hap4, and Gal4 that help in targeting of the complex to promoters (Neely et al. 2002). In humans, BRG/BRM complex can associate with type I and type II nuclear receptors such as glucocorticoid receptor (GR), estrogen receptor (ER), androgen receptor (AR), retinoic acid receptor (RAR), vitamin D3 receptor (VDR), or peroxisome proliferator-activated receptor γ (PPARγ) in a ligand-dependent or independent manner to activate hormone responsive genes(Hsiao et al. 2003). Nuclease sensitivity assays, primer extension, UV cross-linking, and indirect end labeling showed SWI/SNF dependent nucleosome repositioning concomitant with transcript appearance at several genes.

The direct role of SWI/SNF in repression of yeast genes has been less well characterized. *SER3* a serine biosynthesis gene was initially implicated as a target of SWI/SNF mediated repression (Martens and Winston 2003). However, it was later found to be an indirect effect of an intergenic transcript from an adjacent *SRG1* promoter (Martens and Winston 2003). SWI/SNF has also been shown to interact with Hir1/Hir2 repressors, but this interaction activates transcription at the *HTA1-HTB1* genes (Prochasson et al. 2005). SWI/SNF represses heat shock genes under heat stress but its effect is less pronounced (Shivaswamy and Iyer 2008). In *S. pombe*, Snf22 physically associates with $fip1^+$, $frp1^+$, and $str3^+$ promoters and mediates the repression of hexose transport and iron transport genes (Monahan et al. 2008).

In humans, involvement of BRG/BRM complexes in repression is more studied and has important implications in tumor suppression. Knock-down, deletion, or mutations in BRG1, Brm, or hSNF5/Ini1 components produce malignancy (Medina and Sanchez-Cespedes 2008). BRG1/Brm is recruited by tumor suppressors such as prohibitin and retinoblastoma (Rb) protein to mediate repression of E2F-responsive genes (Wang et al. 2004; Lorch et al. 2001). BRG1 forms a repressor complex with Rb to inhibit ▶ cyclin A, D1, and E (Giacinti and Giordano 2006; Zhang et al. 2000). BRG1 represses the expression of *c-fos* proto-oncogene in an Rb-dependent but E2F or GR independent manner (Simpson et al. 1993). BRG1 and Brm associate with Rb to repress ribonucleotide reductase, thymidylate synthase, and dihydrofolate reductase genes by the recruitment of histone deacetylase mSin3B (Gunawardena et al. 2007).

In addition to tumor-suppressor interaction, BRG1 also associates with several corepressor complexes. Interaction with CoREST recruits BRG1 to neuronal genes to assist in REST mediated repression in conjunction with other deacetylases (Battaglioli et al. 2002). BRG1 components were also shown to interact with NCoR corepressor complex (Underhill et al. 2000). Recently, BRG1 was shown to directly interact with HP1α suggesting the involvement of SWI/SNF components in heterochromatin formation (Lavigne et al. 2009; Nielsen et al. 2002).

RSC conditional mutants affected 12% of the yeast genome in contrast to 6% in case of SWI/SNF. Rsc4 bromodomain conditional mutants downregulated 47–111 genes and upregulated 123–194 genes suggesting an important role of RSC in transcription activation and repression respectively. Another *rsc4*-Δ4 mutant (four amino acids from the C-terminus deleted) downregulated 168 genes but upregulated 338 (Kasten et al. 2004; Soutourina et al. 2006). Microarray on Δ*rsc30* showed RSC's role in ribosomal gene repression (Angus-Hill et al. 2001). In support, *rsc4-2* bromodomain mutant affected a large number of ribosomal and histone genes (Kasten et al. 2004). More direct evidence of RSC involvement in transcription activation comes from DNaseI accessibility data suggesting a repressive chromatin structure at *DUT1* and *SMX3* genes (Soutourina et al. 2006). Direct evidence of RSC-mediated repression of *CHA1* was shown by nucleosome positioning in indirect labeling experiments suggesting an open chromatin structure (Moreira and Holmberg 1999). At the *HTA1-HTB1* genes, RSC plays an antagonistic role to SWI/SNF by repressing transcription even though both chromatin remodeling complexes are recruited by the same Hir1/Hir2 repressor (Prochasson et al. 2005; Ng et al. 2002).

Unlike SWI/SNF which affects only polII genes, loss of RSC reduces transcription from polI, polII, and polIII genes as shown by genome-wide Sth1 localization and nucleosome occupancy studies (Ng et al. 2002; Parnell et al. 2008). This could partly be due to the association of Rsc4 with Rpb5, a subunit shared by all three RNA polymerases (Soutourina et al. 2006). Importantly, loss of Sth1 increases nucleosome density at polIII promoters (particularly tRNA genes) while polII promoters show more subtle single nucleosome changes that include gain or movement. This study has

important implications on promoter-specific activity of RSC – nucleosome eviction at polIII vs. sliding at polII (Ng et al. 2002).

Several subunits of the SWI/SNF complex in yeast show genetic interactions with proteins involved in transcription elongation. Prominent among these, Swp29 genetically interacts with TFIIS elongation factor while Swi2, Snf5, and Snf6 with Spt16, a subunit of the elongation complex FACT (Shogren-Knaak et al. 2006; Malone et al. 1991). Drosophila Brahma, Moira, and Osa suppress the rough eye phenotype of *asf1* mutants and physically interact with ASF1, another elongation factor implicated in redeposition of histones in the coding regions of genes (Moshkin et al. 2002). Human Brm is found in *CD44* coding regions where it regulates alternate splicing (Batsche et al. 2006). Similarly, BRG1 is associated with *HSP70* promoter and coding regions following heat shock (Corey et al. 2003). Several yeast SWI/SNF subunits are also localized to coding regions of inducible genes such as *GAL1*/*GAL10, HSP82, HSP104, MET2* and *MET6* under conditions of active transcription, traveling with the elongating RNA polymerase(Schwabish and Struhl 2007). The RSC complex is recruited to acetylated nucleosomal templates and helps in elongation by promoting the passage of stalled PolII. PolII passage through chromatin involves loss of a H2A/H2B dimer forming hexasomes. Complexes promoting dimer loss like SWI/SNF and RSC therefore promote polII passage during elongation. However, in an in vitro transcription assay, RSC was more potent than SWI/SNF in promoting polII passage across nucleosomal barriers (Carey et al. 2006). It has been proposed that ATP-dependent chromatin remodelers may also interact with histone chaperones to facilitate polII passage (Park and Luger 2008).

Transcription memory is the ability of daughter cells to remember the transcription activity of certain genes in the mother. This promotes the efficient "switching on" or "switching off" of genes in the daughter to quickly establish a "mother-like transcription status." In this regard, histone modifications (particularly methylation) of promoter regions have been shown to play a role in recruiting transcription factors that activate or repress transcription (Francis et al. 2004). Recently, SWI/SNF has been implicated as being involved in establishing transcription memory of *GAL1* genes in daughter cells. Daughter cells show fast kinetics of galactose reinduction following up to 4 h of repression with glucose. Normally, cells that have never been exposed to galactose take ~20 min to induce the *GAL1* gene peaking at 1 h. Daughter cells however take <10 min and are able to show this fast kinetics for one round of cell division suggesting epigenetic memory is transient. Transcriptional memory was not dependent on histone modifications, but an ISWI deletion in a Δ*swi2* background reinforced the fast reinduction kinetics. This suggested that SWI/SNF establishes transcriptional memory at *GAL1* by antagonizing ISWI action (Kundu et al. 2007).

SWI/SNF and Histone Acetylation Coordinate to Control Chromatin Structure and Function

Current studies indicate that chromatin is dynamic rather than a passive structural scaffold. The chromatin modification enzymes incorporate post-translational modifications on the histone proteins to create docking sites for specific chromatin associated proteins containing specialized domains. Some of these modifications are temporary while others may persist through cell divisions to confer stable epigenetic memory. This principle of histone marking by covalent modifications forms the basis of a "histone code" (Strahl and Allis 2000).

The yeast SWI/SNF and RSC complexes contain one or several bromodomain(s), which are also found in HAT complexes like SAGA and the general transcription factor $TAF_{II}250$ (Jacobson et al. 2000). Bromodomains are 110 residue protein motifs that specifically interact with acetylated lysine residues in histones as well as in non-histone proteins (Dhalluin et al. 1999). While, SWI/SNF possesses a single bromodomain motif residing in the Swi2/Snf2 subunit, 8 out of the 15 bromodomains in yeast are found in RSC. The ATPase motor subunit, Sth1, has only one bromodomain right at its C-terminus much like Swi2/Snf2. The Rsc1, Rsc2, and Rsc4 subunits each have two bromodomains (Kasten et al. 2004; Cairns et al. 1999). The two bromodomains in RSC4 are essential for cell viability and are located adjacent to each other to form the Rsc4 tandem bromodomain. The eighth bromodomain of RSC resides in its RSC10 subunit.

The presence of bromodomain(s) in RSC and SWI/SNF suggests that recognition of histone acetylation

might play a major role in chromatin targeting and/or in their chromatin remodeling function. The single bromodomain in Swi2/Snf2 is dispensable for SWI/SNF activity and has little or no phenotypic effect upon its own deletion (Hassan et al. 2002). Likewise, the bromodomain in Gcn5, the catalytic subunit of SAGA and ADA HAT complexes, also does not play a significant role as is evident from the modest phenotypes observed in Gcn5 bromodomain deletion mutants (Hassan et al. 2002). The bromodomain in Sth1, on the other hand, is required for wild-type function, and deletion mutants lacking different portions of the Sth1 bromodomain are thermosensitive and arrest with highly elongated buds (Du et al. 1998). However, in combination with Gcn5 bromodomain deletion or Tra1 (a targeting subunit in SAGA) *ts* mutation bromodomain deleted SWI/SNF showed strong phenotypes including a synthetic growth defect on raffinose suggesting complimentary functions of SAGA and SWI/SNF in vivo (Hassan et al. 2002). These results are indeed consistent with other evidence for a functional interplay between histone acetylation and SWI/SNF. For example, prior remodeling by SWI/SNF is required for the recruitment of Gcn5 during late mitosis when chromatin is condensed (Krebs et al. 2000); conversely, the Gcn5 bromodomain is required to stabilize SWI/SNF on promoter nucleosomes (Syntichaki et al. 2000). Studies of several promoters induced during differentiation and development revealed that SWI/SNF and SAGA complexes do function jointly, but the temporal order of these complexes can vary. For instance in the case of *yeast* HO gene, expression that occurs at the end of mitosis absolutely required SWI/SNF action for Gcn5 recruitment while activation of the human interferon beta promoter involves a very different order of events wherein histone acetylation by Gcn5 is followed by SWI/SNF recruitment (Cosma et al. 1999; Agalioti et al. 2002). Transactivation by RAR/RXR heterodimers is found to require histone acetylation prior to SWI/SNF action (Dilworth et al. 2000). Similarly, at the PHO8 promoter histone acetylation is a prerequisite for nucleosome remodeling by SWI/SNF (Reinke et al. 2001). The human α1 antitrypsin promoter is unique in the sense that PIC or preinitiation complex assembly occurs long before transcription activation by hBrm and two HAT complexes CBP and P/CAF are recruited simultaneously after PIC assembly (Soutoglou and Talianidis 2002).

Analogous to SWI/SNF, a series of elaborate genetic experiments established cooperativity between RSC and SAGA complexes. Deletions of SAGA encoding genes like *spt20*Δ (believed to abolish all SAGA function), *gcn5*Δ (required for SAGA's histone acetyl transferase activity), and *spt3*Δ (required for a HAT independent activity of SAGA) when combined with *rsc1*Δ or *rsc2*Δ mutations produced the same pattern of synthetic phenotypes as observed with Gcn5 and Swi2/Snf2 double bromodomain deletion mutants (Cairns et al. 1999). Genetic evidence for a functional link between RSC and histone acetylation was further provided when RSC4's tandem bromodomain *ts* mutation in combination with Gcn5 deletion or with histone H3K14 mutation turned out to be lethal suggesting that histone H3K14 acetylation is critical for Rsc4 function (Kasten et al. 2004).

Recently, using immobilized nucleosome arrays containing a block of four Gal4 binding sites it is shown that prior acetylation of the array with SAGA and NuA4 HAT complexes does not enhance SWI/SNF binding per se, but does allow retention of the SWI/SNF complex even after dissociation of the Gal4-VP16 activator that recruited SWI/SNF to the acetylated nucleosomal arrays. In a subsequent publication, they showed that retention of SWI/SNF on acetylated chromatin templates following removal of the transcription activator required the Swi2/Snf2 bromodomain. They validated the role of Swi2/Snf2 bromodomain in anchoring SWI/SNF to target loci in vivo by showing that the Swi2/Snf2 bromodomain contributes to SWI/SNF occupancy at the *SUC2* promoter (Hassan et al. 2002). In addition to histone tail acetylation, H3-K56 acetylation in the structured globular region of the core histone has been also shown to facilitate SWI/SNF recruitment most likely via the disruption of histone-DNA interactions near the entry-exit site of the nucleosome (Xu et al. 2005). An independent experiment by Carey et al. demonstrated that SAGA acetylated nucleosomes also stimulate binding of RSC under in vitro conditions (Carey et al. 2006).

Targeting of chromatin regulators to particular locations in the genome occurs either through interactions with sequence-specific DNA binding transcription factors or by using specialized protein motifs or domains that recognize specific post-translational modifications on histone tails. While, not yet demonstrated in vitro, since histone acetylation stabilizes SWI/SNF binding to nucleosomes, in principle

SWI/SNF could be recruited to chromatin via bromodomain dependent interaction with acetylated histone tails (Carey et al. 2006; Hassan et al. 2001, 2002). Particularly in the case of RSC based on the preponderance of bromodomains contained within its subunits one might envision that the multiple bromodomains might cumulatively increase the affinity for acetylated histone tails and provide efficient targeting of RSC to its substrates. Alternatively, the individual bromodomains within the RSC complex might not be redundant but may interact with different targets, acetylated histones and non-histone proteins, to localize RSC to different nuclear compartments or to regulate its remodeling activity. This may also explain why some of the bromodomains in RSC are essential and others are not. Further supporting the notion that the bromodomains in RSC have distinct binding partners, a recent finding showed that while one (BD#2) of the two essential tandem bromodomains in RSC4 is involved in histone H3-K14 acetylation recognition, the other (BD#1) participates in the auto-regulation of RSC activity by specifically interacting with acetylated Rsc4-K25 (VanDemark et al. 2007). This mechanism might have evolved to regulate the residence time of RSC at sites of remodeling which is critical for RSC to cater to the needs of ~700 physiological targets in the yeast genome. That individual bromodomains within the RSC complex might not be redundant but could have special functional features is further exemplified by the fact that only one of the two bromodomains (BD#2) of RSC1 and RSC2 is essential for cell viability (Cairns et al. 1999).

Does acetylation induced stabilization of SWI/SNF binding only facilitate recruitment of these complexes or such stabilization also contributes to their catalytic activity is another relevant question that has begun to be addressed recently. Biochemical data from Hassan et al. and Chandy et al. showing the requirement of the Swi2/Snf2 bromodomain for octamer transfer and remodeling of SAGA acetylated nucleosomes suggest that histone acetylation can affect the functional activity of SWI/SNF complexes by acting as binding epitopes for recruitment (Hassan et al. 2006). Thus in vivo we see that Pho5 and co-regulated Pho8 gene activation requires transient acetylation of promoter nucleosomes by SAGA and only the nucleosomes acetylated by SAGA become marked for displacement by SWI/SNF (Reinke and Horz 2003; Barbaric et al. 2003). Additional evidence in supporting that histone acetylation affects chromatin remodeling function of SWI/SNF complexes is provided by Ferreira et al. who demonstrated that RSC catalyzed remodeling is enhanced several fold upon histone H3 tail acetylation via lowering of K_m for acetylated nucleosomes (Ferreira et al. 2007).

Involvement in Double Strand Break Repair and Genome Stability

The maintenance of a stable and unaltered genome is crucial to cell survival and faithful propagation of genetic information to its progeny. Genome stability is threatened by numerous exogenous (ionizing radiation, radiomimetic agents) and endogenous (recombination, transposition, mating type switching, chromosome segregation) factors that introduce mutations and chromosomal rearrangements. A cell bearing an unstable genome is prone to growth arrest, premature ageing, apoptosis, and even malignant transformation. Instability is initiated among others by double strand breaks (DSBs) which are by far the most deleterious outcomes of genome-damaging agents (Paques and Haber 1999). Suppressors of DSBs include a class of *trans* acting factors that encompass replication, repair, and checkpoint proteins. A new addition to this class of factors has been ATP-dependent chromatin remodelers like INO80, SWR1, RSC, and SWI/SNF (van Attikum et al. 2007; Chai et al. 2005). SWI/SNF and RSC mutants (*Δsnf2, Δsnf5, sth1, sfh1*) show sensitivity to DNA damaging agents like hydroxyurea, bleomycin, MMS, and γ-irradiation (Chai et al. 2005; Koyama et al. 2002; Bennett et al. 2001). A more direct role has been established by their recruitment to DSBs by ChIP analysis albeit with different kinetics. RSC is bound to induced DSBs at the *MAT* locus in a localized region (0.2 Kb surrounding the break) within 10 min whereas SWI/SNF is recruited by 40 min with increased binding for over 4 h suggesting a role late in the repair process (Chai et al. 2005).

Cells have adopted two highly regulated damage repair pathways to counter DSBs. The homologous recombination repair (HRR) pathway makes use of long stretches of homologous regions of the sister chromatids that act as templates for faithful replication. Nonhomologous end joining (NHEJ) is more error-prone and joins together the broken ends of DNA by recognizing microhomologies of 1–5 bp. An investigation into the repair pathway affected in

SWI/SNF mutants revealed that they were able to rejoin cut plasmids through NHEJ but were unable to perform HRR. HRR proceeds via five steps: (1) 5′-3′ resection, (2) strand invasion, (3) new DNA synthesis, (4) unwinding from template and annealing, and (5) ligation (Peterson and Cote 2004). In SWI/SNF mutants, specifically the strand invasion step during homology search was affected and mutants decreased Rad51 and Rad52 association at *HMLα* (homologous donor template for *MAT* locus repair) (Chai et al. 2005). Given the remodeling and nucleosome displacement properties of SWI/SNF, it can be speculated that SWI/SNF plays a late but essential role in remodeling the homologous donor template region for efficient synapsis during HRR.

In contrast to SWI/SNF, RSC has been speculated to have roles in both NHEJ and HRR. RSC recruitment to DSBs has been shown to be dependent on yKu70, Mre11, and Rsc30 subunit. In addition, pull-down assays and two-hybrid studies indicate Rsc1 and Rsc2 physically interact with yKu80 and Mre11 (Shim et al. 2005). The yKu70/80 heterodimer is the central component of NHEJ recognizing DNA ends and recruiting other NHEJ factors. Mre11 is a component of the MRX/MRN (Mre11, Rad50, Xrs2/Mre11, Rad50, Nbs1) trimeric complex that is one of the key sensors of damaged DNA. It exhibits 3′ to 5′ exonuclease and endonuclease activities that are stimulated in presence of Rad50 and aid in 3′ end resection during NHEJ and HRR. A Δ*rsc30*Δ*rad52* and Δ*rsc8*Δ*rad52* double mutant showed enhanced hypersensitivity to DNA damaging agents suggesting that these RSC subunits affect a pathway other than that affected by Rad52 (Shim et al. 2005). Rad52 is an essential component in HRR and acts as a cofactor for Rad51 recombinase helping it to overcome competition from Replication Protein A (RPA) to bring about efficient strand exchange. Thus Rsc30 and Rsc8 are required for NHEJ. In contrast to *RSC30* and *RSC8*; *STH1*, *SFH1*, *RSC1*, and *RSC2* mutants are all able to perform NHEJ as efficiently as WT but not HR suggesting differential requirement of RSC subunits in the two modes of DSBR (Chai et al. 2005). Despite its early recruitment to DSB sites, a Δrsc2 mutant is unable to perform the final ligation step in HRR pointing to an early and late role of RSC in DSBR (Chai et al. 2005).

Chromosomal segregation during mitosis and meiosis is yet another cellular event that maintains genomic integrity and stability. From the S phase to anaphase, sister chromatids are held together at the centromeres and arms by a four-subunit cohesin complex comprising two SCC (Sister Chromatid Cohesion) proteins Mcd1/Scc1 and Scc3 and two SMC (Structural Maintenance of Chromosomes) proteins Smc1 and Smc3. Cohesion at centromeres and chromosomal arms is temporally regulated and recently the RSC complex has been implicated in cohesin loading. RSC association with cohesin in the arms is cell-cycle regulated while its association at the centromere is constitutive (Huang et al. 2004). However, *STH1* and *RSC2* mutants impair RSC recruitment specifically at the arms and result in their precocious separation. There are two alternative models proposed to explain the role of RSC in chromosomal arm cohesion. One model suggests RSC-mediated cohesin loading is coordinated with DNA replication. In support, factors involved in replication, Ctf7/Eco1, Ctf18, and Ctf8, interact genetically with RSC (Baetz et al. 2004). In another model, chromosomal arm cohesin loading is coupled to DNA repair and recombination. Cohesin has been found to be involved in post-replicative repair and two subunits Smc1 and Smc3 are components of recombination and repair complex RC1 (Huang and Laurent 2004). It is postulated that RSC remodels chromatin at chromosomal arms and recruits cohesin to maintain sister chromatid cohesion. These sister chromatids on one hand serve as templates for proper recombination and repair events and on the other allow for processive DNA replication by assuming ring structures.

Involvement of RSC and SWI/SNF in Cell Cycle Control, Differentiation, and Development

The temporal sequence of events that takes place in a eukaryotic cell before it undergoes replication to form two daughter cells together comprises the cell cycle or cell-division cycle. A typical cell cycle can be divided into two brief periods: interphase and mitotic or M phase. The interphase can be again divided into three sub-phases: gap1 or G1, synthesis or S, and gap2 or G2 phases while the mitotic M phase can be subdivided into prophase, metaphase, anaphase, and telophase.

As cell cycle controls DNA synthesis and cell proliferation, so the progression of cell cycle is highly regulated with specific checkpoints at particular points in the cell cycle like at the G1/S and G2/M transition points guarded by cyclins and cyclin dependent

kinases (CDKs) to monitor the progress of cell cycle. In budding yeast, the genes regulating cell cycle progression have been studied using temperature sensitive lethal mutants (*cdc* mutants) whose growth is arrested at specific points of the cell cycle at the restrictive temperature (Hartwell et al. 1974). These studies generated substantial evidence for an interconnection between chromatin remodeling and cell division cycle progression. Two such elegant genetic studies conducted in budding yeast demonstrated the requirement of two key subunits of RSC, Sth1 and Sfh1, for cell cycle progression through mitosis. Tsuchiya et al. showed that when Sth1, the Swi2/Snf2 counterpart in RSC, is depleted cells undergo an arrest and accumulate at the large bud stage with a single nucleus containing DNA of G2/M phase (Tsuchiya et al. 1992). Consistent with these results a temperature sensitive mutation in Sfh1, the Snf5 paralog in yeast, was found to induce cell cycle arrest at the G2/M phase (Cao et al. 1997). Both of these studies suggested a functional link between cell cycle control and RSC. Subsequent genetic studies conducted to investigate the actual role that RSC plays in cell cycle regulation isolated a temperature sensitive Sth1 mutant with impaired chromatin structure surrounding the centromeres (Tsuchiya et al. 1998). These results provide the molecular basis of the link between RSC and cell cycle control. The defective chromatin organization in absence of a functional RSC complex around centromeres might be abolishing kinetochore function which ultimately results in cell cycle arrest at G2/M transition. The yeast SWI/SNF complex on the other hand plays no role in the progression of cell division cycle through mitosis. In the contrary, SWI/SNF is required to efficiently exit from mitosis by mediating the expression of some late mitotic genes (Krebs et al. 2000). However, in flies, depletion of BAP (Brahma/BRM associated protein) complex, the *Drosophila* SWI/SNF complex, via RNAi mediated knockdown of its OSA subunit resulted in G2 to M arrest while cells lacking the RSC homolog PBAP (polybromo-containing BAP) complex showed no cell cycle defects (Moshkin et al. 2007). The *Drosophila* BAP complex regulates cell cycle progression by controlling the expression of STRING/► CDC25 which is required for entry into mitosis. This apparent contradiction from *yeast* to *flies* suggests for a functional shift in the course of evolution. In humans, both hBRM and BRG1 (BRM related gene 1) complexes participate in the regulation of cell cycle control by interacting with retinoblastoma proteins (Dunaief et al. 1994). These observations once more reinforces the notion that functional differences between yeast SWI/SNF complexes cannot be directly translated to higher eukaryotes.

The RSC complex also stimulates expression of early meiotic genes and sporulation and thereby facilitates developmental processes in budding yeast (Yukawa et al. 1999). There are numerous examples of involvement of the human SWI/SNF complexes in various developmental programs, such as muscle, heart, blood, skeletal, neuron, adipocite, liver, and T-cell development. The SWI/SNF class of chromatin remodeling complexes has instructive and reprogramming roles during development. Of particular importance are the findings that in vertebrates SWI/SNF complexes are combinatorially assembled for maintaining pluripotency and multipotency. Studies of the mammalian SWI/SNF complex, BAF (Brahma associated factor) indicate that a progressive change in its subunit composition underlies the transition from pluripotent embryonic stem cells (ESCs) to multipotent neuronal progenitors to post-mitotic differentiated neurons during nervous system development (Lessard et al. 2007). Mouse ESCs express a BRG1 containing esBAF complex which also contains BAF155 but not BAF170 (Ho et al. 2009). The esBAF complex is crucial for maintaining pluripotency and self-renewal of mouse ESCs (Ho et al. 2009). As ESCs differentiate into neuronal progenitors, the esBAF complex undergoes dynamic subunit exchanges incorporating BRM and BAF60C and eliminating BAF60B to form the neuronal-progenitor specific BAF (npBAF) complex (Lessard et al. 2007; Ho et al. 2009). BRG1 in npBAF complex ensures self-renewal of the neuronal progenitors and their differentiation into committed neurons (Lessard et al. 2007). The BAF45A subunit in npBAF induces proliferation of neuronal progenitors past their normal mitotic exit point (Lessard et al. 2007). As neuronal progenitors exit from mitosis, the BAF45A and BAF53A subunits in the npBAF complex are replaced by BAF45B and BAF53B subunits to form the neuron-specific BAF (nBAF) complex (Lessard et al. 2007). More recently, it has been shown that BAF45A depletion in post-mitotic neurons is microRNA (miR-9* and miR-124) mediated (Yoo et al. 2009). The nBAF complex is essential for post-mitotic neuronal function including activity-dependent dendritic outgrowth by

interacting with CREST, a Ca^{2+} responsive dendritic regulator (Peterson et al. 2007).

The tissue specific BAF complexes have been also reported to play a role in cardiac fate determination during heart development in vertebrates (Lickert et al. 2004). BAF60C which is selectively expressed in regions of the mouse embryo that gives rise to a heart is required for heart morphogenesis, differentiation of cardiac and skeletal muscle cells (Lickert et al. 2004) and more importantly when injected into the non-cardiogenic regions of the developing embryo BAF60C in coordination with transcription factors GATA4 and TBX5 induces the development of beating cardiomycetes from non-cardiogenic mesoderm (Lickert et al. 2004). These data suggest the existence of a BAF60C containing specialized cBAF complex although till date such a cardiogenic complex could not be isolated. On the other hand, the highly homologous polybromo or BAF180 containing BAF (PBAF) complex which is expressed in the epicardium plays roles in mediating coronary development and cardiac chamber maturation (Huang et al. 2008).

The role of BAF complexes is also implicated in T-cell development in the thymus. However, there is no report of any specialized BAF complexes in the thymocytes. During T-cell differentiation two co-receptors of the T-cell antigen receptor, CD4 and CD8, are differentially expressed. *Cd4* is developmentally repressed by a distantly located silencer ~2 kb from the transcription start site of *Cd4* (Sawada et al. 1994). At an early stage of development BAF complex binds to the distant silencer element via BRG1 and BAF57 and recruits a transcription factor RUNX1 which mediates *Cd4* gene silencing (Wan et al. 2009). This data also suggests fundamental mechanistic differences between yeast SWI/SNF and mammalian BAF complexes as the former is known to regulate its target genes by binding to promoters. In mice, BRG1 is again required at a later stage to activate *Cd4* expression indicating that BAF complexes can both activate and repress the same gene depending on the developmental context (Dey et al. 2003).

Although it remains poorly understood, BRG1 and BAF complexes might also be required for skeletal muscle differentiation as a dominant negative allele of *Brg1* or RNAi mediated depletion of BAF60C appears to be detrimental for myogenic transcription factor (MYOD1 and MEF2D) action (Lickert et al. 2004; de la Serna et al. 2001).

Summary

Clearly, there are many ways that the SWI/SNF chromatin remodelers are involved in regulation of transcription, replication, recombination, and DNA repair. It is also evident that with the large number of SWI/SNF complexes that they are not particularly redundant in terms of function, but rather have very specialized roles in the cell. While much can be learned about the fundamental mechanistic properties of SWI/SNF by studying one form of these complexes in such organisms like yeast, it seems likely that there will be differences in their mode of operation which will be context dependent. Differences between various SWI/SNF complexes will be reflected in part how they are differentially recruited to specific genomic regions, but aside from this the data suggest that they will also probably remodel nucleosomes in distinctive ways. So far progress has been made on cataloging the SWI/SNF complexes involved in different developmental pathways, but still there is no data to indicate how the presence or absence of a particular subunit could alter the mode of remodeling that occurs.

References

Agalioti T, Chen G, Thanos D. Deciphering the transcriptional histone acetylation code for a human gene. Cell. 2002;111(3):381–92.

Angus-Hill ML, et al. A Rsc3/Rsc30 zinc cluster dimer reveals novel roles for the chromatin remodeler RSC in gene expression and cell cycle control. Mol Cell. 2001;7(4):741–51.

Baetz KK, et al. The ctf13-30/CTF13 genomic haploinsufficiency modifier screen identifies the yeast chromatin remodeling complex RSC, which is required for the establishment of sister chromatid cohesion. Mol Cell Biol. 2004;24(3):1232–44.

Barbaric S, Reinke H, Horz W. Multiple mechanistically distinct functions of SAGA at the PHO5 promoter. Mol Cell Biol. 2003;23(10):3468–76.

Batsche E, Yaniv M, Muchardt C. The human SWI/SNF subunit Brm is a regulator of alternative splicing. Nat Struct Mol Biol. 2006;13(1):22–9.

Battaglioli E, et al. REST repression of neuronal genes requires components of the hSWI.SNF complex. J Biol Chem. 2002;277(43):41038–45.

Bennett CB, et al. Genes required for ionizing radiation resistance in yeast. Nat Genet. 2001;29(4):426–34.

Cairns BR, et al. Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. Mol Cell. 1999;4(5):715–23.

Cao Y, et al. Sfh1p, a component of a novel chromatin-remodeling complex, is required for cell cycle progression. Mol Cell Biol. 1997;17(6):3323–34.

Carey M, Li B, Workman JL. RSC exploits histone acetylation to abrogate the nucleosomal block to RNA polymerase II elongation. Mol Cell. 2006;24(3):481–7.

Chai B, et al. Distinct roles for the RSC and Swi/Snf ATP-dependent chromatin remodelers in DNA double-strand break repair. Genes Dev. 2005;19(14):1656–61.

Corey LL, et al. Localized recruitment of a chromatin-remodeling activity by an activator in vivo drives transcriptional elongation. Genes Dev. 2003;17(11):1392–401.

Cosma MP, Tanaka T, Nasmyth K. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell. 1999;97(3):299–311.

de la Serna IL, Carlson KA, Imbalzano AN. Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. Nat Genet. 2001;27(2):187–90.

Dey A, et al. The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. Proc Natl Acad Sci USA. 2003;100(15):8758–63.

Dhalluin C, et al. Structure and ligand of a histone acetyltransferase bromodomain. Nature. 1999;399(6735):491–6.

Dilworth FJ, et al. ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR In vitro. Mol Cell. 2000;6(5):1049–58.

Du J, et al. Sth1p, a Saccharomyces cerevisiae Snf2p/Swi2p homolog, is an essential ATPase in RSC and differs from Snf/Swi in its interactions with histones and chromatin-associated proteins. Genetics. 1998;150(3):987–1005.

Dunaief JL, et al. The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell. 1994;79(1):119–30.

Ferreira H, Flaus A, Owen-Hughes T. Histone modifications influence the action of Snf2 family remodelling enzymes by different mechanisms. J Mol Biol. 2007;374(3):563–79.

Ford J, et al. A SWI/SNF- and INO80-dependent nucleosome movement at the INO1 promoter. Biochem Biophys Res Commun. 2007;361(4):974–9.

Francis NJ, Kingston RE, Woodcock CL. Chromatin compaction by a polycomb group protein complex. Science. 2004;306(5701):1574–7.

Giacinti C, Giordano A. RB and cell cycle progression. Oncogene. 2006;25(38):5220–7.

Gunawardena RW, et al. SWI/SNF activity is required for the repression of deoxyribonucleotide triphosphate metabolic enzymes via the recruitment of mSin3B. J Biol Chem. 2007;282(28):20116–23.

Hartwell LH, et al. Genetic control of the cell division cycle in yeast. Science. 1974;183(120):46–51.

Hassan AH, Neely KE, Workman JL. Histone acetyltransferase complexes stabilize swi/snf binding to promoter nucleosomes. Cell. 2001;104(6):817–27.

Hassan AH, et al. Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. Cell. 2002;111(3):369–79.

Hassan AH, Awad S, Prochasson P. The Swi2/Snf2 bromodomain is required for the displacement of SAGA and the octamer transfer of SAGA-acetylated nucleosomes. J Biol Chem. 2006;281(26):18126–34.

Ho L, et al. An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency. Proc Natl Acad Sci USA. 2009;106(13):5181–6.

Hsiao PW, et al. BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. Mol Cell Biol. 2003;23(17):6210–20.

Huang J, Laurent BC. A Role for the RSC chromatin remodeler in regulating cohesion of sister chromatid arms. Cell Cycle. 2004;3(8):973–5.

Huang J, Hsu JM, Laurent BC. The RSC nucleosome-remodeling complex is required for Cohesin's association with chromosome arms. Mol Cell. 2004;13(5):739–50.

Huang X, et al. Coronary development is regulated by ATP-dependent SWI/SNF chromatin remodeling component BAF180. Dev Biol. 2008;319(2):258–66.

Jacobson RH, et al. Structure and function of a human TAFII250 double bromodomain module. Science. 2000;288(5470):1422–5.

Kasten M, et al. Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. EMBO J. 2004;23(6):1348–59.

Kim SJ, et al. Activator Gcn4p and Cyc8p/Tup1p are interdependent for promoter occupancy at ARG1 in vivo. Mol Cell Biol. 2005;25(24):11171–83.

Koyama H, et al. Abundance of the RSC nucleosome-remodeling complex is important for the cells to tolerate DNA damage in Saccharomyces cerevisiae. FEBS Lett. 2002;531(2):215–21.

Krebs JE, et al. Global role for chromatin remodeling enzymes in mitotic gene expression. Cell. 2000;102(5):587–98.

Kundu S, Horn PJ, Peterson CL. SWI/SNF is required for transcriptional memory at the yeast GAL gene cluster. Genes Dev. 2007;21(8):997–1004.

Lavigne M, et al. Interaction of HP1 and Brg1/Brm with the globular domain of histone H3 is required for HP1-mediated repression. PLoS Genet. 2009;5(12):e1000769.

Lessard J, et al. An essential switch in subunit composition of a chromatin remodeling complex during neural development. Neuron. 2007;55(2):201–15.

Lickert H, et al. Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. Nature. 2004;432(7013):107–12.

Lorch Y, Zhang M, Kornberg RD. RSC unravels the nucleosome. Mol Cell. 2001;7(1):89–95.

Malone EA, et al. Mutations in SPT16/CDC68 suppress cis- and trans-acting mutations that affect promoter function in Saccharomyces cerevisiae. Mol Cell Biol. 1991;11(11):5710–7.

Martens JA, Winston F. Recent advances in understanding chromatin remodeling by Swi/Snf complexes. Curr Opin Genet Dev. 2003;13(2):136–42.

Medina PP, Sanchez-Cespedes M. Involvement of the chromatin-remodeling factor BRG1/SMARCA4 in human cancer. Epigenetics. 2008;3(2):64–8.

Monahan BJ, et al. Fission yeast SWI/SNF and RSC complexes show compositional and functional differences from budding yeast. Nat Struct Mol Biol. 2008;15(8):873–80.

Moreira JM, Holmberg S. Transcriptional repression of the yeast CHA1 gene requires the chromatin-remodeling complex RSC. EMBO J. 1999;18(10):2836–44.

Moshkin YM, et al. Histone chaperone ASF1 cooperates with the Brahma chromatin-remodelling machinery. Genes Dev. 2002;16(20):2621–6.
Moshkin YM, et al. Functional differentiation of SWI/SNF remodelers in transcription and cell cycle control. Mol Cell Biol. 2007;27(2):651–61.
Neely KE, et al. Transcription activator interactions with multiple SWI/SNF subunits. Mol Cell Biol. 2002;22(6):1615–25.
Ng HH, et al. Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. Genes Dev. 2002;16(7):806–19.
Nielsen AL, et al. Selective interaction between the chromatin-remodeling factor BRG1 and the heterochromatin-associated protein HP1alpha. EMBO J. 2002;21(21):5797–806.
Paques F, Haber JE. Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol Mol Biol Rev. 1999;63(2):349–404.
Park YJ, Luger K. Histone chaperones in nucleosome eviction and histone exchange. Curr Opin Struct Biol. 2008;18(3):282–9.
Parnell TJ, Huff JT, Cairns BR. RSC regulates nucleosome positioning at Pol II genes and density at Pol III genes. EMBO J. 2008;27(1):100–10.
Peterson CL, Cote J. Cellular machineries for chromosomal DNA repair. Genes Dev. 2004;18(6):602–16.
Peterson S, et al. NAP1 catalyzes the formation of either positive or negative supercoils on DNA on basis of the dimer-tetramer equilibrium of histones H3/H4. Biochemistry. 2007;46(29):8634–46.
Prochasson P, et al. The HIR corepressor complex binds to nucleosomes generating a distinct protein/DNA complex resistant to remodeling by SWI/SNF. Genes Dev. 2005;19(21):2534–9.
Ransom M, et al. FACT and the proteasome promote promoter chromatin disassembly and transcriptional initiation. J Biol Chem. 2009;284(35):23461–71.
Reinke H, Horz W. Histones are first hyperacetylated and then lose contact with the activated PHO5 promoter. Mol Cell. 2003;11(6):1599–607.
Reinke H, Gregory PD, Horz W. A transient histone hyperacetylation signal marks nucleosomes for remodeling at the PHO8 promoter in vivo. Mol Cell. 2001;7(3):529–38.
Sawada S, et al. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell. 1994;77(6):917–29.
Schwabish MA, Struhl K. The Swi/Snf complex is important for histone eviction during transcriptional activation and RNA polymerase II elongation in vivo. Mol Cell Biol. 2007;27(20):6987–95.
Shim EY, et al. The yeast chromatin remodeler RSC complex facilitates end joining repair of DNA double-strand breaks. Mol Cell Biol. 2005;25(10):3934–44.
Shivaswamy S, Iyer VR. Stress-dependent dynamics of global chromatin remodeling in yeast: dual role for SWI/SNF in the heat shock stress response. Mol Cell Biol. 2008;28(7):2221–34.
Shogren-Knaak M, et al. Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science. 2006;311(5762):844–7.
Simpson RT, et al. Nucleosome positioning and transcription. Cold Spring Harb Symp Quant Biol. 1993;58:237–45.
Soutoglou E, Talianidis I. Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. Science. 2002;295(5561):1901–4.
Soutourina J, et al. Rsc4 connects the chromatin remodeler RSC to RNA polymerases. Mol Cell Biol. 2006;26(13):4920–33.
Strahl BD, Allis CD. The language of covalent histone modifications. Nature. 2000;403(6765):41–5.
Sudarsanam P, et al. Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae. Proc Natl Acad Sci USA. 2000;97(7):3364–9.
Syntichaki P, Topalidou I, Thireos G. The Gcn5 bromodomain co-ordinates nucleosome remodelling. Nature. 2000;404(6776):414–7.
Tomar RS, et al. A novel mechanism of antagonism between ATP-dependent chromatin remodeling complexes regulates RNR3 expression. Mol Cell Biol. 2009;29(12):3255–65.
Tsuchiya E, et al. The Saccharomyces cerevisiae NPS1 gene, a novel CDC gene which encodes a 160 kDa nuclear protein involved in G2 phase control. EMBO J. 1992;11(11):4017–26.
Tsuchiya E, Hosotani T, Miyakawa T. A mutation in NPS1/STH1, an essential gene encoding a component of a novel chromatin-remodeling complex RSC, alters the chromatin structure of saccharomyces cerevisiae centromeres. Nucleic Acids Res. 1998;26(13):3286–92.
Underhill C, et al. A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. J Biol Chem. 2000;275(51):40463–70.
van Attikum H, Fritsch O, Gasser SM. Distinct roles for SWR1 and INO80 chromatin remodeling complexes at chromosomal double-strand breaks. EMBO J. 2007;26(18):4113–25.
VanDemark AP, et al. Autoregulation of the rsc4 tandem bromodomain by gcn5 acetylation. Mol Cell. 2007;27(5):817–28.
Wan M, et al. Molecular basis of CD4 repression by the Swi/Snf-like BAF chromatin remodeling complex. Eur J Immunol. 2009;39(2):580–8.
Wang Y, et al. Beyond the double helix: writing and reading the histone code. Novartis Found Symp. 2004;259:3–17 discussion 17–21, 163–9.
Xu F, Zhang K, Grunstein M. Acetylation in histone H3 globular domain regulates gene expression in yeast. Cell. 2005;121(3):375–85.
Yoo AS, et al. MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. Nature. 2009;460(7255):642–6.
Yukawa M, et al. Nps1/Sth1p, a component of an essential chromatin-remodeling complex of Saccharomyces cerevisiae, is required for the maximal expression of early meiotic genes. Genes Cells. 1999;4(2):99–110.
Zhang HS, et al. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell. 2000;101(1):79–89.

Synembryn

► Ric-8

Sytl (Syanptotagmin-Like)

▶ Slp (Synaptotagmin-Like Protein)

Syx

▶ SYX/PLEKHG5, A Rhoa Guanine Exchange Factor Involved in Cell Migration and Angiogenesis

SYX/PLEKHG5, A Rhoa Guanine Exchange Factor Involved in Cell Migration and Angiogenesis

Arie Horowitz
Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic Foundation and Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH, USA

Synonyms

Plekhg5; Syx; Tech

Historical Background

Plekhg5 first emerged as a partial cDNA clone KIAA0720 in the HUGE (Human Unidentified Gene-Encoded) database of large proteins analyzed by the Kazusa Human cDNA Project in Japan (http://www.kazusa.or.jp/huge). It was initially characterized as a RhoA-specific GEF and a potential oncogen named GEF720 (de Toledo et al. 2003). Independently, the rat cDNA of the same protein (named Tech) was characterized also as a RhoA-specific GEF that was highly expressed in cortical and hippocampal neurons (Marx et al. 2005). Two full-length cDNA clones of the mouse ortholog were identified as splice variants (named Syx1 and Syx2) and characterized as participants in cell motility (Liu and Horowitz 2006).

Splice Variants and Domain Structure

The majority of the known splice-variants of Plekhg5 are produced by the initiation of transcription at alternate 5′ start codons (http://www.uniprot.org/uniprot/O94827). These five transcripts code for variants ranging in length from 930 to 1083 residues. Interestingly, the sixth variant is generated by 3′ splicing, very close to the stop codon. The shorter splice variant lacks the two 3′ codons. The absence of the two carboxy-terminus residues in the shorter splice variant removes the PDZ-binding motif and abolishes binding to the PDZ adaptor proteins synectin (Liu and Horowitz 2006) and Mupp1/Patj (Ernkvist et al. 2009). The expression level of the shorter splice variant is negligible in most cell types, except in lines of glioblastoma multiforme, where it reached close to 10% of the expression level of the longer splice variant (Liu and Horowitz 2006). The absence of the PDZ-binding domain resulted in a diffuse rather than targeted distribution of the overexpressed shorter splice variant. RhoA activity in these cells was similarly diffuse and higher than the basal RhoA activity in cells expressing the longer splice variant.

Plekhg5 conforms to the canonical Dbl structure, where a Dbl homology (DH) domain, which is the site of catalytic activity, is flanked by a pleckstrin homology (PH) domain (Fig. 1). Similar to numerous Rho GEFs (Garcia-Mata and Burridge 2007), the C-terminus of the long splice variant of Plekhg5 (Syx1) consists of a PDZ-binding motif. This motif is absent in the shorter splice variant Syx2 (Liu and Horowitz 2006).

Major Sites of Expression and Subcellular Location

Northern blots of mouse tissue showed that plekhg5 is expressed primarily in the brain and to a lesser extent in the heart (de Toledo et al. 2003). More recent Northern blotting pinpointed the expression of plekhg5 in the brain to the hippocampus and cortex (Marx et al. 2005). Quantitative PCR showed that the highest expression level in the human nervous system was in the peripheral nerves (Maystadt et al. 2007). Gene array studies reported a more widely distributed expression of human plekhg5, with similar expression

SYX/PLEKHG5, A Rhoa Guanine Exchange Factor Involved in Cell Migration and Angiogenesis, Fig. 1 Domain structure of Syx. *DH* dbl homology, *PH* pleckstrin homology, *PDZ* postsynaptic density 95, disk large, zona occludens-1. Numbers denote amino acid positions in the mouse protein

levels in the brain, heart, skeletal muscle, lung, kidney, liver, pancreas, and the prostate (Yanai et al. 2005).

Plekhg5 was observed in three main cellular compartments. Plekhg5 is present in the cytoplasm, associated with endocytic vesicles and with angiomotin, primarily but not exclusively in the perinuclear region. Plekgh5 collocated with Rab13, suggesting it traffics together with tight junction proteins (Wu et al. 2011). It was also observed at tight junctions where it collocated with ZO1, and in the leading edge of endothelial cells.

Function

Plekhg5 (pleckstrin homology domain containing family with Rho GEF domain 5) is a member of the Dbl Rho GEFs. Plekhg5 conforms to the canonic Dbl structure, that is, a Dbl homology (DH) domain that catalyzes GDP removal from Rho GTPases, followed by a pleckstrin homology (PH) domain. The PH domain is commonly involved in GEF auto-regulation and targeting. The carboxy-terminus of Plekhg5 (SEV*) conforms to the consensus sequence of neuronal nitric oxide synthase (nNOS) class 1 PDZ-binding motifs. Plekhg5 is widely expressed and highly conserved in vertebrates, but is not expressed in *Drosophila melanogaster* and in the nematode *Caenorhabditis elegans*. Among vertebrates, it is expressed in the zebrafish (XM_001923778.1, 75% homology), chicken (XP_423692.2, partial sequence, 92% homology), mouse (AY605057.1, 86% homology), rat (AY499658.1, 87% homology), cow (XM_867846.3, 90% homology), horse (XP_001915300.1, 90% homology), dog (XM_536728.2, 91% homology), and chimpanzee (XP_514339, 85% homology). Plekhg5 was characterized as RhoA-specific versus RhoB, RhoC, RhoG, Rac1-3, and Cdc42 (de Toledo et al. 2003). The RhoA-specificity was confirmed in an independent study (Marx et al. 2005).

In vitro studies showed that Plekhg5 is essential in endothelial cell migration and tube formation (Liu and Horowitz 2006). Knockdown of the zebrafish *plekhg5* ortholog resulted in growth arrest of the intersegmental vessels and alteration of the morphology of the tip cells of these vessels (Garnaas et al. 2008). Instead of a tapered end that projected long filopodia, the tip cells in the knockdown zebrafish had a round end from which a dense array of thin filopodia protruded in all directions. The loss of filopodia directionality suggests that the growth arrest of the intersegmental vessels was caused, at least in part, by a guidance defect. The *plekhg5*$^{-/-}$ mouse was viable and fertile, but had a subtle angiogenic defect of a similar nature to the phenotype of the zebrafish *plekhg5* knockdown – sparseness of arterioles and capillaries. Probably because of reduced perfusion, the pumping function of the *plekhg5*$^{-/-}$ heart was significantly lower than the wild-type heart. Further in vitro studies showed that *plekhg5* silencing in endothelial cells was accompanied by loss of the ZO1 scaffold protein from tight junctions, and an increase in monolayer permeability (unpublished data). Accordingly, electron microscopy showed that tight junctions in the *plekhg5*$^{-/-}$ coronary capillaries were missing or not fully formed. In vitro studies showed that *plekhg5* silencing in endothelial cells was accompanied by loss of the ZO1 scaffold protein from tight junctions, and an increase in monolayer permeability (unpublished data).

High throughput screening of genes that activate nuclear factor κ B (NFκB) signaling identified *plekhg5* among a group of 299 genes (Matsuda et al. 2003). In agreement, in vitro studies with epithelial cells found that overexpression of *plekhg5*-produced a tenfold increase in NFκB activity (Maystadt et al. 2007). A point mutation (F703S) in the PH domain of human *plekhg5* was found to be a marker of a previously uncharacterized autosomal recessive lower motor neuron disease prevalent in a large inbred family from sub-Saharan Africa (Maystadt et al. 2007; Maystadt et al. 2006). Further studies revealed that overexpressed mutant *plekhg5* in murine motor neuronal cells (but not in human epithelial cells) formed large cytoplasmic aggregates (Maystadt et al. 2007). Conceivably, and similar to other degenerative diseases of the nervous system, the aggregation of the mutant Plekhg5 could have damaged the motor neurons in the affected individuals (Ross and Poirier 2005).

At this point, no nervous system defects were reported in the *plekhg5*$^{-/-}$ mouse.

Relatively little is known on Plekhg5 regulation. Truncation of the first 248 amino-terminal residues together with the carboxy-terminal region immediately downstream of the PH domain conferred constitutive activity on Plekgh5 (Marx et al. 2005). Since both the amino and carboxy-terminus regions were truncated, it is not yet possible to determine if auto-regulation requires one or both regions. No Plekgh5 phosphorylation sites have been reported yet.

The phenotype of the zebrafish *plekhg5* knockdown has been described above. The loss of filopodia directionality suggests that the growth arrest of intersegmental vessels was caused, at least in part, by a guidance defect. The *Plekhg5*$^{-/-}$ mouse was viable and fertile, but had an angiogenic defect consisting of sparseness of arterioles and capillaries. Probably because of reduced perfusion, the pumping function of the *plekhg5*$^{-/-}$ heart was significantly lower than that of the wild-type heart. Further in vitro studies showed that *plekhg5* silencing in endothelial cells was accompanied by loss of the ZO1 scaffold protein from tight junctions and an increase in monolayer permeability (unpublished observations).

Interactions with Ligands and Other Proteins

Plekhg5 binds RhoA (Marx et al. 2005), in agreement with its specificity for RhoA as an effector. A recent study reported that Plekhg5 binds Rnd3 (alternatively named RhoE), a constitutively active GTPase of intrinsically low GTP hydrolysis rate (Goh and Manser 2010). Rnd3 is thought to act as a RhoA antagonist by activating the GTPase activating protein p190 RhoGAP (Wennerberg et al. 2003). Rnd3 expressed endogenously in mouse embryonic stem cells binds to a 70 amino acid-long domain in the N-terminus region of Plekhg5 that bears similarity to the Raf1 Ras-binding domain (Goh and Manser 2010).

Two PDZ adaptor proteins are known to bind the carboxy-terminus of Plekhg5: synectin (Liu and Horowitz 2006) and Mupp1 (Ernkvist et al. 2009; Estevez et al. 2008) as well as the Mupp1 paralog Patj (Ernkvist et al. 2009). The binding site of Plekhg5 to Mupp1 is either the 10th or 13th PDZ domain, or both (Estevez et al. 2008), whereas Plekhg5 binds to the 10th PDZ domain of Patj (Ernkvist et al. 2009). Both Mupp1 and Patj bind angiomotin, which couples Plekhg5 to members of the Par-6/Par-3 apico-basal polarity complex (Ernkvist et al. 2009; Wells et al. 2006). This angiomotin-associated complex includes the Cdc42-specific GTPase-activating protein Rich1, and the scaffold protein Pals1. In turn, Pals1 and Patj link angiomotin and Plekhg5 to the Crumbs complex. Hence, Plekhg5 is member of a large signaling complex involved in maintaining cell polarity and tight junctions.

Summary

Syx/Plekhg5 is a ubiquitously expressed RhoA guanine exchange factor whose functions are known only partially. Despite its high expression level in the brain, its specific neural function is not fully understood. While the highest site of expression appears to be in cortical and hippocampal neurons of the central nervous system, a point mutation in human PLEKHG5 results in an autosomal recessive lower motor neuron disease. In contrast, animal models suggest that one of the primary functions of Syx/Plekhg5 is in angiogenesis.

References

de Toledo M, Senic-Matuglia F, Salamero J, Uze G, Comunale F, Fort P, Blangy A. The GTP/GDP cycling of rho GTPase TCL is an essential regulator of the early endocytic pathway. Mol Biol Cell. 2003;14(12):4846–56. PMID 12960428.

Ernkvist M, Luna Persson N, Audebert S, Lecine P, Sinha I, Liu M, Schlueter M, Horowitz A, Aase K, Weide T, Borg JP, Majumdar A, Holmgren L. The Amot/Patj/Syx signaling complex spatially controls RhoA GTPase activity in migrating endothelial cells. Blood. 2009;113(1):244–253. PMID 18824598.

Estevez MA, Henderson JA, Ahn D, Zhu XR, Poschmann G, Lubbert H, Marx R, Baraban JM. The neuronal RhoA GEF, Tech, interacts with the synaptic multi-PDZ-domain-containing protein, MUPP1. J Neurochem. 2008;106(3):1287–97. PMID 18537874.

Garcia-Mata R, Burridge K. Catching a GEF by its tail. Trends Cell Biol. 2007;17(1):36–43. PMID 17126549.

Garnaas MK, Moodie KL, Liu ML, Samant GV, Li K, Marx R, Baraban JM, Horowitz A, Ramchandran R. Syx, a RhoA guanine exchange factor, is essential for angiogenesis in vivo. Circ Res. 2008;103(7):710–6. PMID 18757825.

Goh LL, Manser E. The RhoA GEF Syx is a target of Rnd3 and regulated via a Raf1-like ubiquitin-related domain. PLoS One. 2010;5(8):e12409. PMID 20811643.

Liu M, Horowitz A. A PDZ-binding motif as a critical determinant of Rho guanine exchange factor function and cell phenotype. Mol Biol Cell. 2006;17(4):1880–7. PMID 16467373.

Marx R, Henderson J, Wang J, Baraban JM. Tech: a RhoA GEF selectively expressed in hippocampal and cortical neurons. J Neurochem. 2005;92(4):850–8. PMID 15686487.

Matsuda A, Suzuki Y, Honda G, Muramatsu S, Matsuzaki O, Nagano Y, Doi T, Shimotohno K, Harada T, Nishida E, Hayashi H, Sugano S. Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. Oncogene. 2003;22(21): 3307–18. PMID 12761501.

Maystadt I, Zarhrate M, Leclair-Richard D, Estournet B, Barois A, Renault F, Routon MC, Durand MC, Lefebvre S, Munnich A, Verellen-Dumoulin C, Viollet L. A gene for an autosomal recessive lower motor neuron disease with childhood onset maps to 1p36. Neurology. 2006;67(1):120–4. PMID 16728649.

Maystadt I, Rezsohazy R, Barkats M, Duque S, Vannuffel P, Remacle S, Lambert B, Najimi M, Sokal E, Munnich A, Viollet L, Verellen-Dumoulin C. The nuclear factor kappaB-activator gene PLEKHG5 is mutated in a form of autosomal recessive lower motor neuron disease with childhood onset. Am J Hum Genet. 2007;81(1):67–76. PMID 17564964.

Ross CA, Poirier MA. Opinion: what is the role of protein aggregation in neurodegeneration? Nat Rev Mol Cell Biol. 2005;6(11):891–8. PMID 16167052.

Wells CD, Fawcett JP, Traweger A, Yamanaka Y, Goudreault M, Elder K, Kulkarni S, Gish G, Virag C, Lim C, Colwill K, Starostine A, Metalnikov P, Pawson T. A Rich1/Amot complex regulates the Cdc42 GTPase and apical-polarity proteins in epithelial cells. Cell. 2006;125(3):535–48. PMID 16678097.

Wennerberg K, Forget MA, Ellerbroek SM, Arthur WT, Burridge K, Settleman J, Der CJ, Hansen SH. Rnd proteins function as RhoA antagonists by activating p190 RhoGAP. Curr Biol. 2003;13(13):1106–15. PMID 12842009.

Wu C, Agrawal SA, Vasanji A, Sarkaria S, Xie J, Liu M, Anand-Apte B, Horowitz A. Rab13-dependent trafficking of RhoA is required for directional migration and angiogenesis. J Biol Chem. 2011;286(26):23511–20. PMID 21543326.

Yanai I, Benjamin H, Shmoish M, Chalifa-Caspi V, Shklar M, Ophir R, Bar-Even A, Horn-Saban S, Safran M, Domany E, Lancet D, Shmueli O. Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics. 2005;21(5):650–9. PMID 15388519.

T

T

► G Protein Alpha Transducin

T10 Antigen

► CD38

T16

► Fibulins

T200

► CD45 (PTPRC)

T3

► CD3

TAN1

► Notch (Notch1, Notch2, Notch3, Notch4)

TAPA-1

► CD81

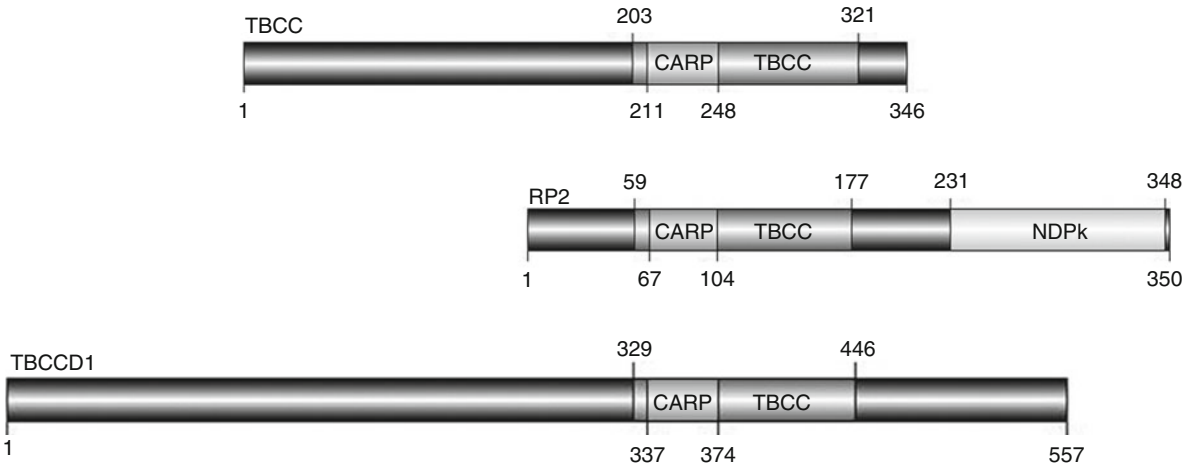
TBCCD1

João Gonçalves[1,2] and Helena Soares[2,3,4]
[1]Centro de Química e Bioquímica, Lisbon, Portugal
[2]Instituto Gulbenkian de Ciência, Oeiras, Portugal
[3]Escola Superior de Tecnologia da Saúde de Lisboa, Lisboa, Portugal
[4]Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

Historical Background

The major microtubule organizing center in animal cells is the centrosome which consists of a pair of centrioles surrounded by the pericentriolar matrix. In interphase cells, centrosomes nucleate and organize the microtubule cytoskeleton and are usually maintained at the cell center in close association with the nucleus (Bettencourt-Dias and Glover 2007). This association constitutes a primordial axis of cytoplasmic compartments organization and organelle positioning (e.g., ER and Golgi apparatus). Centrosomes are also involved in mitotic spindle formation, and an aberrant centrosome number has been linked to multipolar spindles and to cancer cells. In the last years, the ability of centrosomes to become basal bodies and assemble cilia emerged also as a critical role (Bettencourt-Dias and Glover 2007). Cilia are now established as having important roles as movement

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



TBCCD1, Fig. 1 Schematic representation of the functional domains of TBCC domain containing proteins: TBCC, TBCCD1, and RP2. These three proteins share the TBCC and CARP domains

generating and sensory organeles being essential for embryonic development. Critically, cilia malfunction has been associated with abnormal left-right asymmetry, polycystic disease, obesity, neuropathies, blindness, and other ciliopathies (Nigg and Raff 2009).

Proteomic studies have been revealing an increasing number of centrosomal proteins, making it clear that many of them have unknown roles in the cell and can be translocated to different compartments during the cell cycle or in response to environmental cues (Nigg and Raff 2009; Andersen et al. 2003; Keller et al. 2005). This shows that centrosomes concentrate and probably regulate the interactions and the intracellular distribution of a variety of proteins contributing to the establishing of networks of different pathways.

One example of a group of proteins that have been recently localized at the centrosome is the one that comprises several components of the via that promotes the maturation of tubulin heterodimers, the building blocks of microtubules, as well as their regulatory and related proteins. These proteins being critical for the assembly of tubulin heterodimers competent to polymerize and controlling heterodimer quality and degradation (Lopez-Fanarraga et al. 2001) are central factors in regulating microtubule assembly and dynamics. As far as these proteins have been studied in vivo in different biological models, their ability to play other functions, not always related to their role in the tubulin folding pathway, started to emerge. For example, the recently described centrosomal protein TBCCD1 is a protein related, through its functional domains, to the tubulin cofactor C, one of the least studied components of the tubulin folding pathway, that plays a role in centrosome positioning and centrioles' interplay (Feldman and Marshall 2009; Gonçalves et al. 2010).

The Centrosomal/Basal Bodies Localization of TBCCD1

Recently, a new protein related to TBCC (tubulin cofactor C) and RP2 (retinitis pigmentosa 2), called TBCCD1 (TBCC-domain containing 1), was described both in *Chlamydomonas reinhardtii* and human cells (Feldman and Marshall 2009; Gonçalves et al. 2010). These three proteins share two conserved functional domains: TBCC and CARP (Fig. 1). The CARP domain is also described in CAP proteins which regulate actin polymerization. TBCC, together with tubulin cofactor D, acts as β-tubulin GTPase activating proteins (GAP) in the via that leads to mature tubulin heterodimers (Fontalba et al. 1993; Tian et al. 1999). In vitro, RP2 can also act as a GAP for tubulin, but it does not substitute for TBCC in the tubulin-folding pathway. Nevertheless, a functional overlap was further shown by the capacity of RP2 to partially complement the *CIN2* (*tbcc* homologous gene) deletion in budding yeast (Bartolini et al. 2002). RP2 was also shown to bind to and be a GAP for the ADP-ribosylation factor-like 3 (Arl3), a member of the ADP-ribosylation factor family of small GTPases that is highly conserved in eukaryotes, and plays important roles in the regulation of microtubule-dependent

processes. Human Arl3 localizes at centrosomes, midzones, midbodies, and cilia (Veltel et al. 2008; Zhou et al. 2006) and, in the mouse, its knockout causes ciliary problems like: abnormal development of renal, hepatic, and pancreatic epithelial tubule structures, and photoreceptor degeneration (Schrick et al. 2006). It was also shown that Arl3 is necessary for flagellum biogenesis in *Leishmania* (Cuvillier et al. 2000).

RP2 mutations are implicated in the Retinitis Pigmentosa pathology characterized by a progressive photoreceptor cell degeneration leading to the loss of peripheral vision and eventually to blindness. In fact, 15–20% of the cases of Retinitis Pigmentosa result from mutations in the *rp2* gene (Schwahn et al. 1998). The GAP activity of RP2 and *TBCC* relies on the TBCC domain, and many of the RP2 mutations involved in this pathology occur in this domain (Schwahn et al. 1998). For example, this activity is abolished if RP2 is mutated in the only arginine residue conserved between RP2 (Arg-118) and TBCC (Arg-262) and that also abolishes TBCC's GAP activity (Veltel et al. 2008). Human and *Chlamydomonas* TBCCD1 proteins do not contain this critical arginine residue, suggesting that its TBCC domain lost the GAP activity. This hypothesis is supported by the observation that human TBCCD1 is unable to rescue the phenotypes of *CIN2* deletion in yeast (Gonçalves et al. 2010).

Studies of the subcellular localization of TBCCD1 in human cells show that it is localized in the centrosome throughout the cell cycle being also detected in the spindle midzone and in the midbody at the end of cell division (Fig. 2A). TBCCD1 was also found in the basal bodies of primary and motile cilia. Interestingly, *Chlamydomonas* TBCCD1 also localizes at centrioles/basal bodies and in rhyzoplasts, structures that connect them to each other and to the nucleus (Fig. 2B) (Feldman and Marshall 2009).

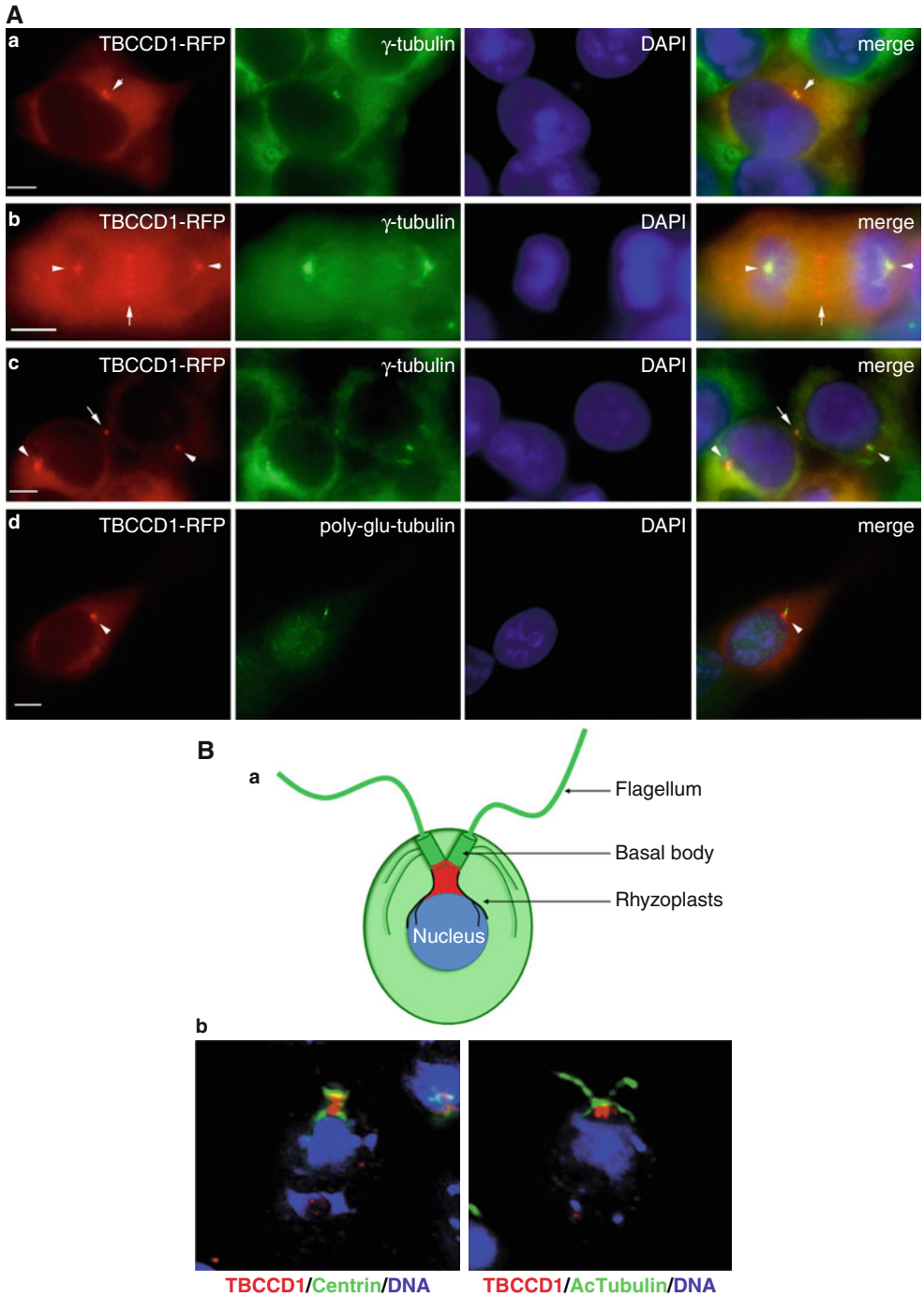
TBCCD1: The Nucleus–Centrosome Connection

TBCCD1 silencing by RNAi in human RPE-1 cells caused a remarkable separation of the centrosome from the nucleus being the former often located at the cell periphery (Gonçalves et al. 2010). Similarly, in *Chlamydomonas*, TBCCD1 loss of function by an insertion disrupting the *TBCCD1* gene caused centriole positioning defects, which leads to the assembly of the mitotic spindle with incorrect orientation (Feldman and Marshall 2009).

In the last years, the importance of the nucleus–centrosome connection has been recognized not only for the intracellular organization of the cytoplasm but also has a key factor implicated, for example, in directed cell migration, adhesion and polarity, crucial events in development and cancer. This connection is dynamic and able to be remodeled in response to several signals, like those involved in cell differentiation (e.g., differentiating neurons). However, the mechanisms underlying the dynamic nucleus–centrosome connection are far from being understood. In this context, TBCCD1 being required for centrosome positioning and correct mitotic spindle orientation is an emerging key factor in the nucleus–centrosome interplay and in the centrosome activities that depend on it. Supporting this view are the observations that in human cells silenced for TBCCD1, the centrosome mispositioning is accompanied by a dramatic disorganization of Golgi apparatus and a G1 cell cycle delay. Moreover, TBCCD1-depleted cells are larger, less efficient in primary cilia assembly, and their migration is slower in wound-healing assays.

Elegant experiments growing mammalian cells in patterned surfaces have shown that geometrical constraints imposed by the substratum play a crucial role in centrosome positioning and cytoplasmic organization (Pouthas et al. 2008). However, this is not the sole responsible factor since microtubules and forces exerted on them by actomyosin and dynein are also critical (Burakov et al. 2003). The existence of a physical link between the centrosome and the nuclear envelope is supported by a variety of data being proteins that are able to bind it to nuclear envelope, such as Zyg-12, Emerin, and Samp1, implicated in it (Malone et al. 2003; Salpingidou et al. 2007; Buch et al. 2009). This interaction seems to be regulated by a group of distinct kinases like the p160ROCK Rho associated and the Polo/Greatwall (Gwl) mitotic kinases (Chevrier et al. 2002; Archambault et al. 2007).

In *Chlamydomonas*, the absence of TBCCD1 can lead to aberrant numbers of centrioles and flagella. TBCCD1 mutant cells (*asq2*) can have up to seven flagella which show that the protein is not essential for the formation of these structures. Additionally, the increased number of centrioles suggests that, in *Chlamydomonas*, TBCCD1 could have a regulatory role in de novo centriole assembly pathway (Feldman



TBCCD1, Fig. 2 *Subcellular localization of TBCCD in human cells and Chlamydomonas*. (**A**) *HEK-293T* Cells were transfected with a plasmid containing TBCCD1-RFP and immunostained using antibodies against γ-tubulin (**a**, **b**, and **c**), or polyglutamylated tubulin (**d**). DNA was stained with DAPI. (**A**) Interphase cell; (**b**) Cell in anaphase; (**c**) Cell in cytokinesis; (**d**) Cell cycle arrested cell after serum starvation for 24 h showing a primary cilium. Arrowheads point to TBCCD1-RFP at centrosomes. Arrows indicate spindle midzone (**b**), midbody (**c**) and basal body (**d**). (**Ba**) Schematic representation of the TBCCD1 localization in *Chlamydomonas*. TBCCD1 cellular localization is showed in red in the region between the rhyzoplasts, structures that are responsible for the connection between the basal bodies and the nucleus. (**Bb**) In wild-type cells, TBCCD1 localizes to the region between the rhizoplasts and to the centrioles (Reproduced from Feldman and Marshall, 2009 with permission)

and Marshall 2009). This phenotype has not been detected in human cells depleted in TBCCD1 which may be related to remaining levels of TBCCD1 in these cells that impair the appearance of more dramatic effects.

Marshal and coworkers (Feldman and Marshall 2009) have also showed that the linkage between centrioles in *asq2* mutant cells has been affected. Interestingly, in moving RPE-1 centrin-GFP knockdown cells analyzed by time-lapse microscopy, the centrioles are less dynamic that in control cells. In fact, in control-moving cells, the centrioles present a dynamic behavior in which they temporarily distance slightly from each other, suggesting that TBCCD1 is also involved in centriole–centriole interaction.

Summary and Perspectives

Both characterizations of TBCCD1 in human cells and *Chlamydomonas* show that TBCCD1 is a centrosomal/basal body protein conserved throughout evolution that is involved in the correct positioning of these organelles in the cell and affecting the activities depending on it.

The roles of TBCCD1 only now started to be revealed, and much more investigation is required to clarify its functions. In the immediate, it would be important to understand if this protein establishes a physical link to the nucleus or, alternatively, if it regulates this interaction. Therefore, the clarification if TBCCD1 presents or not a GAP activity associated to the identification of its partners would be a decisive step to clarify this question.

References

Andersen JS, Wilkinson CJ, Mayor T, Mortensen P, Nigg EA, Mann M. Proteomic characterization of the human centrosome by protein correlation profiling. Nature. 2003;426(6966):570–4.

Archambault V, Zhao X, White-Cooper H, Carpenter ATC, Glover DM. Mutations in Drosophila Greatwall/Scant reveal its roles in mitosis and meiosis and interdependence with Polo kinase. PLoS Genet. 2007;3(11):e200.

Bartolini F, Bhamidipati A, Thomas S, Schwahn U, Lewis SA, Cowan NJ. Functional overlap between retinitis pigmentosa 2 protein and the tubulin-specific chaperone cofactor C. J Biol Chem. 2002;277:14629–34.

Bettencourt-Dias M, Glover DM. Centrosome biogenesis and function: centrosomics brings new understanding. Nat Rev Mol Cell Biol. 2007;8:451–63.

Buch C, Lindberg R, Figueroa R, Gudise S, Onischenko E, Hallberg E. An integral protein of the inner nuclear membrane localizes to the mitotic spindle in mammalian cells. J Cell Sci. 2009;122:2100–7.

Burakov A, Nadezhdina E, Slepchenko B, Rodionov V. Centrosome positioning in interphase cells. J Cell Biol. 2003;162:963–9.

Chevrier V, Piel M, Collomb N, Saoudi Y, Frank R, Paintrand M, Narumiya S, Bornens M. Job D The Rho-associated protein kinase p160ROCK is required for centrosome positioning. J Cell Biol. 2002;157:807–17.

Cuvillier A, Redon F, Antoine JC, Chardin P, DeVos T, Merlin G. LdARL-3A, a Leishmania promastigote-specific ADP-ribosylation factor-like protein, is essential for flagellum integrity. J Cell Sci. 2000;113:2065–74.

Feldman JL, Marshall WF. ASQ2 Encodes a TBCC-like protein required for mother-daughter centriole linkage and mitotic spindle orientation. Curr Biol. 2009;19:1238–43.

Fontalba A, Paciucci R, Avila J, Zabala JC. Incorporation of tubulin subunits into dimers requires GTP hydrolysis, J. Cell Sci. 1993;106:627–32.

Gonçalves J, Nolasco S, Nascimento R, Lopez Fanarraga M, Zabala JC, Soares H. TBCCD1, a new centrosomal protein is required for centrosome and Golgi apparatus positioning. EMBO Rep. 2010;11:194–200.

Keller LC, Romijn EP, Zamora I, Yates JR 3rd, Marshall WF. Proteomic analysis of isolated chlamydomonas centrioles reveals orthologs of ciliary-disease genes. Curr Biol. 2005;15(12):1090–8.

Lopez-Fanarraga M, Avila J, Guasch A, Coll M, Zabala JC. Review: postchaperonin tubulin folding cofactors and their role in microtubule dynamics. J Struct Biol. 2001;135:219–29.

Malone CJ, Misner L, Le Bot N, Tsai MC, Campbell JM, Ahringer J, White JG. The *C. elegans* hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. Cell. 2003;115:825–36.

Nigg EA, Raff JW. Centrioles, centrosomes and cilia in health and disease. Cell. 2009;139:663–78.

Pouthas F, Girard P, Lecaudey V, Ly TB, Gilmour D, Boulin C, Pepperkok R. Reynaud EG In migrating cells, the Golgi complex and the position of the centrosome depend on geometrical constraints of the substratum. J Cell Sci. 2008;121:2406–14.

Salpingidou G, Smertenko A, Hausmanowa-Petrucewicz I, Hussey PJ, Hutchison CJ. A novel role for the nuclear membrane protein emerin in association of the centrosome to the outer nuclear membrane. J Cell Biol. 2007;178:897–04.

Schrick JJ, Vogel P, Abuin A, Hampton B, Rice DS. ADP-ribosylation factor-like 3 is involved in kidney and photoreceptor development. Am J Pathol. 2006;168:1288–98.

Schwahn U, Lenzner S, Dong J, Feil S, Hinzmann B, van Duijnhoven G, Kirschner R, Hemberger M, Bergen AA, Rosenberg T, Pinckers AJ, Fundele R, Rosenthal A, Cremers FP, Ropers HH, Berger W. Positional cloning of the gene for X-linked retinitis pigmentosa 2. Nat Genet. 1998;19:327–32.

Tian G, Bhamidipati A, Cowan NJ, Lewis SA. Tubulin folding cofactors as GTPase-ctivating proteins. GTP hydrolysis and the assembly of the alpha/beta-tubulin heterodimer. J Biol Chem. 1999;274:24054–8.

Veltel S, Gasper R, Eisenacher E, Wittinghofer A. The retinitis pigmentosa 2 gene product is a GTPase-activating protein for Arf-like 3. Nat Struct Mol Biol. 2008;15:373–80.
Zhou C, Cunningham L, Marcus AI, Li Y, Kahn RA. Arl2 and Arl3 regulate different microtubule-dependent processes. Mol Biol Cell. 2006;17:2476–87.

TC4

► Ran

Tcf13

► Tead

Tcf13r1

► Tead

Tcf13r2

► Tead

TCP-1 and Cct1

► CCTα

TCRζ

► CD3ζ

TDAG51

► PHLDA1 (Pleckstrin Homology-like Domain, Family A, Member): Alias: PHRIP; TDAG51; DT1P1B11; MGC131738

TDF

► Sry

TEA Domain Family Member 1

► Tead

TEA Domain Family Member 2

► Tead

TEA Domain Family Member 3

► Tead

TEA Domain Family Member 4

► Tead

Tead

Norman L. Eberhardt
Departments of Medicine and Biochemistry & Molecular Biology, Mayo Clinic, Rochester, MN, USA

Synonyms

[TEAD1] AbaA (*Aspergillus nidulans*); Gtrgeo5; M-CAT binding factor; mTEF-1; Scalloped (Sd) (*D. Melanogaster*); Tcf13; TEA domain family member 1; TEAD-1; TEAD1; TEC1 (*S. cerevisiae*); TEF-1; TEF1
[TEAD2] Etdf; ETF; TEA domain family member 2; TEAD-2; TEAD2; TEF-4; TEF4

[TEAD3] DTEF-1; ETFR-1; Tcf13r2; TEA domain family member 3; TEAD-3; TEAD3; TEF-5
[TEAD4] Rtef-1; Tcf13r1; TEA domain family member 4; TEAD-4; TEAD4; Tef-3; Tefr1a; Tefr1b

Historical Background

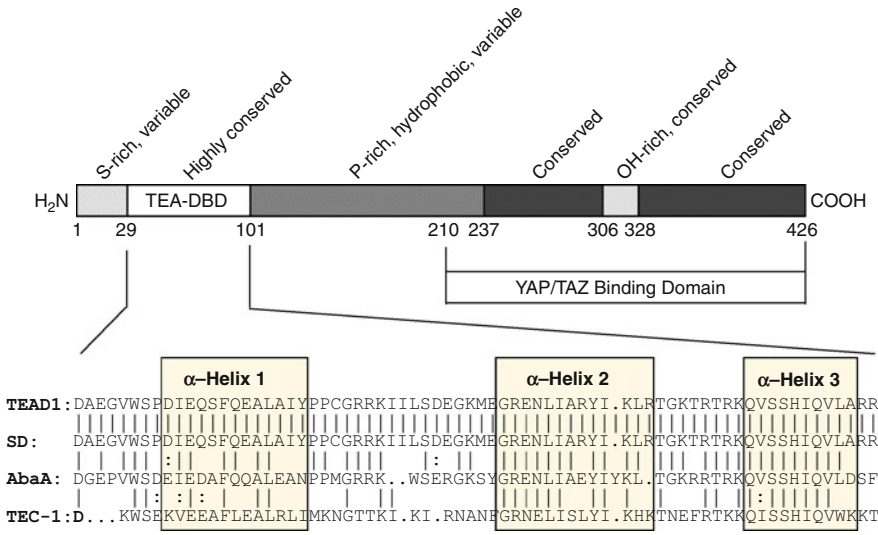
In vertebrates, the TEAD family of transcription factors is comprised of four members that contain a highly conserved (99–100%) DNA-binding domain, designated the TEA/ATTS domain (Burglin 1991) (Fig. 1). This highly conserved domain is comprised of three α helical domains and is not confined to vertebrates, but is also found in transcription factors from multiple organisms, including the TEAD1 homologs AbaA (*Aspergillus nidulans*), TEC1 (*Saccharomyces cerevisiae*), and scalloped (sd, *Drosophila melanogaster*). Thus TEAD family members represent primordial transcription factors, emphasizing their very early evolutionary origins and central importance in regulating gene expression and essential developmental functions. TEAD1 was originally cloned as a transcriptional enhancer factor (TEF) that was involved in mediating the activity of the Simian virus 40 (SV40) enhancer (Xiao et al. 1991). Subsequent studies revealed that TEAD family members bind to the MCAT motif, 5′-CATTCCT-3′, that is found in the promoter–enhancer regions of cardiac, smooth, and skeletal muscle-specific genes (reviewed in Yoshida 2008). In addition, TEAD family members have been found to be involved in the regulation of a diverse array of genes, including the human papilloma virus (HPV) E6 and E7 oncogenes, and human chorionic somatomammotropin, Foxa2, and PAX3 genes (reviewed in Eberhardt et al. 1996; Jacquemin and Davidson 1997; Kaneko and DePamphilis 1998; and Walker et al. 1991). Most knowledge of TEAD regulation of gene expression is derived from studies of muscle-specific genes.

TEAD genes play critical roles in mammalian development as demonstrated by knockout of the TEAD1 and TEAD4 genes, both of which lead to embryonic lethal phenotypes (Chen et al. 1994; Yagi et al. 2007). In addition, dual knockout of TEAD1 and TEAD2 results in embryonic lethality with severe growth defects and morphological abnormalities (Sawada et al. 2008), emphasizing the diverse critical roles TEAD family members play in mammalian development.

TEAD1

The human *TEAD1* gene is located on chromosome 11p15.4. TEAD1, the prototypical member of the TEA/ATTS transcription factor family, is widely expressed in multiple tissues, including skeletal muscle, pancreas, placenta, lung, and heart. TEAD3 and TEAD4 share a similar tissue distribution, suggesting that these proteins have partially redundant functional roles. TEAD1 regulates the expression of several muscle-specific genes, including cardiac troponin T, β-myosin heavy chain, smooth muscle α-actin, skeletal α-actin, and the α1-adrenergic receptor gene in cardiac myocytes (reviewed in Yoshida 2008). In addition, the expression of myocardin, the transcriptional co-activator of serum response factor (SRF), is regulated by an enhancer that is mediated by the combined actions of TEAD1, Mef2, and Foxo transcription factors. TEAD1 is required specifically for myocardin enhancer activation in neural-crest-derived smooth muscle cells and dorsal aorta. TEAD1 represses expression of the gene encoding involucrin in keratinocyte terminal differentiation and regulates early gene expression in mouse embryogenesis. TEAD1 has been implicated in the control of a placental enhancer/silencer driving the expression of the placental lactogens in syncytiotrophoblasts and inhibiting the expression of these genes in pituitary somatolactotrophs (reviewed in Eberhardt et al. 1996; Jacquemin and Davidson 1997; and Walker et al. 1991).

The most compelling evidence for TEAD1 involvement in cardiac development comes from studies of TEAD1 knockout mice, resulting in multiple cardiac defects and embryonic lethality (Chen et al. 1994). The mutant embryos exhibited enlarged pericardial cavity, bradycardia, a dilated fourth brain ventricle, and lethality at days e11.5–12.5. Heart development in the mutant embryos was extensive, indicating that TEAD1 was not required for initiation of heart development, but exhibited a very thin ventricular wall and reduced trabeculation. Transcription of several muscle-specific genes believed to be TEF-1 targets appeared to be normal, suggesting that many of these genes may be regulated by the other TEAD family members. The defect in cardiogenesis has been attributed to diminished transcription of one or several cardiac-specific genes that may be more specifically regulated by TEAD1.



Tead, Fig. 1 Schematic structure of TEAD family members showing the domains with enriched amino acids, serine (S), proline (P), hydrophobic, and hydroxylated (OH) and their relative degree of conservation. The N-terminal serine-rich domain is frequently phosphorylated, providing a mechanism for modulation of TEAD activity. The amino acid sequences of the highly conserved TEA DNA-binding domains (DBD) of TEAD1, AbaA (*Aspergillus nidulans*), TEC1 (*Saccharomyces cerevisiae*), and scalloped (SD, *Drosophila melanogaster*) and the α-helical regions are shown. The YAP/TAZ binding domain is indicated. The x-ray crystal structure of the YAP-TEAD4 complex indicates that the N-terminal region of YAP is folded into two short helices interspersed with a loop containing the PXXΦP motif and the C-terminal domain of TEAD4 has an immunoglobulin-like fold (Chen et al. 2010). The TEAD4-YAP binding is mediated chiefly by the two α-helices on the N-terminus of YAP. Numbers indicate amino acid positions for TEAD1 and are very similar for the other TEAD family members

TEAD2

The human *TEAD2* gene is located on chromosome 19q13.3. The tissue-specific expression pattern of TEAD2 is unique, since it is essentially absent from adult tissues. TEAD2 is expressed during the first 7 days of embryonic development in selected embryonic tissues, including the cerebellum, testis, tail bud, and distal portion of the forelimb and hindlimb buds. TEAD2 is involved in mouse early embryo development and has been implicated along with Ets-1 in the regulation of CTP:phosphocholine cytidylyltransferase activity. Mouse TEAD2 has also been shown to be a regulator of Pax3, a transcription factor that is essential for premigratory neural-crest cells that give rise to the peripheral nervous system, melanocytes, and certain vascular smooth muscle cells. TEAD2 has also been shown to be important in effective muscle regeneration, where it acts in conjunction with MyoD and Fgfr4.

TEAD2 and TEAD1 have been shown to exhibit partially overlapping and redundant functions and are critical for normal development (Sawada et al. 2008). While mice lacking TEAD2 appear to be normal, knock out of both TEAD1 and TEAD2 genes results in an embryonic lethal phenotype at E9.5. Embryos exhibited reduced cell proliferation and increased apoptosis along with multiple severe growth defects and morphological abnormalities, including absence of a closed neural tube, notochord, and somites.

TEAD3

The human *TEAD3* gene is located on chromosome 6p21.2. TEAD3 is expressed primarily in the heart and placenta. TEAD3 binds to the chorionic somatomammotropin gene enhancer and positively regulates hCS gene expression in human and murine placental cells (reviewed in Eberhardt et al. 1996; Jacquemin and Davidson 1997; and Walker et al. 1991). TEAD3 has also been implicated in the regulation of the gene encoding 3β-hydroxysteroid dehydrogenase/isomerase in the placenta. In the heart TEAD3, along with TEAD4, is involved in regulating the expression of muscle-specific genes and is involved in mediating the α1-adrenergic response in cardiac myocytes.

TEAD4

The human *TEAD4* gene is located on chromosome 12p13.2-p13.3. TEAD4 is expressed primarily in cardiac and skeletal muscle, where the protein activates muscle-specific genes through interactions with the muscle-specific MCAT response element. Genes regulated by TEAD4 include cardiac α-myosin heavy chain, skeletal muscle α -actin, and the S100B gene. TEAD4 seems to have a unique function in mediating the α1-adrenergic response in hypertrophic cardiac myocytes. The α1-adrenergic activation of TEAD4 appears to require phosphorylation at Ser 322. TEAD4 has also been implicated in the regulation of vascular endothelial growth factor (VEGF) in the ischemic heart and hypoxic endothelial cells.

Knock out of the mouse TEAD4 gene in mice results in a preimplantation lethal phenotype (Yagi et al. 2007). Specification of mammalian cell lineages begins shortly after fertilization with formation of a blastocyst comprised of trophectoderm and inner cell mass (ICM), giving rise to the placenta and embryo, respectively. TEAD4(−/−) embryos lack expression of trophectoderm-specific genes, including Cdx2, and lack trophoblast stem cells, trophectoderm, or blastocoel cavities with resultant inability to implant into the uterine endometrium. However, conditional knock out of TEAD4 embryos after implantation results in complete development, demonstrating that TEAD4 is the earliest gene required for specification of the trophectoderm lineage.

Co-regulatory Molecules Involved in TEAD Function

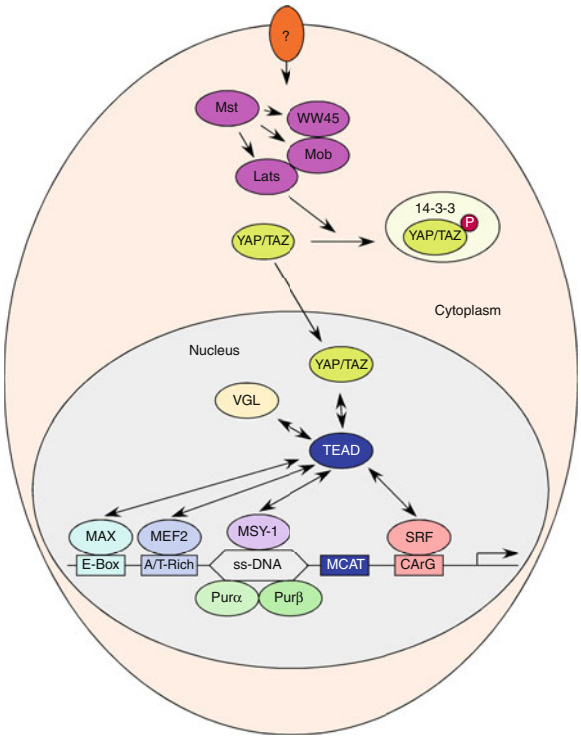
Like most transcription factors, the TEAD family interacts with a number of co-activator molecules that are essential for TEAD function. Two well-studied interactions include the vestigial and Yki/Yap/Taz family of proteins (Fig. 2). In Drosophila, the TEAD1 homolog Scalloped (Sd) interacts with Vestigial (Vg) to form a complex that binds DNA through the TEA/ATTS DNA-binding domain of Sd. The Sd–Vg complex is a critical regulator of wing development. In humans, there is a family of vestigial-related (Vgl) gene products, which interact with TEAD1. Vgl-2 (VITO-1), a member of the SID or scalloped interaction domain-containing gene products, interacts with TEAD1 and is associated with differentiation during embryonic skeletal myogenesis. Vgl-4, a novel member of the vestigial-like family of transcription cofactors, regulates α1-adrenergic activation of gene expression in cardiac myocytes.

Recent studies have implicated the Hippo signaling pathway in the control of organ size and tumorigenesis in both Drosophila and mammals (reviewed in Zhao et al. 2008). The Hippo pathway modulates the nuclear localization of the paralogous Yki/YAP/TAZ transcription co-activators that interact directly with the TEAD family transcription factors. In mammalian cells, the Hippo pathway kinase cascade phosphorylates YAP and TAZ, which prevents their nuclear localization via an interaction with the 14-3-3 protein. All TEAD family members have been shown to bind YAP65, a powerful transcriptional co-activator. It has been suggested that YAP65 regulates TEAD-dependent transcription in response to mitogenic signals.

Several other proteins have been shown to interact with TEAD family members that are important in modulating TEAD function. TEAD1 interacts with poly(ADP-ribose) (PARP) polymerase and this interaction has been proposed to provide muscle-specific gene expression in cardiac myocytes. PARP is thought to be an auxiliary protein required for TEAD1 transcriptional function. TEAD1 interacts with the basic helix–loop–helix zipper protein Max. Max is thought to cooperate with TEAD1 to regulate expression of the gene encoding α-myosin heavy chain. Multiprotein complexes of TEAD1, Max, and PARP have been proposed to be involved in gene expression in slow muscle fibers. TEAD1 binds to the TATA-binding protein (TBP), resulting in the loss of the ability of TBP to bind to the TATA box, and may be partly responsible for TEAD1 transrepressor activity. TEAD1 has been shown to bind the SV40 large T antigen. TEAD1 interacts with SRF and Mef2 in cardiomyocytes. TEAD1 and TEAD2 have been shown to interact with ▶ SRC1 and all members of the p160 family including SRC1, TIF2, and ▶ RAC3, and all of these factors are able to potentiate transcription from a TEF response element in vitro.

Summary

The TEAD family of transcription factors evolved very early and plays critical roles in the regulation of gene



Tead, Fig. 2 Schematic diagram of known components of regulation of TEAD activity and interaction with other transcriptional control elements in the regulation of mammalian gene expression. Within the nucleus, TEAD family members interact with a number of transcription factors indicated by *double-headed arrows*. The indicated pathways emphasize the regulation of muscle-specific genes (Yoshida 2008). Not all interactions are implied to occur simultaneously. Localization of YAP/TAZ within the nucleus is mediated by members of the Hippo intracellular kinase signaling cascade (Zhao et al. 2008). Members of the Hippo pathway in mammals include the hippo (Hpo) homolog Mst (Mst1/2, or STK4/STK3), the Sav homolog WW45, the Mats homolog Mob, the Wts homolog Lats (Lats1/2), and the yorkie (Yki) homologs YAP and TAZ

expression in yeast, flies, plants, and mammals. TEAD function is critical for mammalian development, including cardiac, trophoblast, neural tube, notochord, and somite development. TEAD family members regulate the expression of a large variety of genes including viral, muscle-specific, and developmentally important genes such as Cdx2 and Pax3. Considerable insight into the mechanisms by which TEAD family members regulate gene expression has been obtained, including the interaction of a large number of other transcription factors and/or co-regulators, such as Max, Mef2, SRF, YAP/TAZ, and Vgl. Future studies are anticipated to uncover many additional developmental and regulatory pathways that are mediated by TEAD family members. In addition, increasing detail will be added at the molecular level to our understanding of the discrete mechanisms by which TEAD family members regulate gene expression.

Acknowledgments I wish to extend my apologies to the many authors whose contributions could not be cited due to space limitations. Where possible I have cited review articles to allow readers access to the literature. In addition, the readers may access additional information and references on TEAD via the Nature-UCSD signaling gateway: http://www.signaling-gateway.org/.

References

Burglin TR. The TEA domain: a novel, highly conserved DNA-binding motif. Cell. 1991;66:11–2.

Chen Z, Friedrich GA, Soriano P. Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes Dev. 1994;8:2293–301.

Chen L, Chan SW, Zhang X, Walsh M, Lim CJ, Hong W, Song H. Structural basis of YAP recognition by TEAD4 in the hippo pathway. Genes Dev. 2010;24:290–300.

Eberhardt NL, Jiang SW, Shepard AR, Arnold AM, Trujillo MA. Hormonal and cell-specific regulation of the human growth hormone and chorionic somatomammotropin genes. Prog Nucleic Acid Res Mol Biol. 1996;54:127–63.

Jacquemin P, Davidson I. The role of the TEF transcription factors in cardiogenesis and other developmental processes. Trends Cardiovasc Med. 1997;7:192–7.

Kaneko KJ, DePamphilis ML. Regulation of gene expression at the beginning of mammalian development and the TEAD family of transcription factors. Dev Genet. 1998;22:43–55.

Sawada A, Kiyonari H, Ukita K, Nishioka N, Imuta Y, Sasaki H. Redundant roles of Tead1 and Tead2 in notochord development and the regulation of cell proliferation and survival. Mol Cell Biol. 2008;28:3177–89.

Walker WH, Fitzpatrick SL, Barrera-Saldana HA, Resendez-Perez D, Saunders GF. The human placental lactogen genes: structure, function, evolution and transcriptional regulation. Endocr Rev. 1991;12:316–28.

Xiao JH, Davidson I, Matthes H, Garnier JM, Chambon P. Cloning, expression, and transcriptional properties of the human enhancer factor TEF-1. Cell. 1991;65:551–68.

Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A. Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. Development. 2007;134:3827–36.

Yoshida T. MCAT elements and the TEF-1 family of transcription factors in muscle development and disease. Arterioscler Thromb Vasc Biol. 2008;28:8–17.

Zhao B, Lei QY, Guan KL. The Hippo-YAP pathway: new connections between regulation of organ size and cancer. Curr Opin Cell Biol. 2008;20:638–46.

TEAD1

► Tead

TEAD-1

► Tead

TEAD2

► Tead

TEAD-2

► Tead

TEAD3

► Tead

TEAD-3

► Tead

TEAD4

► Tead

TEAD-4

► Tead

TEC1 (*S. cerevisiae*)

► Tead

Tech

► SYX/PLEKHG5, A Rhoa Guanine Exchange Factor Involved in Cell Migration and Angiogenesis

TEF1

► Tead

TEF-1

► Tead

Tef-3

► Tead

TEF4

► Tead

TEF-4

► Tead

TEF-5

► Tead

Tefr1a

▶ Tead

Tefr1b

▶ Tead

TEP1

▶ PTEN

Testis Determining Factor

▶ Sry

Testosterone/Dihydrotestosterone Receptor

▶ Androgen Receptor (AR)

Tetraspanin-25

▶ CD53

TFE3/Transcription Factor Binding to IGHM Enhancer 3 (AGS11)

▶ Activators of G-Protein Signaling (AGS)

TFEB/Transcription Factor EB (AGS12)

▶ Activators of G-Protein Signaling (AGS)

THBS1

▶ Thrombospondin-1

The 5-HT_3 Receptor

Sarah C. R. Lummis
Department of Biochemistry, University of Cambridge, Cambridge, UK

Synonyms

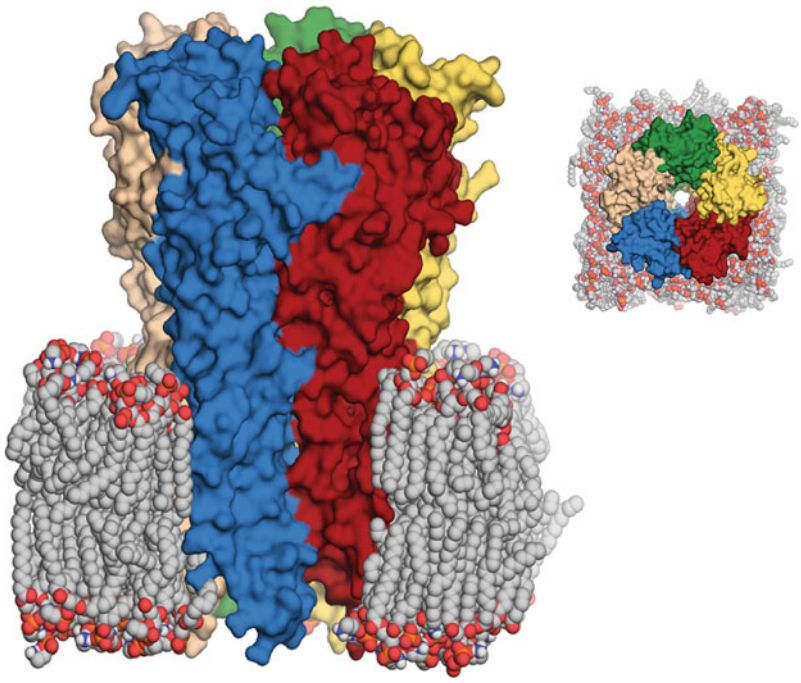
5-HT; 5-Hydroxytryptamine; Serotonin

Historical Background

5-Hydroxytryptamine (5-HT), also known as serotonin, was initially identified as a potent vasoconstrictor present in blood serum (Rapport et al. 1947). It is now clear 5-HT has a multitude of functions including activation or inhibition of muscle, exocrine and endocrine glands, central and peripheral neurons, and cells of the hematopoietic and immune systems. 5-HT receptors were initially subdivided into D and M subtypes, based on their sensitivity to dibenyline or morphine (Gaddum and Picarelli 1957). Currently, 5-HT receptors are divided into seven major families ($5\text{-}HT_{1\text{-}7}$) based on transduction and structural characteristics. All are G-protein-coupled receptors, except for the $5\text{-}HT_3$ receptor which is a ligand-gated ion channel (LGIC), and indeed, it is this receptor which is the original M receptor.

Introduction

The $5\text{-}HT_3$ receptor is a cation-selective member of the Cys-loop family of LGIC. The receptors are located primarily in the central and peripheral nervous systems and in reasonable quantities in the gastrointestinal tract and the immune system. They are also found in lower amounts in many other tissues, suggesting a possible physiological importance in many body regions. Structural details of the $5\text{-}HT_3$ receptor at the molecular level are unresolved, but a wealth of evidence shows that it is closely related to the nACh receptor



The 5-HT$_3$ Receptor, Fig. 1 The 5-HT$_3$ receptor. Homology model based on the structure of GluCl (Hibbs and Gouaux 2011) showing the 5-subunit configuration of this protein. These subunits surround the ion pore (see *inset*, image from above). The extracellular domain contains the ligand binding site which is predominantly β-sheet; the transmembrane domain is mostly α–helical and contains the pore. There is also an intracellular domain whose structure was not determined for GluCl

(see Thompson et al. 2010; Reeves and Lummis 2002 for reviews). Consequently, the 5-HT$_3$ receptor is well represented by cryo-electron microscope images of the nACh receptor and by crystal structures of the acetylcholine binding protein (AChBP), the GluCl receptor, and the bacterial Cys-loop receptor homologues GLIC and ELIC (Brejc et al. 2001; Hilf and Dutzler 2008, 2009; Bocquet et al. 2009; Hibbs and Gouaux 2011). It consists of a large, mostly β-sheet structured, extracellular domain, an α-helical transmembrane domain, and a relatively unstructured intracellular domain. Activation by 5-HT and other agonists opens an integral cation-selective channel. The receptor is inhibited by 5-HT$_3$ receptor-selective antagonists, some of which are useful therapeutic agents, and is also modulated by a wide range of substances, including alcohols, steroids, and anesthetics.

Receptor Heterogeneity

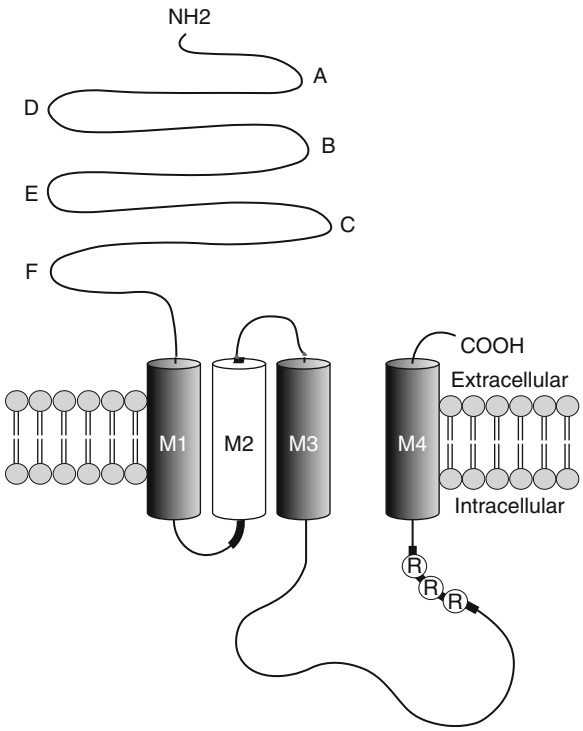
The functional receptor is a pentamer (Fig. 1), and five 5-HT$_3$ receptor subunits have been identified (5-HT3A-E). Only the A subunit can form functional homomeric receptors, while subunits B–E function only as heteromeric receptors in combination with the A subunit (Davies et al. 1999; Niesler et al. 2003). Homomeric 5-HT$_3$A and heteromeric 5-HT$_3$AB receptors have been extensively characterized. 5-HT$_3$AB receptors have an increased single channel conductance, reduced Ca^{2+} permeability, faster kinetics, increased EC$_{50}$s, and decreased Hill slope. There are also some differences in the potency of noncompetitive antagonists. Data from functional expression of 5-HT3A with either 5-HT3C, D, or E subunits suggest their characteristics are similar to those of homomeric 5-HT$_3$A receptors, but there may be some differences in expression levels (Holbrook et al. 2009; Niesler et al. 2007).

The Receptor Binding Site

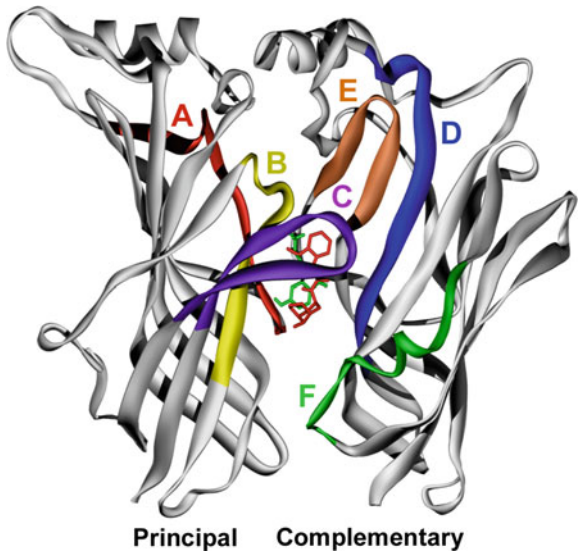
The five subunits surround a central ion-conducting pore (Fig. 1). The extracellular N-terminal region contains the ligand binding site, which lies at the interface of two adjacent subunits and is formed by three loops (A-C) from the "principal" subunit and three β-strands (D–F) from the adjacent or "complementary" subunit (Figs. 2 and 3). A number of studies have identified key residues that are involved in both agonist and antagonist binding (Fig. 4). A comprehensive review of the 5-HT$_3$ ligand binding site can be found in Thompson and Lummis (2006).

The Receptor Transmembrane Domain

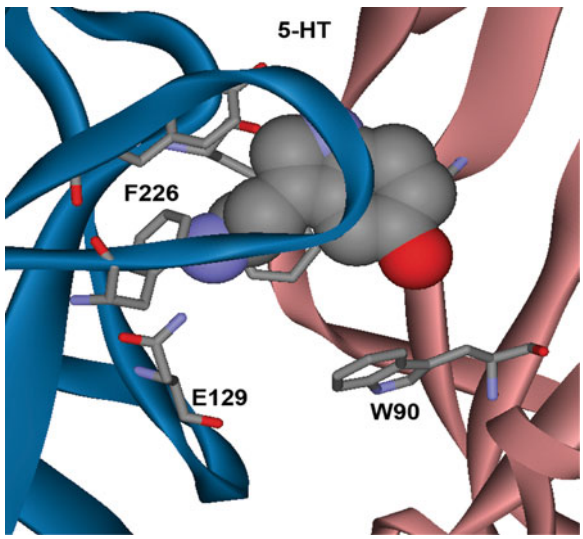
The transmembrane region contains four membrane spanning α-helices (M1–M4) and a short



The 5-HT$_3$ Receptor, Fig. 2 A 5-HT$_3$ receptor subunit. Cartoon representation of a subunit showing the binding loops A–F, the four transmembrane spanning segments and the intracellular domain which contain three functionally important arginine (R) residues



The 5-HT$_3$ Receptor, Fig. 3 The 5-HT$_3$ receptor binding site. Model based on AChBP. The binding site is located between two adjacent subunits and constituted from three loops (A–C) from the principle subunit and three loops (D–F) from the complementary subunit. 5-HT and granisetron are shown docked in the binding pocket



The 5-HT$_3$ Receptor, Fig. 4 5-HT in the binding site. Model showing 5-HT docked into the binding site and several residues known to be important in ligand binding

C-terminus. M2 from each subunit lines the pore and contains regions responsible for channel gating and ion selectivity. In 5-HT$_3$ receptors, this pore is predominantly sodium and potassium selective, and its opening results in a rapidly activating and then desensitizing inward current. The intracellular domain is formed by a loop of residues (~110) between M3 and M4. The structure of this domain remains uncertain, but functionally it has a role in channel conductance and receptor modulation. Homomeric 5-HT$_3$ receptors composed of A subunits alone form functional channels with a conductance that is so small (sub-pS) that it cannot be resolved directly. Although the B subunit cannot form homomeric channels, it can be combined with A subunits to generate functional heteromeric receptors that display a much larger conductance (9–17 pS); this difference is the consequence of three arginine residues that lie within an α-helix in the M3–M4 loop (Fig. 2) (Kelley et al. 2003). The intracellular domain is also known to modulate 5-HT$_3$ receptor function as a result of posttranslational modifications.

Therapeutics

5-HT_3 receptor antagonists are in use clinically, primarily for controlling chemotherapy and radiotherapy-induced nausea and vomiting and in postoperative nausea and vomiting. Alosetron is an effective symptomatic treatment for irritable bowel syndrome. In addition, studies have revealed a diversity of potential disease targets that might be amenable to alleviation by 5-HT_3 receptor selective compounds; these include addiction, pruritis, emesis, fibromyalgia, migraine, rheumatic diseases, and neurological phenomena such as anxiety, psychosis, nociception, and cognitive function (Thompson and Lummis 2007).

Summary

The 5-HT_3 receptor is the only 5-HT receptor that is a ligand-gated ion channel. It is a member of the Cys-loop family of neurotransmitter-gated ion channels, which also includes nACh, glycine, and GABA A receptors. It is a pentamer, and five subunits (A–E) can contribute to a functional receptor, as long as A subunits are present. The large extracellular domain contains the neurotransmitter binding site which is located between two adjacent subunits. The transmembrane pore is lined by α-helices, and the intracellular domain has a role in channel conductance and receptor modulation. A range of diseases may be amenable to treatment with 5-HT_3 receptor antagonists; currently, such compounds are in clinical use as antiemetics and to treat irritable bowel syndrome.

References

Bocquet N, Nury H, Baaden M, Le Poupon C, Changeux JP, Delarue M, Corringer PJ. X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. Nature. 2009;457:111–14.

Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, Sixma TK. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature. 2001;411:269–76.

Davies PA, Pistis M, Hanna MC, Peters JA, Lambert JJ, Hales TG, Kirkness EF. The 5-HT3B subunit is a major determinant of serotonin-receptor function. Nature. 1999;397:359–63.

Gaddum JH, Picarelli ZP. Two kinds of tryptamine receptor. Br J Pharmacol Chemother. 1957;12:323–8.

Hibbs RE, Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature. 2011;474:54–60.

Hilf RJ, Dutzler R. X-ray structure of a prokaryotic pentameric ligand-gated ion channel. Nature. 2008;452:375–9.

Hilf RJ, Dutzler R. Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. Nature. 2009;457:115–18.

Holbrook JD, Gill CH, Zebda N, et al. Characterisation of 5-HT3C, 5-HT3D and 5-HT3E receptor subunits: evolution, distribution and function. J Neurochem. 2009;108:384–96.

Kelley SP, Dunlop JI, Kirkness EF, Lambert JJ, Peters JA. A cytoplasmic region determines single-channel conductance in 5-HT_3 receptors. Nature. 2003;424:321–4.

Niesler B, Frank B, Kapeller J, Rappold GA. Cloning, physical mapping and expression analysis of the human 5-HT3 serotonin receptor-like genes HTR3C, HTR3D and HTR3E. Gene. 2003;310:101–11.

Niesler B, Walstab J, Combrink S, et al. Characterization of the novel human serotonin receptor subunits 5-HT3C,5-HT3D, and 5-HT3E. Mol Pharmacol. 2007;72:8–17.

Rapport MM, Green A, Page IH. Purification of the substance which is responsible for the vasoconstrictor activity of serum. Fed Proc. 1947;6:184.

Reeves DC, Lummis SC. The molecular basis of the structure and function of the 5-HT_3 receptor: a model ligand-gated ion channel (review). Mol Membr Biol. 2002;19:11–26.

Thompson AJ, Lummis SC. 5-HT_3 receptors. Curr Pharm Des. 2006;12:3615–30.

Thompson AJ, Lummis SC. The 5-HT_3 receptor as a therapeutic target. Expert Opin Ther Targets. 2007;11:527–40.

Thompson AJ, Lester HA, Lummis SC. The structural basis of function in Cys-loop receptors. Q Rev Biophys. 2010;43:449–99.

The Cholecystokinin-B Receptor

► Cholecystokinin-2 Receptor

The Leukocyte Common Antigen (LCA)

► CD45 (PTPRC)

Thrombospondin

► Thrombospondin-1

Thrombospondin-1

David R. Soto-Pantoja and David D. Roberts
Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Synonyms

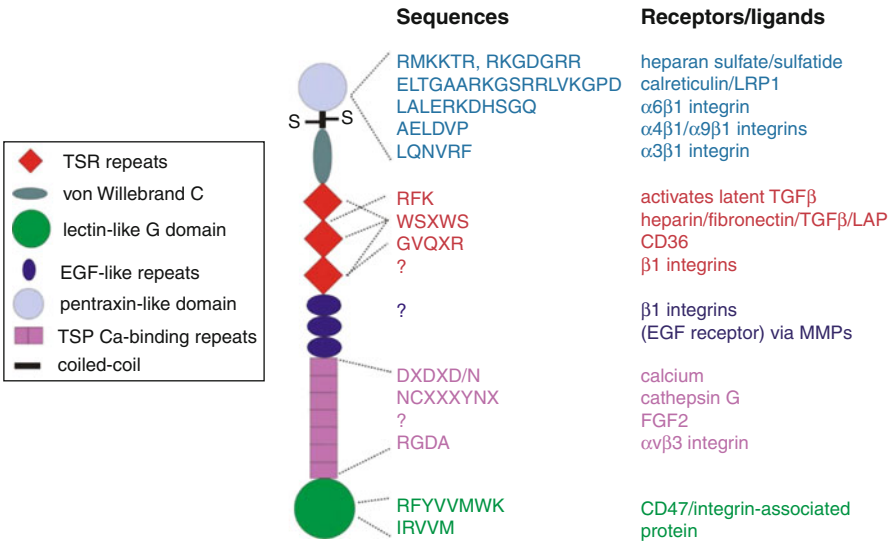
Glycoprotein G; THBS1; Thrombospondin; TSP1

Historical Background

Thrombospondin-1 (TSP1) was first characterized in 1971 by Baenziger et al. as a glycoprotein released from the α-granules of platelets in response to treatment with thrombin (Roberts and Lau 2011). This large protein consists of three 150 kDa disulfide-linked subunits and is highly conserved among vertebrate species (Carlson et al. 2008). Multi-sequence analysis has generated a phylogenetic tree for the evolution of modern TSPs (Bentley and Adams 2010). Duplication of the gene encoding a primordial TSP that is currently found in insects initiated the evolution of two subfamilies containing five members in modern vertebrates (Bentley and Adams 2010). The central feature of all TSPs is the presence of a carboxy-terminal signature domain containing EGF-like modules and seven TSP-type Ca-binding repeats, which wrap around the C-terminal lectin-like globular domain. This domain is about 650 amino acids long and is the most conserved region between the TSPs (Carlson et al. 2008). TSP1 and TSP2 belong to the first subfamily and are trimeric proteins that contain an N-terminal globular domain that binds heparin, followed by a coiled-coil motif that mediates trimer formation, a von Willebrand factor type C domain, three type 1 repeats (TSRs), followed by the signature domain (Carlson et al. 2008) (Fig. 1). Although they share only 25% sequence identify, the N-terminal domains of both TSP1 and TSP2 bind to heparan sulfate proteoglycans, low-density lipoprotein receptor-related protein (LRP1), and α4β1 and α6β1 integrins. However, only TSP1 binds to α3β1 integrin via this domain. The second group is composed of TSP3–5, which are pentameric proteins that are most abundant in bone and cartilage extracellular matrix (Carlson et al. 2008; Roberts and Lau 2011). The members of this subgroup lack a von Willebrand factor type C domain and type 1 repeats and contain four copies of the type 2 EGF repeats (Carlson et al. 2008; Roberts and Lau 2011). Gene deletion studies in mice have demonstrated little redundancy between the functions of each family member, indicating that each TSP has specific roles in vertebrates (Roberts and Lau 2011).

Function

In certain contexts, TSP1 mediates cell-to-cell and cell-to-matrix interactions, but its major physiological functions involve modulation of signaling by other extracellular factors and direct signaling through its cell surface receptors (Fig. 2). Stable proteolytic fragments of TSP1 were used in early studies to uncover activities of several regions of the molecule. The amino-terminal heparin-binding domain interacts with proteins in the extracellular matrix and on the cell surface. This unit contains binding sites for heparin, sulfated glycolipids, α3β1, α4β1, α6β1, and α9β1 integrins, calreticulin, and LRP1 (Carlson et al. 2008). The von Willebrand factor C module, also termed the pro-collagen module, has been implicated in the anti-angiogenic effects of TSP1, but this needs confirmation by mutagenesis. The TSRs mediate interactions with β1 integrins, fibronectin, the latency-associated protein of TGFβ, and the scavenger receptor CD36. The latter interaction mediates an anti-angiogenic activity of TSP1, and binding to latency-associated protein causes activation of latent TGFβ. The three epidermal growth factor-like (EGF) modules indirectly activate the EGF receptor and contain additional β1 integrin-binding sites. The calcium-binding modules when ligated by calcium wrap around the C-terminal lectin-like (or G) module of TSP1, binds fibroblast growth factor, and the last of these contains a RGD integrin-binding motif (Carlson et al. 2008). The C-terminal G module interacts with the other major receptor for TSP1, ▶ CD47, and mediates a second major anti-angiogenic activity of TSP1 as well as its modulation of vascular smooth muscle tone and inhibition of T-cell activation (Frazier et al. 2010).

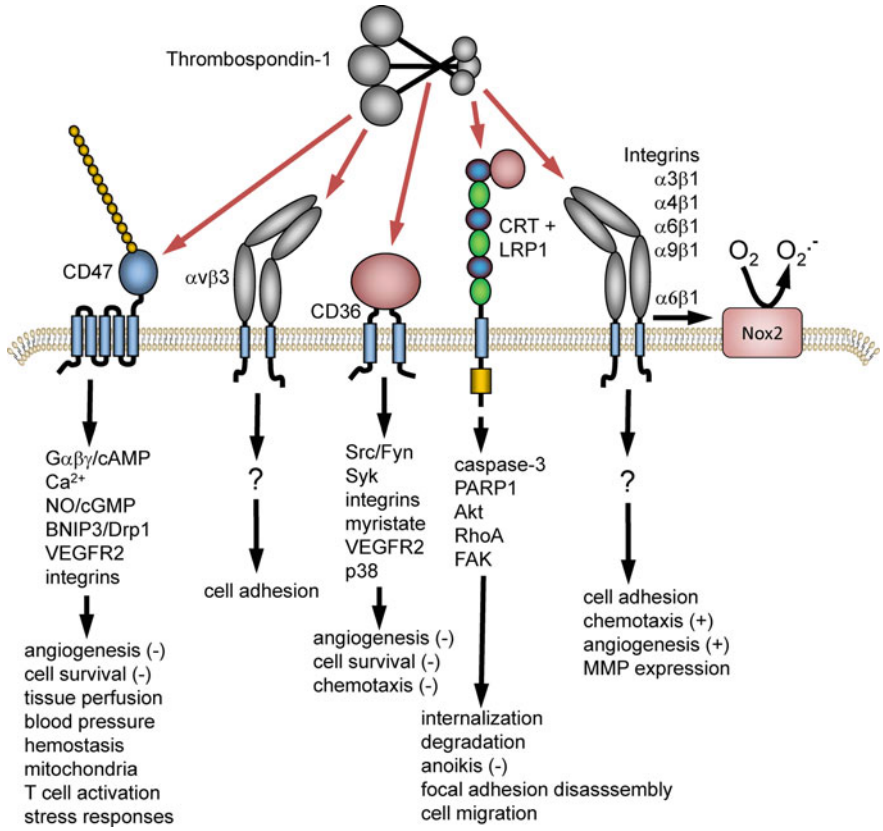


Thrombospondin-1, Fig. 1 Schematic representation of the structure and functional sequences of TSP1. TSP1 is a ~450 kDa trimeric glycoprotein composed of three identical 150 kDa polypeptide chains linked by disulfide bonds. Each subunit of TSP1 contains the following structural elements: an N-terminal globular domain, an α-helical region that forms a parallel homotrimeric coiled coil, a von Willebrand factor type C module, three TSP type 1 (TSR) repeats, and the TSP "signature" domain, which consists of three epidermal growth factor (EGF)-like TSP type 2 repeats, seven calcium-binding type 3 repeats (the last of which contains an RGD integrin-binding sequence), and a COOH-terminal domain. Known receptors and ligands are mapped to their respective binding domains in TSP1 and to specific recognition sequences where known

Expression and Regulation

TSP1 is produced by many cell types upon stimulation by cytokines, growth factors, and stress (Isenberg et al. 2009). TSP1 is expressed during early embryogenesis particularly on the cells surface of embryonic cells (Bornstein et al. 1990). Treatment of with antibodies inhibited blastocyst outgrowth, suggesting a role of TSP1 during early development. In normal adults, the expression of TSP1 is low but detectable in muscle cells, bone marrow cells, among others (Isenberg et al. 2009). TSP1 expression is regulated at the level of transcription, mRNA stability, translation, and post-translational modification. Posttranslational modifications have been demonstrated in several domains of this protein and may be important for some TSP1 functions (Roberts and Lau 2011). Expression of TSP1 is elicited by stress, injury, and inflammatory responses (Lopez-Dee et al. 2011). During the acute phase of inflammation, TSP is transiently expressed, and multiple factors seem to modulate the release of TSP1 during this process. TSP1 is strongly expressed in neutrophils, inducing an intense chemotactic response to injured tissues (Lopez-Dee et al. 2011). Ischemia/reperfusion injury results in a marked elevation of expression of TSP1 and is associated with an increase in cell death (Isenberg et al. 2008, 2009).

Tissue levels of TSP1 also tend to increase during aging and are associated with the onset of chronic diseases such as atherosclerosis, Alzheimer's, and type II diabetes (Roberts and Lau 2011). Consistent with the latter clinical finding, TSP1 is regulated by glucose. Transcription of TSP1 is activated by the hexosamine pathway of glucose catabolism (Roberts and Lau 2011). Specific inhibitors of glutamine:fructose 6-phosphate amidotransferase (GFAT), an enzyme controlling the hexosamine pathway, as well as direct inhibitors of protein glycosylation, efficiently inhibited glucose-stimulated TSP1 transcription (Roberts and Lau 2011). Hyperglycemia increases TSP1 transcription in kidney mesangial cells by the stimulation of USF2 protein accumulation this is negatively regulated by an increase in cGMP. Moreover, the regulation of USF2 is mediated by an angiotensin II–dependent mechanism, indicating that expression of TSP is also regulated by the rennin angiotensin system (Visavadiya et al. 2011). TSP1 levels were elevated in adipocytes of rats fed a high fat diet to induce obesity and insulin



Thrombospondin-1, Fig. 2 Signaling functions of TSP1 receptors. TSP1 has over eight known receptors. TSP1 interacts with several integrins (α3β1, α4β1, α6β1, and α9β1) that mediate cell adhesion, migration, and the pro-angiogenic activity of the N-terminal domain. TSP1 signaling through α6β1 in activated macrophages activates the NADPH oxidase Nox2 to produce superoxide (O_2^-). The N-terminal domain of TSP1 also modulates endothelial cell focal adhesions and vascular permeability by engaging calreticulin (CRT) and low density lipoprotein receptor-related protein (LRP1). The central type 1 repeats of TSP1 inhibit angiogenesis by stimulating apoptosis and inhibiting chemotaxis of microvascular endothelial cells through engaging the receptor CD36. CD36 is a class B scavenger receptor and fatty acid translocase. TSP1 inhibits uptake of myristate via CD36. CD47 is a high affinity cell surface receptor for the C-terminal domain of TSP1. CD47 is ubiquitously expressed and also plays an important role in the regulation of cell survival, stress responses, systemic blood pressure, and immune regulation. TSP1 binding to CD47 modulates intracellular calcium, heterotrimeric G protein, and cyclic nucleotide signaling in various cell types

resistance. Expression of TSP1 was increased in adipocytes treated in vitro with high glucose, indicating that TSP1 expression and secretion is modulated by glucose and in models of insulin resistance in vivo and in vitro. Levels of ATP are also responsible for secretion and expression of TSP1 from dendritic cells. This may be regulated by the increase in cAMP, but other mechanisms may be involved (Roberts and Lau 2011). Uptake by receptors is also important for the regulation of extracellular TSP1 levels. The uptake is mediated by the amino terminus of TSP1 binding to heparan sulfate proteoglycans and LRP1/calreticulin (Roberts and Lau 2011).

Expression of TSP1 is also altered in malignant cells (Isenberg et al. 2009). For a majority of cancers, expression of TSP1 decreases with tumor progression. Expression of TSP1 is generally higher in the early stages, and its reduction over time is implicated in the angiogenic switch and precedes the increase expression of VEGF-A (Isenberg et al. 2009). However, not all cancer types follow this pattern, and higher circulating levels of TSP1 were reported in patients with malignant colorectal carcinoma even though levels in the tumor cells are decreased (Isenberg et al. 2009; Lopez-Dee et al. 2011). Several oncogenes and tumor suppressor genes regulate the transcription or

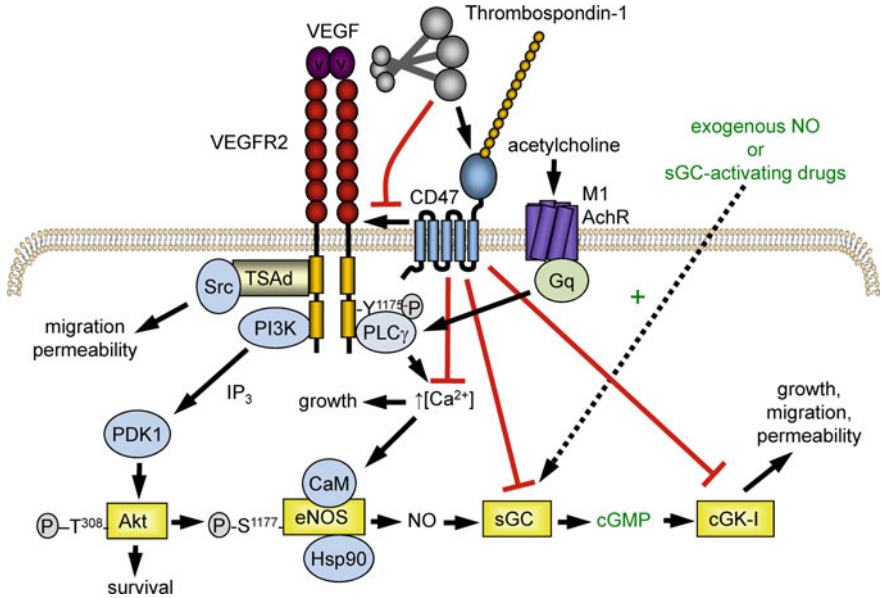
translation of TSP1 including N-Ras, K-Ras, R-Ras, ▶ Myc, ▶ p53, p73, nm23, U19/EAF2, and WT1 (Isenberg et al. 2009; Su et al. 2010). TSP1 is also subject to silencing by hypermethylation in some cancers.

Physiology

To study the physiological role of TSP1, Lawler et al. generated TSP1 deficient mice using homologous recombination to disrupt the mouse gene (Roberts and Lau 2011). Despite its expression in embryonic tissue, TSP1 null mice are viable, fertile and do not show gross abnormalities (Lopez-Dee et al. 2011; Roberts and Lau 2011). A reduction in reproductive fitness is associated with altered ovarian follicle morphology and with a deficiency in clearing VEGF by internalizing LRP1 (Roberts and Lau 2011). The original colony of null mice showed in some cases lordotic curvature of the spine and chronic lung inflammation, the latter associated with regulation of latent TGFβ activation. However, rederived TSP1 null mice in C57Bl/6 background do not exhibit this phenotype (Roberts and Lau 2011). Investigators have noticed that TSP1 null mice are leaner than their wild-type counterparts, which is consistent with their increased mitochondrial density and may result from its regulation of mitochondrial biogenesis by modulation of the cGMP and cAMP pathways (Frazier et al. 2011). Although TSP1 was originally isolated from and is highly expressed in the α-granules of platelets, the *tsp1* null mice showed no defects in aggregation of washed platelets. However, subsequent studies showed that TSP1 plays a role in platelet function by regulated activity of von Willebrand factor by inhibiting its proteolytic cleavage of ADAM13 (Roberts and Lau 2011). TSP1 also inhibits soluble ▶ guanylate cyclase and cGMP-dependent protein kinase activation in platelets. In the presence of physiological levels of NO or its physiological precursor arginine, TSP1 is necessary for thrombin-induced platelet aggregation, and this activity is mediated through its receptors ▶ CD47 and CD36 (Isenberg et al. 2009; Frazier et al. 2010).

The first physiological function identified for TSP1 was inhibition of angiogenesis (Isenberg et al. 2009; Lopez-Dee et al. 2011). TSP1 inhibits the growth and migration of endothelial cells and induces apoptosis. TSP1 blocks endothelial cell growth and migration stimulated by pro-angiogenic factors such as fibroblast growth factor-2 (▶ FGF2) and VEGF in vitro and in vivo (Isenberg et al. 2009). Anti-angiogenic activities are mediated by two TSP1 receptors: CD36 and ▶ CD47 (Fig. 2). TSP1 binding to CD36 on endothelial cells alters ▶ Src and Fyn kinase activities, with downstream effects on Akt, p38 MAPK, and Syk (Kazerounian et al. 2011) (Fig. 2). TSP1 binding to CD47 alters heterotrimeric G protein activation, calcium, cGMP, and cAMP signaling in vascular cells (Bauer et al. 2010; Frazier et al. 2010; Yao et al. 2011). TSP1 was recently discovered to potently inhibit VEGF receptor-2 (VEGFR2) signaling through engaging its receptor ▶ CD47 in endothelial cells, which is dissociated from its constitutive association with VEGFR2 (Kaur et al. 2010). Pro-angiogenic activity of TSP1 has also been reported, which is mediated by the N-terminal domain interacting with α3β1, α4β1, and α9β1 integrins (Fig. 2).

One of main functions of TSP1 is the regulation of nitric oxide (NO) signaling (Fig. 3). NO is a bioactive gas that serves as a signaling molecule with several physiological functions. NO is the endothelium derived relaxing factor that controls arterial smooth muscle tone by relaxing vascular smooth muscle cells (VSMC). NO diffuses rapidly through tissue and binds to its most responsive target soluble guanylate cyclase to stimulate the production of cGMP, which in VSMC triggers their relaxation (Isenberg et al. 2009). Endogenous TSP1 functions as a tonic antagonist of NO and signaling in endothelial cells, VSMC, and platelets. The inhibition of NO signaling by TSP1 is mediated primarily by ▶ CD47 and under some conditions by CD36. TSP1 type 1 repeats and synthetic peptides derived from this domain engage CD36, which blocks its ability to uptake myristate required for translocation of ▶ Src kinases to the plasma membrane (Isenberg et al. 2009). TSP1 blocks myristate-stimulated cGMP synthesis in an endothelial NO synthase (eNOS)-dependent manner. This mechanism is associated with the inhibition by TSP1 of angiogenesis and the regulation of endothelial cell chemotaxis. Binding of CD36 is sufficient for the inhibition of NO/cGMP signaling but it is not necessary since TSP1 strongly inhibits NO/cGMP signaling in cells that lack CD36. The C-terminal domain of TSP1 binds ▶ CD47, and this unit or peptides derived from it potently and redundantly inhibit NO/cGMP signaling at the level of eNOS, soluble guanylate cyclase, and



Thrombospondin-1, Fig. 3 TSP1 inhibition of NO/cGMP signaling through CD47. Binding of TSP1 signals via CD47 to inhibit NO-mediated activation of soluble guanylate cyclase (sGC) in endothelial cells, VSMC, and platelets and cGMP-mediated activation of cGMP-dependent protein kinase (cGK-I) in platelets. TSP1 interaction with CD47 directly inhibits VEGFR2 signaling in endothelial cells by displacing CD47 from its constitutive complex with VEGFR2. TSP1 also inhibits vasodilation mediated by acetylcholine by limiting the duration of calcium flux stimulated by acetylcholine in endothelial cells, which activates endothelial nitric oxide synthase (eNOS) via calmodulin (CaM)

cGMP-dependent protein kinase. Notably, TSP1 also inhibits activation of soluble guanylate cyclase by heme-dependent and heme-independent pharmacological activators (Miller et al. 2010). Thus, these drugs may not be effective for overcoming deficiencies in NO/cGMP signaling in pathological conditions where TSP1 expression is elevated. The expression of ▶ CD47 is also essential for CD36-mediated inhibition of NO signaling. Moreover, a 100-fold lower concentration of TSP1 is sufficient to inhibit NO signaling in vascular cells through ▶ CD47 when compared to CD36 (Isenberg et al. 2009). TSP1 and ▶ CD47 null mice, but not CD36 null mice, show elevated cGMP levels in vascular cells and muscle tissue.

The regulation of NO by TSP1 has many physiological repercussions especially in the vascular system. TSP1 null mice are hypersensitive to vasorelaxation induced by NO donors. Furthermore, TSP1 null mice exhibit decreased central pulse pressure and lower blood pressure than their wild-type counterparts, and these mice have exaggerated hypotensive responses to anesthetics and vasorelaxants (Bauer et al. 2010). TSP1 also inhibits the acetylcholine-mediated activation of eNOS in the endothelium through ▶ CD47. TSP1 inhibits eNOS activation through the inhibition of calcium signaling and serine-1177 phosphorylation of eNOS. Intravenous treatment with TSP1 and ▶ CD47 antibodies acutely increases blood pressure (Bauer et al. 2010). Therefore, TSP1 regulation of NO has vasopressor functions that regulate systemic blood pressure. NO also plays an important role in mediating angiogenic signaling stimulated by VEGF, and TSP1 inhibits angiogenesis in part by inhibiting cGMP synthesis stimulated by VEGF. NO is also an important inhibitor of platelet activation, and TSP1 promotes platelet activation by blocking this activity of NO.

High NO levels can reduce fixed ischemic and ischemia/reperfusion injuries by improving blood flow and limiting an inflammatory response. Expression of TSP1 is strongly induced during reperfusion of ischemic cardiac muscle, kidney, and liver tissues in mouse, rat, and porcine I/R injury models (Isenberg et al. 2009). Pretreatment and posttreatment of these injuries decreases I/R injury. This indicates that TSP1

levels are also important in the regulation of tissue perfusion during stress situations.

Inflammation is another important physiological response regulated by TSP1. TSP1 plays important roles in regulating innate and adaptive immunity through its effects on monocytes, macrophages, dendritic cells, T cells, and NK cells. TSP1 is released during acute inflammation and can exert both proinflammatory activities in several cell types. This duality can be explained in part by differential regulation of CD36 by peroxisome proliferator-activated receptor (PPAR) and TGFβ (Lopez-Dee et al. 2011). PPAR expression elicits potent anti-inflammatory responses however in the absence of PPAR in leukocytes causes the increased secretion of TSP1 and stimulates chemotaxis of these cells along with neutrophils (Lopez-Dee et al. 2011). On the other hand, it is well known that TSP1 causes activation of latent TGFβ by releasing or changing the conformation of its latency-associated protein (Lopez-Dee et al. 2011). TGFβ negatively regulates CD36 which can limit the recruitment of macrophages (Lopez-Dee et al. 2011). Moreover, its interaction with ▶ CD47 is also essential for the recruitment of polymorphonuclear cells. However, in a model of retinal injury TSP1 limited the migration of microglial cells (Roberts and Lau 2011). Levels of TSP1 regulate migration of phagocytic cells such as macrophages and neutrophils to areas of injury, lack of TSP1 in model of wound healing wound closure was delayed due to lack of macrophages to the wound, however lack of TSP1 was also found to be protective in liver ischemic injuries (Isenberg et al. 2009). TSP1 also regulates the activation of macrophages, and M1 differentiated macrophages are increased in tumors that overexpress TSP1 (Isenberg et al. 2009). Furthermore, TSP1 acutely induces superoxide production via NADPH oxidase-2 in macrophages via its binding to α6β1 integrin (Isenberg et al. 2009).

TSP1 is also a potent regulator of T cells. TSP1 stimulates T-cell adhesion, matrix metalloproteinase expression, and migration via α4β1 integrin. Interaction of TSP1 with ▶ CD47 inhibits ▶ CD3-dependent T-cell activation and regulates differentiation of CD4+ CD25+ T cells. Inhibition of T cell receptor signaling by TSP1 requires a proteoglycan isoform of ▶ CD47 (Kaur et al. 2011).

TSP1 as a Therapeutic Target

The diverse physiological effects of TSP1 make it an attractive therapeutic target. Since the discovery of the anti-angiogenic properties of this molecule, research has focused on the use of TSP1 analogs as anticancer treatments. ABT-510 is a synthetic nonapeptide derived from the TSR repeats of TSP that interacts with CD36. Preclinical data indicates that ABT-510 inhibits VEGF and ▶ FGF2 signaling and limits endothelial cell migration, proliferation, tube formation and neovascularization, and induces endothelial and tumor cell apoptosis (Isenberg et al. 2009). ABT-510 inhibits tumor growth in mouse xenograft models. However, ABT-510 therapy did not demonstrate clear clinical efficacy in a clinical trial of patients with stage IV melanoma. Further dose escalation or combination with cytotoxic therapy may be more effective therapeutically (Markovic et al. 2007). However, ABT-510 is well tolerated and was studied in combination with other chemotherapies (Nabors et al. 2010).

Blockade of TSP1 signaling through ▶ CD47 may also be beneficial in certain pathologies. Blockade of TSP1 or CD47 results in improved recovery from ischemia and I/R injuries in several animal models (Isenberg et al. 2009). Blocking TSP1 also overcomes the deleterious effects of aging on survival of ischemic stress in a mouse model. Moreover, lack of TSP1 in combination with radiation enhanced tumor growth delay in a syngeneic melanoma model. Blockade of TSP1/CD47 signaling in wild-type mice simultaneously protected muscle and bone marrow tissue from radiation while markedly reducing tumor growth (Maxhimer et al. 2009).

Summary

TSP1 is a multifunctional signaling protein that engages at least eight distinct signaling receptors on endothelial cells and subsets of these receptors on other cell types. Studies in mice lacking TSP1 and several of its receptors have identified physiological functions for TSP1 in regulating platelet hemostasis, angiogenesis, mitochondrial homeostasis, local and systemic regulation of blood flow, and innate and adaptive immunity. Elevated TSP1 expression contributes to the pathogenesis of acute injuries and several chronic diseases of

aging. Conversely, loss of TSP1 expression in some cancers promotes tumor angiogenesis and impairs innate antitumor immunity. Agents targeting specific TSP1 receptors have shown benefit in treating these diseases in animal models and early clinical trials, and further study of the signaling mechanisms regulated by TSP1 could lead to development of additional therapeutics for these conditions.

References

Bauer EM, Qin Y, Miller TW, Bandle RW, Csanyi G, Pagano PJ, et al. Thrombospondin-1 supports blood pressure by limiting eNOS activation and endothelial-dependent vasorelaxation. Cardiovasc Res. 2010;88:471–81.

Bentley AA, Adams JC. The evolution of thrombospondins and their ligand-binding activities. Mol Biol Evol. 2010;27:2187–97.

Bornstein P, Alfi D, Devarayalu S, Framson P, Li P. Characterization of the mouse thrombospondin gene and evaluation of the role of the first intron in human gene expression. J Biol Chem. 1990;265:16691–8.

Carlson CB, Lawler J, Mosher DF. Structures of thrombospondins. Cell Mol Life Sci. 2008;65:672–86.

Frazier WA, Isenberg JS, Kaur S, Roberts DD. CD47. Nature signaling gateway. 2010. Available from: http://www.signaling-gateway.org/molecule/query?afcsid=A002870. Accessed 16 Feb 2010.

Frazier EP, Isenberg JS, Shiva S, Zhao L, Schlesinger P, Dimitry J, et al. Age-dependent regulation of skeletal muscle mitochondria by the thrombospondin-1 receptor CD47. Matrix Biol. 2011;30:154–61.

Isenberg JS, Frazier WA, Krishna MC, Wink DA, Roberts DD. Enhancing cardiovascular dynamics by inhibition of thrombospondin-1/CD47 signaling. Curr Drug Targets. 2008;9:833–41.

Isenberg JS, Martin-Manso G, Maxhimer JB, Roberts DD. Regulation of nitric oxide signalling by thrombospondin 1: implications for anti-angiogenic therapies. Nat Rev Cancer. 2009;9:182–94.

Kaur S, Martin-Manso G, Pendrak ML, Garfield SH, Isenberg JS, Roberts DD. Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47. J Biol Chem. 2010;285:38923–32.

Kaur S, Kuznetsova SA, Pendrak ML, Sipes JM, Romeo MJ, Li Z, et al. Heparan sulfate modification of the transmembrane receptor CD47 is necessary for inhibition of T cell receptor signaling by thrombospondin-1. J Biol Chem. 2011;286:14991–5002.

Kazerounian S, Duquette M, Reyes MA, Lawler JT, Song K, Perruzzi C, et al. Priming of the vascular endothelial growth factor signaling pathway by thrombospondin-1, CD36, and spleen tyrosine kinase. Blood. 2011;117:4658–66.

Lopez-Dee Z, Pidcock K, Gutierrez LS. Thrombospondin-1: multiple paths to inflammation. Mediators Inflamm. 2011;2011:296069.

Markovic SN, Suman VJ, Rao RA, Ingle JN, Kaur JS, Erickson LA, et al. A phase II study of ABT-510 (thrombospondin-1 analog) for the treatment of metastatic melanoma. Am J Clin Oncol. 2007;30:303–9.

Maxhimer JB, Soto-Pantoja DR, Ridnour LA, Shih HB, Degraff WG, Tsokos M, et al. Radioprotection in normal tissue and delayed tumor growth by blockade of CD47 signaling. Sci Transl Med. 2009;1:3ra7.

Miller TW, Isenberg JS, Roberts DD. Thrombospondin-1 is an inhibitor of pharmacological activation of soluble guanylate cyclase. Br J Pharmacol. 2010;159:1542–7.

Nabors LB, Fiveash JB, Markert JM, Kekan MS, Gillespie GY, Huang Z, et al. A phase 1 trial of ABT-510 concurrent with standard chemoradiation for patients with newly diagnosed glioblastoma. Arch Neurol. 2010;67:313–9.

Roberts DD, Lau L. Matricellular proteins. In: Mecham RP, editor. Biology of extracellular matrix: an overview. Berlin/Heidelberg: Springer; 2011. p. 369–413.

Su F, Pascal LE, Xiao W, Wang Z. Tumor suppressor U19/EAF2 regulates thrombospondin-1 expression via p53. Oncogene. 2010;29:421–31.

Visavadiya NP, Li Y, Wang S. High glucose upregulates upstream stimulatory factor 2 in human renal proximal tubular cells through angiotensin II-dependent activation of CREB. Nephron Exp Nephrol. 2011;117:e62–70.

Yao M, Roberts DD, Isenberg JS. Thrombospondin-1 inhibition of vascular smooth muscle cell responses occurs via modulation of both cAMP and cGMP. Pharmacol Res. 2011;63:13–22.

TI-241

► ATF3 Activating Transcription Factor 3

TICAM-1

► Toll-like Receptor Adaptor Protein Family Members

TICAM-2

► Toll-like Receptor Adaptor Protein Family Members

TIF2

► Steroid Receptor Coactivator Family

TIF5 (eIF5)

Umadas Maitra and Romit Majumdar
Department of Developmental and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA

Synonyms

Tif5p (in yeast)

Introduction

The translation initiation process is mediated by a series of partial reactions each of which requires the participation of a large number of protein factors collectively called eukaryotic (translation) initiation factors (eIFs). The process leads to the assembly of an 80S ribosome-bound initiator Met-tRNA$_i$ at the start AUG codon of an mRNA that is active in peptidyl transfer. According to the currently accepted view of translation initiation, the first step is the binding of the initiator Met-tRNA$_i$ to the heterotrimeric GTP-binding initiation factor eIF2 to form the Met-tRNA$_i$•eIF2•GTP ternary complex. The ternary complex then binds to the 40S ribosomal subunit containing bound initiation factor eIF3, a process that requires two other initiation factors eIF1 and eIF1A. This interaction leads to the formation of a 43S preinitiation complex (40S•eIF3•eIF1•eIF1A•Met-tRNA$_i$•eIF2•GTP) which is then recruited to the 5′-capped end of the mRNA via protein-protein interaction between the cap-bound initiation factor eIF4F and eIF3 bound to the 43S preinitiation complex. The resulting 43S complex then binds two RNA-helicases eIF4A and eIF4B and scans the mRNA in a 5′ → 3′ direction until it is positioned at the initiating codon (usually an AUG codon) by base-pairing interaction between the AUG codon and the anticodon of Met-tRNA$_i$ to form the 48S initiation complex (mRNA•40S•eIF3•eIF1•eIF1A•Met-tRNA$_i$eIF2•GTP). Concomitant with the formation of the 48S initiation complex, eIF1 is released from its binding site in the 48S complex. Subsequently, another initiation factor eIF5 interacts with eIF2 in the 48S initiation complex, and acting as a GTPase activating protein (GAP), activates the intrinsic GTPase activity of eIF2 to promote the hydrolysis of bound GTP. Hydrolysis of GTP leads to the immediate release of P_i and eventually of inactive eIF2•GDP (and other bound factors) during eIF5B-mediated joining of the 60 ribosomal subunit to the 48S ribosomal complex to form an 80S initiation complex (80S•Met-tRNA$_i$•mRNA) that is competent to form the first peptide bond during the elongation phase of protein synthesis. (For a review, see Pestova et al. 2007; Hinnebusch et al. 2007; Sonenberg and Hinnebusch 2009; Lorsch and Dever 2010).

Historical Background

Isolation and Initial Characterization of eIF5

Initiation factor eIF5 was one among the seven distinct eIFs that were originally isolated in many laboratories from a variety of eukaryotic sources including rabbit reticulocyte lysates on the basis of its ability to stimulate in vitro translation of globin mRNA in a partially purified reconstituted system. The purified protein was shown to be a monomeric protein of apparent M_r = 150,000–170,000 (For a review, see Maitra et al. 1982 and references therein). Functional characterization of eIF5 in the translation initiation assay showed that eIF5 did not play a role in the in vitro formation of the 48S initiation complex. However, the protein was essential for the subsequent joining of the 60S ribosomal subunit to the 48S complex to form a functional 80S initiation complex. It was further shown that prior to or concomitant with the subunit joining reaction, eIF5 promoted the hydrolysis of GTP bound to eIF2 in the 48S initiation complex. Hydrolysis of GTP was shown to be a prerequisite for the joining of the 60S ribosomal subunit to the 48S complex to form the active 80S initiation complex (reviewed by Maitra et al. 1982). However, later, using a more direct and a specific assay that measured the ability of eIF5 to mediate the joining of the 60S ribosomal subunit to a preformed 48S initiation complex formed with an AUG trinucleotide in lieu of an entire mRNA, a new activity of about 50 kDa was isolated from calf liver extracts and rabbit reticulocyte lysates that migrated on SDS-polyacrylamide-gels with an apparent M_r of 58,000 (reviewed by Das and Maitra 2001).

Subsequently, a similar activity was purified from the yeast *Saccharomyees cerevisiae* as a protein of about 45 kDa that migrated in SDS-polyacrylamide-gels as a protein of about 54,000–56,000 (Das and Maitra 2001).

This anomaly in molecular size of eIF5 was resolved when later work (Pestova et al. 2000) showed that the higher molecular weight polypeptide detected in earlier eIF5 preparations reported by others is another distinct initiation factor, designated eIF5B, that is required along with eIF5 for the joining of the 60S ribosomal subunit to the 48S initiation complex formed with a natural mRNA as the template. However, eIF5B has no role to play in the hydrolysis of GTP bound to eIF2 in the 48S initiation complex formed either with AUG or a natural mRNA as a template.

Biochemical characterization of the 50 kDa protein, designated as eIF5, showed that eIF5 promotes the hydrolysis of GTP only when the nucleotide is bound as a Met-tRNA$_i$•eIF2•GTP ternary complex on the 40S ribosomal subunit. eIF5 fails to promote hydrolysis of either free GTP or GTP bound to eIF2 as a ternary complex. The reaction is unaffected by the presence of 60S ribosomal subunits, indicating that GTP hydrolysis reaction precedes the joining of the 60S ribosomal subunit to the 40S complex. Finally, following eIF5-promoted GTP hydrolysis, even though P_i is released almost instantaneously from the 40S ribosomes, eIF2•GDP complex formed remains initially bound to the ribosomal complex and can be released by prolonged incubation at 37°C or by sucrose gradient centrifugation (Raychaudhuri and Maitra 1986).

Molecular Genetic Characterization eIF5

To show that the 50 kDa protein functions as a canonical translation initiation factor essential for translation of mRNAs in vivo, both the mammalian cDNA and the single copy essential yeast gene *TIF5* encoding eIF5 were cloned and expressed in *Escherichia coli* (For a review see, Das and Maitra 2001). In each case, the purified recombinant mammalian protein (calculated molecular mass 48,926) or the yeast protein (calculated molecular mass 45,436) mimics eIF5 isolated from natural sources in molecular size, in specific activity, and in its ability to promote the hydrolysis of GTP bound to eIF2 in the 48S initiation complex. Additionally, to understand the function of eIF5 in vivo in yeast cells, a conditional mutant yeast strain in which a functional but a rapidly degradable form of eIF5 fusion protein was synthesized from the glucose repressible *GAL* promoter was constructed. Depletion of eIF5 from this mutant yeast strain resulted in inhibition of cell growth and the rate of in vivo protein synthesis. Analysis of the polysome profiles of eIF5-depleted cells showed greatly diminished polysomes with simultaneous increase in the pool of free 80S ribosomes and free 60S and 40S ribosomal subunits. Such a polysome/ribosome profile is characteristic of cells lacking an essential translation initiation factor. Furthermore, lysates of cells depleted of eIF5 were inactive in translation of both total yeast poly(A)$^+$ and luciferase mRNAs in vitro. Addition of 45-kDa purified yeast eIF5 restored translation in these cell lysates. Similar observation was also made when a thermosensitive mutant yeast strain was used. These results show that the *TIF5* gene product, a protein of 45,346 Da, is indeed an initiation factor required for initiation of protein synthesis. Additionally, mammalian eIF5 can functionally substitute for yeast eIF5 in maintaining yeast cell viability and growth, indicating that the mechanism of eIF5-promoted GTP hydrolysis is conserved from yeast to mammals.

eIF5 Is Not a GTPase Protein, but Rather Functions as a GTPase Activating Protein (GAP)

GTP-binding domains, G1-G4, are characteristic of members of the GTPase superfamily. An important feature of these proteins is that they not only bind GTP but also possesses an intrinsic GTPase activity, which is often stimulated by their interaction with an effector molecule that acts as a GTPase activating protein (GAP). Both mammalian and yeast eIF5 contain imperfect G1-G4 domains. The presence of imperfections in the G1-G4 domains of eIF5 explains why eIF5, by itself, does not bind or hydrolyze free GTP (Das and Maitra 2001). In contrast, the heterotrimeric eIF2 binds GTP and its γ subunit contains the conserved GTP-binding domains (G1-G4) (Hinnebusch et al. 2007; Lorsch and Dever 2010). Since interaction of eIF5 with Met-tRNA•eIF2•GTP bound in the 48S initiation complex is necessary for hydrolysis of GTP, the question arises whether eIF5 functions as a specific

GAP to eIF2-GTPase (reviewed by Das and Maitra 2001; Hinnebusch et al. 2007; Pestova et al. 2007).

Binding studies show that eIF5 forms a complex with eIF2, the GTP-binding protein, by specifically interacting with the eIF2β subunit (Das et al. 1997; Asano et al. 1999). It was further demonstrated that the N-terminal lysine-rich region of eIF2β (designated K-boxes 1, 2, and 3) is involved in binding the C-terminal region of eIF5 containing a glutamic acid-rich bipartite motif (amino acids 345-347 and 384-386) (Asano et al.; 1999; Das and Maitra 2000). Mutational analysis showed that yeast eIF5 mutants E346A, E347A and E384A, E385A exhibited a marked decrease in binding to eIF2, and were also severely defective in eIF5-mediated hydrolysis of GTP bound to the 48S initiation complex (Das and Maitra 2000). These mutant eIF5 proteins were also defective in stimulating translation in eIF5-depleted yeast cell-free translation extracts, indicating that the eIF5•eIF2 interaction is essential for eIF5 function in vitro and in vivo.

eIF5 also binds to the γ subunit of eIF2. In fact, it has been demonstrated the amino terminal domain (NTD) of yeast eIF5 (residues 1-279) is as active as full-length eIF5 in activating the intrinsic GTPase activity of eIF2 (Hinnebusch et al. 2007). This observation led to the hypothesis that interaction between eIF2β and eIF5 does not activate the intrinsic GTPase eIF2γ, rather it may merely serve to recruit eIF5 to eIF2 in the 48S ribosomal complex. However, mutational studies in the γ and β subunits of yeast eIF5 are also consistent with an alternative hypothesis that eIF5-eIF2β interaction derepresses the GTPase activity of eIF2γ (reviewed by Pestova et al. 2007).

Further deletion analysis studies showed that an invariant arginine residue at position 15 at the N-terminal end of eIF5 is also essential for its GTP hydrolysis-promoting activity (Das et al. 2001; Paulin et al. 2001. This property of eIF5 is reminiscent of typical GAPs like RasGAPs and RhoGAPs that contain "arginine finger" motifs consisting of an invariant arginine residue at the N-terminus of their catalytic domains that is necessary for their GTPase-stimulating activity, in addition to motifs required for interacting with their respective G proteins. In typical GAPs, a secondary arginine residue stabilizes the finger loop carrying the primary arginine residue (reviewed by Das and Maitra 2001). In analogy with RasGAP and RhoGAP, it appears that either Lys33 or Lys55, or both in rat eIF5 constitute the "secondary" element required for eIF5 GAP function. (Das et al. 2001; Paulin et al. 2001).

However, it should be emphasized that while interactions between eIF5 and eIF2β and eIF5 and eIF2γ are necessary for activation of GTPase activity of eIF2γ, these interactions alone are not sufficient since binding of eIF2 as a Met-tRNA$_i$•eIF2•GTP ternary complex to the 40S ribosomal subunit is essential for the GAP activity of eIF5 toward eIF2. Therefore, it has been postulated that the 40S ribosomal subunit also interacts with eIF2γ and stabilizes the switch regions of eIF2γ, which is required for the GTPase activity of eIF2 (Das and Maitra 2001; Das et al. 2001). In summary, during translation initiation, the γ subunit of eIF2 which contains the consensus GTP-binding domains G1-G4, is the presumed GTPase, while eIF5 and the 40S ribosome act as GAPs. It should be noted that as a GAP for eIF2, eIF5 increases the rate of GTP hydrolysis in the ribosomal preinitiation complex by over six orders of magnitude (Algire et al. 2005).

Role of eIF5 in AUG Start Codon Selection and the Link Between eIF5-Promoted GTP Hydrolysis and AUG Selection

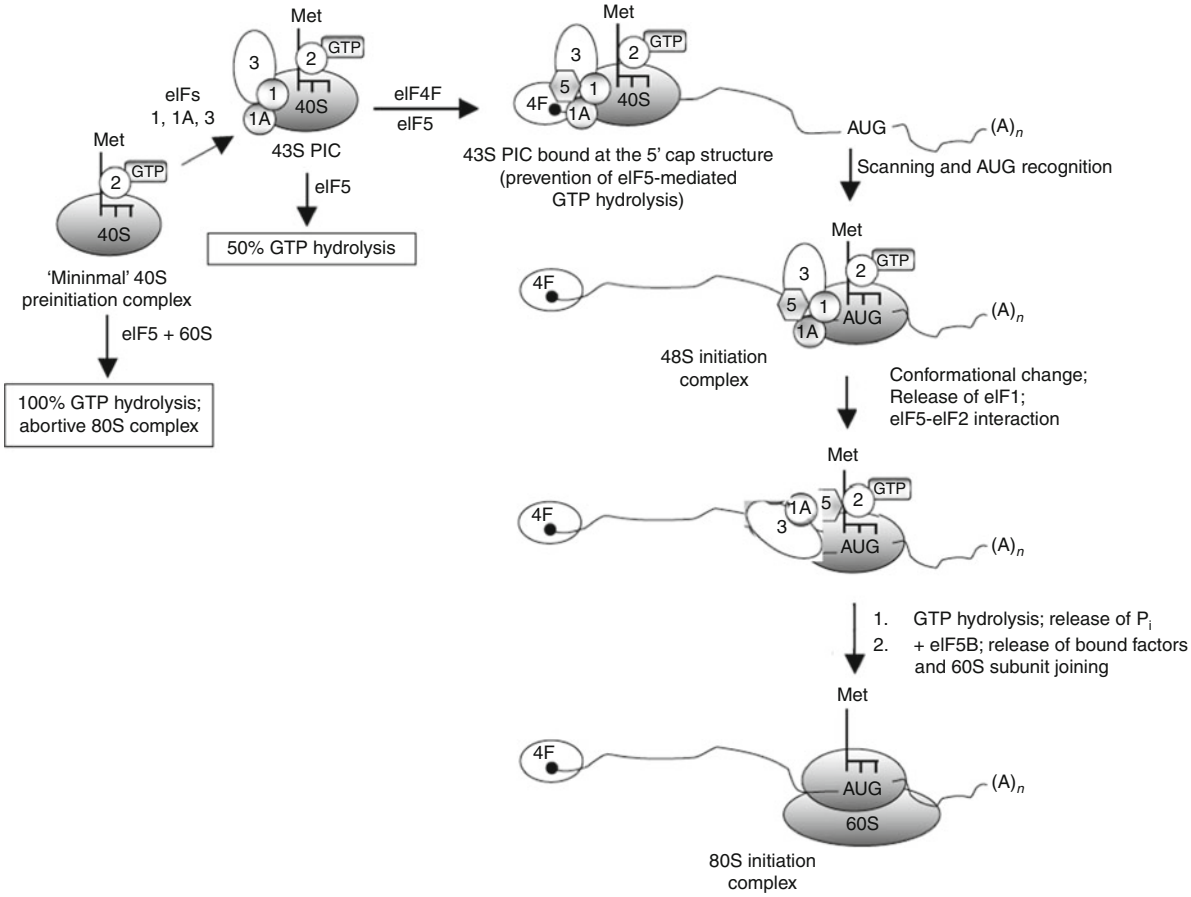
In eukaryotes, translation is known to be initiated almost exclusively from AUG codons. This is in sharp contrast to prokaryotes where non-AUG codons such as UUG or GUG codons also serve as efficient sites for translation initiation from about 10% of mRNAs. To understand the mechanism by which the stringency of start site selection in eukaryotes is maintained, a series of elegant yeast genetic experiments was carried out by Donahue and his colleagues (Huang et al. 1997; Donahue 2000). It was shown that translation can be initiated from a UUG codon only in the presence of complementary suppressor mutations in several initiation factors – eIF1, α, β, and γ subunits of eIF2, and eIF5. The identification, in this screen of eIF2, a GTP-binding initiation factor, and eIF5, a protein known to be a GAP, indicated that a defect in GTP binding/hydrolysis may be the cause for the breakdown in the fidelity of translation start site selection. Indeed, subsequent biochemical assays (Huang et al. 1997) demonstrated that in each of the *Sui* mutants, either an elevated level of GTP hydrolysis by eIF2 or an aberrant dissociation of Met-tRNA$_i$

releases bound initiation factors from the 43S preinitiation complex prematurely and leaves the initiator Met-tRNA at the P site of the 40S subunit. This allows the translation machinery to initiate from a UUG codon. These data suggest that in eukaryotes, in order to ensure translation fidelity, hydrolysis of GTP must be suppressed during scanning of the mRNA by the 43S preinitiation complex. It can only occur when the 43S preinitiation complex has selected the initiation AUG codon and that the process of AUG selection and eIF5-promoted GTP hydrolysis is likely to be coupled. Since all the components required for GTP hydrolysis, namely eIF2, eIF5, and 40 ribosome, are present in the 43S preinitiation complex, there must exist a mechanism by which premature GTP hydrolysis and therefore aberrant initiation is prevented prior to AUG selection. The possibility exists that the coupling of eIF5-promoted GTP hydrolysis to the AUG selection process may be modulated by other initiation factors bound in the 43S preinitiation complex.

Biochemical support for the role of multiple initiation factors in AUG selection stemmed from a variety of protein-protein interaction studies. It was observed that in addition to its interaction with eIF2, eIF5 also interacts with eIF3, eIF4G, and eIF1 (reviewed by Hinnebusch et al. 2007; Pestova et al. 2007). While the N-terminal domain of eIF5 interacts with the G-domain of eIF2γ, the C-terminal domain of eIF5 interacts with the K-boxes of eIF2β, as well as with eIF1, eIF3, and eIF4G. Additionally, work in the yeast *S. cerevisiae* has shown that eIF5 is a component of the multifactorial protein complex consisting of eIF2, eIF3, eIF1, and stoichiometric amount of Met-tRNA$_i$. (Asano et al. 2000) that may be important for coordinating eIF5-promoted GTP hydrolysis to the AUG selection process. In fact, eIF5 was found to be associated with the 43S preinitiation complex bound to eIF4F at the 5′-cap structure of mRNA (Majumdar and Maitra 2005). It may, thus, be possible that the eIF5•eIF2 interaction, which is essential for GTP hydrolysis, is physically blocked in the 43S preinitiation complex prior to AUG selection via protein-protein interactions involving one or more bound initiation factors, and this block is released once AUG is selected by the ribosomal complex.

Subsequent in vitro biochemical studies on eIF5-mediated GTP hydrolysis demonstrated that addition of eIF5 to the 43S preinitiation complex containing bound ternary complex, as well as bound initiation factors eIF1, eIF1A, and eIF3 did not result in complete inhibition of hydrolysis of the GTP bound to the 43S preinitiation complex (Majumdar and Maitra 2005). However, near-complete abolition of GTP hydrolysis was observed when eIF4F protein (containing a bound 5′ mRNA cap structural analog) was added to the 43S preinitiation complex containing bound eIF1, eIF1A, and eIF3 (Majumdar and Maitra 2005). Similar results were obtained when the 43S preinitiation complex was positioned at the 5′-capped end of an mRNA via binding to eIF4F (Majumdar and Maitra 2005). Surprisingly, although both cap analog-bound eIF4F as well as eIF4F free of the cap analog-bound the 43S preinitiation complex with comparable efficiency, eIF4F could exert its effect on eIF5-promoted GTP hydrolysis only when it was bound to the cap analog. Taken together, these results indicate that the specific cap-bound conformation of the eIF4F protein was, perhaps, required to fully block eIF5-promoted GTP hydrolysis in the 43S preinitiation complex. Since, eIF5 was found to be stably associated with 43S preinitiation complex as well, it strongly favors the hypothesis that eIF5 was completely prevented from interacting with eIF2 in the 43S preinitiation complex in the presence of the other bound initiation factors, eIF1, eIF1A, eIF3, and eIF4F and as a result aberrant GTP hydrolysis (i.e., GTP hydrolysis prior to positioning of the 43S preinitiation complex at the AUG start codon) was prevented under these conditions.

However, when the 43S preinitiation complex was allowed to scan the 5′-UTR (in presence of additional factors required for scanning – eIF4A, -4B, and ATP) to reach the start AUG codon, there was a complete restoration of eIF5-promoted GTP hydrolysis. Under these conditions, toe-printing analysis revealed the 40S ribosomal complex to be positioned at the AUG codon of the mRNA (Majumdar and Maitra 2005). It has been demonstrated that codon-anticodon base-pairing between the initiator Met-tRNA$_i$ and the AUG codon of an mRNA leads to a marked conformational change in the 48S complex resulting in the release of bound eIF1 and possibly reorientation of the bound factors within the complex (Nanda et al. 2009). As a result, eIF5 may then be able to interact with eIF2, which triggers the GAP activity of eIF5 resulting in the hydrolysis of GTP bound in the 48S initiation complex. Support for this model came from determination of structures of N-terminal and C-terminal domains of



TIF5 (eIF5), Fig. 1 Schematic representation for the role of eIF5 in AUG start codon selection and the link between eIF5-promoted GTP hydrolysis and AUG selection. By virtue of its ability to interact with multiple eIFs (eIF1, eIF1A, eIF3, and eIF4F), eIF5 can bind to the 43S preinitiation complex bound to eIF4F at the 5′-cap structure. However, bound eIF5 is prevented from interacting with the 40S-bound eIF2•GTP due to the presence of other bound initiation factors. In particular, due to the structural similarity between eIF1 and the NTD of eIF5, this domain of eIF5 that must interact with the GTP-binding domain of eIF2γ to act as a GAP for eIF2 and is excluded from the eIF1 binding site in the 43S preinitiation complex (PIC) (Lorsch and Dever 2010), preventing premature GTP hydrolysis prior to AUG selection. Positioning of the 43S PIC at the AUG start codon causes a conformational change of the 48S initiation complex that releases eIF1 from the 40S ribosome and allowing the NTD of eIF5 to occupy the eIF1 binding site in the PIC. eIF5 can now interact with eIF2 thereby leading to GTP hydrolysis. Although shown bound to the 5′-cap structure, whether eIF4F remains bound to the cap or is a component of the scanning 43S ribosomal complex is not yet clear (Adapted from Majumdar and Maitra 2005)

eIF5 (Pestova et al. 2007; Lorsch and Dever 2010). It has been observed that the N-terminal domain of eIF5 is structurally similar to eIF1 (Conte et al. 2006). This finding along with the observation that eIF5 and eIF1 antagonizes each other's binding to the 43S preinitiation complex (Nanda et al. 2009) has suggested a model (Nanda et al. 2009) in which eIF1 is bound in the scanning 43S complex in competition with the NTD of eIF5 thus preventing interaction between the NTD of eIF5 and eIF2. Release of eIF1 upon start codon recognition of the 43S complex may allow eIF5 to move to the eIF1 binding site in the preinitiation complex and allow interaction with eIF1A as well as with eIF2 to trigger its GAP activity leading to GTP hydrolysis and Pi release. At any rate, it is clear that the process of AUG selection is intimately connected to the hydrolysis of bound GTP in the 48S initiation complex. The overall sequence of events in the link between AUG section and GTP hydrolysis is summarized in Fig. 1.

Summary

eIF5, a single-subunit protein of about 49 kDa in mammals and 45 kDa in the yeast *Saccharomyces cerevisiae*, in conjunction with GTP and other translation initiation factors, plays an essential role in initiation of protein synthesis in eukaryotic cells. Following scanning of the 5′-UTR of an mRNA by 43S ribosomal preinitiation complex (40S•eIF3•mRNA•Met-tRNA•eIF2•GTP•eIF1•eIF1A) and positioning of the preinitiation complex at the AUG start codon of the mRNA to form the 48S initiation complex, eIF5 interacts with eIF2 bound as a Met-$tRNA_i$•eIF2•GTP ternary complex in the ribosomal complex to promote the hydrolysis of bound GTP. Hydrolysis of GTP is a stringent prerequisite for the eventual release of eIF2•GDP and other bound initiation factors during eIF5B-mediated joining of the 60 ribosomal subunit to the 48S complex to form the functional 80S initiation complex. Extensive biochemical studies have shown that eIF5 physically interacts with eIF2 and acts a GTPase-activating protein (GAP) for eIF2, increasing the intrinsic GTPase activity of eIF2 by over six orders of magnitude. Additionally, recent genetic, biochemical, structural analysis of eIF5 and other initiation factors have provided compelling evidence that eIF5, by virtue of its interaction with other initiation factors, is also an essential component of the scanning 43S ribosomal complex and plays an important role in the stringency of the start codon selection. Specific single-site mutation of eIF5 has been shown to alter the ability of the scanning 43S preinitiation complex to utilize UUG codons as start sites in addition to AUG codons. Thus, in addition to its function as a constitutive GAP for eIF2, the initiative factor eIF5, in conjunction with other initiation factors in the ribosomal scanning complex, plays a direct role in start codon selection.

References

Algire MA, Maag D, Lorsch JR. Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation. Mol Cell. 2005; 20:251–62.

Asano K, Clayton J, Shalev J, Hinnebusch AG. A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and the initiator tRNA (Met) is an important translation intermediate in vivo. Genes Dev. 2000;14:2534–46.

Asano K, Krishnamoorthy T, Phan L, Pavitt GD, Hinnebusch AG. Conserved bipartite motifs in yeast eIF5 and eIF2B epsilon, GTPase-activating and GDP-GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2. EMBO J. 1999;18:1673–88.

Conte MR, Kelly G, Babon J, Sanfelice D, Youell J, Smerdon SJ, Proud CG. Structure of the eukaryotic initiation factor eIF5 reveals a fold common to several translation factors. Biochemistry. 2006;45:4550–8.

Das S, Maitra U. Mutational analysis of mammalian translation initiation factor 5 (eIF5): role of interaction between the beta subunit of eIF2 and eIF5 in eIF5 function in vitro and in vivo. Mol Cell Biol. 2000;20:3941–50.

Das S, Maitra U. Functional significance and mechanism of eIF5-promoted GTP hydrolysis in eukaryotic translation initiation. Prog Nucleic Acid Res Mol Biol. 2001;70: 207–31.

Das S, Maiti T, Das K, Maitra U. Specific interaction of eukaryotic translation initiation factor 5 (eIF5) with the beta-subunit of eIF2. J Biol Chem. 1997;272:31712–8.

Das S, Ghosh R, Maitra U. Eukaryotic translation initiation factor 5 functions as a GTPase-activating protein. J Biol Chem. 2001;276:6720–6.

Donahue TF. Genetic approaches to translation initiation in *Saccharomyces cerevisiae*. Genes Dev. In: Soneberg N, Hershey JWB, Matthews MB, editors. Translational Control of Gene Expression. New York: Cold Spring Harbor Press; 2000. p. 487–502.

Hinnebusch AG, Dever TE, Asano K. Mechanism of translation initiation in the yeast *Saccharomyces cerevisiae*. In: Mathews M, Sonneberg N, Hershey JWB, editors. Translational Control in Biology and Medicine. New York: Cold Spring Harbor Laboratory Press; 2007. p. 225–68.

Huang HK, Yoon H, Hannig EM, Donahue TF. GTP hydrolysis controls stringent selection of the AUG start codon during translation initiation in *Saccharomyces cerevisiae*. Genes Dev. 1997;11:2396–413.

Lorsch JR, Dever TE. Molecular view of 43S complex formation and start site selection in eukaryotic translation initiation. J Biol Chem. 2010;285:21203–7.

Maitra U, Stringer EA, Chaudhuri A. Initiation factors in protein biosynthesis. Annu Rev Biochem. 1982;51:869–900.

Majumdar R, Maitra U. Regulation of GTP hydrolysis prior to ribosomal AUG selection during eukaryotic translation initiation. EMBO J. 2005;24:3737–46.

Nanda JS, Cheung YN, Takacs JE, Martin-Marcos P, Saini AK, Hinnebusch AG, Lorsch JR. eIF1 controls multiple steps in start codon recognition during eukaryotic translation initiation. J Mol Biol. 2009;394:268–85.

Paulin FE, Campbell LE, O'Brien K, Loughlin J, Proud CG. Eukaryotic translation initiation factor 5 (eIF5) acts as a classical GTPase-activator protein. Curr Biol. 2001;11:55–9.

Pestova TV, Lomakin IB, Lee JH, Choi SK, Dever TE, Helen CUT. The joining of ribosomal subunits in eukaryotes requires eIF5B. Nature. 2000;403:332–5.

Pestova TV, Lorsch JR, Helen CUT. The Mechanism of translation initiation in eukaryotes. In: Mathews M, Sonenberg N, Hershey JWB, editors. Translational Control in Biology and

Medicine. New York: Cold Spring Harbor Laboratory Press; 2007. p. 87–128.
Raychaudhuri P, Maitra U. Identification of ribosome-bound eukaryotic initiation factor 2•GDP binary complex as an intermediate in polypeptide chain initiation reaction. J Biol Chem. 1986;261:7723–8.
Sonenberg N, Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell. 2009;136:731–45.

Tif5p

▶ TIF5 (eIF5)

TIF6 (eIF6)

Arunima Biswas, Avik Choudhuri and Umadas Maitra
Department of Developmental and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA

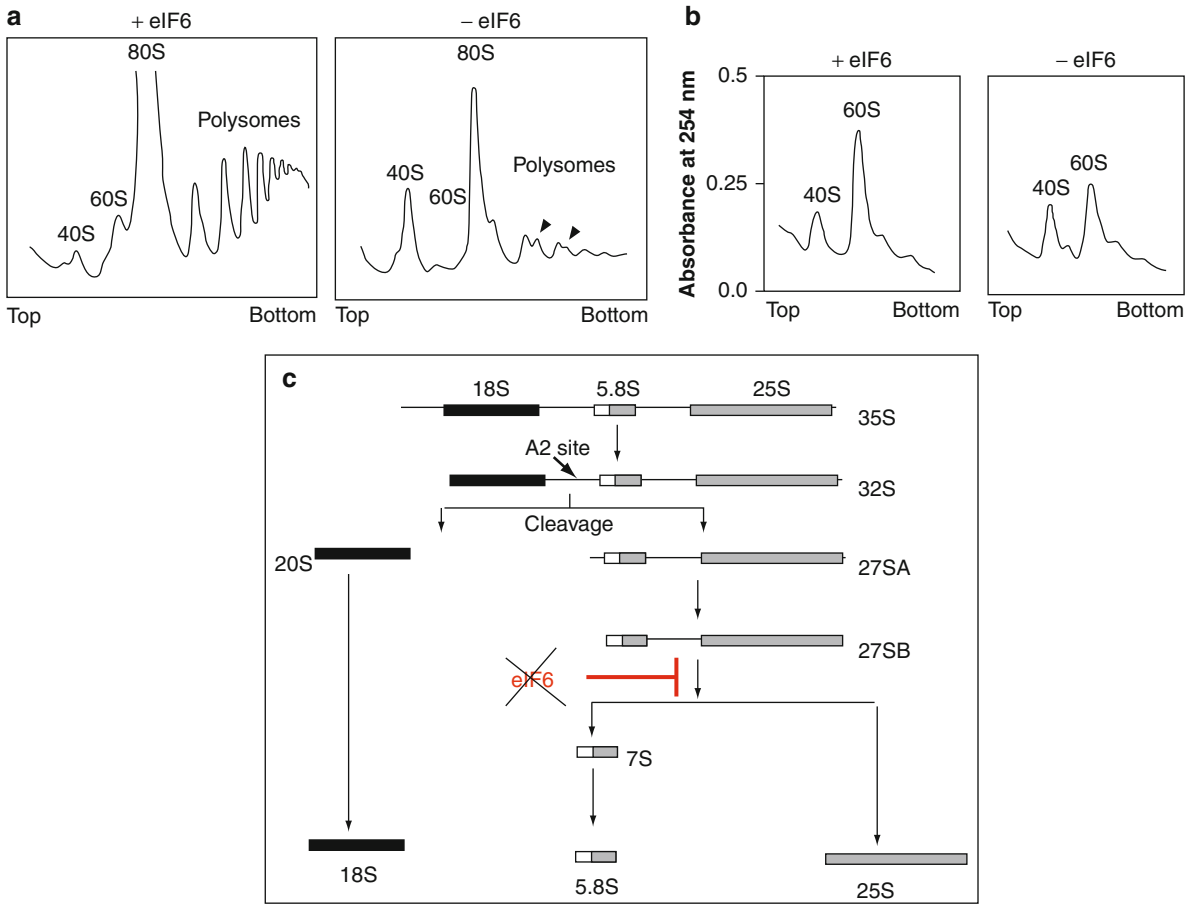
Synonyms

aIF6 in Archae; Tif6p (in yeast)

Historical Background

(a) *Isolation and initial characterization of eIF6* – Eukaryotic initiation factor 6 (eIF6), a monomeric protein of about 26.5 kDa, was originally isolated from the postribosomal supernatant of both wheat germ (Russel and Spremulli 1979) and mammalian cell extracts (Valenzuela et al. 1982) based on an in vitro assay that measured the ability of the protein to bind specifically to the 60S ribosomal subunit and to prevent the association of the 60S subunit to the 40S ribosomal subunit to form the 80S ribosomes. Because, the assembly of the 80S initiation complex during initiation of protein synthesis requires a cellular pool of free 40S and 60S ribosomal subunits, eIF6 was thought to play a direct role in the provision of free ribosomal subunits required for initiation of protein synthesis. The protein was therefore classified as a eukaryotic translation initiation factor (eIF), although its function in translation was poorly characterized. Notably, it was shown that eIF6-bound 60S ribosomal subunit is incapable of joining the 40S (48S) initiation complex to form the 80S initiation complex (Valenzuela et al. 1982). It should be noted that recent cryo-electron microscopic studies reveal that eIF6 and the 40S ribosomal subunit share a common binding region on the 60S subunit and cannot bind simultaneously. Thus the ribosomal subunit anti-association activity of eIF6 is due to its ability to physically block intersubunit bridge formation (Gartmann et al. 2010).

(b) *Functional characterization of eIF6* using m*olecular genetic analysis in yeast*: To facilitate further characterization of eIF6, first a human cDNA (Si et al. 1997) and then the single-copy essential *Saccharomyces cerevisiae* gene (Si and Maitra 1999; Sanvito et al. 1999) encoding functionally active eIF6, each of 245 amino acids (calculated M_r,26,558 for human eIF6 and 25,550 for yeast eIF6) were cloned and expressed in *Escherichia coli*. The two proteins are 72% identical. A molecular genetic analysis in yeast was therefore used by investigators to elucidate the cellular function of eIF6 (Si and Maitra 1999; Sanvito et al. 1999). Using a mutant yeast strain having a conditional eIF6 expression system, these investigators showed that depletion of eIF6 from this mutant strain resulted in inhibition of both cell growth and rate of in vivo protein synthesis. Analysis of the polysome profiles of wild-type and eIF6-depleted cells showed that eIF6-depletion caused a marked reduction in the number of polyribosomes compared with that in wild-type cells (Fig. 1a). However, in contrast to cells depleted of an essential initiation factor, where a reduction in the size of polyribosomes is always accompanied by a marked increase in the number of 80S ribosomes and both free 40S and 60S ribosomal subunits, the decrease in polyribosome content in eIF6-depleted cells is accompanied by a decrease (not increase) in the number of both 80S and 60S ribosomes and concomitant accumulation of half-mer polyribosomes (representing stalled 43S initiation complexes at the 5'-UTR and at the AUG



TIF6 (eIF6), Fig. 1 (**a**) Analysis of the polyribosome profile of eIF6-depleted mutant yeast cells. Lysates of wild-type (wt) and eIF6-depleted mutant yeast cells were subjected to 7–47% (wt/vol) sucrose gradient centrifugation and the A_{254} profile of the gradient fractions was analyzed in a UV-absorbance monitor. The positions of free 40S, 60S, 80S ribosomes, polyribosomes, and half-mer polyribosomes are indicated. Details of the procedures are in the reference, Si and Maitra (1999). (**b**), eIF6 depletion results in a decrease in 60S ribosomal subunits. Total ribosome isolated from wild-type and eIF6-depleted cells were dissociated into 40S and 60S ribosomal subunits, and sedimented through 15–40% sucrose gradient and ribosome profiles were determined. (The figures in panels (**a**) and (**b**) are reproduced with permission from Si and Maitra 1999). Copyright 1999, the American Society of Microbiology. (**c**) Schematic representation of the organization and processing sites of 35S pre-rRNA. The 35S precursor RNA contains the sequences for 18S, 25S, and 5.8S rRNAs that are separated by transcribed spacer regions that are removed at various processing steps. The major cleavage sites, the endonucleolytic and exonucleolytic processing steps, processing intermediates, and pathways leading to mature rRNAs are indicated. The processing step that is blocked by eIF6 depletion is also shown (adapted from the reference Basu et al. 2001)

codon of mRNAs awaiting association with 60S ribosomal subunits). Direct determination of total 40S and 60S subunit content of eIF6-depleted cells confirmed that there was a selective reduction of total 60S with respect to total 40S ribosomal subunits, causing a stoichiometric imbalance in the 60S/40S subunit ratio (Fig. 1b) resulting in the formation of half-mer polysomes. These results, along with the observation that lysates of yeast cells lacking eIF6 remained active in translation of mRNA in vitro (Si and Maitra 1999), led to the conclusion that eIF6 does not function as a canonical translation initiation factor for global protein synthesis. Additionally, it was shown that the stability of mature 60S ribosomal particles, synthesized in yeast cells in the presence of eIF6, was not significantly affected following removal of eIF6 from these cells (Basu et al. 2001). Rather evidence

clearly indicated that in eIF6-depleted cells, the reduction of 60S ribosomal subunits is due to severe inhibition in the biogenesis of 60S ribosomal subunits. To understand how depletion of eIF6 affects 60S ribosome biogenesis, it is necessary to review very briefly the salient features of ribosome biogenesis.

eIF6 is Essential for the Biogenesis of 60S Ribosomal Subunits

Ribosome biogenesis (reviewed in Kressler et al. 2010; Venema and Tollervey 1999) is a highly conserved process from yeast to mammals and occurs primarily in the nucleolus, where four ribosomal rRNAs (25S in yeast or 28S in mammals), 18S, 5.8S, and 5S are formed, modified co-transcriptionally by pseudouridylation and methylation, and processed during their assembly with 78 (in yeast) and 79 (in mammals) ribosomal proteins into mature 40S and 60S ribosomal subunits. In yeast, where the process has been best characterized, the 18S rRNA of the 40S subunit and the 25S (28S in mammals) and 5.8S rRNAs of the 60S subunit are transcribed in the nucleolus from the rDNA transcription unit by RNA polymerase I as a single large precursor RNA known as the 35S pre-RNA in yeast and 45S pre-rRNA in mammals. The fourth rRNA 5S, also a constituent of the 60S subunit, is transcribed independently from 5S DNA by RNA polymerase III. Immediately following the synthesis of the 35S pre-rRNA, many ribosomal proteins (mostly of the 40S subunits) as well as a large number of transacting nonribosomal proteins and small nucleolar RNPs (SnoRNPs) associate with the 35S pre-rRNA to form the 90S ribonucleoprotein (RNP) particle. Association of the transacting nonribosomal proteins as well as snoRNAs with the preribosomal RNP particles are required for accurate pre-RNA processing, pre-rRNA modification, and ribosome assembly. The 35S pre-rRNA present in the 90S RNP then undergoes a sequence of ordered exonucleolytic trimming and endonucleolytic cleavage reactions (see Fig. 1c) giving rise to several intermediate RNP particles of decreasing size in the nucleus and eventually to a mature 40S ribosomal subunit containing 18S rRNA and a mature 60S ribosomal subunit containing 5.8S and 25S rRNAs. As shown in Fig. 1c, the very first endonucleolytic cleavage at the A_2 site results in the formation of pre-40S (43S) and pre-60S (66S) particles. Each precursor particle then follows an independent pathway for subsequent biogenesis in the nucleolus and nucleoplasm, nuclear export and final maturation in the cytoplasm. It should be noted that not all transacting assembly proteins associate simultaneously with the 90S or intermediate pre-40S and pre-60S RNP particles. Rather, these proteins associate with preribosomal RNP particles at different steps of the processing reactions. As the biogenesis and maturation of the pre-60S and pre-40S particles proceed first in nucleolus and then in the nucleoplasm, most of the bound transacting proteins (not the ribosomal proteins) are sequentially released along the biogenesis pathway and recycled in the nucleolus for new rounds of biogenesis. The pre-40S and pre-60S particles that emerge from the nuclear pore complex to the cytoplasm contain only a few bound transacting proteins (reviewed in Kressler et al. 2010; Panse and Johnson 2010). These bound proteins are released in sequence from the preribosomal particles in the cytoplasm to form translation-competent mature ribosomal subunits.

eIF6 is one of the essential 60S ribosomal assembly proteins that associates with the pre-60S ribosomal particles in the nucleolus and is required for the subsequent maturation of the 60S ribosomal particles. In eIF6-depleted cells, while the 20S precursor RNA present in the pre-40S particles is processed to 18S rRNA quite efficiently, most of the 27S pre-rRNA present in the pre-60S particles is degraded without forming 25S and 5.8S rRNAs (Basu et al. 2001). Specifically, depletion of eIF6 blocks the processing of the intermediate 27SB pre-rRNA to 7S and 25.5S pre-rRNAs that are the precursors of 5.8S and 25S mature rRNAs, respectively (Basu et al. 2001 and Fig. 1C). Thus, eIF6 is necessary for the formation of 60S ribosomal subunits because it is necessary for the formation of 25S and 5.8S rRNAs, constituents of the 60S ribosomal subunit.

eIF6 (Tif6p) is Phosphorylated In Vitro and In Vivo in Mammalian and Yeast Cells

In earlier studies (Basu et al. 2003; Ray et al. 2008), it was observed that in both mammalian and yeast cells, eIF6 (Tif6p) is phosphorylated at Ser-174 (major site) and Ser-175 (minor site) by the nuclear isoform of CK1 (CK1α or δ in mammals and Hrr25p in yeast). In yeast

cells, these are the only sites that are phosphorylated in vivo. Mutation of Ser-174 alone to alanine abolishes phosphorylation of yeast eIF6 by >75% while mutation of both Ser-174 and Ser-175 to alanine or depletion of Hrr25 from yeast cells causes total abolition of eIF6 phosphorylation. More importantly, failure to phosphorylate eIF6 in vivo either by depletion of Hrr25p from yeast cells or alanine replacement of Ser-174 and Ser-175 of Tif6p inhibited efficient processing of preribosomal RNA to form the mature 25S and 5.8S rRNAs and thus 60S ribosome biogenesis in yeast (Ray et al. 2008). Conversely, mutation of Ser-174 alone to alanine abolishes yeast cell growth and viability. Taken together, these results suggest that phosphorylation of Tif6p at Ser-174 and Ser-175 plays an important regulatory role in the function of Tif6p. However, the molecular basis of phosphorylation of eIF6 (Tif6p), is not apparent from these studies.

Nuclear Export of eIF6 (Tif6p) Bound to the pre-60S Ribosomal Particles and its Subsequent Release

Although the nascent pre-60S particles are mostly assembled in the nucleolus and to a lesser extent in the nucleoplasm, they are exported out of the nucleus to the cytoplasm where final maturation of the pre-60S ribosomal particles occurs to form the mature translationally competent 60S ribosomal subunits. Most of the transacting protein factors that associate with the preribosomal particles during their nucleolar assembly are released in the nucleus prior to the export of the pre-60S particles in the cytoplasm. However, a small number of protein factors including eIF6 (Tif6p) remain bound as the pre-60S particles exit the nucleus and enter the cytoplasm. The cytoplasmic ribosomal maturation pathway involves sequential and ordered release of these bound protein factors by the action of specific energy-consuming cytoplasmic ATPases or GTPases, each of which associates with the preribosomal particles to affect the release of a specific bound factor. In addition, some critical ribosomal proteins are also added at this stage to the 60S particles to make functional 60S ribosomes. The released factors are recycled back to the nucleus for another round of pre-60S ribosome assembly and export. In the ordered release of the bound factors, Tif6p and the nuclear export adapter Nmd3 are the last proteins to be released. Following their release, the pre-60S particles become mature 60 ribosomal subunits and competent to participate in translation.

Mechanism of Release of eIF6 (Tif6p) from the pre-60S Particles

In earlier studies it was reported that in mammalian cells, release of eIF6 from the pre-60S particles is triggered by phosphorylation of eIF6 at Ser-235 by protein kinase C (PKC) and RACK1 (receptor for activated kinase C) (Ceci et al. 2003). However, molecular genetic analysis in yeast cells provided compelling evidence that two cytoplasmic proteins – the GTPase elongation factor-like 1 (Efl1p) (Becam et al. 2001; Senger et al. 2001) and Sdo1, the yeast ortholog of highly conserved mammalian Shwachman–Bodian–Diamond Syndrome (SBDS) protein that is mutated in the inherited bone marrow failure and predisposition to leukemia disorder – genetically interact with the pre-60S particles containing bound eIF6 in the cytoplasm, and act cooperatively to facilitate the release of eIF6 from the pre-60S particles (Menne et al. 2007).

In yeast cells, deletion of either *EFL1* or *SDO1* confers a very slow growth phenotype. It was also observed that while in wild-type yeast cells, eIF6 (Tif6p) localized predominantly to the nucleolus, there was a large accumulation of Tif6p bound to the pre-60S particles in the cytoplasm of *efl1*Δ and *sdo1*Δ cells. Importantly, multiple gain of function *TIF6* alleles (having a missense mutation in *TIF6)* that rescued the growth defect of either *sdo1*Δ or *efl1*Δ cells also restored both the nuclear export defect and nucleolar localization of Tif6p. Biochemical analysis showed that in contrast to wild-type Tif6p, which binds to 60S ribosomal subunits with a relatively high affinity, mutant eIF6 in the suppressor strains has a much reduced affinity for 60S subunits and can thus bypass the requirement of Sdo1 and Efl1p for eIF6 release (Menne et al. 2007).

Direct biochemical evidence in support of the requirement of SDO1 and EFL1 in the release of eIF6 from the pre-60S particles came from the recent elegant work of Finch et al. (2011) using a

reconstituted in vitro system. These investigators isolated stalled pre-60S ribosomal particles containing bound eIF6 from *sbds*-deleted mouse liver. Incubation of these eIF6-bound pre-60S particles with purified recombinant human SBDS and EFL1 proteins resulted in the release of eIF6 from the pre-60S particles. This reaction requires GTP binding to EFL1 and subsequent energy of hydrolysis of GTP coupled to eIF6 release. EFL1 alone has a low intrinsic GTPase activity which is markedly stimulated by the addition of purified mature mammalian 60S ribosomal subunits (Senger et al. 2001). Addition of SBDS stimulated further 60S-dependent GTP hydrolysis by EFL1 (Finch et al. 2011). However, despite its 60S ribosome-dependent GTPase activity, EFL1 alone in the absence of SBDS is unable to promote the release of eIF6 from the 60S-found. eIF6 complex although GTP hydrolysis still occurs under these conditions. Further mechanistic studies showed that the essential role of SBDS is to tightly couple the activation of EFL1-mediated GTP hydrolysis on the 60S ribosome to eIF6 release. A conserved lysine residue at position 151 of SBDS, which is mutated in SDS disorder, is required for cooperativity with EFL1 and is essential for this coupling process.

Taken together, these observations of Menne et al. (2007) and Finch et al. (2011) provide compelling evidence that the mechanism of eIF6 release from the pre-60S particles in the cytoplasm during the final maturation of 60S ribosomal subunits is highly conserved between yeast and mammals and involves cooperative interaction of SBDS and EFL1 in mediating the GTP hydrolysis-dependent release of eIF6 from the pre-60S particles. In contrast to the report of Ceci et al. (2003), there was no evidence for the requirement of phosphorylation of eIF6 at Ser-235 for its release.

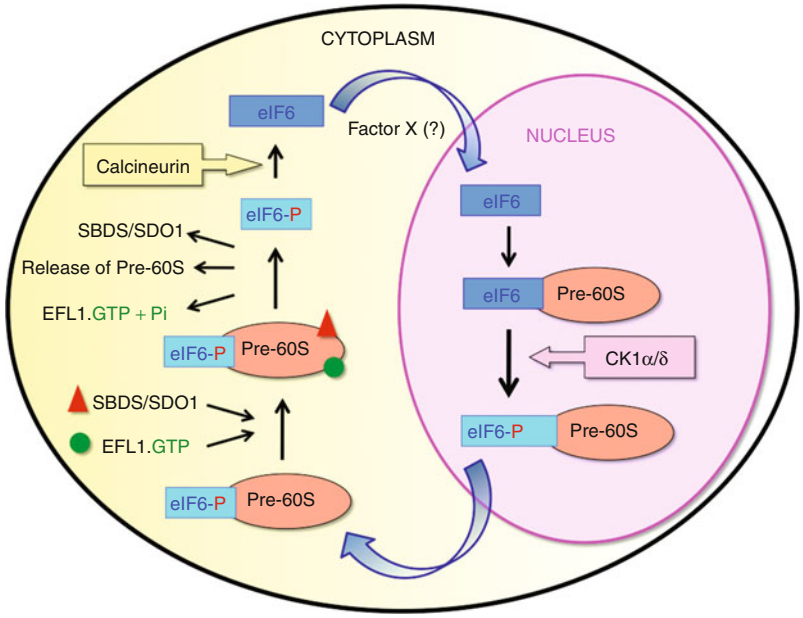
Recycling of eIF6: Opposing Action of Casein Kinase 1 and Calcineurin Phosphatase in Nucleo-Cytoplasmic Shuttling of eIF6

After eIF6 is released from the pre-60 particles, it must be imported back to the nucleus for continued 60S ribosome biogenesis. eIF6 does not appear to have a nuclear localization signal (NLS) or a nuclear export signal (NES). However, as mentioned before, both the yeast and mammalian eIF6 are phosphorylated by the nuclear isoform of CK1 at Ser-174 and Ser-175 and phosphorylation of eIF6 plays an important physiological role in its function in yeast cells. Additionally, examination of the highly conserved amino acid sequence of eIF6 surrounding the CK1 phosphorylation sites at Ser-174 and Ser-175 shows that in all nucleated species examined so far, the protein also possesses a sequence motif LQVP that is known to be a binding motif for the Ca^{2+}-regulated protein phosphatase calcineurin as shown below (reviewed in Biswas et al. 2011).

^{168}E D Q D E L**S S** L L Q V P L V^{182}	Human
Q D Q E E L**S S** L L Q V P L V	*S. cerevisiae*
Q D Q D E L**S S** L L Q V P L V	*Drosophila melanogaster*
D E L E F LK S LF K V E Y I	*Methanococcus jannaschii*

The presence of a biologically important CK1 phosphorylation site and a calcineurin docking motif in nucleated species gave rise to the hypothesis that the nuclear entry and export of eIF6 might be regulated by CK1 phosphorylation and dephosphorylation by calcineurin as was described for NFAT family of transcription factors in vertebrates and a stress-responsive transcription factor, Crz1p, in yeast (reviewed in Biswas et al. (2011)).

In a recent report, Biswas et al. (2011) presented several lines of evidence which suggest that eIF6 shuttles back and forth between the nucleus and the cytoplasm of mammalian cells. This process is dependent on phosphorylation and dephosphorylation of eIF6 at Ser-174 and Ser-175 mediated by CK1 and the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin, respectively. Using COS7 cells, it was shown that the nuclear export of eIF6, bound to the pre-60S particles, requires phosphorylation of Ser-174 and Ser-175. Failure to phosphorylate at these sites either by mutation of the serine residues to alanine or treatment of cells with a CK1 inhibitor causes a significant fraction (>90%) of eIF6 to be retained in the nucleus. These observations also suggest that it is the phosphorylated form of eIF6 that is exported to the cytoplasm and is presumably released from the pre-60S particles. For nuclear import of eIF6, the protein phosphatase



TIF6 (eIF6), Fig. 2 Schematic representation of the functional pathway of eIF6 in 60S ribosome biogenesis. In the nucleolus, eIF6 associates with the pre-60S particles along with >100 transacting protein factors and is essential for pre-60S ribosome assembly and pre-rRNA processing. eIF6 remains associated with the pre-60S particles during pre-60S maturation in the nucleoplasm as well as during the nuclear export of the pre-60S particles. Nuclear export of eIF6 bound to the pre-60S particles requires phosphorylation of eIF6 at Ser-174 and Ser-175 by the nuclear isoform of CK1. In the cytoplasm, during the final maturation process, two cytoplasmic proteins SBDS/Sdo1 and EFL1/Efl1p interact with the pre-60S particles and catalyze the release of eIF6 coupled to GTP hydrolysis by EFL1. The released eIF6 that is presumably in the phosphorylated form then interacts with Ca^{2+}/calmodulin-regulated protein phosphatase calcineurin and the dephosphorylated form of eIF6, either by itself or by interaction with another as yet unidentified protein factor X containing the NLS signal, is imported to the nucleolus to participate in another round of 60S ribosome biogenesis

calcineurin, following its activation by Ca^{2+}, associates with eIF6 that is presumed to be in the phosphorylated form, and appears to play an essential role in eIF6 nuclear import as the localization of eIF6 changes from cytosolic to nuclear subsequent to calcineurin activation in vivo. This event is blocked by the immunosuppressive drug cyclosporin A, that is known to be a specific calcineurin inhibitor suggesting that the dephosphorylated form of eIF6 is imported to the nucleus. At present, it is not known whether nuclear import of dephosphorylated eIF6 requires its association with any cofactor (providing the NLS signal) or eIF6 can enter the nucleus by itself due to its low molecular weight. Additionally, nuclear import and export of eIF6 occur even in the presence of a potent protein synthesis inhibitor cycloheximide and is therefore a reflection of dynamics of import and export of preexisting eIF6 molecules rather than de novo new eIF6 synthesis. The release of eIF6 from the pre-60S particles and its recycling to the nucleus are summarized schematically in Fig. 2.

Summary

eIF6, a highly conserved protein from yeast to mammals, binds to the 60S ribosomal subunit and functions as a ribosomal subunit anti-association factor by preventing premature association with the 40S ribosomal subunit. Molecular genetic analysis in yeast has provided compelling evidence that eIF6 is not a canonical translation initiation factor. Rather, it is essential for 60S ribosome biogenesis and assembly. Specifically, eIF6 associates with the pre-60S particles in the nucleolus along with many other transacting protein factors and is essential for 60S ribosome assembly and pre-rRNA processing to form the mature

rRNAs, constituents of the 60S ribosomal particle. Association of eIF6 with the pre-60S particles is also required for the export of the assembled pre-60S particles from the nucleus to the cytoplasm where the release of eIF6 occurs. Nuclear export of the pre-60S particles containing bound eIF6 requires phosphorylation of eIF6 at Ser-174 and Ser-175 by the nuclear isoform of CK1. In the cytoplasm, during the final maturation of the pre-60S particles, two cytoplasmic proteins SBDS/Sdo1 and EFL1/Efl1p interact with the pre-60S particles and catalyze the release of eIF6 coupled to hydrolysis of GTP by EFL1. The released eIF6 that is presumably in the phosphorylated form then interacts with Ca^{2+}/calinodulin-dependent protein phosphatase calcineurin and the dephosphorylated form of eIF6 is imported to the nucleus to participate in another round of 60S ribosome assembly and biogenesis. These observations suggest eIF6 plays an important regulatory role in 60S ribosome biogenesis in eukaryotic cells.

Concluding Remarks

Since the original isolation of eIF6 as a ribosomal subunit anti-association factor, it is now clear that this activity of eIF6 is not utilized to generate free 40S and 60S ribosomal subunits required for initiation of protein synthesis. Rather, association of eIF6 with the pre-60S particles in the nucleolus prevents their premature interaction with the pre-40S particles both in the nucleus and cytoplasm. eIF6 is released only when all other bound factors dissociate from the pre-60 particle leading to the formation of mature 60S ribosomal subunit. Secondly, the essential requirement of SBDS (along with the GTPase EFL1) in the release of eIF6 from the pre-60S particles underscores the importance of this protein in 60S ribosome biogenesis. Mutation of the SBDS gene has been shown to be associated in the inherited leukemia predisposition disorder Shwachman–Diamond syndrome (SDS). Some of the disease-causing mutant proteins are unable to catalyze the release of eIF6 from the pre-60S particles causing defects in 60S ribosome maturation and biogenesis. Thus, SDS has been defined as a "ribosomopathy" by Finch et al. (2011). Finally, the requirement of Ca^{2+}-activated calcineurin phosphatase in the nuclear import of eIF6 could provide a means by which changes in intracellular Ca^{2+} levels could modulate nuclear import of eIF6, and consequently, nucleolar 60S ribosome biogenesis. Clearly, a detailed molecular investigation of these steps is necessary to understand various quality control mechanisms in the pathway of ribosome biogenesis.

Cross-References

▶ NFAT

References

Basu U, Si K, Warner JR, Maitra U. The *Sacchamomyces cerevisiae* TIF6 gene encoding translation initiation factor 6 is required for 60S ribosomal subunit biogenesis. Mol Cell Biol. 2001;21:1453–62.

Basu U, Si K, Deng H, Maitra U. Phosphorylation of mammalian translation initiation factor 6 and its *Saccharomyces cerevisiae* homologue Tif6p: evidence that phosphorylation of Tif6p regulates its nucleocytoplasmic distribution and is required for yeast cell growth. Mol Cell Biol. 2003;23:6187–99.

Becam AM, Nasr F, Racki WJ, Zagulski M, Herbert CJ. Ria1p (ynl163c), a protein similar to elongation factor 2, is involved in the biogenesis of 60S subunit of the ribosome in *Saccharomyces cerevisiae*. Mol Genet Genomics. 2001;266:454–62.

Biswas A, Mukherjee S, Das S, Shields D, Chow CW, Maitra U. Opposing action of casein kinase 1 and calcineurin in nucleo-cytoplasmic shuttling of mammalian translation initiation factor eIF6. J Biol Chem. 2011;286:3129–38.

Ceci M, Gaviraghi C, Gorrini C, Sala LA, Offenhauser N, Marchisio PC, Biffo S. Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. Nature. 2003;426:579–84.

Finch AJ, Hilcenko C, Basse N, et al. Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes Shwachman-Diamond Syndrome. Genes Dev. 2011;25:917–29.

Gartmann M, Blau M, Armache J-P, Mielke T, Topf M, Beckmann R. Mechanism of eIF6-mediated inhibition of ribosomal subunit joining. J Biol Chem. 2010;285:14848–51.

Kressler D, Hurt E, Bassler J. Driving ribosome assembly. Biochem Biophys Acta. 2010;1803:673–83.

Menne TF, Goyenechea B, Sanchez-Puig N, Wong CC, Tonkin LM, Ancliff PJ, Brost RL, Costanzo M, Boone C, Warren AJ. The Shwachman-Bodian-Diamond syndrome protein mediates translational activation of ribosomes in yeast. Nat Genet. 2007;39:486–95.

Miluzio A, Beugnet A, Volta V, Biffo S. Eukaryotic initiation factor 6 mediates a continuum between 60S ribosome biogenesis and translation. EMBO Rep. 2009;10:459–65.

Panse VG, Johnson AW. Maturation of eukaryotic ribosomes: acquisition of functionality. Trends Biochem Sci. 2010;35:260–6.
Ray P, Basu U, Ray A, Majumdar R, Deng H, Maitra U. The *Saccharomyces cerevisiae* 60S ribosome biogenesis factor Tif6p is regulated by Hrr25p-mediated phosphorylation. J Biol Chem. 2008;283:9681–91.
Russel DW, Spremulli LL. Purification and characterization of a ribosome dissociation factor (eukaryotic initiation factor 6) from wheat germ. J Biol Chem. 1979;254:8796–800.
Sanvito F, Piatti S, Villa A, Bossi M, Lucchini G, Marchisio PC, Biffo S. The beta 4 integrin interactor p27 (BBP/eIF6) is an essential nuclear matrix protein involved in 60S ribosomal subunit assembly. J Cell Biol. 1999;144:823–37.
Senger B, Lafontaine DLJ, Graindorge J-S, Gadal O, Camasses A, Sanni A, Garnier J-M, Breitenbach M, Hurt E, Fasiolo F. The nucle(ol)ar Tif6p and Efl1p are required for a late cytolasmic step of ribosome synthesis. Mol Cell. 2001;8:1363–73.
Si K, Maitra U. The *Saccharomyces cerevisiae* homologue of mammalian translation initiation factor 6 does not function as a translation initiation factor. Mol Cell Biol. 1999;19:1416–26.
Si K, Chaudhuri J, Chevesich J, Maitra U. Molecular cloning and functional expression of a human cDNA encoding translation initiation factor 6. Proc Natl Acad Sci USA. 1997;94:14285–90.
Valenzuela DM, Chaudhuri A, Maitra U. Eukaryotic ribosomal subunit anti-association activity of calf liver is contained in a single polypeptide chain protein of M_r=25,500 (eukaryotic initiation factor 6). J Biol Chem. 1982;257:7712–19.
Venema J, Tollervey D. Ribosome synthesis in *Saccharomyces cerevisiae*. Annu Rev Genet. 1999;33:261–311.

Tif6p

► TIF6 (eIF6)

TIP3 (Tec Interacting Protein-3 = SOCS1)

► SOCS

TIRAP

► Toll-like Receptor Adaptor Protein Family Members

TIR-Domain-Containing Adapter Molecule 1

► Toll-like Receptor Adaptor Protein Family Members

TIR-Domain-Containing Adapter Molecule 2

► Toll-like Receptor Adaptor Protein Family Members

TIR-Domain-Containing Adapter Protein Inducing IFN-Beta

► Toll-like Receptor Adaptor Protein Family Members

TIRP

► Toll-like Receptor Adaptor Protein Family Members

Tlr4

► TLR4, Toll-Like Receptor 4

TLR4, Toll-Like Receptor 4

Jayalakshmi Krishnan and Sangdun Choi
Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Synonyms

ARMD10; CD284; hToll; Lps; Ly87; Ran/M1; Rasl2-8; Tlr4; Toll; Toll-like receptor 4; Toll-like receptor, type 4

Historical Background

Overproduction of the cytokine profile (cytokine tsunami or storm) can be caused by both infectious and non-infectious diseases. During this process, inflammatory responses are activated either to provide a protective mechanism or to damage tissues when excessively produced cytokines attempt to overwhelm the cause of their production. This system is activated whenever there is a foreign invasion of the body. Foreign invasion is attributed to microbial associated molecules known as Pathogen Associated Molecular Patterns (PAMPS), which activate our immune system through Pattern Recognition Receptors (PRRs) present in the body fluids, cell membranes, and cytoplasm. PRRs not only identify PAMPs, but also molecules released from damaged cells known as Damage Associated Molecular Patterns (DAMPs). There is a great deal of evidence that DAMPs such as products of necrosis or breakdown components of extracellular matrix are originated or produced by the host species (Yang et al. 2010; Schaefer 2010), even though this would seem to contradict the original belief that the immune system only responded to non-self compounds.

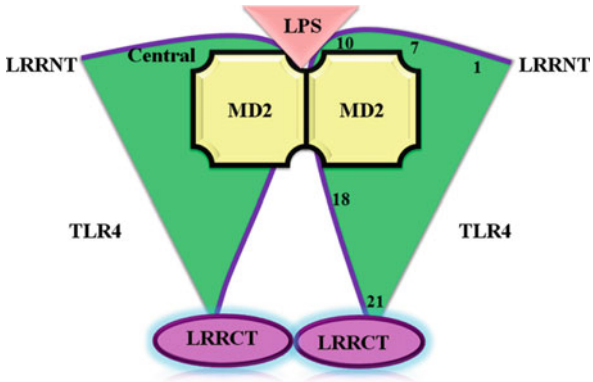
One group of transmembrane PRRs located on the cell membranes and endosomes are known as Toll-like Receptors (TLRs). TLRs are involved in the primary response against invaders, connecting the innate and adaptive immune responses. Recently, three major classes of PRRs were identified in addition to TLRs, C-type lectin receptors (CLR), retinoic acid-inducible gene (RIG)-1-like receptors (RLR), and nucleotide-binding oligomerization domain protein-1- like receptors (NLR). These systems augment the response of TLRs to invading agents. Originally, the activation of any of the aforementioned receptors was thought to be associated with the production of pleotrophic inflammatory cytokines such as TNF, IL-1b, and IL-6. However, type I IFNs are also produced by TLR activation and these compounds enable the cells to resist viral infection, leading to acquired immune responses as well as hematopoietic stem cell production and turnover (Beutler 2009; Kawai and Akira 2009).

Prima facie TLRs were reported in *Drosophila melanogaster* as Toll1 receptor in 1985 (Hansson and Edfeldt 2005). In this fly, Toll gene performs nonimmune functions such as establishing dorsoventral polarity, synaptogenesis, and axon path finding during embryogenesis (Anderson et al. 1985; Halfon et al. 1995; Rose et al. 1997). TLRs are Type I transmembrane glycoproteins that are characterized by the presence of Leucine Rich Repeats (LRRs) and TIR (Toll/Interleukin 1 Receptor) domains. To date, 10 TLRs have been identified in humans, while 13 have been identified in mice. Various TLRs bind with different PAMPs located in the outer wall of microbes or within their cytoplasm. Among the PAMPs, bacterial lipopolysaccharide (LPS) binds with TLR4, while lipopeptides are recognized by TLR2 in conjunction with TLR1 and TLR6, triacylated lipopeptides are bound by TLR1/2 heterodimers, diacylated lipopeptides interact with TLR2 and TLR6, ▶ TLR5 interacts with flagellin, dsRNA binds with TLR3, ssRNA interacts with TLR7 and TLR8, and ▶ TLR9 binds with unmethylated CpG islands. Innate immunity through the TLR system is known to be very important for vaccine design (Hashiguchi et al. 2010). In this entry, we discuss TLR4, its structure and associated receptors, signaling pathways, and the mechanism by which it is activated.

TLR4 Structural Features with MD2

The TLR4 gene, which is also known as Lps; Ly87; Ran/M1; and Rasl2-8, is located in chromosome 4 in mice and chromosome 9 in humans. TLR4 belongs to the LRR family and is composed of an N-terminal, central, and C-terminal domain. TLRs are characterized by two conserved regions, the extracellular leucine rich region (LRR) and the cytoplasmic Toll/IL-1 receptor (TIR) domain. The LRR, which is involved in recognition of the ligand, is composed of 19–25 tandem repeats of 24–29 amino acids folded in strands and helices that are linked by loops. TLR4 is composed of a 608 residue extracellular domain (Medzhitov et al. 1997) and is highly polymorphic when compared with the transmembrane and cytosolic domains (Schmitt et al. 2002; Gay and Gangloff 2007). The TIR domain, which shares homology with the interleukin 1 receptor (IL-1R), modulates protein–protein interactions between the TLRs and the adaptor proteins involved in the signal transduction cascade (O'Neill and Bowie 2007). The TIR domain, which provides a scaffold for the recruitment of MyD88 (myeloid differentiation primary response gene 88), is composed of three highly conserved motifs known as Box-1, Box-2, and Box-3 (Xu et al. 2000). In these three boxes, Box-2

forms loops known as BB loops by connecting β-strands and α-helixes. These loops are indispensable for signaling, as a single point mutation in proline residue (P712H) render C3H/HeJ mice hyporesponsive to LPS (Poltorak et al. 1998). Ligand induced dimerization is one of the hallmarks of TLR4 activation as it is the first line of regulation. CD14, MD2, and TLR4, which are receptosomes, are very important for ligand (LPS) recognition by TLR4. The role of CD14 is to enhance LPS binding to MD2, whereas MD2 is indispensable in the formation of stable receptor-ligand complexes. Interaction between TLR4 and MD2 is mediated in the concave surface at the amino-terminal region of TLR4, especially Glu (24) and Pro (34) and central domains that are critical for MD2 binding (Nishitani et al. 2005) (Fig. 1). Following ligand binding, the TIR domain initiates downstream signaling events starting with adaptor proteins. After recognition of LPS complexes and signaling, the TLR4, MD2, CD14, and LPS complex is ubiquitinated and sorted to late endosomes/lysosomes (dynamin and clathrin dependent mechanism) for degradation, after which the antigens are presented to $CD4^{+}$T cells and signaling is terminated.



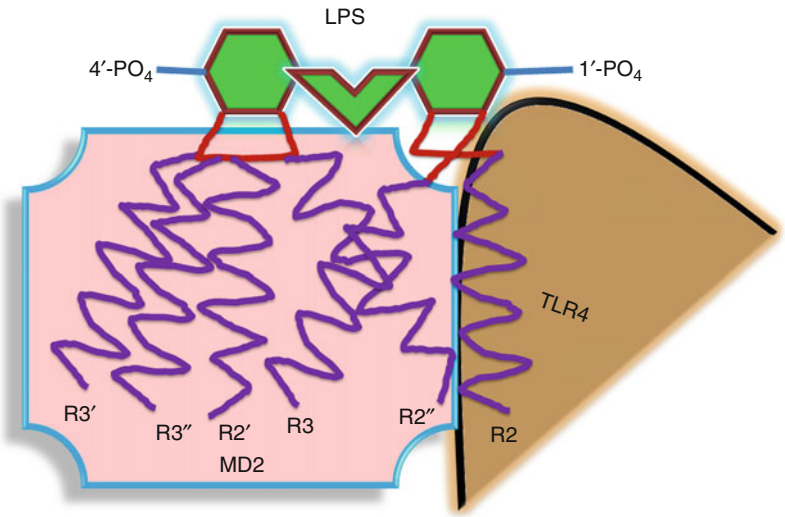
TLR4, Toll-Like Receptor 4, Fig. 1 Diagrammatic representation of the structure of the TLR4-MD2-LPS complex. TLR4 is composed of outer extracellular domain and intracellular domain known as TIR domain. Outer structure is formed of leucine rich repeats and divided as N-terminal, central, and C-terminal domains. MD2 binds with the amino and central domains. After ligand binding and dimerization of TLR4, carboxy terminals fuse together to conduct signaling

Single Nucleotide Polymorphisms of TLR4

Several studies of mice and humans have demonstrated an increased susceptibility to various infections when TLR genes display mutations. Single nucleotide polymorphisms (SNPs) have been reported in various TLR genes, such as TLR2, ▶ TLR4, TLR5, TLR6, TLR9, and TLR10, which are associated with increased disease susceptibility (Schroder and Schumann 2005). TLR4 is unable to function when it shows single nucleotide polymorphisms in its expression on Asp299Gly and Thr399IIe, and is unresponsive to LPS (Douville et al. 2010). TLR4 SNPs (single nucleotide polymorphisms) have been investigated in an extent way. It shows two nonsynonymous SNPs, an A/G transition at SNP rs4986790 that causes the substitution of aspartic acid by glycine at amino acid position 299 (Asp299Gly), and a C/T transition at SNP rs4986791 that causes the substitution of threonine by isoleucine at amino acid position 399 (Thr399Ile). These two SNPs occur in serious infections in HIV-1 infected patients with a history of nadir CD4 cell count of <100 cells/cubic mm (Papadopoulos et al. 2010). Such SNPs also display extensive damage to the system during infections with various diseases and in response to various conditions such as Gram-negative bacteria, septic shock, endotoxemia, disseminated candidiasis, invasive aspergillosis, respiratory syncytial virus infection, meningeal disease, bacterial vaginosis, premature birth, brucellosis, coronary heart disease, asthma, hematogenous osteomyelitis, and *Plasmodium falciparum* infections.

Binding of LPS with MD2 and TLR4

LPS, a TLR4 agonist, is used in several adjuvants that have been shown to be highly effective in both experimental and clinical settings (Thompson et al. 2005; Przetak et al. 2003; Kundi 2007; Baldridge et al. 2004). When LPS interacts with MD2, five of the six lipid chains of LPS are buried deeply in the hydrophobic pocket in MD2, whereas the remaining chain interacts with the conserved phenylalanine in the TLR4 receptor through a hydrophobic interaction (Fig. 2) (Park et al. 2009; Liu et al. 2008; Jin et al. 2007). Park et al. (2009) determined the crystal structure of TLR4-MD2, which helped explain its binding mechanisms. When this complex binds, it provides an "M"- shaped architecture. TLR4 consists of LRRNT (leucine rich repeats N-terminal), central, and LRRCT (leucine rich repeat

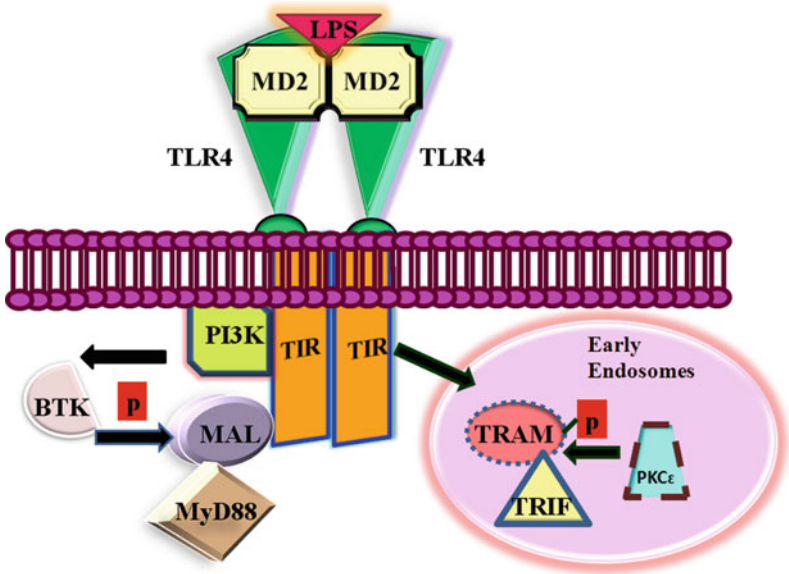


TLR4, Toll-Like Receptor 4, Fig. 2 Binding region of LPS with TLR4 and MD2. Schematically shown is the structure of TLR4-MD2-LPS complex. The receptosomes form an "M" shaped structure. Five acyl chains of LPS named R3′, R3″, R2′, R3, and R2″ form hydrophobic interaction with MD2 and bound in deep interior pockets whereas one chain R2 is exposed to bind with TLR4

C-terminal) domains. MD2 binds with the amino and central domains through their two antiparallel beta-sheets, which forms hydrophobic interactions with LPS. LPS is composed of lipids as well as carbohydrates, the former being referred as endotoxin and the latter being composed of O-antigens. For LPS, it is necessary to have six lipid acyl chains (R3′, R3″, R2′, R3, R2″, and R2) and two phosphate groups to elicit its endotoxic effects. Five of the six acyl chains (R3′, R3″, R2′, R3, R2″) are buried deeply in MD2, whereas the remaining chain (R2) is exposed to the surface to bind with TLR4. The two phosphate groups, 4′-PO_4 and 1′-PO_4, are critical for LPS induced dimer formation, where they form ionic interactions with positively charged amino acids such as lysine and arginine. In addition, the binding of LPS to MD2 causes notable changes in Phe^{126}, which forms the hydrophilic interaction that is critical for MD2 and TLR4 receptor stabilization as well as activation. Eritoran (or E5564, a lipid A antagonist) is a synthetic molecule produced from non-pathogenic *Rhodobactor sphaeroids* (Mullarkey et al. 2003). The crystal structure of TLR4-MD2 complex bound with Eritoran has recently been described, which has shed light into how Eritoran makes its connection with the large hydrophobic internal pocket in MD2 without binding to TLR4 (Kim et al. 2007). Further, in vitro studies have shown that Eritoran blocks the production of cytokines and downregulates intracellular generation of proinflammatory cytokines (Mullarkey et al. 2003; Czeslick et al. 2006). Moreover, there is pharmacological evidence that the drug must be administered every 12 h for its optimum response (Rossignol et al. 2008). Due to its action as an antagonist, Eritoran blocks signaling and subsequent ▶ NF-κB activation. Elucidation of the action of agonists or antagonists on TLR receptors permits design of new therapeutic interventions.

Signaling Components Associated with TLR4

Bruton's tyrosine kinase (BTK) is a TIR-domain-binding protein that participates in NF-κB activation during TLR4 signaling (Fig. 3). BTK binds with Box-1 and Box-2 of the TIR domain and phosphorylates ▶ MAL (MyD88-adaptor-like) at its tyrosine residue. TLR4 interacts directly with Nox4 (NAD(P)H oxidase 4), which is a protein related to gp91phox (Nox2) of phagocytic cells. This binding is involved in the production of ROS and NF-κB activation during LPS induced activation. TLR4 also binds with high mobility group box 1 (HMG1). This binding is mediated by a B-box within one of the two DNA-binding regions in the HMG1 protein. Specifically, HMG1-induced cytokine production through TLR4 stimulation requires the presence of cysteine in position 106 within the HMG1 (Yang et al. 2010). Another key kinase involved in TLR signaling is the p85 subunit of phosphoinositide 3-kinase (PI3K), which binds through its SHC (Src homology 2 domain containing) domain to the phosphotyrosine residue. There are two putative ▶ PI3K binding sites, Tyr674 and Tyr630 in TLR4, and PI3K is



TLR4, Toll-Like Receptor 4, Fig. 3 Signaling components associated with TLR4. TLR4-mediated signaling is identified as MyD88-dependent and independent. MyD88-independent signaling is mediated by TRAM and TRIF. TRIF signaling occurs in early endosomes where it gets phosphorylated by PKCε. PI3K and BTK are also involved in MAL and MyD88-dependent signaling

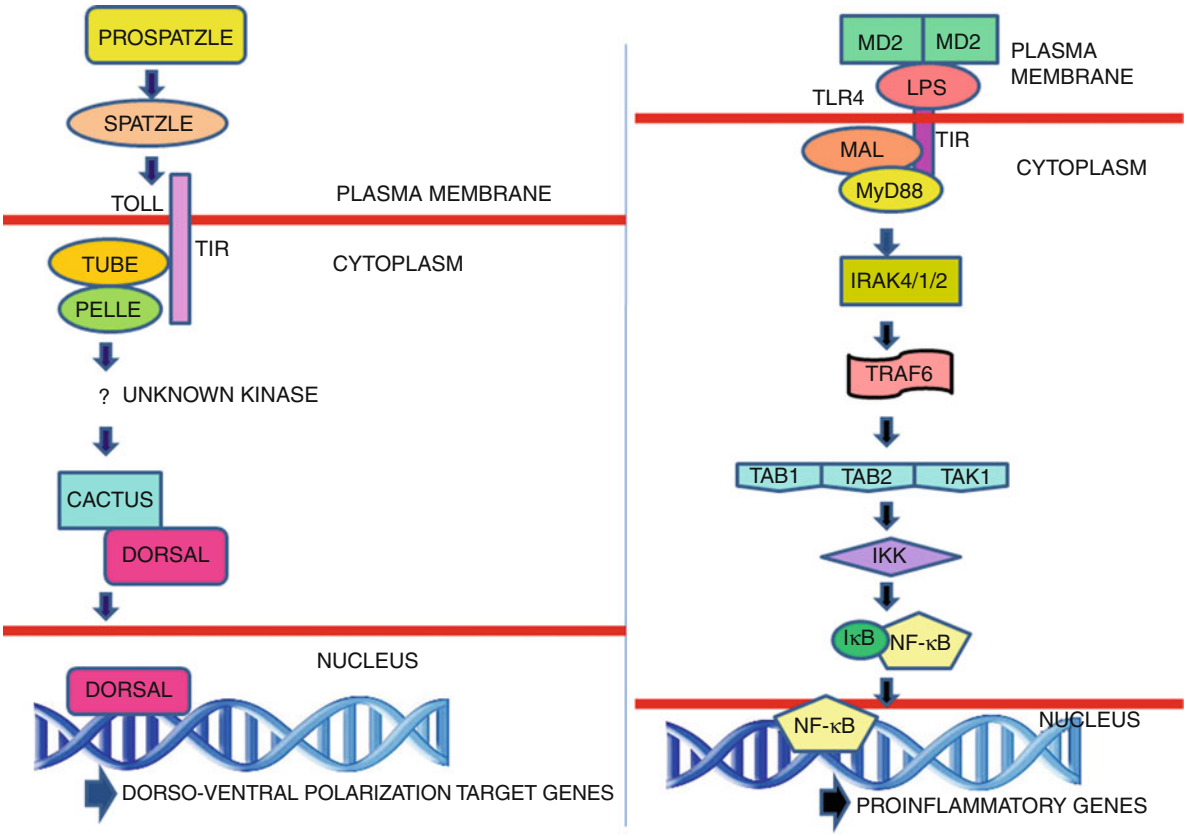
involved in the phosphorylation of TLR2 and TLR3 phosphotyrosine residues as well. ▶ TRAM (TRIF-related adaptor molecule) is phosphorylated by PKCε at its serine-16 residue when stimulated with LPS, which attenuates the response of *Irf3* (Interferon regulatory factor 3) and *Ccl5* (Chemokine (C-C) motif ligand 5). TRAM is trafficked to early endosomes, where it binds with ▶ TRIF (TIR-domain-containing adapter-inducing interferon-β).

Comparison of Toll (*Drosophila*) Signaling with TLR4 Signaling

As discussed earlier, Toll was first identified in *Drosophila* as a protein that mediates dorsoventral polarity during embryonic development (Fig. 4). However, in 1996, Jules A. Hoffman showed that Toll is involved in defense against fungal infection in *Drosophila* (Lemaitre et al. 1996). At the same time, another group searching for LPS-binding protein found a similar structural sequence of Toll in the human genome that resembled the IL1 receptor extracellular domain (Gay and Keith 1991). Thereafter, Janeway group showed that a receptor similar to TLR induced the expression of certain genes involved in adaptive immunity when stimulated with artificial antibodies (Medzhitov et al. 1997). Finally, Poltorak et al. (1998) discovered the current TLR4 and its association with LPS for immune responses. The *Drosophila* genome is composed of nine TLR genes. In *Drosophila*, Toll-1 is maternally transmitted. Spatzle, which is generated from prospatzle during positional signaling in the ventral region of embryo, binds with Toll-1. In turn, Toll-1 recruits and activates ▶ MyD88 homologue Pelle and Tube. Pelle, which is a serine/threonine kinase, phosphorylates itself and Tube as well. The subsequent signaling by an unknown kinase activates cactus (a homologue of IκB proteins) and releases Dorsal (a homologue of NF-κB), which in turn translocates to the nucleus to turn on genes associated with dorsoventral polarity (similar to TLR signaling leading to proinflammatory responses).

Signaling Pathways of TLR4

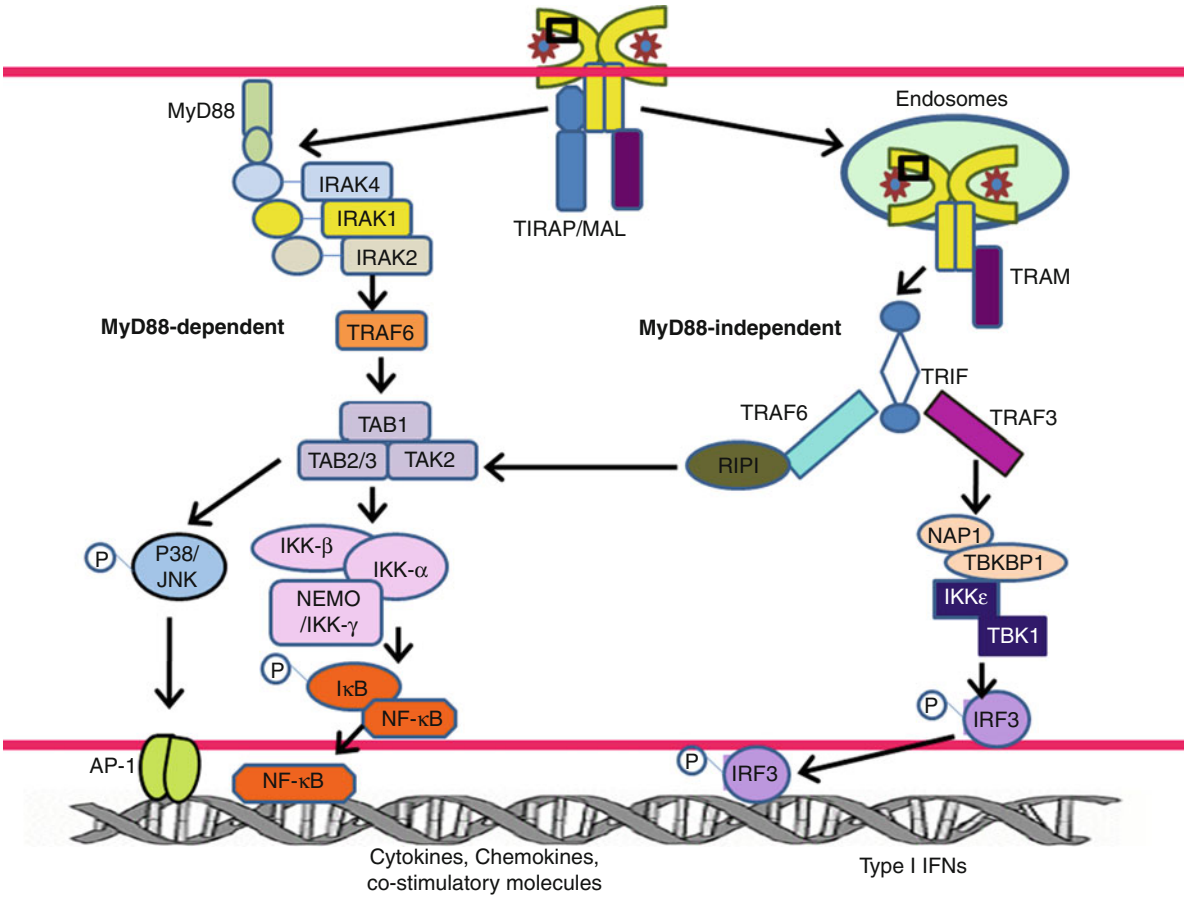
After ligation of the TLR ligands, TLRs dimerize and transmit signals throughout the cell, which leads to the activation of various signaling mechanisms and causes the transcriptional upregulation of distinct genes depending on the cell type and the nature of the stimuli. TLRs use five TIR-domain-containing adaptors, MyD88, Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β signal adaptor protein (TRIF also known as TICAM-1), ▶ TIRAP (TIR-domain-containing adaptor protein)/Mal, TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM). These adaptors brace the lacunae between the receptor and



TLR4, Toll-Like Receptor 4, Fig. 4 Comparison of Toll of *Drosophila* with human TLR4. Toll4 resembles Toll of *Drosophila melanogaster*. Both the receptors look alike in their extracellular domains as well as intracellular domains. Toll mediated signaling through cactus and dorsal gives rise to the genes involved in dorsoventral polarization. TLR4 mediated signaling through IκB and NF-κB leads to the robust induction of the genes involved in proinflammatory responses, which connects between innate and adaptive immunity

signaling molecules to elicit biological responses, which are essential for innate immunity.

TLR signaling is classified into two distinct pathways based on the use of MyD88 (widely used receptor) and TRIF. Among various TLR receptors, TLR4 signaling is unique in that it signals via two different pathways, the MyD88-dependent pathway, which is dependent on the MyD88 adaptor, and the MyD88-independent pathway, which depends on TRIF (Fig. 5). The first pathway is crucial for the rapid production of the proinflammatory cytokines IL-6 and ▶ TNF-α. Even though the MyD88-dependent signaling is very important for most infections, it is not involved in resisting the secondary lethal challenge caused by influenza virus (Seo et al. 2010). MyD88 deficient mice display increased risk for obesity associated diabetes (Hosoi et al. 2010). MyD88 is composed of an N-terminal death domain (DD) separated from the C-terminal TIR domain by a short linker region. MyD88-TIR is composed of three sites that are related to conserved boxes 1–3 of the domain and is important for the LPS/TLR4 pathway (Ohnishi et al. 2009). Two of these sites are located at opposite surfaces of the molecule and mediate direct interaction with Mal-TIR. Several knockout studies have provided evidence that MyD88 is essential for various TLR-responses, with the exception of TLR3 (Kawai et al. 1999; Adachi et al. 1998; Schnare et al. 2000). In general, MyD88 serves as a signaling adaptor by communicating signals to downstream kinases. In contrast, MAL acts as a sorting or connecting adaptor that only promotes interaction between MyD88 and TLR4. MyD88 interacts with IL-1R associated kinase (IRAK4) through its N-terminal DD, after which IRAK4 activates other family members such as IRAK1 and IRAK2. Recent studies have identified



TLR4, Toll-Like Receptor 4, Fig. 5 Signaling of TLR4. TLR4 uses four adaptor proteins such as MyD88, TRIF, TRAM, and MAL. Based on the usage of MyD88, TLR4 signaling is classified as MyD88-dependent or independent pathway. Both the pathways use TRAF6. MyD88-dependent pathway activates MAP kinases and IKK leading to NF-κB and AP1 nuclear translocation, thus causing proinflammatory cytokine production. PKCε phosphorylates TRAM, which in turn activates TRIF. TRIF activates both TRAF6 and TRAF3. TRAF6 leads to MAPK and IKK activation through RIP1. TRAF3 leads to IRF3/7 nuclear translocation through TBK1 and IKKε. The translocated IRF complex causes the expression of type I IFNs

critical residues in MyD88 DD as being important for its interaction with IRAK family members, as the substitution of these residues impaired propagation of downstream signaling responses (Loiarro et al. 2009). The activated IRAKs dissociate from MyD88 and interact with TRAF6, an E3 ubiquitin protein ligase. TRAF6 recruits another E2 ubiquitin-conjugating enzyme complex composed of Ubc13 and Uev1A, which catalyze the formation of conjugated lysine 63-linked polyubiquitin chain on TRAF6 itself as well as the formation of unconjugated free polyubiquitin chain (Xia et al. 2009). This macromolecular complex ultimately impinges on TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), and TAB2/3. TAB3 is activated by free polyubiquitin chain and phosphorylates IKK complex composed of IKK-α, IKK-β, and NF-κB essential modulator (NEMO). These activated complexes phosphorylate IκB-α, an NF-κB inhibitory protein, followed by freeing NF-κB in the cytoplasm. This freed NF-κB translocates to the nucleus, where it binds with κB sequences to activate various cytokines, chemokines, and costimulatory molecules. Other notable kinases activated by this signaling mechanism and p38 and JNK, which belong to the MAP kinase family and lead to activation of the AP-1 family of transcription factors that further add to the expression of cytokine genes (Kawai and Akira 2006). The ultimate goal of this pathway is to activate the underlying signaling mechanisms/cascades that use NF-κB as a central

vigilance controller and concomitant activation of AP-1 to turn the pleiotropic genes involved in many physiological (cell process and cell proliferation) and inflammatory response on and off.

In the MyD88-independent pathway, TLR4 makes use of TRIF. The presence of well conserved TIR domain and several TRAF6-binding regions makes TRIF the unique adaptor for signal transduction (Yamamoto et al. 2003) as it activates both IRF3/7 and NF-κB (Fitzgerald et al. 2003). However, because TLR4-TRIF is unable to act alone, this recruits TRAM, and it is myristoylated at N-terminus to be associated with plasma membrane and requires PKCε phosphorylation for its activation (Rowe et al. 2006; McGettrick et al. 2006). TRIF-dependent pathway activates TRAF3, and it is important to activate two IKK related kinases, IKKε and TBK1. Various proteins interact with IKKε and TBK1, such as TBK-binding protein 1 (TBKBP1) and NAK-associated protein 1 (NAP1). This complex phosphorylates IRF3/7 at its C-terminus initiating type I IFN production. In addition, TLR4 has recently been shown to recognize not only PAMPS, but also the viral molecules that bind indirectly with viral activated DAMPs following infection with H5N1 avian influenza virus, thus modulating its effects (Imai et al. 2008).

Summary

Toll was first identified in *Drosophila melanogaster* as a gene that controls dorsoventral polarity. Further, continuous investigations by various research groups to identify the receptor responsible for binding of LPS in mammalian cells revealed that a gene very similar to the IL1 receptor extracellular domain had a sequence similar to that of *Drosophila melanogaster* Toll-1, which was named TLR. LPS is captured by LPS-binding protein and presented to the MD2-CD14 and TLR4 complex. Since MD2 lacks an intracellular signaling domain, it presents LPS to TLR4 for further action. Of the six acyl chains of LPS, five chains bind in the deep pocket of MD2 through hydrophobic interactions, while the remaining chain is exposed to the surface to bind with TLR4. The two phosphate groups of LPS aid in the formation of receptor dimer. The signaling responses of TLR4 to LPS are divided into MyD88-dependent and independent events, in which the former uses MyD88 as adaptor and occurs in the cytoplasm and the latter uses TRIF as the major adaptor for signaling mechanisms and occurs in the early endosomes. The gene expression mediated by TLR4 connects innate and adaptive immune responses, which form the first line of defense against microbes. The binding of LPS to TLR4 is determined by the crystal structure, but little research has been conducted to investigate the binding mechanism of various other ligands, such as taxol and heat shock proteins. Dimerization and underlying signaling mechanisms have been elucidated by the identification of crystal structures of TLR1-TLR2, TLR3, and TLR4. Such identification is indispensable for all other TLR families; hence, additional research should be conducted in that area. Overall, TLR4 is a key elicitor of immune responses that is also involved in various types of physiological and pathological events. Post-translational modifications, ubiquitination mechanisms such as mono and linear ubiquitinations, and phosphorylation events make it more arduous to delineate the signaling networks; however, elucidation of the TLR4 structure, extracellular and intracellular binding mechanisms, and signaling events indicate that it is a promising candidate for therapeutics related to various immune-related disorders.

Acknowledgments This work was supported by the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (2012016803).

References

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998;9:143–50.

Anderson KV, Jurgens G, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. Cell. 1985;42:779–89.

Baldridge JR, McGowan P, Evans JT, Cluff C, Mossman S, et al. Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Expert Opin Biol Ther. 2004;4:1129–38.

Beutler B. Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. Immunol Rev. 2009;227:248–63.

Czeslick E, Struppert A, Simm A, Sablotzki A. E5564 (Eritoran) inhibits lipopolysaccharide-induced cytokine production in human blood monocytes. Inflamm Res. 2006;55:511–5.

Douville RN, Lissitsyn Y, Hirschfeld AF, Becker AB, Kozyrskyj AL, et al. TLR4 Asp299Gly and Thr399Ile polymorphisms: no impact on human immune responsiveness to

LPS or respiratory syncytial virus. PLoS One. 2010;5: e12087.
Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med. 2003;198:1043–55.
Gay NJ, Gangloff M. Structure and function of Toll receptors and their ligands. Annu Rev Biochem. 2007;76:141–65.
Gay NJ, Keith FJ. Drosophila Toll and IL-1 receptor. Nature. 1991;351:355–6.
Halfon MS, Hashimoto C, Keshishian H. The Drosophila toll gene functions zygotically and is necessary for proper motoneuron and muscle development. Dev Biol. 1995;169:151–67.
Hansson GK, Edfeldt K. Toll to be paid at the gateway to the vessel wall. Arterioscler Thromb Vasc Biol. 2005;25:1085–7.
Hashiguchi S, Yamaguchi Y, Takeuchi O, Akira S, Sugimura K. Immunological basis of M13 phage vaccine: Regulation under MyD88 and TLR9 signaling. Biochem Biophys Res Commun. 2010;402:19–22.
Hosoi T, Yokoyama S, Matsuo S, Akira S, Ozawa K. Myeloid differentiation factor 88 (MyD88)-deficiency increases risk of diabetes in mice. PLoS One. 2010;5:e12537.
Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, et al. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. Cell. 2008;133:235–49.
Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell. 2007;130:1071–82.
Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity. 1999;11:115–22.
Kawai T, Akira S. TLR signaling. Cell Death Differ. 2006;13:816–25.
Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol. 2009;21:317–37.
Kim HM, Park BS, Kim JI, Kim SE, Lee J, et al. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell. 2007;130:906–17.
Kundi M. New hepatitis B vaccine formulated with an improved adjuvant system. Expert Rev Vaccines. 2007;6:133–40.
Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996;86:973–83.
Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, et al. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science. 2008;320:379–81.
Loiarro M, Gallo G, Fanto N, De Santis R, Carminati P, et al. Identification of critical residues of the MyD88 death domain involved in the recruitment of downstream kinases. J Biol Chem. 2009;284:28093–103.
McGettrick AF, Brint EK, Palsson-McDermott EM, Rowe DC, Golenbock DT, et al. Trif-related adapter molecule is phosphorylated by PKC{epsilon} during Toll-like receptor 4 signaling. Proc Natl Acad Sci USA. 2006;103:9196–201.
Medzhitov R, Preston-Hurlburt P, Janeway Jr CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388:394–7.
Mullarkey M, Rose JR, Bristol J, Kawata T, Kimura A, et al. Inhibition of endotoxin response by e5564, a novel Toll-like receptor 4-directed endotoxin antagonist. J Pharmacol Exp Ther. 2003;304:1093–102.
Nishitani C, Mitsuzawa H, Hyakushima N, Sano H, Matsushima N, et al. The Toll-like receptor 4 region Glu24-Pro34 is critical for interaction with MD-2. Biochem Biophys Res Commun. 2005;328:586–90.
Ohnishi H, Tochio H, Kato Z, Orii KE, Li A, et al. Structural basis for the multiple interactions of the MyD88 TIR domain in TLR4 signaling. Proc Natl Acad Sci USA. 2009;106:10260–5.
O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol. 2007;7:353–64.
Papadopoulos AI, Ferwerda B, Antoniadou A, Sakka V, Galani L, et al. Association of toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms with increased infection risk in patients with advanced HIV-1 infection. Clin Infect Dis. 2010;51:242–7.
Park BS, Song DH, Kim HM, Choi BS, Lee H, et al. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature. 2009;458:1191–5.
Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282:2085–8.
Przetak M, Chow J, Cheng H, Rose J, Hawkins LD, et al. Novel synthetic LPS receptor agonists boost systemic and mucosal antibody responses in mice. Vaccine. 2003;21:961–70.
Rose D, Zhu X, Kose H, Hoang B, Cho J, et al. Toll, a muscle cell surface molecule, locally inhibits synaptic initiation of the RP3 motoneuron growth cone in Drosophila. Development. 1997;124:1561–71.
Rossignol DP, Wong N, Noveck R, Lynn M. Continuous pharmacodynamic activity of eritoran tetrasodium, a TLR4 antagonist, during intermittent intravenous infusion into normal volunteers. Innate Immun. 2008;14:383–94.
Rowe DC, McGettrick AF, Latz E, Monks BG, Gay NJ, et al. The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc Natl Acad Sci USA. 2006;103:6299–304.
Schaefer L. Extracellular matrix molecules: endogenous danger signals as new drug targets in kidney diseases. Curr Opin Pharmacol. 2010;10:185–90.
Schmitt C, Humeny A, Becker CM, Brune K, Pahl A. Polymorphisms of TLR4: rapid genotyping and reduced response to lipopolysaccharide of TLR4 mutant alleles. Clin Chem. 2002;48:1661–7.
Schnare M, Holt AC, Takeda K, Akira S, Medzhitov R. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. Curr Biol. 2000;10:1139–42.
Schroder NW, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis. 2005;5:156–64.
Seo SU, Kwon HJ, Song JH, Byun YH, Seong BL, et al. MyD88 signaling is indispensable for primary influenza A virus infection but dispensable for secondary infection. J Virol. 2010;84:12713–22.
Thompson BS, Chilton PM, Ward JR, Evans JT, Mitchell TC. The low-toxicity versions of LPS, MPL adjuvant and RC529, are efficient adjuvants for CD4+ T cells. J Leukoc Biol. 2005;78:1273–80.

Xia ZP, Sun L, Chen X, Pineda G, Jiang X, et al. Direct activation of protein kinases by unanchored polyubiquitin chains. Nature. 2009;461:114–9.
Xu Y, Tao X, Shen B, Horng T, Medzhitov R, et al. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature. 2000;408:111–5.
Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. 2003;301:640–3.
Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, et al. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc Natl Acad Sci USA. 2010;107:11942–7.

TLR4AP

▶ Toll-like Receptor Adaptor Protein Family Members

TLR5

Shaikh M. Atif and Stephen J. McSorley
Center for Comparative Medicine, University of California Davis, Davis, CA, USA

Synonyms

Myd88: Myeloid differentiation primary response gene (88); Toll-like receptor 5; TRIF: TIR domain–containing adaptor-inducing interferon β

Introduction

An infected host can quickly recognize an invading pathogen via germ-line-encoded pattern recognition receptors (PRRs) that detect microbe-associated molecular patterns (MAMPs) commonly expressed by many pathogens. Recognition of MAMPs by their corresponding receptor allows the host to initiate a rapid inflammatory response that can limit initial pathogen replication (Janeway and Medzhitov 2002). At least four different groups of PRRs have been described with varying cellular location and ligand specificity. Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are membrane-associated, while RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) are found in the cytosol. Among the 13 known TLRs, only TLR5 and TLR11 exclusively recognize protein microbial ligands (Kawai and Akira 2010). TLR5 detects flagellin, a protein MAMP that is highly conserved and expressed by both gram-negative and gram-positive bacteria (Hayashi et al. 2001).

Monomeric flagellin is the major subunit of bacterial flagella, which is a whiplike structure that helps bacteria to resist intestinal peristalsis and establish productive infection. Polymerized flagellin forms the majority of the flagellar structure and is therefore one of the most abundant proteins expressed by flagellated bacteria. Furthermore, *Salmonella sp.* is known to secrete monomeric flagellin in response to lyso phosphatidylcholine, a product of intestinal epithelial cells (Subramanian and Qadri 2006). Thus, flagellin represents a major bacterial product, especially within the intestine. After recognition of flagellin, TLR5 forms a homodimer and induces proinflammatory signaling through the recruitment of adaptor molecules. Induction of proinflammatory genes by TLR5 detection of flagellin leads to recruitment of immune cells to the site of infection, thereby allowing the host to contain initial infection.

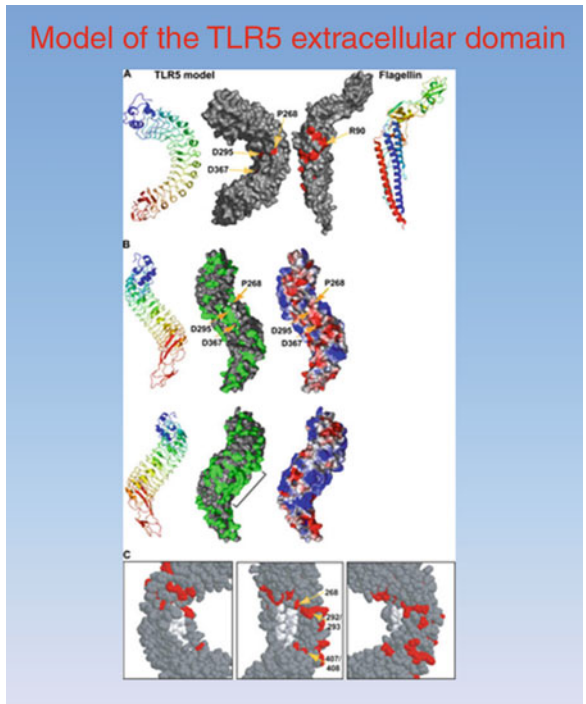
Background

In 1997, Rock et al. cloned and characterized human TLR5 and mapped this gene to chromosome 1. The corresponding TLR5 gene in mouse was reported 2 years later by Sebastiani et al. (Rock et al. 1998; Sebastiani et al. 2000). Murine TLR5 gene bears 81% homology to human TLR5 gene and around 40% homology to other TLR gene sequences (Sebastiani et al. 2000). In 2001, Hayashi et al. reported that bacterial flagellin is a ligand for TLR5 and requires ▶ Myd88 to produce inflammatory cytokines in response to flagellin stimulation (Hayashi et al. 2001). Further studies on TLR5 demonstrated that it is able to modulate adaptive immune response by inducing the expression of co-stimulatory molecules on antigen-presenting cells and secretion of immunomodulatory cytokines such as IL-12 (Iwasaki and Medzhitov 2004). Because of these stimulatory properties, TLR5 has been shown to function as an effective adjuvant (Mcsorley et al. 2002).

Structure of TLR5 and Flagellin

TLR5 is a type I transmembrane glycoprotein composed of 858 amino acids (aa) in humans and 859 aa in mice with a large extracellular domain, a transmembrane domain, and intracellular domain (Andersen-Nissen et al. 2007). The extracellular domain contains leucine-rich repeats (LRRs) at the N-terminal region and forms a horseshoe-shaped 3D solenoid structure (Werling et al. 2009). This region consists of a conserved sequence, LxxLxxLxxNxxL (where L is a leucine and x is any amino acid). The crystal structure of TLR5 has not yet been solved, but mathematical modeling by the Aderem laboratory (Fig. 1) has provided critical information about the structure of the extracellular domain (Andersen-Nissen et al. 2007). The binding site for flagellin was mapped to the highly conserved lateral concave region that forms the central core (174–401) of the solenoidal structure encoded by multiple LRRs within the extracellular domain of TLR5 (Andersen-Nissen et al. 2007). This concave region consists of β-sheets which provide stability to the solenoid and also allows a large surface area for flagellin binding. Three residues in the concave region of TLR5 molecule, P268, D295, and D367, take part in the direct interaction with flagellin molecules (Andersen-Nissen et al. 2007). Alterations in the amino acid (aa) composition of TLR5 within this region between different hosts result in differential ability of the receptor to recognize flagellin (Fig. 1) (Andersen-Nissen et al. 2007). It is not yet clear whether flagellin monomers bind to a single TLR5 receptor, which then dimerizes with another flagellin-bound TLR5 molecule like other cell surface receptors, or if flagellin binds to already dimerized TLR5 molecule leading to conformational change in the receptor to initiate the signaling program (Andersen-Nissen et al. 2007).

The N- and C-terminal regions of flagellin together form a hydrophobic core that brings the ends of the molecule together and allows binding to the extracellular domain of TLR5 (Smith et al. 2003). These amino acids are located in the highly conserved D1 domain of the flagellin protein and cluster on the convex surface that contacts adjacent flagellin monomers in the flagellar protofilament (Smith et al. 2003). Thus, mutation of individual residues within this TLR5 recognition site significantly reduced or completely abolished bacterial motility (Andersen-Nissen et al. 2007), suggesting that evolving functional flagellin that can evade TLR5 detection requires a complex series of mutations. The TLR5 recognition site on flagellin is therefore conserved among a wide variety of flagellated bacteria,



TLR5, Fig. 1 Model of the TLR5 extracellular domain. (**a**) Ribbon representations (*far left* and *far right*) and molecular surface representations (*middle left* and *middle right*) of the best model of the TLR5 ectodomain (*left*) and the structure of flagellin (PDB lucu; *right*). The ribbon is colored sequentially from the N-terminus (*blue*) to the C-terminus (*red*). Amino acids important for flagellin recognition are shown on the TLR5 model in red. Amino acids on flagellin previously determined to be important for TLR5 recognition are shown in red on the flagellin structure. (**b**) Ribbon representations (*left*) and molecular surface representations (*middle* and *right*) of the best model of the TLR5 ectodomain, oriented ~90° to the view in (**a**). Views 180° apart are shown in the top and bottom rows. The ribbon is colored sequentially from the N-terminus (*blue*) to the C-terminus. Molecular surface representations are colored by conservation (*center*: residues ≥90% similar among TLR5 sequences are *green*) or by electrostatic potential (*right*: positive, *blue*; negative, *red*). The conserved concavity and lateral patch regions are indicated with a dotted white oval and bracket, respectively. Positions of residues important for flagellin recognition are indicated with *arrows*. (**c**) Space-filling representation of the TLR5 model. Residues of the conserved concavity (*white*) and residues that differ between human and mouse TLR5 in the central 228-amino-acid region, as well as 407 and 408 (*red*), are highlighted. Amino acids surrounding the concavity that were mutated are indicated with *arrows*

although there are select bacterial species that possess unique flagellin molecules able to evade TLR5 recognition (Andersen-Nissen et al. 2007).

Distribution of TLR5 in Mammals

TLR5 has been reported to be expressed exclusively on the surface of the human epithelial cells present in the airway, spleen, lung, liver, intestine, kidney, and urogenital tract (Nishimura and Naito 2005; Ochiel et al. 2010). In the airway epithelium, Zhang et al. demonstrated the importance of TLR5 during *Pseudomonas aeruginosa* infection by showing that an aflagellate mutant of Pseudomonas was poorly controlled in host lungs due to a deficient inflammatory response (Zhang et al. 2005). A similar protective role for TLR5 has also been reported for pneumococcal lung infection (Munoz et al. 2010).

In the murine intestine, TLR5 expression has been reported to be confined to the basolateral surface within the ileum and colon and on apical surface of the follicle-associated epithelium (FAE) (Bogunovic et al. 2007; Gewirtz et al. 2001). TLR5 expression in the intestine is thought to be important for the detection of intestinal pathogens, but may also be important in maintaining intestinal homeostasis via recognition of intestinal flora. Indeed, an imbalance in the detection of intestinal microbiota via TLR5 has been reported to cause intestinal inflammation (Vijay-Kumar et al. 2008).

Although flagellin recognition by TLR5 can activate splenic dendritic cells (Mcsorley et al. 2002), the expression of TLR5 by conventional dendritic cells is somewhat controversial (Salazar-Gonzalez and McSorley 2005). While human dendritic cells can be activated by flagellin in vitro (Means et al. 2003), there are conflicting accounts of murine dendritic cell activation via TLR5 (Means et al. 2003; Salazar-Gonzalez et al. 2007; Didierlaurent et al. 2004). A report by Uematsu et al. has suggested that murine TLR5 expression may be limited to dendritic cells from the intestinal lamina propria (Uematsu and Akira 2009). However, TLR5-specific effects have been detected using purified murine splenic dendritic cells in vitro (Letran et al. 2011a), although it should be noted that this primarily involved an effect of TLR5 on antigen presentation to CD4 T cells, rather than innate immune activation.

TLR5 Signaling

Recognition of flagellin by TLR5 activates a signaling cascade that leads to the activation of nuclear factor kappa B (NF-kB) (Kawai and Akira 2010). As noted above, the binding site for flagellin is found on the LRR within the extracellular domain of TLR5. It is thought that this binding induces conformational change in the intracellular domain that allows homodimerization and initiation of downstream signaling (Hayashi et al. 2001; Mizel et al. 2003). TLR5 is also able to form a heterodimer with TLR4, and this heterodimerization induced nitric oxide production in COS-1 cells (Mizel et al. 2003). Activation of both homo- and heterodimers mobilizes NF-kB and induces tumor necrosis factor alpha production (Moors et al. 2001). Upon ligand binding, TLR5 uses myeloid differentiation factor 88 (▶ Myd88) and IL-1 receptor-associated kinase 4 (IRAK4) to activate a series of molecules that includes the key intermediate TNF receptor–associated factor 6 (TRAF6) (Kawai and Akira 2010). TLR5 signaling has mostly been thought to be ▶ Myd88-dependent (Kawai and Akira 2010), but recent evidence is emerging that it can also function in a Myd88-independent manner (Letran et al. 2011a, b; Kawai and Akira 2011).

Myd88-Dependent Pathway

After TLR5 dimerization, ▶ Myd88 is recruited to the C-terminus of the conserved cytoplasmic TIR domain. ▶ Myd88 has a bipartite structure composed of an amino-terminal death domain and a carboxyl-terminal TIR domain with a short intervening linker segment (McGettrick and O'Neill 2004). It is able to recruit IRAK 4 through the interaction of death domains in the N-terminus of ▶ Myd88 and IRAK4 to form a DD complex (Lin et al. 2010). This ▶ Myd88 and IRAK4 complex further recruits IRAK4 downstream partners IRAK2 or IRAK1 and forms a complex termed "the myddosome" (Lin et al. 2010).

The IRAK complex allows phosphorylation of Ser/Thr molecules within the C-terminus of IRAK molecules leading to activation (Lin et al. 2010). After activation, IRAK1 and IRAK2 can dissociate from the complex and interact with TRAF6, an E3 ubiquitin ligase which polyubiquitinates and activates the transforming growth factor β-activated kinase

TAK1/TAB1/TAB2/TAB3 complex (Kawai and Akira 2010). This activated TAK1 complex causes phosphorylation and activation of the IKK complex consisting of IKK-α IKK-β, and IKK-γ/NEMO (Kawai and Akira 2010). The IkB complex is strongly associated with NF-kB in the cytosol and upon phosphorylation undergoes degradation by the proteasome pathway, allowing free NF-kB to translocate to the nucleus and induce transcription of proinflammatory genes (Kawai and Akira 2010). TAK1 also simultaneously activates the MAP kinase pathway, by directly interacting with MEKK, p38, and JNK, which results in phosphorylation and activation of NF-kB and AP-1 transcription factors (Kawai and Akira 2011). NF-kB and AP-1 control inflammatory responses by inducing proinflammatory cytokines and increase expression of co-stimulatory molecules on antigen-presenting cells (Iwasaki and Medzhitov 2004). A schematic pathway of the factors involved in TLR5 signaling is presented in Fig. 2.

Myd88-Independent Functions of TLR5

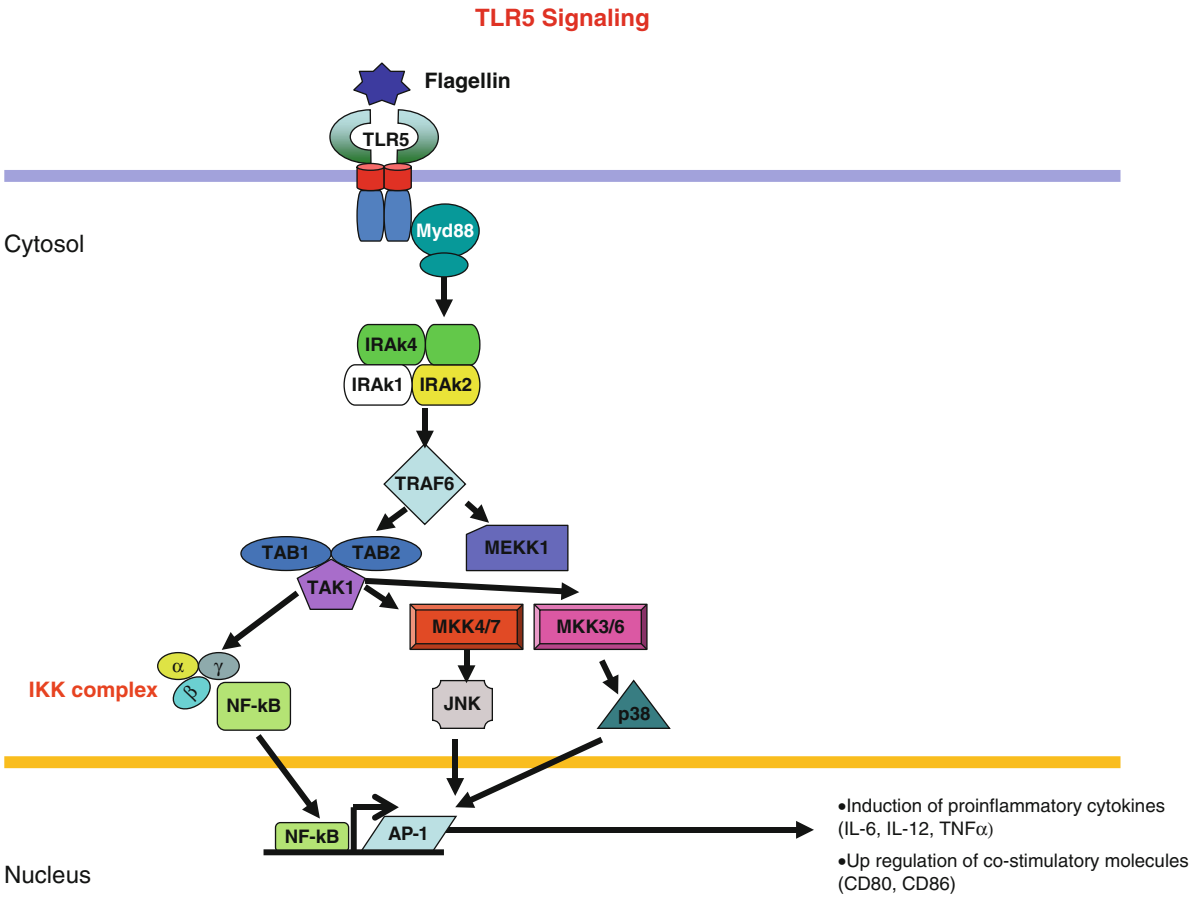
Other TLRs such as TLR3 and TLR4 can signal independently of ▶ Myd88 using the adaptor molecule TRIF, which can also efficiently activate NF-kB (Kawai and Akira 2011). Although TLR5 does not signal through TRIF (Choi et al. 2010), TLR5 signaling can be inhibited by TRIF via a proteolytic effect that requires the extracellular domain of TLR5 and the C-terminus region of TRIF (Choi et al. 2010). A direct interaction of TRIF with the extracellular domain of TLR5 is not thought to occur, but rather requires the activity of caspases (Choi et al. 2010). Activation of caspases on the proteolytic breakdown of TLR9 has also been described during maturation (Choi et al. 2010). Further insight into the mechanism of action of caspases on TLR5 induced by TRIF should increase understanding of the cross talk between TRIF and TLR5 and may also help illuminate that nature of Myd88-independent signaling.

Recent data also suggest that TLR5 can play a major role in promoting the antigen presentation of flagellin epitopes to CD4 T cells (Letran et al. 2011a, b; Kawai and Akira 2011). Intriguingly, this effect seems to be independent of Myd88 and TRIF (Letran et al. 2011a). A Myd88-independent function of TLR5 was demonstrated to promote humoral and adaptive immune responses to flagellin immunization and suggest that TLR5 might function as an endocytic receptor in similar fashion to C-type lectin receptors (Letran et al. 2011a). It is not yet clear what molecules are involved downstream to TLR5 to promote this biological effect. It seems possible that signaling molecules present in other endocytic receptor pathways could be involved, or that molecules promoting autophagy could direct endocytosed flagellin to the lysosome complex (Fig. 3). Further understanding of Myd88-independent functions of TLR5 is required and may be beneficial in understanding the targeting of flagellin during inflammatory bowel disease (Lodes et al. 2004).

Pathologies Associated with TLR5 Signaling

The intestine harbors a large and diverse microbiological community, and the host immune system has developed mechanisms to prevent inappropriate responses to these organisms (Santaolalla et al. 2011). Given that the intestine harbors flagellated bacteria, TLR5-expressing intestinal epithelial cells, and dendritic cells, the role of TLR5 signaling in intestinal immunity is likely to be of some importance. As noted above, there is evidence that TLR5 is tightly associated with the basolateral surface of epithelial cells, suggesting that recognition of flagellin might only occur after penetration of the epithelial layer by a pathogen (Gewirtz et al. 2001). However, this would also mean that any perturbation in the epithelial layer could give rise to a massive inflammatory response against commensal organisms (Vijay-Kumar et al. 2008). It is possible that this process plays a role in the initiation or progression of inflammatory bowel disease.

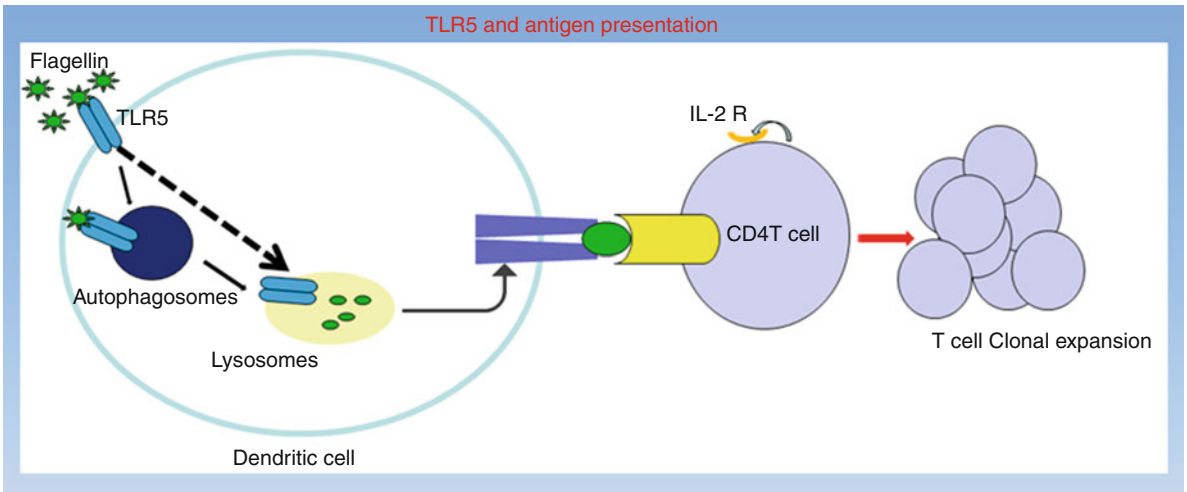
In contrast, several studies have described an important role for TLRs in promoting intestinal homeostasis and wound healing (Rakoff-Nahoum et al. 2004). Indeed, TLR signaling has been shown to promote epithelial cell proliferation, IgA production, maintenance of tight junctions, and antimicrobial peptide expression, thus maintaining a healthy epithelial barrier (Santaolalla et al. 2011). Indeed, studies by the Gewirtz laboratory point to an important role for TLR5 signaling in maintaining intestinal homeostasis (Vijay-Kumar et al. 2008). TLR5-deficient mice displayed no resistance to the development of intestinal inflammation, and conversely, a proportion of these



TLR5, Fig. 2 TLR5 signaling pathway. Flagellin recognition by the extracellular domain of TLR5 leads to the recruitment of adaptor molecule Myd88 to the TIR domain to initiate the signaling cascade. Myd88 forms a complex with IRAK4 and IRAK1/2 called as "myddosome" that allows the phosphorylation of the IRAK molecules. IRAK1/2 upon phosphorylation interacts with E3 ubiquitin ligase and TRAF6 which then polyubiquitinates TAK1 and activates MEK kinases to activate downstream molecules MAPK, p38, and JNK. TAK1 subsequently phosphorylates IKK complex, resulting in the release of NF-kB. TRAF6 signaling leads to the activation of NF-kB and AP-1 transcription factor which then induces the transcription of proinflammatory genes and upregulates co-stimulatory molecules

mice develop spontaneous colitis manifested by rectal prolapse, splenomegaly, anemia, and enlarged mesenteric and sublingual lymph nodes (Vijay-Kumar et al. 2010). However, this phenotype is not consistently observed, and the same line housed in other animal facilities did not develop intestinal inflammation (Letran et al. 2011b). It therefore seems likely that specific bacterial flora underlie the reported susceptibility of TLR5-deficient mice to the development of colitis.

There are natural polymorphisms in human TLR5 that allow analysis of this receptor in human disease (Hawn et al. 2003). Four single-nucleotide polymorphisms (SNPs) have been identified (Hawn et al. 2003). The TLR5-stop SNP (cytosine$_{1174}$ – thymidine transition converting Arg$_{392}$ to a stop codon) causes loss of the signaling TIR domain, thus abolishing TLR5 activity (Hawn et al. 2003). Approximately 10% of the world's population across a variety of ethnicities carries the TLR5-stop as heterozygotes and have a 75% reduction in TLR5 function (Hawn et al. 2005). Although TLR5-stop homozygotes have not been reported to be associated with health problems, heterozygous carriage of TLR5-stop increases the likelihood of developing clinical disease upon exposure to *Legionella* (Hawn et al. 2003), but not *Salmonella typhi* (Dunstan et al. 2005), perhaps suggesting that TLR5 is more important in protection



TLR5, Fig. 3 TLR5-mediated antigen presentation. Recognition of flagellin by TLR5 on dendritic cell promotes its uptake and presentation independent of ▶ Myd88

against pathogens that colonize mucosal surfaces than against those that breech mucosal surfaces to cause systemic disease. The TLR5-stop polymorphism, but not other TLR5 alleles, is associated with protection from developing SLE, and the protection was most pronounced in individuals who are seronegative for anti-dsDNA autoantibodies (Hawn et al. 2005). Further analysis of this cohort of patients should allow greater understanding of the role of TLR5 in humans.

Sepsis is a systemic inflammatory disorder caused by excessive inflammation induced by the activation of innate immune receptors. It is one of the leading causes of mortality in surgical intensive care units. Hemodynamic studies have shown that septic shock induced by TLR5 signaling results in hypotension-associated cardiac failure and multiple organ dysfunctions (Liaudet et al. 2002). Thus, targeting flagellin/TLR5 axis could help in preventing the debilitating effects of septic shock.

Conclusion

TLR5 is an important receptor that can direct innate and adaptive immune responses to flagellated bacterial pathogens. The role of TLR5 in the development of inflammatory disorders of the intestine requires further investigation, but available data suggest that this receptor may be important for maintaining gut homeostasis. Although the downstream signaling pathways through ▶ Myd88 are well described, the role of TLR5 in antigen presentation is Myd88-independent and requires greater analysis to uncover whether this represents a novel signaling pathway.

References

Andersen-Nissen E, Smith KD, Bonneau R, Strong RK, Aderem A. A conserved surface on toll-like receptor 5 recognizes bacterial flagellin. J Exp Med. 2007;204(2):393–403. Epub 2007/02/07.

Bogunovic M, Dave SH, Tilstra JS, Chang DT, Harpaz N, Xiong H, et al. Enteroendocrine cells express functional toll-like receptors. Am J Physiol Gastrointest Liver Physiol. 2007;292(6):G1770–83. Epub 2007/03/31.

Choi YJ, Im E, Pothoulakis C, Rhee SH. TRIF modulates TLR5-dependent responses by inducing proteolytic degradation of TLR5. J Biol Chem. 2010;285(28):21382–90. Epub 2010/05/11.

Didierlaurent A, Ferrero I, Otten LA, Dubois B, Reinhardt M, Carlsen H, et al. Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. J Immunol. 2004;172(11):6922–30. Epub 2004/05/22.

Dunstan SJ, Hawn TR, Hue NT, Parry CP, Ho VA, Vinh H, et al. Host susceptibility and clinical outcomes in toll-like receptor 5-deficient patients with typhoid fever in Vietnam. J Infect Dis. 2005;191(7):1068–71. Epub 2005/03/05.

Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol. 2001;167(4):1882–5. Epub 2001/08/08.

Hawn TR, Verbon A, Lettinga KD, Zhao LP, Li SS, Laws RJ, et al. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with

susceptibility to legionnaires' disease. J Exp Med. 2003;198(10):1563–72. Epub 2003/11/19.

Hawn TR, Wu H, Grossman JM, Hahn BH, Tsao BP, Aderem A. A stop codon polymorphism of toll-like receptor 5 is associated with resistance to systemic lupus erythematosus. Proc Natl Acad Sci USA. 2005;102(30):10593–7. Epub 2005/07/20.

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by toll-like receptor 5. Nature. 2001;410(6832): 1099–103. Epub 2001/04/27.

Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004;5(10):987–95. Epub 2004/09/30.

Janeway Jr CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197–216. Epub 2002/02/28.

Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat Immunol. 2010;11(5):373–84. Epub 2010/04/21.

Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637–50. Epub 2011/05/28.

Letran SE, Lee SJ, Atif SM, Uematsu S, Akira S, McSorley SJ. TLR5 functions as an endocytic receptor to enhance flagellin-specific adaptive immunity. Eur J Immunol. 2011a;41(1):29–38. Epub 2010/12/25.

Letran SE, Lee SJ, Atif SM, Flores-Langarica A, Uematsu S, Akira S, et al. TLR5-deficient mice lack basal inflammatory and metabolic defects but exhibit impaired CD4 T cell responses to a flagellated pathogen. J Immunol. 2011b; 186(9):5406–12. Epub 2011/04/01.

Liaudet L, Deb A, Pacher P, Mabley JG, Murthy KG, Salzman AL, et al. The Flagellin-TLR5 axis: therapeutic opportunities. Drug News Perspect. 2002;15(7):397–409. Epub 2003/04/05.

Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signaling. Nature. 2010; 465(7300):885–90. Epub 2010/05/21.

Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, et al. Bacterial flagellin is a dominant antigen in Crohn disease. J Clin Invest. 2004;113(9):1296–306. Epub 2004/05/05.

McGettrick AF, O'Neill LA. The expanding family of MyD88-like adaptors in toll-like receptor signal transduction. Mol Immunol. 2004;41(6–7):577–82. Epub 2004/06/29.

McSorley SJ, Ehst BD, Yu Y, Gewirtz AT. Bacterial flagellin is an effective adjuvant for CD4+ T cells in vivo. J Immunol. 2002;169(7):3914–9. Epub 2002/09/24.

Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. The toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol. 2003;170(10):5165–75. Epub 2003/05/08.

Mizel SB, Honko AN, Moors MA, Smith PS, West AP. Induction of macrophage nitric oxide production by Gram-negative flagellin involves signaling via heteromeric toll-like receptor 5/toll-like receptor 4 complexes. J Immunol. 2003;170(12):6217–23. Epub 2003/06/10.

Moors MA, Li L, Mizel SB. Activation of interleukin-1 receptor-associated kinase by gram-negative flagellin. Infect Immun. 2001;69(7):4424–9. Epub 2001/06/13.

Munoz N, Van Maele L, Marques JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal administration of flagellin protects mice from Streptococcus pneumoniae lung infection. Infect Immun. 2010;78(10):4226–33. Epub 2010/07/21.

Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. Biol Pharm Bull. 2005;28(5):886–92. Epub 2005/05/03.

Ochiel DO, Ghosh M, Fahey JV, Guyre PM, Wira CR. Human uterine epithelial cell secretions regulate dendritic cell differentiation and responses to TLR ligands. J Leukoc Biol. 2010;88(3):435–44. Epub 2010/04/14.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004;118(2):229–41. Epub 2004/07/21.

Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA. 1998;95(2):588–93. Epub 1998/01/22.

Salazar-Gonzalez RM, McSorley SJ. Salmonella flagellin, a microbial target of the innate and adaptive immune system. Immunol Lett. 2005;101(2):117–22. Epub 2005/06/25.

Salazar-Gonzalez RM, Srinivasan A, Griffin A, Muralimohan G, Ertelt JM, Ravindran R, et al. Salmonella flagellin induces bystander activation of splenic dendritic cells and hinders bacterial replication in vivo. J Immunol. 2007;179(9): 6169–75. Epub 2007/10/20.

Santaolalla R, Fukata M, Abreu MT. Innate immunity in the small intestine. Curr Opin Gastroenterol. 2011;27(2): 125–31. Epub 2011/01/21.

Sebastiani G, Leveque G, Lariviere L, Laroche L, Skamene E, Gros P, et al. Cloning and characterization of the murine toll-like receptor 5 (Tlr5) gene: sequence and mRNA expression studies in Salmonella-susceptible MOLF/Ei mice. Genomics. 2000;64(3):230–40. Epub 2000/04/11.

Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol. 2003;4(12):1247–53. Epub 2003/11/20.

Subramanian N, Qadri A. Lysophospholipid sensing triggers secretion of flagellin from pathogenic salmonella. Nat Immunol. 2006;7(6):583–9. Epub 2006/05/02.

Uematsu S, Akira S. Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection. J Gastroenterol. 2009;44(8):803–11. Epub 2009/06/24.

Vijay-Kumar M, Aitken JD, Gewirtz AT. Toll like receptor-5: protecting the gut from enteric microbes. Semin Immunopathol. 2008;30(1):11–21. Epub 2007/12/11.

Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking toll-like receptor 5. Science. 2010;328(5975):228–31. Epub 2010/03/06.

Werling D, Jann OC, Offord V, Glass EJ, Coffey TJ. Variation matters: TLR structure and species-specific pathogen recognition. Trends Immunol. 2009;30(3):124–30. Epub 2009/02/13.

Zhang Z, Louboutin JP, Weiner DJ, Goldberg JB, Wilson JM. Human airway epithelial cells sense *Pseudomonas aeruginosa* infection via recognition of flagellin by toll-like receptor 5. Infect Immun. 2005;73(11):7151–60. Epub 2005/10/22.

TLR9

▶ Toll-Like Receptor 9

TM14

▶ Fibulins

TMEM85 (Transmembrane Protein 85)

Michael T. Greenwood
Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Kingston, ON, Canada

Synonyms

Cell proliferation-inducing gene 17; Proliferation-inducing gene 17

Historical Background

Prior to 2008, there were no publications on the TMEM85 gene or protein. The GenBank and Entrez Gene databases described TMEM85 as an evolutionary conserved transmembrane domain containing protein (DUF1077 superfamily motif) found in all eukaryotes examined (TMEM85-gene). In the yeast *Saccharomyces cerevisiae*, the TMEM85 orthologue is called *YGL231c*. In 2003, it was reported that yeast cells lacking *YGL231c* showed an increased sensitivity to the toxic effects of ectopically expressed Parkinson's disease associated gene α-synuclein (Willingham et al. 2003). Finally, the analysis of a GFP tagged genomic copy of *YGL231c* suggests that the protein is a resident of the endoplasmic reticulum (ER) (YGL231c-gene).

TMEM85, an Anti-apoptotic Gene

By screening a human heart cDNA expression library in yeast cell undergoing apoptosis due to the ectopic expression of a cDNA encoding the pro-apoptotic Bax protein, Ring et al. (2008) identified human TMEM85 as a Bax suppressor. Bax is the pro-apoptotic member of the ▶ Bcl-2 family of proteins. Such yeast-based screens have been useful in identifying a variety of different anti-apoptotic sequences (Greenwood and Ludovico 2010). For example, the ceramide utilizing sphingomyelin synthase was originally identified in such a screen.

Ectopic expression of TMEM85 also protected yeast from the apoptotic inducing effects of Reactive Oxygen Species (ROS). This suggests that TMEM85 is not simply a Bax suppressor but an anti-apoptotic gene. Overexpression of the yeast TMEM85 orthologue also protected yeast cells from apoptotic inducing stress (Ring et al. 2008).

TMEM85 Structure and Function

Human TMEM85 is a complex alternatively spliced gene that is reported to produce four different transcripts and proteins (Ring et al. 2008). The predicted proteins range in size from 183, 149, 97, and 66 residues. The largest protein is predicted to contain two transmembrane domains and it likely corresponds to the yeast 190 residue *YGL231c* ortholog. The 149 residue TMEM85 variant (v2) is identical to the 183 residue protein at its N-terminal but it contains a unique C-terminal region and a single transmembrane domain. It represents the sequence identified as having anti-apoptotic properties. The other variant proteins are predicted to be soluble proteins.

There are other discernable functional sequences present within the TMEM85 proteins. Thus the structure provides no insight to what its function could be in the ER or how it may function as an anti-apoptotic protein (UniProt page). Although many anti-apoptotic proteins, such as heat shock proteins and Bcl-2, belong to large family of proteins many others are unique or small groups of proteins, such as heme oxygenase 1, whose anti-apoptotic effects cannot be readily deciphered from their sequence. This lack of functional information is somewhat surprising considering that TMEM85 is highly conserved and is present in species from yeast to man ranging all way from worms to plants. There are a large number of web sites that have compiled the TMEM85 sequences from different species (http://omabrowser.org/cgi-bin/gateway.pl?f=DisplayEntry&p1=0061323).

One interesting study, using a two hybrid screen, identified TMEM85 as protein that interacts with protein prestin (Zheng et al. 2009). Prestin is a component of the outer hair cells (OHC) that are housed in the organ of Corti and play a mechanosensing role important for hearing. Knockout mouse models of prestin clearly show that they play an important role in the process of hearing. The fact that TMEM85 is highly conserved and is present in unicellular organisms like yeast suggest that it must play a fundamental role in the cell, while its potential interaction with more specialized proteins like preston suggests that TMEM85 may also have multiple functions in protozoans.

TMEM85 Expression

Very little is currently published regarding the expression or regulation of the mammalian TMEM85 gene. RT-PCR analysis of the RNA isolated from a variety of tissues suggests that all four transcripts are expressed in the heart while variant 1 and 4 are commonly observed in most tissues (Ring et al. 2008). A few other reports that have studied global gene expression patterns have identified TMEM85 expression to be increased in the Caudate Nucleus of Parkinson's patients as well as being more highly expressed in the brain white matter compared to the spinal cord (Bossers et al. 2009; Yan et al. 2009).

Function of the TMEM85 Orthologue in Yeast

Nothing else is known about mammalian TMEM85, so further clues as to the potential function of TMEM85 may be obtained from a recent study in yeast that carried out a global analysis to identify proteins that are required for protein folding in the ER (Jonikas et al. 2009). Of interest, the yeast orthologue of TMEM85, *YGL231c*, was identified as being part of tight multiprotein transmembrane complex of six poorly characterized proteins. Although the function of the complex was not determined, all six members were renamed as EMC1 to 6 proteins (*YGL231c* is *EMC4p*) to reflect that they are part of an ER membrane protein complex that is likely to have a functional significance. Cells lacking the complex show increased accumulation of misfolded ER proteins. Given that misfolded proteins leads to ER stress and to the induction of apoptosis, the proposed function of the EMC complex is in line with the reported anti-apoptotic function of TMEM85.

Summary

Human TMEM85 represents a complex alternatively spliced gene that encodes highly conserved transmembrane domain proteins of largely unknown function. TMEM85 was identified as a protein with anti-apoptotic properties in a screen of yeast cells ectopically expressing a pro-apoptotic mammalian Bax sequence. Analysis of the yeast ortholog of TMEM85, *YGL231c*, suggests that it is a protein that resides in the endoplasmic reticulum (ER). Further analysis suggests that *YGL231c* forms a tight complex with five other also largely functionally uncharacterized proteins. *YGL231c* was renamed *ECM4* to reflect the fact that it is found in this ER membrane protein complex. The proposed role of the ECM complex in regulating folding of ER proteins is consistent with the reported anti-apoptotic function of human TMEM85. A significant amount of work is still required in order to understand the role of TMEM85.

References

Bossers K, Meerhoff G, Balesar R, van Dongen JW, Kruse CG, Swaab DF, Verhaagen J. Analysis of gene expression in Parkinson's disease: possible involvement of neurotrophic support and axon guidance in dopaminergic cell death. Brain Pathol. 2009;19:91–107.

Greenwood MT, Ludovico P. Expressing and functional analysis of mammalian apoptotic regulators in yeast. Cell Death Differ. 2010;17:737–45.

Jonikas MC, Collins SR, Denic V, Oh E, Quan EM, Schmid V, Weibezahn J, Schwappach B, Walter P, Weissman JS, Schuldiner M. Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum. Science. 2009;323:1693–7.

Ring G, Khoury CM, Solar AJ, Yang Z, Mandato CA, Greenwood MT. Transmembrane protein 85 from both human (TMEM85) and yeast (YGL231c) inhibit hydrogen peroxide mediated cell death in yeast. FEBS Lett. 2008;582:2637–42.

Saccharomyces Genome Database for the YGL231c gene. http://www.yeastgenomeorg/cgi-bin/locusfpl?locus=YGL231C. Accessed 5 April 2011.

The Entez Gene web page for the TMEM85 gene. http://www.ncbinlmnihgov/gene/51234. Accessed 5 April 2011.

The Universal Protein Resource (UniProt) web page for the human TMM85 protein http://www.uniprot.org/uniprot/Q5J8M3. Accessed 5 April 2011.
Willingham S, Outeiro TF, DeVit MJ, Lindquist SL, Muchowski PJ. Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. Science. 2003;302:1769–72.
Yan Z, Lathia KB, Clapshaw PA. Murine spinal cord transcriptome analysis following reduction of prevalent myelin cDNA sequences. Cell Mol Neurobiol. 2009;29:1109–19.
Zheng J, Anderson CT, Miller KK, Cheatham M, Dallos P. Identifying components of the hair-cell interactome involved in cochlear amplification. BMC Genomics. 2009;10:127.

tm-PTPe

▶ PTPe (RPTPe and Cyt-PTPe)

TNFRSF12A

▶ Fn14

TNFRSF5

▶ CD40

TNFα (Tumor Necrosis Factor α)

▶ p38 MAPK Family of Signal Transduction Proteins

Tnk2

▶ ACK1

TOB1 (TOB, Transducer of ERBB2)

▶ BTG/TOB

TOB2 (Transducer of ERBB2 2)

▶ BTG/TOB

Toll

▶ TLR4, Toll-Like Receptor 4

Toll/Interleukin-1 Receptor Domain-Containing Protein

▶ Toll-like Receptor Adaptor Protein Family Members

Toll-Interleukin 1 Receptor (TIR) Domain-Containing Adapter Protein

▶ Toll-like Receptor Adaptor Protein Family Members

Toll-Interleukin-1 Receptor Domain-Containing Adapter Protein Inducing Interferon Beta

▶ Toll-like Receptor Adaptor Protein Family Members

Toll-Like Receptor 3

Aisha Qasim Butt and Sinéad M. Miggin
Department of Biology, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

Synonyms

CD283; CD283 antigen

Historical Background

The innate immune system of mammals is equipped with various kinds of cells, such as macrophages and dendritic cells (DCs), which provides the first line of defense to the host in recognizing various kinds of pathogens. These cells have developed different classes of protein based receptors for recognizing numerous kinds of pathogen associated molecular patterns (PAMPs) (Miggin and O'Neill 2006). These different classes of pathogen recognition receptors (PRRs) includes, *Membrane bound PRRs* such as Toll-like receptors (TLRs), Receptor kinases, Mannose receptors and *Cytoplasmic PRRs* such as Nucleotide oligomerization domain (NOD) receptors, the Retinoic acid inducible gene I (RIG-I)-like receptor (RLR) family, and the recently described AIM2 and DAI cytosolic DNA receptors. All these receptor proteins play a crucial role in "danger" recognition and induction of the innate immune responses against a variety of bacterial and viral infections at the cytoplasmic, endosomal, intercellular, and extracellular level (Kumar et al. 2009; Siednienko et al. 2011)

To date, ten human TLRs have been identified and equivalent forms of many of these have been found in other mammalian species (Kumar et al. 2009). Each member of the TLR family recognizes different kinds of PAMPs. For example, TLR1 in combination with TLR2 or TLR6 recognizes triacyl lipopeptides and diacyl lipopeptides, respectively. TLR4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria and TLR5 recognizes flagellin from bacterial flagellum. In contrast to the above-mentioned plasma-membrane-localized TLRs, the so-called antiviral TLRs, TLR3, TLR7/8, and TLR9 are located on endocytic vesicles, and recognize double-stranded (ds)RNA and single-stranded (ss)RNA, respectively, whereas, murine TLR11 recognizes uropathogenic *Escherichia coli* (Kumar et al. 2009).

After recognition of PAMPs, activation of TLRs is initiated by the formation of multi-protein complexes containing the TLR and the recruitment of proximal cytoplasmic Toll/IL-1 receptor (TIR) domain-containing adaptor proteins. To date, four activating TLR adaptor proteins have been identified, namely, Myeloid differentiation factor 88 (▶ MyD88), ▶ MyD88 adaptor-like (Mal) (also known as TIR domain containing adaptor protein (TIRAP)), TIR domain-containing adaptor inducing interferon (IFN)-beta (TRIF) (also known as TICAM-1) and TRIF-related adaptor molecule (TRAM). In addition, an inhibitory TLR adaptor protein called sterile alpha and TIR motif-containing protein (SARM) has also been identified (Vercammen et al. 2008). Upon recruitment of the activationary adaptor proteins to the TLRs via TIR domain interactions, a series of TLR signaling cascades are elicited which culminates in the production of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β, and IL-12. In addition, activation of TLR3, -7, -8, and -9 induces the production of antiviral type I interferons (IFN-β and IFN-α). Moreover, TLRs induce DC maturation, which is essential for the activation of pathogen-specific adaptive immune responses (Miggin and O'Neill 2006; Kumar et al. 2009).

In spite of various similarities between all TLRs in terms of signal transduction pathways and the production of proinflammatory cytokines, there is rigid specificity with respect to their adaptor usage (Miggin and O'Neill 2006; Kumar et al. 2009). ▶ MyD88 is the common downstream adaptor that is recruited by all TLRs except TLR3 and leads to activation of nuclear factor (NF)-κB. Mal is required for signaling by TLR4 and TLR2 (Miggin and O'Neill 2006; Kenny et al. 2009). TRIF mediates TLR3 and TLR4 signaling and induces the activation of transcription factors IRF3 and IRF7 which leads to the expression of Type I IFNs. Finally TRAM regulates TLR4 signaling exclusively acting as a bridging adaptor to recruit TRIF to the TLR4 complex (Kumar et al. 2009).

In recent years, researchers have gained a more in depth understanding of the signaling pathways that are modulated upon TLR engagement. This essay focuses on *Toll-like receptor 3*, a key molecule that recognizes viral double-stranded (ds) RNA originating from dsRNA viruses such as reovirus. TLR3 also recognizes dsRNA produced during the replication of single-stranded (ss) RNA viruses, such as West Nile virus (WNV), respiratory syncytial virus (RSV), and encephalomyocarditis virus (EMCV). In addition, TLR3 also recognizes a synthetic analogue of dsRNA known as polyriboinosinic:polyribocytidylic acid (poly(I:C)) (Kumar et al. 2009). Herein, *Toll-like receptor 3* will be described in terms of its structure, expression, subcellular localization, and its ligands. In addition, the signaling pathways that are activated by TLR3 and its curtailment by various molecules thus limiting proinflammatory cytokines and Type I IFN

production will also be described. Also, major perspective applications of *Toll-like receptor 3* in the context of antiviral and cancer immunotherapies will also be described.

Properties of TLR3

Discovery of the first human Toll-like receptor by Nomura and colleagues in 1994 (Nomura et al. 1994) has paved the way for a breakthrough in Immunobiology. Since then, a family of ten human TLRs with detailed crystal structures has been identified and they have been proved to be pivotal elements in the human innate immune system.

Structure of TLR3

Human TLR3 consists of an extracellular domain containing 23 leucine-rich repeats (LRRs), N- and C-terminal flanking regions, a transmembrane domain, and an intracellular TIR domain (Bell et al. 2006). Analysis of the TLR3-ectodomain (ECD) revealed that the LRRs form a large horseshoe shaped structure which has an inner diameter of approximately 42 Å, an outer diameter of 90 Å, and a thickness of 35 Å. The TLR3-ECD is highly glycosylated with 15 predicted N-glycosylation sites, while the lateral surface is completely carbohydrate free and offers a large surface area for interaction with other molecules. It has been demonstrated that amino acid residues H539 and N541 located on the glycosylation-free lateral surface of the LRR domain of TLR3 are critical for ligand binding (Bell et al. 2006). TLR3-ECD bind as dimeric units to dsRNA oligonucleotides of at least 45 bp in length, the minimal length required for the transduction of signal through the receptor (Botos et al. 2009). A LRR-deletion study on TLR3 has shown that the C-terminal LRRs control receptor signaling and dimerization; the intermolecular contacts between the C-terminal domains of two TLR3-ECD stabilize the dimer and position the C-terminal residues closely, which is considered to be essential for signal transduction across plasma membrane in intact TLR3 molecules (Botos et al. 2009).

Expression and Subcellular Localization of TLR3

The expression and subcellular localization of TLR3 is regulated in a cell-type specific manner and is modulated by the activation status of the cell. TLR3 expression has been detected in immune cells including macrophages, DCs, γδ T cells, NK cells, T cells, and mast cells (Siednienko and Miggin 2009; Gauzzi et al. 2010). TLR3 expression has also been detected in nonimmune cells, including fibroblasts, epithelial cells, keratinocytes, glial cells, and neuronal cells (Gauzzi et al. 2010; Siednienko et al. 2010).

Unlike the other viral RNA sensor proteins whose localization is exclusively cytoplasmic, TLR3 has been found localized intracellularly within endosomal vesicles and also localized at the cell surfaces (Siednienko et al. 2010). For example, in DCs, TLR3 is predominantly detected in the intracellular endocytic vesicles (Siednienko et al. 2010). In contrast, while fibroblasts and epithelial cells express intracellular TLR3, its expression predominates on the cell surface. Likewise, peritoneal and bone marrow-derived murine mast cells express TLR3 both in the cytoplasm and on the cell surface. In keratinocytes, high levels of intra-cytoplasmic TLR3 expression and low levels of surface TLR3 are detected (Gauzzi et al. 2010). Interestingly, Arg740 and Val741 are involved in the intracellular localization and expression of TLR3 in humans, while in murine TLR3, no crucial structural motif residues have been identified to date (Funami et al. 2004). The expression of TLR3 in both DCs and macrophages is upregulated by viral infection and exogenous addition of poly(I:C) or type I IFN (Gauzzi et al. 2010; Siednienko et al. 2010).

Ligands and its Delivery to TLR3

The primary ligand for TLR3 is dsRNA. As previously stated, TLR3 senses dsRNA viruses such as reovirus and dsRNA produced during the replication of ssRNA viruses, such as WNV, RSV, and EMCV. In addition to dsRNA from viral origin, endogenous dsRNA released from dying cells also activates TLR3 (Gauzzi et al. 2010). Interestingly, poly(I:C) in a cell-associated form is reported to be more efficient in triggering TLR3 than soluble dsRNA, suggesting that dsRNA derived from dying cells is a more potent and physiologically relevant TLR3 ligand than dsRNA from live cells (Gauzzi et al. 2010). In the laboratory, the stable synthetic dsRNA analogue, poly(I:C) is extensively used as an artificial activator of TLR3. It must be noted that beyond TLR3, dsRNA is also recognized by several other cytosolic sensors, such as protein kinase R, 20–50-oligoadenylate synthetases, and the more recently identified RLR RNA helicases, RIG-I and melanoma

differentiation-associated gene5 (Mda5) (Gauzzi et al. 2010). In certain cell types, TLR3 is predominantly expressed intracellularly, thus the TLR3 ligand is transfected into the cells with cationic lipososmes such as lipofectin and DOTAP and these ligand-liposomes complexes are believed to be delivered to the endosome to activate TLR3 (Gauzzi et al. 2010). It has been found that TLR3 provides several sites to accommodate ligand binding depending on the glycosylation status of the extracellular domain and the structural characteristics of the ligand (example: modification and ligand length). Notably, TLR3 signaling has been shown to be more potently induced by longer duplex length of poly(I:C) rather than shorter ones (de Bouteiller et al. 2005).

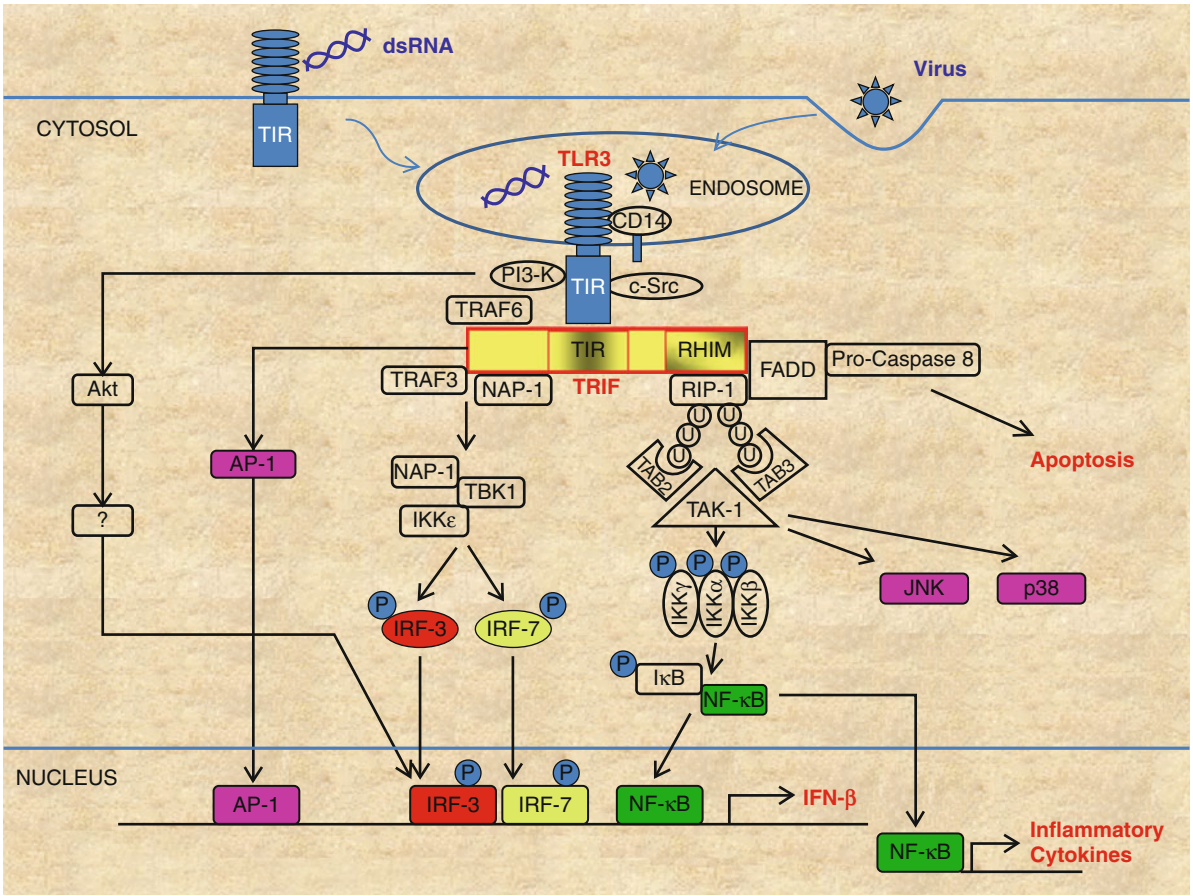
Regarding the delivery mechanism of dsRNA to the TLR3 containing endocytic vesicle, it has been demonstrated that CD14 directly binds to extracellular poly(I:C) and enhances dsRNA-mediated TLR3 activation (Gauzzi et al. 2010) by direct internalization and co-localization of CD14 with TLR3 which then facilitates the transfer of the dsRNA to the intracellular TLR3 (Gauzzi et al. 2010). Supporting this concept is the fact that intracellular co-localization of TLR3 and CD14 in multiple compartments, including the endoplasmic reticulum and the Golgi apparatus has been demonstrated (Gauzzi et al. 2010). Interestingly, human DCs, which do not express CD14 and do not express TLR3 on their surface, produce type I IFN and IL-12p70 in response to exogenously added dsRNA and antibodies against TLR3 do not inhibit dsRNA-induced IFN-β production by DCs (Gauzzi et al. 2010). This suggests that the extracellular dsRNA must be internalized via a putative uptake receptor (e.g., clathrin-mediated endocytosis) or by the uptake of the apoptotic bodies derived from virally infected cells containing viral dsRNA in order to activate intracellularly localized TLR3. Thus, the dsRNA may originate from many sources including dying cells, apoptotic bodies, and viruses and the mechanisms that facilitate association of the dsRNA with TLR3 largely depends on the cell type and concomitant subcellular distribution of TLR3.

Signaling Pathway

TRIF-Mediated TLR3 Signaling

The transduction of TLR3 signaling is mediated by the TIR domain-containing adaptor protein, TRIF, which binds to the intracellular TIR domain of TLR3 (Fig. 1) leading to the activation of various transcription factors such as interferon regulatory factor (IRF) 3, ▶ NF-κB, and activator protein (AP)-1, and concomitant induction of inflammatory cytokines, type I IFN, DC maturation, and activation of the adaptive immune system. The mechanisms by which TRIF activates ▶ NF-κB and IRF3 have been analyzed extensively and research have shown that mice deficient in TRIF display impaired responses to dsRNA (Kumar et al. 2009; Siednienko et al. 2010), indicating the indispensible role played by TRIF in TLR3 signaling.

Regarding TRIF, the proline-rich N-terminal domain of TRIF mediates IRF3 activation by facilitating the phosphorylation of IRF3 at critical residues via a number of auxiliary molecules. NAK-associated protein 1 (NAP-1) forms part of the active kinase complex for IRF3 and facilitates the association of TRIF with TANK-binding kinase 1 (TBK1; also called NAK or T2K) and IκB kinase related kinase ε (IKKε; also called IKKι) (Gauzzi et al. 2010). TBK1 and IKKε also interact with TANK (TRAF family member-associated ▶ NF-κB activator) which also facilitates the activation of IRF3 (Kumar et al. 2009). Furthermore, TNF receptor-associated factor 3 (TRAF3) plays a crucial role in TLR3 signaling as various independent studies show that TRAF3 forms a complex with NAP-1 and TRIF and TRAF3-deficient mice exhibit impaired IFN-β in response to poly(I:C) (Fig. 1) (Oganesyan et al. 2006). It must be noted that TRAF3 and NAP-1 are also critical adaptor proteins in RIG-I/Mda5-mediated IRF3 activation (Oganesyan et al. 2006). TRAF6 also interacts with TRIF, though its role in IFN-β production is largely non-critical (Yamamoto and Takeda 2010). The RNA helicase, DEAD box protein 3 (DDX3) has also been shown to play a role in TLR3 signaling (Schröder et al. 2008). Upon TKB1/IKKε-mediated phosphorylation and homo/heterodimerization of IRF3/7, the complex subsequently translocates into the nucleus and binds to the IFN-β enhanceosome (Schröder et al. 2010) and IFN-stimulated response elements (ISREs) to induce the transcription of responsive genes including the Type I IFN-β and CCL5 genes (Siednienko et al. 2011) (Fig. 1). Thus, TRAF3, NAP-1, TBK1/IKKε, and the N-terminal region of TRIF play an important role in TLR3-mediated IRF3/7 activation (Oganesyan et al. 2006).



Toll-Like Receptor 3, Fig. 1 TLR3 signaling pathway

While the N-terminal region of TRIF mediates IRF3/7 activation, the leucine-rich C-terminal region of TRIF is involved in ▶ NF-κB activation via receptor-interacting protein 1 (RIP1) (Fig. 1). RIP1 interacts with TRIF through the RIP homolytic interaction motif (RHIM) domain of TRIF, followed by polyubiquitination (U) of RIP1, which recruits the ubiquitin receptor protein transforming growth factor β-activating kinase (TAK) binding proteins 2 and 3 (TAB2 & TAB3) and TAK1 to form a TAK1 complex (Fig. 1) (Gauzzi et al. 2010; Yamamoto and Takeda 2010). TAK1 then phosphorylates IKKα and IKKβ, which consequently phosphorylates the ▶ NF-κB inhibitor IκB, concomitantly leading to its degradation and nuclear translocation of ▶ NF-κB and induced expression of IFN-β (Fig. 1) (Gauzzi et al. 2010). TAK1 also activates two other classes of kinase, JNK and p38 and these activate the transcription factor, AP-1 (Jin et al. 2010). In addition, another protein, TRAF6 also mediates ▶ NF-κB activation, though this involves binding of TRAF6 to the N-terminal region of TRIF (Fig. 1). Studies have suggested that the participation of TRAF6 in TRIF-mediated ▶ NF-κB induction is cell-type specific as TRAF6 is essential for ▶ NF-κB activation in mouse embryonic fibroblasts, whereas poly(I:C) induced ▶ NF-κB activation is not impaired in TRAF6 deficient macrophages (Gauzzi et al. 2010; Sasai et al. 2010). The TAK1 complex also activates the mitogen-activated protein kinases (MAPKs) including *c-jun* N-terminal kinase, p38, and extracellular signal related kinase (ERK) to control mRNA expression and the stability of mRNA for inflammatory genes by mediating phosphorylation of the AP-1 transcription family proteins (Yamamoto and Takeda 2010).

Of all the TLR adaptor proteins, TRIF is the only adaptor that is able to engage mammalian cell death signaling pathways whereby TRIF interacts with

Fas-associated cell death domain (FADD) through RIP1 and activates procaspase-8 to initiate cell apoptosis (Vercammen et al. 2008; Jin et al. 2010); this is an important host defense mechanism for limiting the spread of viral infection. Moreover, TRIF also plays a role in TLR3 induced proliferative responses in B cells (Vercammen et al. 2008).

PI3K and c-src in TLR3 Signaling

While the TIR domain of TLR3 serves to recruit the adaptor molecule, TRIF, additional molecules are also capable of interacting with TLR3 and modulating its signaling. For example, studies have shown that phosphatidylinositol 3-kinase (PI3K) is capable of interacting with phosphorylated tyrosine residues Tyr^{759} and Tyr^{858} located within the TIR domain of TLR3. This interaction serves to activate the TLR3 pathway toward IRF3 and concomitant induction of IFN-stimulated genes while impairing ▶ NF-κB-dependent proinflammatory signaling (Fig. 1) (Vercammen et al. 2008). Another tyrosine kinase molecule known as c-Src has also been shown to interact with endosomally localized TLR3 and has been shown to play a crucial role in dsRNA-mediated TLR3 signaling (Vercammen et al. 2008).

In summary, TLR3 is distributed on the cell surface and the endosome in a cell-type-dependent manner. The adaptor protein TRIF is localized in the cytoplasm. Upon stimulation of TLR3 with synthetic dsRNA or upon recognition of an invading virus, TRIF is recruited to the endosomal/cell surface TLR3 via TIR domain interactions and thereafter moves away from the receptor to speckle-like structures that also include downstream signaling molecules. The interaction of TRIF with RIP1 results in polyubiquitination (U) of RIP1 and its binding to the ubiquitin receptors TAB2 and TAB3 which activates TAK1. Activated TAK1 induces phosphorylation of IKKα and IKKβ leading to phosphorylation and degradation of IκB and release and translocation of NF-κB to the nucleus. TAK1 also activates two other classes of kinase, JNK and p38 and these activate the transcription factor, AP-1. Also, TRIF activates TBK1 and IKKε through NAP1 and this results in phosphorylation and nuclear translocation of IRF3 and IRF7 resulting in IFN-β production. TRAF3 also binds with the TBK1/IKKε complex inducing IRF3 activation. PI3K is also recruited to the TIR domain of TLR3 and activates kinase Akt for full phosphorylation and activation of IRF3 in the nucleus. Tyrosine kinase c-Src also plays a role in TLR3 signaling. TRIF interacts with FADD through RIP1 and activates procaspase-8 to initiate cell apoptosis. Many of the mechanisms by which TLR3/TRIF is activated and transmits signals remain largely unknown. Phosphorylation and activation of the various transcription factors such as IRF3, IRF7, NF-κB, and AP-1 molecules leads to IFN-β and proinflammatory cytokine production and consequent DC maturation and complete activation of the innate and adaptive immune system.

Negative Regulation of TLR3 Signaling

Excessive or sustained activation of TLR3 signaling, leading to elevated levels of proinflammatory cytokines and IFN-β is potentially harmful or even fatal for the host cell. Clinically, this may present as systemic inflammatory response syndromes such as inflammation-associated myopathies, WNV-driven CNS inflammation and viral or autoimmune liver diseases. Given this, the body has evolved several mechanisms for modulating and curtailing TLR3-mediated cellular responses. For example, protein inhibitor of activated signal transducers and activators of transcription (PIASy), TRAF1, and TRAF4 have been shown to inhibit TLR3 ligand induced activation of IRF3 and ▶ NF-κB (Vercammen et al. 2008). Interestingly, PI3K has been shown to inhibit TLR3-mediated ▶ NF-κB activation while activating IRF3. Similarly, another kinase known as RIP3 has been shown to inhibit TLR3 induced ▶ NF-κB activation by competing with RIP1 for TRIF binding (Vercammen et al. 2008). In contrast, suppressor of IKKε (SIKE) has been shown to inhibit TLR3-mediated IRF3 activation, without affecting ▶ NF-κB activation (Vercammen et al. 2008).

The TLR adaptors Mal and ▶ MyD88 were not thought to be involved in TLR3 signaling until a recent study demonstrated that ▶ MyD88 negatively regulates TLR3/TRIF-induced corneal inflammation through a mechanism involving JNK phosphorylation, but not p38, IRF3, or ▶ NF-κB (Johnson et al. 2008; Siednienko et al. 2011). ▶ MyD88 has also been shown to inhibit TLR3-dependent phosphorylation of IRF3 and thus curtail TLR3-mediated IFN-β and RANTES production (Siednienko et al. 2011). ▶ MyD88 also inhibits TLR3-dependent IL-6 induction, but not IκB degradation nor p38 activation in murine macrophages (Kenny et al. 2009). The TLR adaptor Mal has also been shown to inhibit TLR3-dependent IFN-β production through

a mechanism distinct from ▶ MyD88 whereby Mal inhibits TLR3 ligand-mediated IRF7 activation (Siednienko et al. 2010). Mal has also been shown to inhibit TLR3-dependent IL-6 induction (Kenny et al. 2009). In addition, the fifth TLR adaptor protein, SARM which contains a sterile α-(SAM) and a HEAT/armadillo (ARM) motif has been shown to inhibit TLR3 signaling. While SARM does not induce IRF3 or ▶ NF-κB activation itself, it inhibits TRIF-mediated TLR3 signaling (Carty et al. 2006) with both the TIR and SAM domains proving vital for SARM functionality.

Thus, it is clear that TLR3 inhibitory molecules have the ability to curtail global TLR3 signaling and/or to inhibit a specific pathway on the TLR3 signaling cascade in a cell-type specific manner. These molecules may serve to alter the balance between ▶ NF-κB and IRF3/7 following TLR3 activation, thus providing an immune response that it tailored to a specific cell-type/ligand/biological milieu. Moreover, the vast array of molecules and mechanisms that have evolved to curtail TLR3 signaling highlights the importance of TLR3 immunomodulation.

Therapeutic Manipulation of TLR3

Once microbes breach the physical barriers of the body such as skin or the intestinal tract mucosa, they are recognized by TLRs which activates the innate immune cell response.

Antiviral Function

It is well established that dsRNA-induced activation of TLR3 instigates a series of signaling cascade events which leads to the induction of *IFN-β* gene transcription and the production of antiviral IFN-β, IL-12, and various inflammatory cytokines (Vercammen et al. 2008; Gauzzi et al. 2010). Thus, TLR3 plays a key role in antiviral immune responses.

TLR3 is involved in the sensing of dsRNA derived from reovirus (Vercammen et al. 2008). TLR3 also recognizes dsRNA produced during the replication of many viruses including single-stranded (ss)RNA viruses, such as WNV, RSV and EMCV (Vercammen et al. 2008). It must be noted that TLR3 is dispensable for the detection of certain viruses (Vercammen et al. 2008). Regarding the therapeutic value of TLR3 manipulation, it has been shown that activation of TLR3 using poly(I:C) inhibits human immunodeficiency virus (HIV) infection by increasing the expression of Type I IFN antiviral factors thus restricting HIV expression and replication (Zhou et al. 2010). Also, poly(I:C) has proven beneficial as a mucosal adjuvant for an influenza virus vaccine in a murine infection model (Vercammen et al. 2008). Interestingly, another TLR3 agonist, Poly(I:C_{12}U), has been shown to have antiviral activity against hepatitis B virus, several flaviviruses, coxsackie B_3 virus, and Punta Toro virus. Given that excessive TLR3 signaling that is associated with many viral based infections, the use of TLR3 antagonists to curtail excessive TLR3 signaling would also be of therapeutic value.

Antitumor Effect

TLR agonists have the capacity to prime and shape adaptive immunity and so have aroused significant interest in the development of cancer immunotherapies (Cheng and Xu 2010). Regarding TLR3, its activation by dsRNA induces dual antitumoral activity, either an anticancer immune response or apoptosis of the cancer cells via TLR3 which is expressed by the cancer cell (Jin et al. 2010); these two modes of action work synergistically against cancer. Given that cancer cells are malignantly transformed cells of the host, they express antigens that are either not expressed or only expressed in trace amounts by the healthy host and so are referred to as tumor associated antigens (TAAs). Thus, induction of TLR3-mediated apoptosis of the cancer cells presents the immune system with a new repertoire of TAAs in a TLR3 activation context that is favorable to the development of long-term anticancer immune responses (Jin et al. 2010). In addition, activation of TLR3 using agonists leads to production of type I IFN and other inflammatory mediators, which aid in the phenotypic and functional maturation of DCs. For example, immunization with the melanoma peptide trp2 and adjuvants consisting of cationic liposomes complexed with TLR3/9 agonists has been shown to control the growth of established B16 melanoma tumors in a therapeutic tumor vaccine model (Vercammen et al. 2008). Despite increased expression of TLR3 on many tumors and the immunostimulatory and protective properties of dsRNA, many questions remain to be answered regarding TLR3 in the context of its antitumor activity.

Summary

Much information has come to light in the last decade providing a convincing picture of the important, yet complex, interplay between TLR3, the TLR adaptor proteins, the signaling pathways and the role of TLR3 in antiviral and antitumor therapies. It must be noted that although it is clear that several viruses stimulate TLR3-dependent signaling, the importance of TLR3 signaling in the antiviral immune response is not definitive. For example, an absence of TLR3 does not affect the outcome of a viral infection in some instances; this may be attributed to redundancy with other dsRNA sensors, such as the RLRs, RIG-I, and Mda5. Another complication in understanding TLR3 functionality is the absence of a physiological ligand for TLR3. Most studies use poly(I:C), a synthetic agonist, but the identity of the viral RNA sequences that actually activate TLR3 are poorly understood. Moreover, the potential of endogenous RNA (e.g., from dying cells) to mediate TLR3 signaling requires further study. Regarding the cellular localization of TLR3, it is important to consider that the subcellular localization of TLR3 may affect subcellular signaling events. Lastly, to fulfill the therapeutic potential of TLR3, studies must be performed to further elucidate the signaling pathways and proteins that can positively and/or negatively regulate TLR3 signaling. These molecules could potentially be used to modulate inappropriate TLR3 signaling in a number of clinical contexts and to develop enhanced vaccine adjuvant therapy against tumor and viral infectious diseases.

References

Bell JK, Askins J, Hall PR, Davies DR, Segal DM. The dsRNA binding site of human Toll-like receptor 3. Proc Natl Acad Sci USA. 2006;103:8792–7.

Botos I, Liu L, Wang Y, Segal DM, Davies DR. The toll-like receptor 3:dsRNA signaling complex. Biochim Biophys Acta. 2009;1789:667–74.

Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat Immunol. 2006;7:1074–81.

Cheng YS, Xu F. Anticancer function of polyinosinic-polycytidylic acid. Cancer Biol Ther. 2010;10:1219–23.

de Bouteiller O, Merck E, Hasan UA, Hubac S, Benguigui B, Trinchieri G, et al. Recognition of double-stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH. J Biol Chem. 2005; 280:38133–45.

Funami K, Matsumoto M, Oshiumi H, Akazawa T, Yamamoto A, Seya T. The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. Int Immunol. 2004; 16:1143–54.

Gauzzi C, Del Corno M, Gessani S. Dissecting TLR3 signalling in dendritic cells. Immunobiology. 2010;215:713–23.

Jin B, Sun T, Yu XH, Liu CQ, Yang YX, Lu P, et al. Immunomodulatory effects of dsRNA and its potential as vaccine adjuvant. J Biomed Biotechnol. 2010;2010:690438.

Johnson AC, Li X, Pearlman E. MyD88 functions as a negative regulator of TLR3/TRIF-induced corneal inflammation by inhibiting activation of c-Jun N-terminal kinase. J Biol Chem. 2008;283:3988–96.

Kenny EF, Talbot S, Gong M, Golenbock DT, Bryant CE, O'Neill LA. MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. J Immunol. 2009; 183:3642–51.

Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. Biochem J. 2009;420:1–16.

Miggin SM, O'Neill LA. New insights into the regulation of TLR signaling. J Leukoc Biol. 2006;80:220–6.

Nomura N, Nagase T, Miyajima N, Sazuka T, Tanaka A, Sato S, et al. Prediction of the coding sequences of unidentified human genes. II. The coding sequences of 40 new genes (KIAA0041-KIAA0080) deduced by analysis of cDNA clones from human cell line KG-1 (supplement). DNA Res. 1994; 1:251–62.

Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, et al. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature. 2006;439:208–11.

Sasai M, Tatematsu M, Oshiumi H, Funami K, Matsumoto M, Hatakeyama S, et al. Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the Toll-like receptor 3/4 pathway. Mol Immunol. 2010;47:1283–91.

Schröder M, Baran M, Bowie AG. Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKe-mediated IRF activation. EMBO J. 2008;27:2147–57.

Siednienko J, Miggin SM. Expression analysis of the Toll-like receptors in human peripheral blood mononuclear cells. Methods Mol Biol. 2009;517:3–14.

Siednienko J, Halle A, Nagpal K, Golenbock DT, Miggin SM. TLR3-mediated *IFN-beta* gene induction is negatively regulated by the TLR adaptor MyD88 adaptor-like. Eur J Immunol. 2010;40:3150–60.

Siednienko J, Gajanayake T, Fitzgerald KA, Moynagh P, Miggin SM. Absence of MyD88 results in enhanced TLR3-dependent phosphorylation of IRF3 and increased IFN-(beta) and RANTES production. J Immunol. 2011;186:2514–22.

Vercammen E, Staal J, Beyaert R. Sensing of viral infection and activation of innate immunity by toll-like receptor 3. Clin Microbiol Rev. 2008;21:13–25.

Yamamoto M, Takeda K. Current views of toll-like receptor signaling pathways. Gastroenterol Res Pract. 2010; 2010:240365.

Zhou Y, Wang X, Liu M, Hu Q, Song L, Ye L, et al. A critical function of toll-like receptor-3 in the induction of anti-human immunodeficiency virus activities in macrophages. Immunology. 2010;131:40–9.

Toll-Like Receptor 4

▶ TLR4, Toll-Like Receptor 4

Toll-Like Receptor 4 Adapter Protein

▶ Toll-like Receptor Adaptor Protein Family Members

Toll-like receptor 5

▶ TLR5

Toll-Like Receptor 9

Felipe Francisco Tuon
Division of Infectious and Parasitic Diseases, Hospital Universitario Evangelico de Curitiba, Curitiba, PR, Brazil

Synonyms

TLR9

Historical Background

Toll receptors are transmembrane proteins that are evolutionarily conserved. These receptors were first recognized in *Drosophila*, as an essential molecule for embryogenic patterning (Anderson 2000).

Activation of these receptors in *Drosophila* initiates an intracellular kinase cascade that produces a translocation of transcription factors from cytoplasm to nucleus. These factors activate a variety of inflammatory mediators and cytokines, initiating an immune response (Imler and Hoffmann 2000).

Considering these facts, some researchers began a search for toll-related proteins in humans. In 1997, they identified the first human homologue, initially termed "human toll" and subsequently termed ▶ TLR4 (Medzhitov et al. 1997). After this, more toll-like receptors were discovered, as TLR1, TLR2, TLR3, ▶ TLR5, and TLR6.

In 2000, Du et al. examined the human genomic sequence database in an effort to identify novel TLRs (Du et al. 2000). He found more three TLRs, which were designated TLR7, TLR8, and TLR9. Two of these TLRs are X-linked and lie in close opposition to one another at Xp22. A third is located at human chromosome 3p21.3.

Structure

Molecular Cloning and Sequence Analysis of TLR9

All the information about structure of TLR9 was well described by Chuang et al. (Chuang and Ulevitch 2000). TLR9 exhibit features of type I transmembrane proteins. Type I proteins are single pass molecules anchored to the lipid membrane with a stop-transfer anchor sequence and have their N-terminal domains targeted to the endoplasmic reticulum lumen during synthesis.

TLR9 contains two hydrophobic regions: (1) a transmembrane domain, and (2) a signal peptide at the amino-terminus. The transmembrane regions separate TLR9 into two domains: an extracellular domain with more than 800 amino acid residues and a cytoplasmic domain with around 200 amino acid residues. External domain contains copies of leucine-rich repeats, more than found in other TLRs. Following the leucine-rich repeats-flanking region, there is a sequence of about 60 amino acid containing cysteine residues, which are conserved in all TLRs. This conserved area can be found in other proteins such as platelet glycoprotein IX and 1b-alpha (Hickey et al. 1989). The cytoplasmic domains of TLR9 share sequence homology with other members of human TLR family, although there is some variation within these regions.

Tissue Distribution of TLR9

Toll-like receptors can be found in different tissues. In contrast to all of the other TLRs, the TLR9 was initially found in immune-cell-rich tissue, including spleen, lymph node, bone marrow, and peripheral blood leukocytes. However, the TLR9 can also be found in the skin and endothelial cells (Tuon et al. 2010a, b).

Activation of TLR9

Some TLRs recognize protein and lipid structures found in microorganisms. However, toll-like receptor 3, 7, 8, and 9 recognize nucleic acid fragments from microorganisms. TLR3 respond to specific double-stranded RNA segments, TLR9 respond to DNA-specific oligonucleotides-bearing unmethylated CpG base pairs (CpG motifs), and TLR7 and TLR8 recognize specific single-stranded RNA segments (Barton et al. 2006).

The TLR9 can recognize CpG motifs which can be found in many bacterial and viral genomes (Gangloff and Gay 2008). After the recognition of the CpG motifs, several intracellular enzymatic reactions occur and the translocation of NF-kB to the nucleous induces the production of cytokines, initiating the innate immune response.

The role of TLR9 in CpG motifs detection was suggested by the observation that the activation of CpG-induced responses requires components of TLR signaling pathways, including ▶ MyD88 and ▶ TRAF6 (Hacker et al. 2000; Schnare et al. 2000). TLR9 was identified as a receptor by demonstrating that responses to CpG DNA are abrogated in mice lacking TLR9 (Hemmi et al. 2000). Furthermore, sensitivity to CpG DNA can be reconstituted by expression of TLR9, without the co-expression of CD14 (Bauer et al. 2001).

Unlike other TLRs, the TLR9 is localized in intracellular compartments such as the endosome and lysosome (Fig. 1). Thus, to stimulate an innate immune response the non-self nucleic acid must be internalized by phagocytosis. This occurs in macrophages, dendritic cell, as well as endothelial cells. Endosomal acidification is prerequisite for the activation of the signaling pathway mediated by CpG DNA. This data suggests that generation of the CpG DNA signal takes place inside the endosome (Hacker et al. 1999). Binding of DNA containing unmethylated CpG motifs to TLR9 causes a conformational shift in the receptor, which is thought to result in recruitment of the adapter protein MyD88. All TLRs share the same signaling molecule, the MyD88, attached in the cytoplasmatic portion of the receptors. After the activation, MyD88 recruits the serine/threonine kinase IRAK (IL-1 associated protein kinase). IRAK is phosphorylated and dissociated from the receptor complex and associates with TRAF6 (TNF receptor–activated factor 6). This process results in the activation of three pathways: JNK, MAPK, and NF-κB. NF-κB attaches to DNA in the promoter region of target genes as a dimmer, inducing the synthesis of several cytokines.

Although NF-kB is known to function as an activator of transcription, its role in the differential transcription of cytokines is less well defined. Some interactions between NF-kB and other activated transcription factors are important for determining the transcription rates of cytokine genes. NF-kB may also produce preferential binding, which could favor the production of certain cytokines. For example, cooperative binding with the transcription factor NF–IL-6 (a variance of NF-kB) is required for the transcriptional activation of IL-8 and IL-6 (Baeuerle and Baltimore 1996). In addition, NF-kB may have direct protein–protein interactions with other transcription factors, such as the glucocorticoid receptor, that alter the ability of NF-kB to bind to DNA.

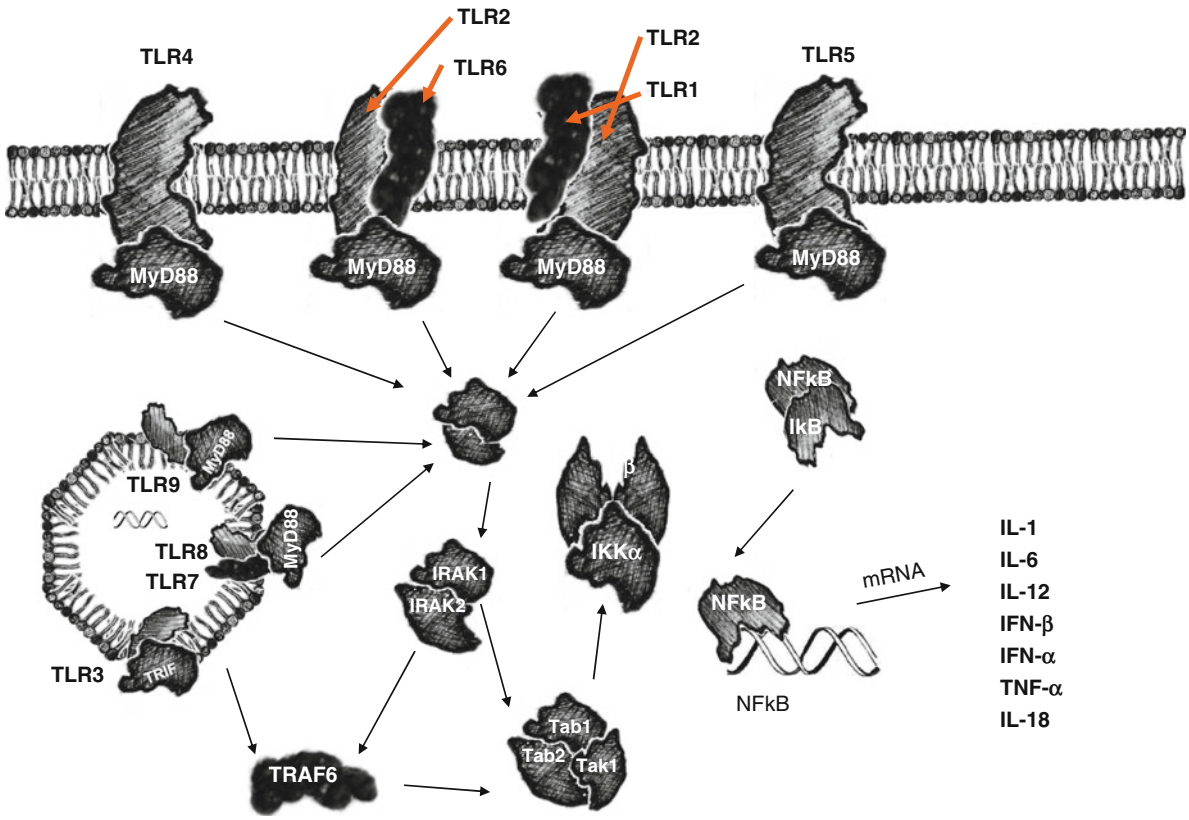
The activation of TLR9 is associated with the expression of IL-1, IL-8, TNF-alpha, IFN-gamma, CD80 and CD86.

Application

TLR9 agonists are currently in clinical trials for use in lung cancer, as antiviral therapy, as adjuvants, and as immune modulators in asthma and allergies. TLR9 antagonists, such as the antimalarial drug chloroquine, have been used since the 1950s to treat immune-mediated inflammatory disorders such as rheumatoid arthritis, systemic lupus erythematosus, and Sjögren's syndrome. However, the use of antimalarials is limited due to the side effects or suboptimal efficacy (Sun et al. 2007).

Cancer

TLR9 agonists have shown activity against several neoplasms. Phase I and II clinical trials have indicated that these agents have antitumor activity as single agents and enhance the development of antitumor T-cell responses when used as therapeutic vaccine adjuvants. The efficacy of these anticancer agents is being tested in several tumor types (Krieg 2008). The activation of the innate response has direct antitumor effects and the enhanced presentation of the tumor antigen in a Th1-like cytokine and can promote an better antitumor immune response.



Toll-Like Receptor 9, Fig. 1 Toll-like receptors in the human cell – The TLR9 is shown in endosome as well as TLR7, TLR8, and TLR3. All toll-like receptors are linked with MyD88, except TLR3. After the activation, MyD88 recruits the IRAK4. IRAK is phosphorylated and dissociated from the receptor complex and associates with TRAF6. This process results in the activation of NF-κB which attaches to DNA in the promoter region of target genes inducing the synthesis of some cytokines

Synthetic oligodeoxynucleotide (ODN) agonists for TLR9 are currently in development for the treatment of cancer. Because the phosphodiester bond of native DNA is rapidly degraded by endonucleases, these investigational CpG ODNs use a nuclease-resistant phosphorothioate backbone that improves the half-life in the body. These agonists have been tested in cervical carcinoma, sarcoma, breast cancer, chronic lymphocytic leukemia, lymphomas, lung and renal cancer, and glioblastoma.

Although the results are encouraging evidence of the potential clinical benefits of TLR9 activation, the greatest improvement in patient outcomes is likely to result from the use of this approach in combination with other therapies that work in a synergistic manner. However, two phase III trials of PF-3512676 administered concurrently with chemotherapy for patients with lung cancer have reported a lack of incremental efficacy.

Autoimmune Disorders

The exacerbation of inflammatory response by TLRs cytokine increases the production of antibodies, and several studies have been done to evaluate this overexpression of cytokines as a cause of autoimmune disorders.

Murine studies of lupus have examined whether CpG ODN would induce or flare lupus. These studies have shown increase in anti-DNA antibodies, proteinuria, and glomerulonephritis. Treatment with CpG ODN also exacerbated disease in mice, perhaps because of a more sustained immune stimulatory effect (Krieg and Vollmer 2007).

There are controversial findings of the role of TLR9 in central nervous system autoimmune disorders, including multiple sclerosis. CpG ODN accelerated the development of multiple sclerosis. Furthermore, TLR9 −/− and MyD88 −/− mice were resistant and partially resistant to multiple sclerosis development,

respectively. These findings suggest an association of TLR9 with autoimmune disorders in central nervous system. Nevertheless, in some in vivo models, TLR9 activation has decreased the severity of multiple sclerosis, once mice injected with CpG ODN starting 4 days before multiple sclerosis model induction had a marked reduction in the subsequent disease severity, compared with mice treated with a control ODN (Krieg and Vollmer 2007).

Several investigators have examined the effects of CpG DNA on experimental models of arthritis. Because disease in these models results from Th1-mediated inflammation, TLR9 stimulation would be expected to aggravate the disease process. When injected into joints, CpG ODNs induce a transient self-limited inflammatory arthritis with a peak severity at 3 days, followed by resolution. However, systemic injection of CpG does sensitize the mouse, resulting in an exaggerated arthritis when the mice are subsequently given an intra-articular injection of a low dose of CpG DNA.

TLR9 activation on intestinal epithelial cells suppresses gut inflammation, in contrast to the immune stimulatory effects in immune cells. Mice genetically deficient in TLR9 are susceptible to acute colitis. TLR9 activation promotes the development of T regulatory cells that protect against gut inflammation. These studies suggest that in this anatomic region, TLR9 activation has a constitutive role in preventing inflammation.

Recent evidence has suggested that selective, specific antagonists for TLR9 might be beneficial in certain diseases, such as lupus. Thus, the use of suppressive ODN or novel small molecule TLR inhibitors with a larger safety window and differentiated selectivity may potentially have significant clinical utility.

Infectious Diseases

Therapeutic treatment with TLR9 agonists might protect against intracellular microorganisms. Studies in mice have demonstrated that the innate immune defenses activated by CpG ODNs can protect against a wide range of viral, bacterial, and even some parasitic pathogens (Krieg 2007). The adjuvant therapy increases the pro-inflammatory cytokine synthesis, improving the antigen presentation, macrophage activation, and adequate Th1-like adaptive immune response. Animal models have been shown efficacy in chronic viral infection as hepatitis C and B, and mycobacterial infection by *M. tuberculosis*.

However, the major application of TLR9 agonist is related with the gold standard vaccine adjuvant, capable of inducing powerful antigen-specific antibody and Th1 cellular immune responses. This strong adjuvant activity results from synergy between TLR9 and B-cell receptor, inhibition of B-cell apoptosis, enhanced IgG class switch DNA recombination, and dendritic cell maturation and differentiation, resulting in enhanced activation of Th1 cells and strong cytotoxic T lymphocyte generation.

In humans, CpG ODNs have been used as adjuvants in controlled randomized trials for hepatitis B vaccination, anthrax vaccine, and influenza vaccine.

Summary

In summary, TLR9 exhibit structural features shared by all the other TLRs. TLR9 activate NF-kappaB through the same signal transduction pathway used by other TLRs. TLR9 activation induces powerful Th1-like innate and adaptive immune responses and is emerging as a promising therapeutic approach in several disease fields. TLR9 agonists can provide a temporary protection against diverse pathogen and have become a powerful vaccine adjuvant, inducing faster and stronger humoral and cellular immune responses. However, the cause of some autoimmune disorders has been attributed to TLR9 stimulus or, at least, worse the clinical evolution of autoimmune diseases.

References

Anderson KV. Toll signaling pathways in the innate immune response. Curr Opin Immunol. 2000;12:13–9.

Baeuerle PA, Baltimore D. NF-*k*B: ten years after. Cell. 1996;87:13–20.

Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol. 2006;7:49–56.

Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci USA. 2001;98:9237–42.

Chuang TH, Ulevitch RJ. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur Cytokine Netw. 2000;11:372–8.

Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw. 2000;11:362–71.
Gangloff M, Gay NJ. Baseless assumptions: activation of TLR9 by DNA. Immunity. 2008;28:293–4.
Hacker H, Mischak H, Hacker G, et al. Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. EMBO J. 1999;18:6973–82.
Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptorassociated factor (TRAF)6. J Exp Med. 2000;192:595–600.
Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo HA. Toll-like receptor recognizes bacterial DNA. Nature. 2000;408:740–5.
Hickey MJ, Williams SA, Roth GJ. Human platelet glycoprotein IX: an adhesive prototype of leucine-rich glycoproteins with flank-center-flank structures. Proc Natl Acad Sci USA. 1989;86:6773–7.
Imler JL, Hoffmann JA. Signaling mechanisms in the antimicrobial host defense of Drosophila. Curr Opin Microbiol. 2000;3:16–22.
Krieg AM. Antiinfective applications of toll-like receptor 9 agonists. Proc Am Thorac Soc. 2007;4:289–94.
Krieg AM. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. Oncogene. 2008;27:161–7.
Krieg AM, Vollmer J. Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. Immunol Rev. 2007;220:251–69.
Medzhitov R, Preston-Hurlburt P, Janeway Jr CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388:394–7.
Schnare M, Holt AC, Takeda K, Akira S, Medzhitov R. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. Curr Biol. 2000;10:1139–42.
Sun S, Rao NL, Venable J, Thurmond R, Karlsson L. TLR7/9 antagonists as therapeutics for immune-mediated inflammatory disorders. Inflamm Allergy Drug Targets. 2007;6:223–35.
Tuon FF, Fernandes ER, Duarte MI, Amato VS. The expression of TLR2, TLR4 and TLR9 in the epidermis of patients with cutaneous leishmaniasis. J Dermatol Sci. 2010a;59:55–7.
Tuon FF, Fernandes ER, Pagliari C, Duarte MI, Amato VS. The expression of TLR9 in human cutaneous leishmaniasis is associated with granuloma. Parasite Immunol. 2010b;32:769–72.

Toll-Like Receptor Adaptor Protein 3

▶ Toll-like Receptor Adaptor Protein Family Members

Toll-like Receptor Adaptor Protein Family Members

Enda Shevlin and Sinéad M. Miggin
Department of Biology, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

Synonyms

Mal: Myd88-adapter-like; TLR4AP; TIRAP; Toll-interleukin 1 receptor (TIR) domain-containing adapter protein; Toll-like receptor 4 adapter protein; Wyatt
MyD88: Myeloid differentiation primary response gene 88
SARM: MyD88-5; Sterile alpha and TIR motif-containing protein
TRIF: TICAM-1; TIR-domain-containing adapter molecule 1; TIR-domain-containing adapter protein inducing IFN-beta; Toll-interleukin-1 receptor domain-containing adapter protein inducing interferon beta
TRAM: TICAM-2; TIRP; TIR-domain-containing adapter molecule 2; TRIF-related adapter molecule; Toll-like receptor adaptor protein 3; Toll/interleukin-1 receptor domain-containing protein

Historical Background

Toll-like receptors (TLRs) play a critical role in innate immunity by providing a frontline defense mechanism against invading pathogens such as bacteria, fungi and viruses. They accomplish this by recognising evolutionarily conserved pathogen-associated molecular patterns (PAMPs) which are unique to pathogens are allow the host immune system to distinguish non-self from self. For example, TLR2 and TLR4 recognise bacterial cell wall components such as lipopeptides and lipopolysaccharide (LPS) respectively, whereas TLR3 and TLR9 recognise signature viral and bacterial nucleotide sequences (Miggin and O'Neill 2006). TLRs are also activated by sterile inflammatory mediators known as danger-associated molecular patterns

(DAMPs), for example hyaluronan - an extracellular matrix fragment (Chen and Nunez 2010).

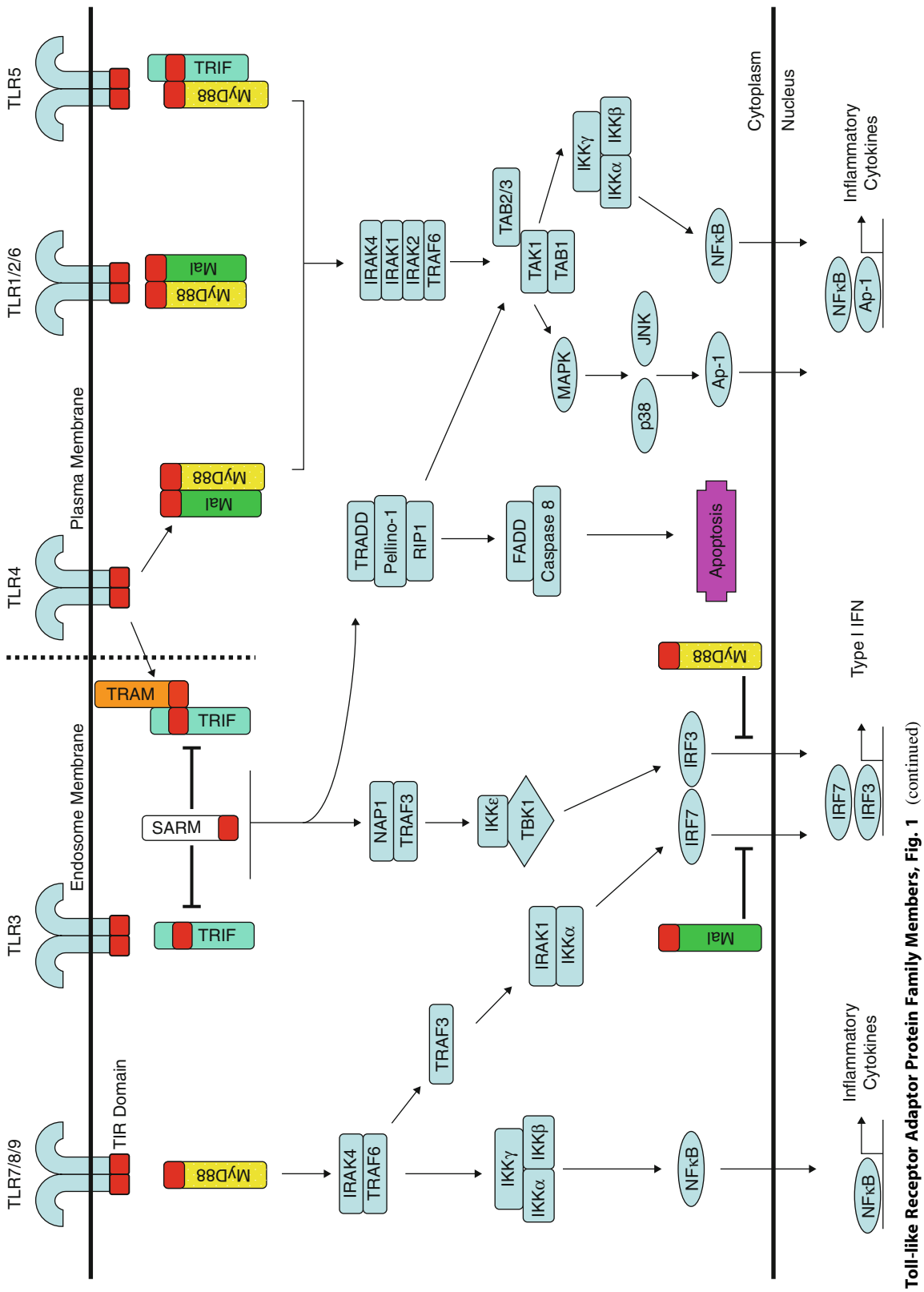
The existence of the TLRs, their adaptor proteins, as well as their functionality in the context of innate immunity commenced in 1998 and was proceeded by rapid advancements in the field thereafter. This concurred with the revival of the idea that the innate immune system and its role in inflammation were the central pivot upon which the early immune response is dictated. Each member of the TLR family senses different PAMPs and DAMPs which leads to the activation of TLR signaling, the downstream dissemination of which involves recruitment of appropriate TLR adaptor proteins to the TLR complex. The TLR adaptor proteins then couple to downstream protein kinases that ultimately lead to the activation of transcription factors such as nuclear factor-κB (▶ NF-κB) and members of the interferon (IFN)-regulatory factor (IRF) family.

A critical domain common to all five of the TLR adaptors as well as to the TLRs themselves is the Toll/interleukin-1 (IL-1) receptor (TIR) domain. This is located on the intracellular portion of all TLRs and allows binding to the reciprocal TIR domain on the adaptor's exposed surface. The IL-1 receptor also contains a TIR domain hence the existence of the TLR/IL-1R superfamily (Miggin and O'Neill 2006). Despite the TLRs having somewhat similar signal transduction pathways, there is specificity with regard to their adaptor usage (Akira and Takeda 2004) (Fig. 1). There are four *activating* TLR adaptor proteins myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (Mal); also known as Toll-IL-1 adaptor protein (TIRAP), TIR-domain-containing adaptor inducing IFN-β (TRIF; also known as TICAM-1) and TRIF-related adaptor molecule (TRAM; also known as TICAM-2 and TIRP). Despite the TLRs having somewhat similar signal transduction pathways, there is specificity with regard to their adaptor usage (Akira and Takeda 2004). MyD88 is the common downstream adaptor that is recruited by all TLRs, except TLR3 (Kawai and Akira 2006). Mal is required for TLR4, and to a lesser extent, TLR2 signaling (Yamamoto et al. 2002; Kenny et al. 2009). TRIF mediates TLR3 and TLR4 signaling (Fitzgerald et al. 2003). Finally, TRAM mediates TLR4 signaling exclusively (Fitzgerald et al. 2003) acting as a bridging adaptor to recruit TRIF to the TLR4 complex. In addition, an *inhibitory* TLR adaptor protein called sterile alpha and TIR motif-containing protein (SARM) has also been identified (Carty et al. 2006) which negatively regulates TRIF mediated signaling. TLR4 is the only TLR whose activation utilises all five TLR adaptor proteins and as a result, its signalling is split into two broad categories according to their dependency on MyD88. The 'MyD88-dependent' pathway utilises Mal and MyD88 and is responsible for early stage inflammation. It is used by TLR2/4/7/8 and TLR9. The 'MyD88 independent' pathway uses TRAM and TRIF to activate anti-viral and late-stage inflammatory responses and is used by TLR3 and TLR4. Thus, the function of the adaptor proteins is to provide specificity to TLR signalling in order to tailor the resulting cytokine profile to best defend against the infectious agent.

To summarise, TLR engagement in response to a PAMP or DAMP instigates the recruitment of the relevant TLR adaptor protein(s) which provides a docking platform for downstream effector signaling molecules. This culminates in the production of pro-inflammatory cytokines, chemokines, and antiviral type I IFNs (IFN-β and IFN-α) which serve to trigger an inflammatory and/or antiviral immune response to limit the infectious agent. The remainder of the chapter will provide a more detailed discussion of the functionality of each of the TLR adaptors.

MyD88

As previously stated, ▶ MyD88 is the common downstream adaptor that is recruited by all TLRs, except TLR3. MyD88 was first identified in 1990 as a protein that was induced during the terminal differentiation of $M1D^{+}$ myeloid precursors in response to IL-6 (O'Neill and Bowie 2007). Early studies indicated that ▶ MyD88 was a TLR adaptor molecule that functioned to recruit IL-1R-associated protein kinase (IRAK-1) to the interleukin-1 receptor (IL-1R) complex following IL-1 stimulation which resulted in the activation of ▶ NF-κB. Human ▶ MyD88 is 296 amino acids in length and contains three domains: a N-terminal death domain which enables interactions with the IRAKs, an interdomain, and a C-terminal TIR domain which facilitates homotypic interaction with other



Toll-like Receptor Adaptor Protein Family Members, Fig. 1 (continued)

TIR-containing proteins (Jenkins and Mansell 2010). In 1998, MyD88 was also implicated in TLR signaling (Medzhitov et al. 1998). In contrast to the other TLR adaptor proteins, ▶ MyD88 also mediates signaling through the IL-1 receptor. Further elaboration on ▶ MyD88 and its role in TLR signaling can be found within this encyclopedia as a separate entry.

Mal

Mal, the second TLR adaptor to be identified, was simultaneously discovered by two independent labs in 2001 (Fitzgerald et al. 2001; Horng et al. 2001). Having observed late stage ▶ NF-κB and Jun N-terminal kinase (JNK) activation in MyD88-deficient mice, Fitzgerald and colleagues (Fitzgerald et al. 2001) speculated that another, as yet unidentified TIR-domain-containing adaptor protein was mediating this effect. High-throughput sequencing of a human DC EST cDNA library identified Mal – a TIR-domain-containing protein, 235 amino acids in length, that was capable of activating ▶ NF-κB (via IRAK2) and JNK as well as extracellular signal-regulated kinase (ERK) -1 and -2. Mal was shown to homodimerize and heterodimerize with ▶ MyD88. It was also shown that a dominant-negative form of Mal inhibited TLR4 (but not IL-1R or IL-18R) mediated ▶ NF-κB activation (Fitzgerald et al. 2001; Horng et al. 2001). It is generally accepted that Mal acts a bridging adaptor between MyD88 and TLR4/2.

Mal Localization

Mal is localized primarily to the plasma membrane, although Mal-positive, actin-negative vesicles can be found throughout the cell (Kagan and Medzhitov 2006). Mal is concentrated at the leading edge of murine embryonic fibroblasts (MEFs). In macrophages, Mal is localized to discrete regions of the plasma membrane called membrane ruffles which are biochemically similar to the leading edge of fibroblasts (Kagan and Medzhitov 2006).

Mal interacts with the TIR domain of TLR2 and TLR4. This association is facilitated by the phosphatidylinositol 4,5-bisphosphate (PIP_2)-binding domain contained within the N-terminal region of Mal (Kagan and Medzhitov 2006). This allows Mal to target to PIP_2-rich regions of the plasma membrane which contain high levels of TLR2 and TLR4, thus facilitating their association with Mal (O'Neill and Bowie 2007). Notably, Mal is not involved in the recruitment of MyD88 to other compartments, including endosomal compartments devoid of PIP_2 (Kagan and Medzhitov 2006). Further, in Mal/MyD88 double-deficient cells, transfection of a mutant construct which incorporates a PIP_2 binding site to the C-terminus of MyD88 directs MyD88 to the plasma membrane and restores lipopolysaccharide (LPS) signaling *via* TLR4 (Kagan and Medzhitov 2006).

Surface charge distribution models of Mal, MyD88, and TLR4 have shown that the TIR domains of TLR4 and MyD88 are electropositive – and would thus be expected to repel each other under normal circumstances. However, the TIR domain of Mal is electronegative which would facilitate binding of TLR4 to MyD88 in order to transduce TLR4 signaling (O'Neill and Bowie 2007). Moreover, molecular docking experiments have suggested that Mal binds to a homodimer of TLR4 and that Mal actually competes with TRAM for TLR4 binding (Nunez Miguel et al. 2007).

Toll-like Receptor Adaptor Protein Family Members, Fig. 1 *Role of TLR adaptors in TLR signaling pathways.* MyD88 is the central adaptor in TLR signaling, capable of transducing signals mediated by all TLRs except TLR3. MyD88 does however negatively regulate TLR3-induced IRF3 activation. Regarding TLR1/2, TLR2/6, TLR4, and TLR5 signaling, MyD88 interacts with the TLR though their TIR domains (red). IRAK4 is then recruited to the complex which in turn recruits IRAK1 and/or IRAK2 via death domain (DD) interactions. TRAF6 is then recruited to activate TAK1, leading to subsequent activation of MAPK and ▶ NF-κB. In the case of TLR7/8/9 signaling, the MyD88 complex can also recruit TRAF3 which activates IRF7. TRIF is the sole adaptor involved in TLR3 signaling and recruits NAP1 and TRAF3 to activate the noncanonical IKKs, TBK1, and IKKε. These in turn phosphorylate IRF3 and IRF7 causing their translocation into the nucleus where they bind to the type I IFN (IFN-α and IFN-β) gene promoter. TRIF is also required to mediate maximal TLR5-induced ▶ NF-κB and MAPK activation by binding directly to TLR5. As part of a suspected negative feedback loop, TRIF can also mediate caspase-dependent TLR5 degradation. Mal acts as a bridge to allow TLR4 and to a lesser extent TLR2, to signal via the MyD88 pathway. Mal also negatively regulates TLR3-mediated IRF7 activation. TRAM, like Mal, is another bridging adaptor and links TLR4 to the TRIF pathway. The Mal:MyD88 and TRAM:TRIF complexes facilitate bifurcation of the TLR4 pathway to allow for the synthesis of both inflammatory cytokines and type I IFN, respectively. The fifth adaptor SARM interacts with TRIF and impairs TRIF signaling

Mal and TLR4 Signaling

Ligand engagement of TLR4, e.g., binding of LPS via MD-2 and CD14, causes TLR4 dimerization and nonexclusive interaction with Mal. Docking experiments have predicted that the Mal (and TRAM) interaction surfaces on the TLR4 dimer interface are at either side of the structure rather than at the top, a region that would be sterically hindered by the membrane (Nunez Miguel et al. 2007). The TLR4 dimer:Mal complex provides a platform allowing MyD88 to bind which then facilitates the recruitment of IRAK1 and IRAK4. Tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) is subsequently recruited and activated via an oligomerisation/auto-ubquitination event. Activated TRAF6 then recruits transforming growth factor activated kinase 1 (TAK1) and TAK1 binding protein 2 (TAB2). This complex interacts with the inhibitor of ▶ NF-κB kinase (IKK) complex, which consists of IKKα, IKKβ, and IKKγ (also known as NEMO), leading to the activation of ▶ NF-κB and subsequent activation of ▶ NF-κB-dependent genes, including the pro-inflammatory cytokines IL-1,IL-6, and TNFα (Moynagh 2008).

Mal and TLR2 Signaling

The role of Mal in TLR2 signaling is complicated by the fact that TLR2 can heterodimerize with both TLR1 and TLR6 to recognize tri- and diacylated lipopeptides, respectively. Overexpression studies have shown that Mal interacts with TLR1 and TLR2, but not TLR6 (Kenny et al. 2009). Although Mal was originally suspected to be essential for TLR2 signaling (Horng et al. 2002), more recent studies have shown that Mal plays a lesser role here when compared to TLR4 (Kenny et al. 2009). Specifically, whilst Mal is required for TLR2 signaling when exposed to low levels of *Salmonella typhimurium*, Mal is redundant at high concentrations of ligand or in response to high levels of *S. typhimurium* (Kenny et al. 2009). This suggests that the physiological role of Mal in the context of TLR2 signaling is to prime or amplify low strength bacterial signals.

Modulators of Mal Functionality

Additional levels of specificity and control are added to TLR signaling by virtue of the fact that the TLR adaptors themselves are subject to a myriad of regulatory mechanisms. Mal contains a proline, glutamic acid, serine, and threonine (PEST) domain, located at amino acids 32–72 in human Mal. PEST domains are found in short-lived proteins which undergo phosphorylation, polyubiquitination of lysine residues, and subsequent degradation via the 26S proteasome. The presence of a PEST domain in Mal would therefore suggest that it may be a target for degradation. Interestingly, suppressor of cytokine signaling 1 (SOCS-1), has been shown to inhibit LPS signaling by ubiquitinating Mal and thus targeting it for proteosomal degradation (O'Neill and Bowie 2007). The ubiquitination of Mal is facilitated by Bruton's tyrosine kinase (Btk) – a protein which is the case of Mal, performs two important functions. Specifically, Btk induces tyrosine phosphorylation of Mal, thus potentiating TLR2/4-driven ▶ NF-κB signaling. However, the same phosphorylation event provides a platform for the aforementioned SOCS-1 mediated ubquitination/degradation of Mal – thus serving to limit the over-activation of the inflammatory immune response (O'Neill and Bowie 2007). IRAK1 and IRAK4 have also been shown to phosphorylate Mal, thereby facilitating its TLR4-ligand-mediated ubiquitination and degradation; IRAK1 and IRAK4 inhibitors blocked this effect (Dunne et al. 2010). Mal has also been shown to interact with caspase-1, with cleavage of Mal by caspase-1 being required to modulate Mal functionality (Miggin et al. 2007; Ulrichts et al. 2010).

A number of studies have been carried out on a variant of Mal that contains a leucine at position 180 instead of a serine (O'Neill and Bowie 2007; Jenkins and Mansell 2010). It has been reported that Mal Ser180Leu does not associate with TLR2 and confers a protective phenotype in malaria and tuberculosis by inhibiting the inflammatory response (O'Neill and Bowie 2007). Other groups dispute this claim (Jenkins and Mansell 2010). Overall, the studies to date indicate an association between heterozygosity at Mal Ser180Leu and protection against multiple infections.

TRIF

Initially, Mal was thought to mediate the MyD88-independent pathway following TLR4 engagement, leading to IRF3 activation and delayed/late activation of ▶ NF-κB. However, given that Mal was instead shown to act as a bridging adaptor in the

MyD88-dependent pathway which was activated following TLR4/2 engagement, it remained unclear how TLR4 might mediate IFN-β induction (O'Neill and Bowie 2007).

In 2003, a third TLR adaptor, TRIF, was identified by two separate groups, by one employing database screening to identify novel TIR-domain containing proteins with the other employing a yeast two-hybrid screen using TLR3 as a bait (O'Neill and Bowie 2007). It was found that overexpression of TRIF, 712 amino acids in length, leads to the induction of the IFN-β promoter. In TRIF-deficient mice, whilst impaired TLR3 and TLR4 ligand induced IRF3 activation and concomitant IFN-β induction was observed, TLR2, TLR7 and TLR9 signaling was unaffected (O'Neill and Bowie 2007). Notably, TLR4 ligand induced ▶ NF-κB activation is completely abolished in cells deficient in both MyD88 and TRIF, indicating that TRIF is essential for "MyD88-independent" TLR4 signaling (Jenkins and Mansell 2010). Further, a germline mutation in mice termed Lps2 confirmed the role of TRIF in mediating "MyD88-independent" signaling (Jenkins and Mansell 2010).

TRIF Localization

TRIF is expressed at low levels in most tissues and cells and is diffusely localized in the cytoplasm of resting cells (Tatematsu et al. 2010). When endosomal TLR3 is activated by double strand (ds) RNA, TRIF transiently colocalizes with TLR3 and then dissociates from the receptor forming speckled structures that colocalize with downstream signaling molecules (Tatematsu et al. 2010). Upon stimulation of TLR4 with LPS, TRIF is activated by endosomal TRAM, which associates with the internalized TLR4 complex (Tatematsu et al. 2010). Thus, TRIF is indirectly recruited to TLR4 via TRAM. Also, overexpression of TRIF leads to homo-oligomerization through the TIR domain and the C-terminus, forming a complex called the TRIF signalosome (Jenkins and Mansell 2010; Tatematsu et al. 2010).

TRIF and TLR3/4 Signaling

TRIF, like Mal, has consensus TRAF6-binding motifs in the N-terminal region as well as a TIR domain (O'Neill and Bowie 2007). TRIF also has a TRAF2-binding site in the N-terminal region (Tatematsu et al. 2010) and a C-terminal receptor-interacting protein (RIP) homotypic interaction motif (RHIM) domain (O'Neill and Bowie 2007). The TIR domain of TRIF is essential for binding to the TIR domain of TLR3 and to TRAM. All of these domains serve to facilitate TRIF-mediated signaling, with each domain playing a distinct role therein.

The N-terminal region of TRIF participates in IRF3/7 activation by recruiting the IRF3-activating kinases, TANK-binding kinase 1 (TBK1), and inhibitor of ▶ NF-κB kinase ε (IKKε, also known as IKKι). NAK-associated protein 1 (NAP-1) forms part of the active kinase complex for IRF3 and serves to facilitate the association of TRIF with TBK1 and IKKε (Gauzzi et al. 2010). Upon TBK1/IKKε-mediated phosphorylation and homo/heterodimerization of the IRF3/7 complex, translocation of the phosphorylated IRF complex to the nucleus occurs. Here, it binds to both the IFN-β enhanceosome (Siednienko et al. 2010) and the IFN-stimulated response elements (ISREs) to induce the transcription of responsive genes including the type I IFN and CCL5 genes (Siednienko et al. 2011) (Fig. 1). TNF receptor-associated factor 3 (TRAF3) plays a crucial role in TLR3 signaling as various independent studies show that TRAF3 forms a complex with NAP-1 and TRIF (Fig. 1) (O'Neill and Bowie 2007).

Two separate ▶ NF-κB activation pathways bifurcate from TRIF, and these map to distinct sites at the N- and C-termini. The binding motifs in the N-terminal region of TRIF serve to recruit TRAF6 although its role in TRIF signaling remains controversial (O'Neill and Bowie 2007). Studies suggest that the participation of TRAF6 in TRIF-mediated ▶ NF-κB induction is cell type specific as TRAF6 is essential for ▶ NF-κB activation in mouse embryonic fibroblasts (MEFs), whereas poly(I:C), a TLR3 ligand, induced ▶ NF-κB activation is not impaired in TRAF6-deficient macrophages (Gauzzi et al. 2010; Sasai et al. 2010). There is a separate route to ▶ NF-κB activation involving the RHIM domain of TRIF whereby TRIF facilitates the recruitment of both RIP1 and RIP3 through this domain. Adding credence to the importance of RIP1 and RIP3 in TRIF signaling is the fact that poly(I:C)-induced ▶ NF-κB activation is completely blocked in RIP-1-deficient MEFs. In contrast, RIP3 has been shown to negatively regulate the TRIF–RIP1–▶ NF-κB pathway. Studies are ongoing to further define the role of RIP1 and RIP3 in TLR signaling (O'Neill and Bowie 2007).

TRIF also mediates the induction of apoptosis through TLR3 and TLR4. This is facilitated by direct recruitment of RIP1 to the C-terminal RHIM domain of TRIF, and involves activation of a complex containing TRADD, FADD, and caspase-8 (O'Neill and Bowie 2007). This apoptotic pathway is believed to be responsible for bacterial-induced apoptosis of infected macrophages, bacterial-induced DC apoptosis and DC maturation (O'Neill and Bowie 2007; Jenkins and Mansell 2010).

TRIF and TLR5 Signaling

Although it was thought that TRIF mediated TLR3 and TLR4 signaling only, a number of recent studies have shown that TRIF also plays an important role in TLR5 signaling. Stimulation of human colonic epithelial cells with the TLR5 ligand flagellin, allows TLR5 and TRIF, but not TRAM, to interact and mediate TLR5-induced ▶ NF-κB and mitogen-activated protein kinase (MAPK) activation in intestinal epithelial cells (IEC) (Rhee 2011). TRIF-deficient IECs stimulated with flagellin exhibit decreased inflammatory cytokine expression when compared to their wild-type counterparts (Rhee 2011). Furthermore, TRIF-deficient mice are resistant to flagellin-mediated exacerbation of colonic inflammation and dextran sulfate sodium–induced experimental colitis. Moreover, studies have shown that TRIF-induced caspase activity causes the degradation of TLR5 (Rhee 2011) indicating that TRIF can participate in the proteolytic modification of TLR functionality at the posttranslational level. These recent findings therefore suggest that TRIF plays an important role in regulating host-microbial communication *via* TLR5 in the gut epithelium.

TRIF and Cytosolic dsRNA Detection

A further role for TRIF in innate immune signaling, independent of the TLRs, has recently been identified whereby TRIF appears to be an essential component of a novel dsRNA sensing pathway in DCs (Zhang et al. 2011). More specifically, the RNA helicases DDX1, DDX21, and DHX36 form a complex which enables the sequestration of cytosolic dsRNA which is then followed by binding to TRIF and subsequent induction of type I IFN and inflammatory cytokine responses. It has been shown that DDX1 binds dsRNA via its helicase A domain and that DHX36 and DDX21 bind to TRIF via their HA2-DUF and PRK domains, respectively. The resulting complex triggers the innate antiviral response (Zhang et al. 2011).

Negative Regulation of TRIF

Numerous strategies exist to curtail TRIF signaling, either directly, or via inhibition of downstream signaling molecules. In terms of direct inhibition of TRIF, a number of molecules have been identified. For example, the inhibitory TLR adaptor protein SARM contains a TIR domain and serves to inhibit TRIF-mediated signaling. SARM has been shown to interact with TRIF and both the TIR and SAM domains of SARM are vital for SARM's functionality in this regard. While the exact mechanism of inhibition has not been elucidated, it is suspected that SARM and TRIF interact via their TIR domains, thus preventing the binding of downstream molecules such as RIP1. Alternatively, the SAM domain of SARM may facilitate recruitment of an, as yet unidentified, inhibitory molecule (Carty et al. 2006; Jenkins and Mansell 2010).

Consistent with a role for TRIF in restricting viral replication through type I IFN induction, at least two viruses have been shown to contain proteins that antagonize TRIF. The vaccinia virus (VACV) encoded proteins, A46R and A52R, differentially affect TRIF signaling. More specifically, A46R interacts directly with TRIF and inhibits TRIF mediated TLR3 signaling. Notably, A46R also interacts with the other TLR adaptors and also inhibits TLR4 signaling. In contrast, A52R acts downstream of the TLR adaptors by targeting TRAF6 and IRAK2 (O'Neill and Bowie 2007). Hepatitis C (HCV) virus contains a serine protease NS3-4A that causes the proteolysis of TRIF. The cleavage of TRIF by NS3-4A inhibits both ▶ NF-κB and IRF3 activation by TLR3, thus disabling the innate immune response to the virus (Jenkins and Mansell 2010). The above examples illustrate the importance of TRIF in mediating the anti-viral signalling pathway such that specific inhibition by VACV and HCV confers an advantage to the viruses in vivo.

TRAM

In 2003, the fourth TIR-domain-containing adaptor, TRAM, 235 amino acids in length, was identified following a bioinformatic search of the human genome database (Jenkins and Mansell 2010). It was initially

thought that TRAM was involved in both TLR and IL-1R mediated ▶ NF-κB activation, but not IFN-β induction (Bin et al. 2003). Subsequently, a definitive description of TRAM showed that it interacts with TLR4 and TRIF to regulate TLR4-mediated IRF3 and IRF7 activation (Fitzgerald et al. 2003). TRAM-deficient cells have impaired TLR4-mediated cytokine production and B cell activation, as was observed with TRIF, thus supporting the notion that in the case of TLR4, both the "MyD88-dependent" and "MyD88-independent" pathways are integral for maximal production of pro-inflammatory cytokines. It is now accepted that TRAM acts as bridging adaptor between TLR4 and TRIF in the "MyD88-independent" pathway (Jenkins and Mansell 2010).

TRAM Localization and Involvement in TLR4 Signaling

TRAM exclusively mediates TLR4 signaling. It activates the "MyD88-independent" pathway by facilitating the association of TRIF with TLR4 - similar to the way in which Mal links TLR4 and MyD88. To date, it serves no other known role in TLR signaling. Regarding localization, the N-terminal region of TRAM undergoes constitutive myristoylation, thus facilitating its association with the plasma membrane (Jenkins and Mansell 2010). Moreover, mutation of the myristoylation motif in TRAM abolishes its ability to signal (O'Neill and Bowie 2007).

A distinct requirement for TRAM signaling to occur is the phosphorylation of TRAM on serine 16 by protein kinase Cε (PKCε) (Jenkins and Mansell 2010). TRAM has also been shown to contain a bipartite sorting signal that modulates its trafficking between the plasma membrane and the endosomes. In fact, TRAM must be delivered to the endosomes in a complex with TLR4 to facilitate the activation of IRF3 (Jenkins and Mansell 2010). Thus, activation of TLR4 sequentially induces two signaling pathways from two different cellular locations. The "MyD88-dependent" pathway is induced from the plasma membrane, whereas the "MyD88-independent" pathway is induced from endosomes. Further, these findings suggest that the ability of TLRs to induce an IFN response is dependent on their intracellular localization (Jenkins and Mansell 2010).

Negative Regulation of TRAM

VACV is capable of modulating TRAM functionality. Specifically, an 11-aa-long peptide derived from A46R (termed viral inhibitor peptide of TLR4, or VIPER) has been shown to interact with TRAM (and Mal), thus inhibiting TLR4 signaling. It has been postulated that masking of the critical binding sites on Mal and TRAM specifically inhibits TLR4 signaling (Lysakova-Devine et al. 2010). Also, a splice variant of TRAM, termed TRAM adaptor with GOLD domain (TAG), has been shown to competitively bind TRAM and displace TRIF during LPS-mediated signaling, leading to decreases in RANTES cytokine production without affecting ▶ NF-κB activation (Palsson-McDermott et al. 2009).

SARM

SARM was initially identified in 2001 as a human gene encoding an orthologue of a *Drosophila melanogaster* protein. Structurally, SARM is 690 amino acids in length and contains two sterile alpha motifs (SAM) domains as well as a HEAT/Armadillo repeat motif (ARM) domain (O'Neill and Bowie 2007). Both the SAM and ARM domains are known to be involved in the formation of protein complexes. It was initially shown that the *Caenorhabditis elegans* SARM homologue, TIR1, was important in the efficient immune response against fungal and bacterial infection (O'Neill and Bowie 2007). However, in human cells, it was also shown that unlike the other TIR-domain adaptors, overexpression of SARM failed to induce ▶ NF-κB or activate IRF3-dependent reporter genes and in fact inhibited their activation and expression. (Carty et al. 2006). Human SARM was identified as a negative regulator of TRIF mediated signalling and was therefore the first TIR-domain-containing adaptor shown to be involved in the negative regulation of TLR signaling (Carty et al. 2006). In contrast, a later study showed that macrophages from SARM knockout mice responded normally to TLR3, TLR4, and TLR9 ligands, suggesting that mouse SARM has a redundant role in regulating macrophage responses to these TLR ligands (Jenkins and Mansell 2010). Further research must be undertaken to definitively assign a role for SARM in TLR signaling.

SARM and TLR3/4 Signaling

Although disputed, it appears that in humans, SARM inhibits TRIF-mediated TLR3 and TLR4 signaling by selectively targeting TRIF. In unstimulated cells,

SARM and TRIF are weak interactors, but stimulation with LPS or poly(I:C) induces SARM protein expression and enhances the interaction between SARM and TRIF (Carty et al. 2006). SARM overexpression serves to inhibit TRIF-dependent, but not MyD88- or Mal-dependent, ▶ NF-κB activation. SARM also inhibits poly(I:C)-mediated CCL5 and IFN-β promoter activity. The exact mechanism utilized by SARM to impair TRIF functionality requires further investigation, however it is speculated that SARM may use its TIR domain to bind TRIF and use its SAM domains to recruit an as-yet-unidentified inhibitor. Alternatively, SARM may competitively block the ability of TRIF to directly interact with downstream signal transducers such as TBK1, RIP1, and TRAF6.

Negative Regulation of TLR Signaling by TLR Adaptors

A number of recent studies have highlighted the role of the TLR adaptors themselves in the curtailment of TLR signaling. For example, MyD88 has been shown to negatively regulate TLR3-TRIF-induced corneal inflammation through a mechanism involving JNK phosphorylation, but not p38, IRF3, or ▶ NF-κB (Johnson et al. 2008) and to inhibit TLR3-dependent IL-6 induction (Kenny et al. 2009). MyD88 has also been shown to inhibit TLR3-dependent phosphorylation of IRF3 and thus curtail TLR3-mediated IFN-β and RANTES production (Siednienko et al. 2011). Furthermore, Mal has been shown to inhibit TLR3-dependent IFN-β production through a mechanism that is distinct from MyD88 whereby Mal inhibits TLR3 ligand–mediated IRF7 activation (Siednienko et al. 2010). Mal has also been shown to inhibit TLR3-dependent IL-6 induction (Kenny et al. 2009). As already stated, SARM has been shown to inhibit TRIF-dependent TLR3 and TLR4 signaling (Carty et al. 2006).

Summary

The TLR adaptor proteins are integral modulators of TLR signaling, and consequentially, of innate and adaptive immunity. The specificity of their usage, combined with their broad downstream effects on both autocrine and paracrine immune signaling, highlights them as potential targets for therapeutic immunomodulation (O'Neill et al. 2009).

Many recent advances have been made towards a greater understanding of how TLR adaptors function in the context of innate immunity. It is evident that the TLRs and their adaptor molecules have evolved to respond appropriately to a pathogenic challenge while at the same time, retaining the ability to limit their excessive activation which could otherwise cause detrimental or deleterious damage to the host system. Indeed, many intricate mechanisms by which TLR signaling may be regulated, including adaptor sequestration, differential adaptor utilization, protein degradation, and compartmentalization, have been identified (Akira and Takeda 2010).

While TLR adaptor functionality in the context of signal initiation is well described, the mechanisms that serve to negatively regulate or control them are poorly understood. Evidence suggests that the adaptors may require tight regulation to control the immune response and thus prevent chronic inflammation. Adding credence to this hypothesis is the fact that the TLR adaptors have been shown to bifurcate between activationary and inhibitory roles in TLR signaling as seen with Mal and MyD88. Further study is therefore required to expand on the current understanding of the role played by the TLR adaptors under normal physiological conditions and whether perturbations occur during chronic inflammatory diseases.

References

Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4:499–511.

Bin LH, Xu LG, Shu HB. TIRP, a novel Toll/interleukin-1 receptor (TIR) domain-containing adapter protein involved in TIR signaling. J Biol Chem. 2003;278:24526–32.

Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat Immunol. 2006;7:1074–81.

Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol. 2010;10:826–37.

Dunne A, Carpenter S, Brikos C, Gray P, Strelow A, Wesche H, et al. IRAK1 and IRAK4 promote phosphorylation, ubiquitination, and degradation of MyD88 adaptor-like (Mal). J Biol Chem. 2010;285:18276–82.

Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. Mal (MyD88-adapter-like)

is required for toll-like receptor-4 signal transduction. Nature. 2001;413:78–83.
Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med. 2003;198:1043–55.
Gauzzi C, Del Corno M, Gessani S. Dissecting TLR3 signalling in dendritic cells. Immunobiology. 2010;215:713–23.
Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the toll signaling pathway. Nat Immunol. 2001;2:835–41.
Horng T, Barton GM, Flavell RA, Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for toll-like receptors. Nature. 2002;420:329–33.
Jenkins KA, Mansell A. TIR-containing adaptors in toll-like receptor signalling. Cytokine. 2010;49:237–44.
Johnson AC, Li X, Pearlman E. MyD88 functions as a negative regulator of TLR3/TRIF-induced corneal inflammation by inhibiting activation of c-Jun N-terminal kinase. J Biol Chem. 2008;283:3988–96.
Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls toll-like receptor signaling. Cell. 2006;125:943–55.
Kawai T, Akira S. TLR signaling. Cell Death Differ. 2006;13:816–25.
Kenny EF, Talbot S, Gong M, Golenbock DT, Bryant CE, O'Neill LA. MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. J Immunol. 2009;183:3642–51.
Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. Biochem J. 2009;420:1–16.
Lysakova-Devine T, Keogh B, Harrington B, Nagpal K, Halle A, Golenbock DT, et al. Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule. J Immunol. 2010;185:4261–71.
Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, et al. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell. 1998;2:253–8.
Miggin SM, O'Neill LA. New insights into the regulation of TLR signaling. J Leukoc Biol. 2006;80:220–6.
Miggin SM, Pålsson-McDermott E, Dunne A, Jefferies C, Pinteaux E, Banahan K, et al. NF-kB activation by the Toll-IL-1 receptor domain protein MyD88 adapter-like is regulated by caspase-1. Proc Natl Acad Sci USA. 2007;104:3372–7.
Moynagh PN. The Pellino family: IRAK E3 ligases with emerging roles in innate immune signalling. Trends Immunol. 2008;30:33–42.
Nunez Miguel R, Wong J, Westoll JF, Brooks HJ, O'Neill LA, Gay NJ, et al. A dimer of the toll-like receptor 4 cytoplasmic domain provides a specific scaffold for the recruitment of signalling adaptor proteins. PLoS One. 2007;2:e788.
O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol. 2007;7:353–64.
O'Neill LA, Bryant CE, Doyle SL. Therapeutic targeting of toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol Rev. 2009;61(2):177–97.
Palsson-McDermott EM, Doyle SL, McGettrick AF, Hardy M, Husebye H, Banahan K, et al. TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway. Nat Immunol. 2009;10:579–86.
Rhee SH. Basic and translational understandings of microbial recognition by toll-like receptors in the intestine. Neurogastroenterol Motil. 2011;17:28–34.
Sasai M, Tatematsu M, Oshiumi H, Funami K, Matsumoto M, Hatakeyama S, et al. Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the toll-like receptor 3/4 pathway. Mol Immunol. 2010;47:1283–91.
Siednienko J, Halle A, Nagpal K, Golenbock DT, Miggin SM. TLR3-mediated IFN-beta gene induction is negatively regulated by the TLR adaptor MyD88 adaptor-like. Eur J Immunol. 2010;40:3150–60.
Siednienko J, Gajanayake T, Fitzgerald KA, Moynagh P, Miggin SM. Absence of MyD88 results in enhanced TLR3-dependent phosphorylation of IRF3 and increased IFN-(beta) and RANTES production. J Immunol. 2011;186:2514–22.
Tatematsu M, Ishii A, Oshiumi H, Horiuchi M, Inagaki F, Seya T, et al. A molecular mechanism for toll-IL-1 receptor domain-containing adaptor molecule-1-mediated IRF-3 activation. J Biol Chem. 2010;285:20128–36.
Ulrichts P, Bovijn C, Lievens S, Beyaert R, Tavernier J, Peelman F. Caspase-1 targets the TLR adaptor Mal at a crucial TIR-domain interaction site. J Cell Sci. 2010;123:256–65.
Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature. 2002;420:324–9.
Zhang Z, Kim T, Bao M, Facchinetti V, Jung SY, Ghaffari AA, et al. DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. Immunity. 2011;34:866–78.

Toll-Like Receptor, type 4

► TLR4, Toll-Like Receptor 4

TP53

► p53

Tpd52 (Mouse)

► TPD52 (Tumor Protein D52)

TPD52 (Tumor Protein D52)

Austin Della-Franca, Yuyan Chen and Jennifer Anne Byrne
Children's Cancer Research Unit, Kids Research Institute, The Children's Hospital at Westmead, Westmead, NSW, Australia

Synonyms

CRHSP-28 (rat); CSPP28 (rabbit); hD52 (human); mD52 (mouse); N8 (human); PC-1 (human); PrLZ (human); R10 (quail); Tpd52 (mouse)

Historical Background and Overview

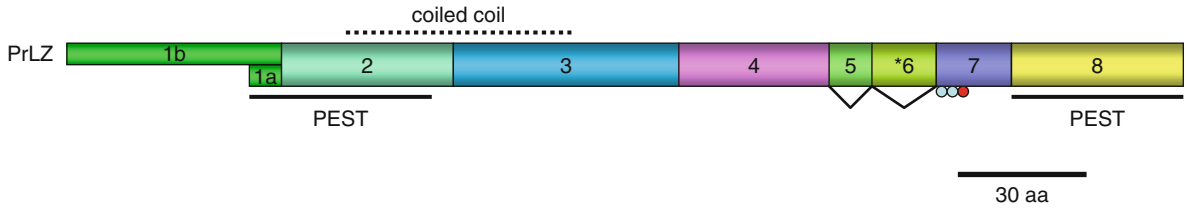
Mammalian *TPD52* sequences were first reported in the mid-1990s, through a number of independent approaches. Two early reports identified *TPD52* sequences through their increased expression in human cancer tissue or cell lines, relative to either nonmalignant tissues or fibroblast cell lines. Orthologous sequences from other species were identified as proteins phosphorylated in response to increased intracellular calcium, or as a retroviral target gene. Early reports also highlighted the existence of paralogous sequences in human and mouse, and *TPD52* is now known to be part of a four-member mammalian gene family. While TPD52-like sequences are well conserved both within and between species, they show little homology with those of other proteins.

Human TPD52 isoforms are approximately 200 residues in length, and represent largely hydrophilic peptides that lack predictors of catalytic activity. The sequences feature a coiled-coil motif of approximately 50 amino acids, and N- and C-terminally located PEST sequences, which have been linked with regulating protein stability (Fig. 1). A number of alternatively spliced *TPD52* transcripts have been either isolated or predicted. Notably, the use of an alternate gene promoter results in the inclusion of an alternate first exon, which produces a longer N-terminal domain (Fig. 1), and such isoforms have been termed PrLZ (prostate leucine zipper) or PC-1 (human prostate and colon-1). A number of internal exons may also be alternatively spliced, including an exon that encodes a putative 14-3-3 binding site (Fig. 1). Most alternative splicing events are not predicted to change protein molecular weights significantly, or to alter isoform detection through the use of C-terminally targeted TPD52 antisera. *TPD52* transcripts are also characterized by long 3'-untranslated regions, thus alternative splicing of small exons might not produce obvious changes to transcript length. Many expression microarray, Northern and Western blot analyses may have thus examined a number of TPD52 transcripts or isoforms simultaneously. For this reason, the term TPD52 will be used generically in this chapter, and isoform-specific identifiers will only be used when discussing functions proposed to be isoform specific. This chapter will also focus upon literature published from 2008, and references to other articles can be found in previous reviews (Boutros et al. 2004; Shehata et al. 2008).

Expression in Normal and Neoplastic Tissues

TPD52 is a predominantly cytoplasmic protein that is partitioned between soluble and insoluble cellular protein fractions, although a nuclear subcellular localization for TPD52 has been reported in some cell types. TPD52 is broadly expressed within normal tissues, with higher expression levels noted within epithelial tissues and mature B cells. While TPD52 expression is characteristic of differentiated cell types, TPD52 expression is also significantly increased in neoplastic tissues relative to corresponding normal tissues.

TPD52 protein is overexpressed in the majority of breast carcinoma samples, and present at increased copy number in a proportion of these cases. While TPD52 appears to be broadly overexpressed within breast carcinoma subtypes, TPD52 overexpression may be enriched in ERBB2-associated breast tumors (Natrajan et al. 2010). TPD52 overexpression has also been repeatedly identified in prostate cancer, with recent studies continuing to confirm this finding (Ummanni et al. 2008). TPD52 protein expression and gene copy number have been reported to be significantly increased in both primary and metastatic prostate cancer, and high TPD52 expression has also been detected in high-grade prostatic intraepithelial neoplasias. Multiple TPD52 isoforms have been



TPD52 (Tumor Protein D52), Fig. 1 Exon and domain organization of TPD52 isoforms. Sequence regions encoded by each exon are numbered and colored individually. The coiled-coil motif is shown as a broken line above the box diagram, whereas the PEST motifs are shown by solid lines below the diagram. Alternatively spliced exons are shown in shades of *green* (electronic version only), these being exons 1a, 1b, 5, and 6. Exon 1b encodes the longer N-terminal region characteristic of PrLZ isoforms, as indicated. Exons 5 and 6 are alternatively spliced in a cassette fashion, as indicated by diagonal lines below the sequence representation. Exon 6 encodes a putative 14-3-3 binding site, indicated by an asterisk. Examples of phosphorylation sites predicted by proteomics studies are shown as *blue* circles (electronic version, light grey in print version), and the confirmed phosphorylation site at Ser136 is shown by a *red* circle (electronic version, dark grey in print version). Note that these sites cluster after the insertion site of exons 5 and/or 6. The scale bar indicates 30 amino acid residues

shown to be overexpressed in prostate cancer, notably the alternative isoform PrLZ which derives from the use of an alternative first *TPD52* exon (Fig. 1). A number of studies have now associated increased TPD52 expression or copy number with poor patient survival in breast and other cancers. However, whereas TPD52 is also overexpressed in the majority of ovarian carcinomas, increased TPD52 expression is associated with significantly improved patient prognosis (Byrne et al. 2010). Understanding the nature of this paradox may highlight different cellular functions for TPD52 in different cancer types.

In addition to solid tumors, TPD52 overexpression has been reported in a number of different hematological malignancies, including multiple myeloma, where *TPD52* may represent an amplification target. Viral integrations within or upstream of *TPD52* genes have been identified in animal models of lymphomagenesis. Recently, increased *TPD52* copy number was reported in human cell lines of both solid and hematopoetic tumor origin, and viral integration sites were noted upstream of the *Tpd52* gene in lymphomas induced by the murine leukemia virus (Mattison et al. 2010).

Immunotherapeutic Targeting

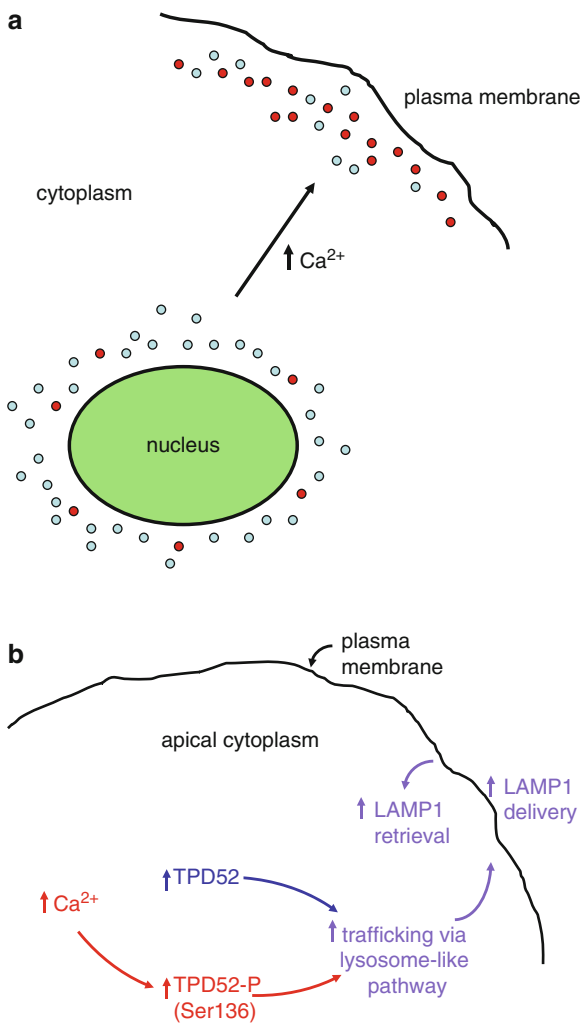
Widespread TPD52 overexpression in different cancer types, combined with the identification of TPD52 as a breast tumor antigen, suggests that TPD52 overexpression in cancer cells may produce immune responses that could be harnessed for cancer therapy. As a consequence, TPD52 overexpression has been targeted in different preclinical models using a number of vaccination strategies. The first such study employed recombinant glutathione S-transferase-TPD52 as a protein vaccine, and assessed the capacity of vaccinated animals to reject challenges of mKSA kidney tumor or 3T3.mD52 fibroblastic cells that endogenously or exogenously express *Tpd52*, respectively (Payton et al. 2008). A significant proportion of animals that had been immunized with a recombinant TPD52 protein vaccine rejected mKSA or 3T3.mD52 tumor challenges, and produced tumor-specific cytotoxic T lymphocyte responses (Payton et al. 2008). The same group then compared their previous recombinant TPD52 vaccine with a *TPD52* cDNA vaccine, in terms of rejection of subcutaneous TRAMP-C1 tumor cell challenges (Lewis et al. 2009). The DNA-based vaccine proved superior, in that immunized mice remained tumor-free for longer. Survivors of the initial tumor challenge rejected a second tumor challenge, and cytokine secretion patterns indicated that a T(H)1-type cellular immune response was involved in producing this tumor protection (Lewis et al. 2009). An independent group has employed overlapping synthetic peptides covering the TPD52 sequence to vaccinate inbred and outbred mice, which elicited specific CD8(+) and CD4(+) T-cell responses (Mirshahidi et al. 2009).

When TPD52-vaccinated BALB/c mice were then challenged with mouse breast carcinoma cells expressing endogenous TPD52, vaccinated animals showed significantly improved survival relative to unvaccinated controls (Mirshahidi et al. 2009). Notably, in all three studies, TPD52 vaccination was not accompanied by evidence of autoimmunity (Payton et al. 2008; Lewis et al. 2009; Mirshahidi et al. 2009), and thus TPD52-based vaccination may be a safe therapeutic strategy to test in human patients.

Cellular Functions

TPD52 expression has been associated with apparently opposing cellular functions typical of differentiated and cancer cells, respectively. On one hand, TPD52 positively regulates exocytosis, a physiological function of differentiated cell types, whereas TPD52 expression also promotes cell proliferation, anchorage-independent growth, and tissue invasion. A challenge for future research will be to determine whether the same or different molecular functions underpin these divergent cellular effects.

Numerous studies have linked TPD52 with exocytosis in different cell types. TPD52 orthologs were identified through being phosphorylated in response to secretory stimuli of gastric parietal and pancreatic acinar cells. Introduction of recombinant TPD52 protein into rat pancreatic acinar cells stimulated the secretion of amylase, and treatment of different cell types with secretagogue produces rapid translocation of TPD52 from supranuclear to subapical cytoplasmic compartments (Fig. 2a). However, the accumulation of TPD52 below the plasma membrane did not correspond with increased TPD52 being detected in membrane fractions (Thomas et al. 2010). TPD52 was found to promote lysosomal-associated membrane protein 1 (LAMP1) trafficking within a lysosome-like secretory pathway, and TPD52 overexpression could also enhance LAMP1 endocytosis (Thomas et al. 2010) (Fig. 2b). TPD52 has been previously found to co-localize with early endosomal markers, and has been reported in close proximity to structures resembling early endosomes in rat pancreatic acinar cells. Co-localization studies also suggest that TPD52 may be associated with endocytic compartments beyond that of the early endosome (Thomas et al. 2010).



TPD52 (Tumor Protein D52), Fig. 2 Diagrammatic representations of (**a**) the consequences of increased intracellular calcium in terms of TPD52 phosphorylation at Ser136 and subcellular localization, and (**b**) TPD52's role in LAMP1 trafficking via a lysosomal-like secretory pathway in cultured cells (from Thomas et al. 2010). (**a**) Increased intracellular calcium produces TPD52 translocation from the perinuclear and/or apical cytoplasm toward the plasma membrane, and increased TPD52 phosphorylation at Ser136. *Red* and *blue* circles (electronic version, dark and light circles in print version) represent TPD52 phosphorylated or not at Ser136, respectively. (**b**) Increased TPD52 expression and/or its calcium-dependent phosphorylation at Ser136 enhanced LAMP1 trafficking to the cell surface and retrieval. TPD52 was found to promote LAMP1 trafficking within a lysosome-like secretory pathway, and TPD52 expression also enhanced LAMP1 endocytosis. *Purple* text highlights functions which may be regulated by both increased TPD52 levels (shown in *blue*) and increased intracellular calcium and/or TPD52 phosphorylated at Ser136 (shown in *red*) Coloured text is visible in electronic version only

Cell line studies show that ectopic TPD52 expression in fibroblasts results in a transformed, metastatic phenotype, and increased PrLZ levels in prostate cancer cell lines further enhance proliferation and tumorigenicity. Increased PrLZ expression in LnCaP cells was shown to increase invasive but not migratory capacity using transwell assays, and was associated with increased cellular MMP-2 levels (Li et al. 2009). Similarly, while TPD52 knockdown reduced both migration and invasion by carcinogen-transformed bronchial epithelial cells, the effects on cellular invasion were more pronounced (Zhao et al. 2010). However, reduction in TPD52 levels also produces significant levels of apoptotic cell death in some cell lines, and so reported effects of TPD52 knockdown upon cellular migration and invasion may be indirect.

Phosphorylation

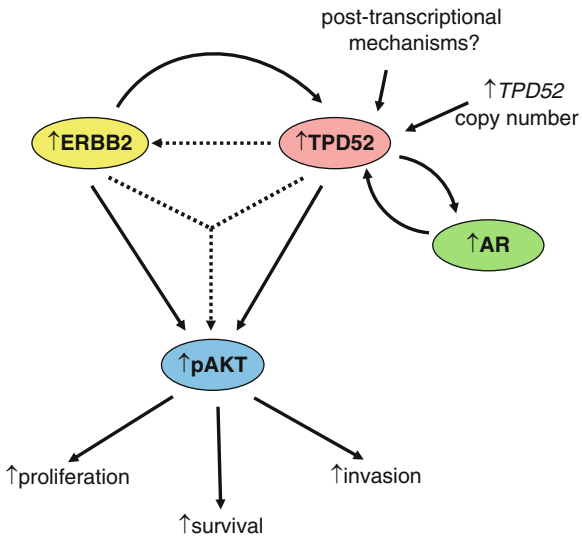
The identification of rabbit and rat TPD52 orthologs as phosphoproteins underscores the likely importance of protein phosphorylation in regulating their functions. Numerous studies indicate that TPD52 proteins from different species are phosphorylated on serine residues, and that phosphorylation occurs in response to increased intracellular calcium (Fig. 2a). Calmodulin kinase II or casein kinase II were both postulated as kinases responsible for TPD52 phosphorylation, with a calmodulin kinase II-like enzyme seeming the most likely candidate. Chew et al. (2008) identified rapid phosphorylation of TPD52 residue Ser136 (Fig. 1) in response to increased intracellular calcium, and presented evidence that the responsible kinase is a previously uncharacterized calmodulin kinase II isoform. The rapid phosphorylation of Ser136 in both HEK293 cells and mouse gastric glands was postulated to be related to exocytosis, which occurs over a similar time frame Chew et al. (2008). The identity of Ser136 as a key phosphorylation site was confirmed by Thomas et al. (2010), and accumulation of TPD52 at the plasma membrane in response to increased intracellular calcium was dependent upon Ser136 phosphorylation (Thomas et al. 2010) (Fig. 2b). Expression of a Ser136Ala TPD52 mutant also enhanced LAMP1 trafficking to the cell surface, but was not responsive to increased intracellular calcium (Thomas et al. 2010).

Signalling Functions and Binding Partners

TPD52 expression has been reproducibly associated with increased cell proliferation, and this may underpin reported associations between increased TPD52 expression in cancer, and poor patient prognosis. Numerous studies have now reported that *TPD52* transcripts are hormone inducible in breast and prostate cancer cell lines, although only isoforms produced from the alternative (*PrLZ* or *PC-1*) promoter may be hormonally regulated (Dutertre et al. 2010). Increased PrLZ expression has been previously associated with an androgen-resistant phenotype in prostate cancer cells. More recently, increased PrLZ expression in LnCaP cells was reported to increase the levels of the androgen receptor (AR) and secretion of prostate-specific antigen (Li et al. 2009). Increased TPD52 expression could contribute to an autocrine loop whereby androgen induces TPD52 expression and activity, which in turn increases AR expression (Fig. 3). In this way, increased TPD52 expression could contribute to the development of androgen resistance in prostate cancer.

A number of independent studies report positive associations between TPD52 expression and that of the receptor tyrosine kinase ERBB2 (Whiteaker et al. 2007; Natrajan et al. 2010) (Fig. 3). Accordingly, a meta-analysis of gene expression studies examining ERBB2-positive breast cancers and cell lines identified increased *TPD52* expression (Kourtidis et al. 2010). Subsequent TPD52 knockdown was associated with reduced viability of breast cancer cell lines, although these effects were not confined to ERBB2-positive cell lines (Kourtidis et al. 2010). A positive association between ERBB2 and TPD52 expression suggests that co-expression of these molecules may be more favorable than the expression of either independently (Fig. 3). That increased TPD52 levels may contribute to increased signalling downstream of ERBB2 is supported by PrLZ overexpression in LnCaP cells having been associated with increased Akt phosphorylation (Fig. 3) on Ser473 (Ummanni et al. 2008), and Thr308, as well as phosphorylation of the downstream targets GSK-3β and Raf.

How TPD52 might exert signalling functions at the molecular level is poorly understood. The primary sequence of TPD52 is largely uninformative in terms of predicting protein function, and protein partners identified to date (such as MAL2, and other



TPD52 (Tumor Protein D52), Fig. 3 Possible relationship between ERBB2, androgen receptor (AR), and TPD52 expression in cancer cells. Findings supported by the literature are shown by solid arrows, whereas proposed relationships are shown using broken arrows. Increased ERBB2 and TPD52 expression have been independently linked with increased Akt signalling. The positive relationship between ERBB2 and TPD52 expression identified through gene expression and proteomic studies is also shown. Concurrently increased ERBB2 and TPD52 expression suggests that these proteins might cooperatively increase Akt signalling, and its downstream effects. TPD52 could also regulate ERBB2 function through endocytotic mechanisms, although this is yet to be demonstrated. In addition, TPD52 expression is hormone responsive, and further elevated by other mechanisms such as gene amplification. Elevated TPD52 expression may also feed back to increase AR expression (Li et al. 2009), potentially creating an autocrine loop which contributes toward the development of androgen independence in prostate cancer

TPD52-like proteins) have undefined signalling functions themselves. TPD52 is known to associate with annexin VI, a negative regulator of EGFR signalling. Interactions between TPD52 and annexin VI have been shown to be calcium dependent, and as increased intracellular calcium also increases TPD52 phosphorylation at Ser136 Chew et al. (2008; Thomas et al. 2010), annexin VI might also bind TPD52 when phosphorylated at Ser136. While it is also tempting to speculate that TPD52 potentiates growth factor receptor signaling by negatively regulating annexin VI, such a mechanism might have more physiological than pathological relevance, as annexin VI expression may be reduced or lost in cancer cells.

While the molecular mechanism is currently unclear, a reasonable working hypothesis might be that TPD52 influences growth factor receptor signalling by modulating endocytosis (Fig. 3). Endocytosis is widely recognized as a mechanism by which receptor-mediated signalling may either be enhanced (by reducing the uptake of receptor–ligand complexes from the cell membrane, and/or promoting their recycling), or attenuated (by enhancing receptor–ligand degradation).

Summary

Despite being first identified over 15 years ago, the signalling functions of TPD52 remain largely elusive. Its expression is characteristic of both differentiated cell types and malignant cancer cells, and this may reflect different functions which predominate in physiological as opposed to pathological settings. Similarly, the differing clinical significance of TPD52 overexpression in different cancer types may reflect multiple functions for TPD52 proteins which may be selected under particular conditions. TPD52 appears to regulate both exo- and endocytosis, possibly by regulating a common function within these pathways, such as vesicle docking or fusion. That TPD52 overexpression plays a key role in tumorigenesis is evidenced by its representing an amplification target in tumors of diverse cellular origins, and a viral integration site in models of leukemia and lymphoma. To date, few studies have directly interrogated the signaling functions of this protein. More such studies will undoubtedly shed valuable light on the mechanisms by which increased TPD52 expression contributes to cellular transformation and tumor progression.

References

Boutros R, Fanayan S, Shehata M, Byrne JA. The tumor protein D52 family: many pieces, many puzzles. Biochem Biophys Res Commun. 2004;325:1115–21.

Byrne JA, Maleki S, Hardy JR, Gloss BS, Murali R, Scurry JP, et al. MAL2 and tumor protein D52 (TPD52) are frequently overexpressed in ovarian carcinoma, but differentially associated with histological subtype and patient outcome. BMC Cancer. 2010;10:497.

Chew CS, Chen X, Zhang H, Berg EA, Zhang H. Calcium/calmodulin-dependent phosphorylation of tumor protein

D52 on serine residue 136 may be mediated by CAMK2delta6. Am J Physiol Gastrointest Liver Physiol. 2008;295:G1159–72.
Dutertre M, Gratadou L, Dardenne E, Germann S, Samaan S, Lidereau R, et al. Estrogen regulation and physiopathologic significance of alternative promoters in breast cancer. Cancer Res. 2010;70:3760–70.
Kourtidis A, Jain R, Carkner RD, Eifert C, Brosnan MJ, Conklin DS. An RNA interference screen identifies metabolic regulators NR1D1 and PBP as novel survival factors for breast cancer cells with the ERBB2 signature. Cancer Res. 2010;70:1783–92.
Lewis JD, Sullivan LA, Byrne JA, de Riese W, Bright RK. Memory and cellular immunity induced by a DNA vaccine encoding self antigen TPD52 administered with soluble GM-CSF. Cancer Immunol Immunother. 2009;58:1337–49.
Li L, Zhang D, Zhang L, Zhu G, Sun Y, Wu K, et al. PrLZ expression is associated with the progression of prostate cancer LNCaP cells. Mol Carcinog. 2009;48:432–40.
Mattison J, Kool J, Uren AG, de Ridder J, Wessels L, Jonkers J, et al. Novel candidate cancer genes identified by a large-scale cross-species comparative oncogenomics approach. Cancer Res. 2010;70:883–95.
Mirshahidi S, Kramer VG, Whitney JB, Essono S, Lee S, Dranoff G, et al. Overlapping synthetic peptides encoding TPD52 as breast cancer vaccine in mice: prolonged survival. Vaccine. 2009;27:1825–33.
Natrajan R, Weigelt B, Mackay A, Geyer FC, Grigoriadis A, Tan DS, et al. An integrative genomic and transcriptomic analysis reveals molecular pathways and networks regulated by copy number aberrations in basal-like, HER2 and luminal cancers. Breast Cancer Res Treat. 2010;121:575–89.
Payton LA, Lewis JD, Byrne JA, Bright RK. Vaccination with metastasis-related tumor associated antigen TPD52 and CpG/ODN induces protective tumor immunity. Cancer Immunol Immunother. 2008;57:799–811.
Shehata M, Weidenhofer J, Thamotharampillai K, Hardy JR, Byrne JA. Tumor protein D52 overexpression and gene amplification in cancers from a mosaic of microarrays. Crit Rev Oncog. 2008;14:33–55.
Thomas DD, Martin CL, Weng N, Byrne JA, Groblewski GE. Tumor protein D52 expression and Ca2+−dependent phosphorylation modulates lysosomal membrane protein trafficking to the plasma membrane. Am J Physiol Cell Physiol. 2010;298:C725–39.
Ummanni R, Teller S, Junker H, Zimmermann U, Venz S, Scharf C, et al. Altered expression of tumor protein D52 regulates apoptosis and migration of prostate cancer cells. FEBS J. 2008;275:5703–13.
Whiteaker JR, Zhang H, Zhao L, Wang P, Kelly-Spratt KS, Ivey RG, et al. Integrated pipeline for mass spectrometry-based discovery and confirmation of biomarkers demonstrated in a mouse model of breast cancer. J Proteome Res. 2007;6:3962–75.
Zhao P, Zhong W, Ying X, Yao B, Yuan Z, Fu J, et al. Comparative proteomic analysis of anti-benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide-transformed and normal human bronchial epithelial G0/G1 cells. Chem Biol Interact. 2010;186:166–73.

TPL2

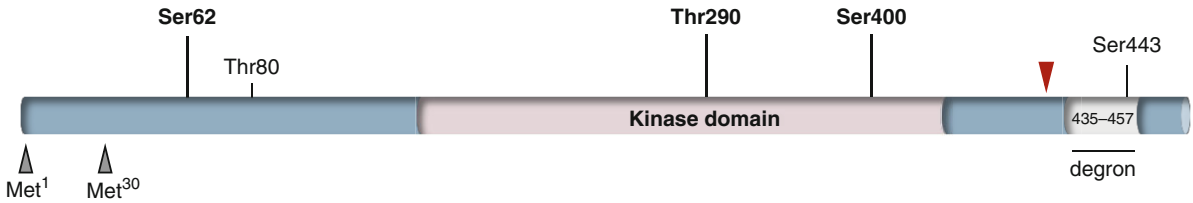
Dimitra Virla[1], Christos Tsatsanis[3] and Aristides G. Eliopoulos[1,2]
[1]Molecular & Cellular Biology Laboratory, Division of Basic Sciences, University of Crete Medical School, Heraklion, Crete, Greece
[2]Institute for Molecular Biology & Biotechnology, Foundation of Research & Technology Hellas (FORTH), Heraklion, Crete, Greece
[3]Department of Clinical Chemistry, University of Crete Medical School, Heraklion, Crete, Greece

Synonyms

Cancer Osaka thyroid oncogene; COT; Map3k8; Mitogen-activated protein kinase kinase kinase 8; Tumor progression locus 2

Historical Background

The *Tpl2* gene, also known as Cot and MAP3K8 (Vougioukalaki et al. 2011), was independently discovered by three research teams in the early 1990s. Cot (Cancer Ozaka Thyroid) was initially described by Miyoshi et al. as a putative human proto-oncogene found to have a rearranged 3′ end in the last coding exon (Miyoshi et al. 1991). The rat homologue of Cot, named *Tumor progression locus* (Tpl2) was identified by Tsichlis et al. as a proto-oncogene that is activated by provirus integration in Moloney murine leukemia (MoMuLV) virus-induced T-cell lymphomas (Patriotis et al. 1993). In mice, the *Tpl2* gene locus is also targeted by provirus insertion in mouse mammary tumor virus (MMTV)-associated mammary carcinomas (Erny et al. 1996). In both cases, provirus insertion occurs in the last intron of the gene and gives rise to a carboxy-terminally truncated protein with transforming capacity. In 1993, the normal *cot* gene was cloned and found to encode for an ORF of 467 aa that generates two protein isoforms of 58 and 52 kDa (Fig. 1) through the utilization of alternative translation initiation sites at methionine 1 and methionine 30 (Aoki et al. 1993). The homology of Tpl2 serine/threonine kinase domain to that of *Saccharomyces*



TPL2, Fig. 1 *Tpl2 primary structure and phosphorylation sites.* The normal *Tpl2* gene locus encodes two different cytoplasmic proteins (58 and 52 kDa) through the utilization of alternative translation initiation sites at methionine 1 and methionine 30. The catalytic domain of Tpl2 kinase activity is located in the center of the protein, bordered by the N-terminus and C-terminus. Several phosphorylation sites of Tpl2 have been identified within its kinase domain, including Thr^{290} and Ser^{400}. Both Thr^{290} and Ser^{400} phosphorylations are necessary but not sufficient for Tpl2 activation and occur prior to its dissociation from NF-κB1/ABIN2 complex. Ser^{62} becomes autophosphorylated and has been proposed to contribute to maximal Tpl2 activation. The C-terminus carries a reported "degron" sequence (aa 435–457) that targets Tpl2 for proteasomal degradation. NF-κB1 binds to and masks "degron" thus regulating Tpl2 stability and activity. C-terminal truncations that occur as a result of provirus insertion in MoMuLV-induced T-cell lymphomas and MMTV-induced mammary adenocarcinomas in rats (*red* arrowhead, aa 424) also remove the "degron" sequence, increasing the stability of Tpl2. The function of N-terminal region is still largely unknown

cerevisae gene product STE11 which is a mitogen-activated protein kinase kinase kinase (MAP3K) was the first indication that Tpl2 may act as a MAP3K.

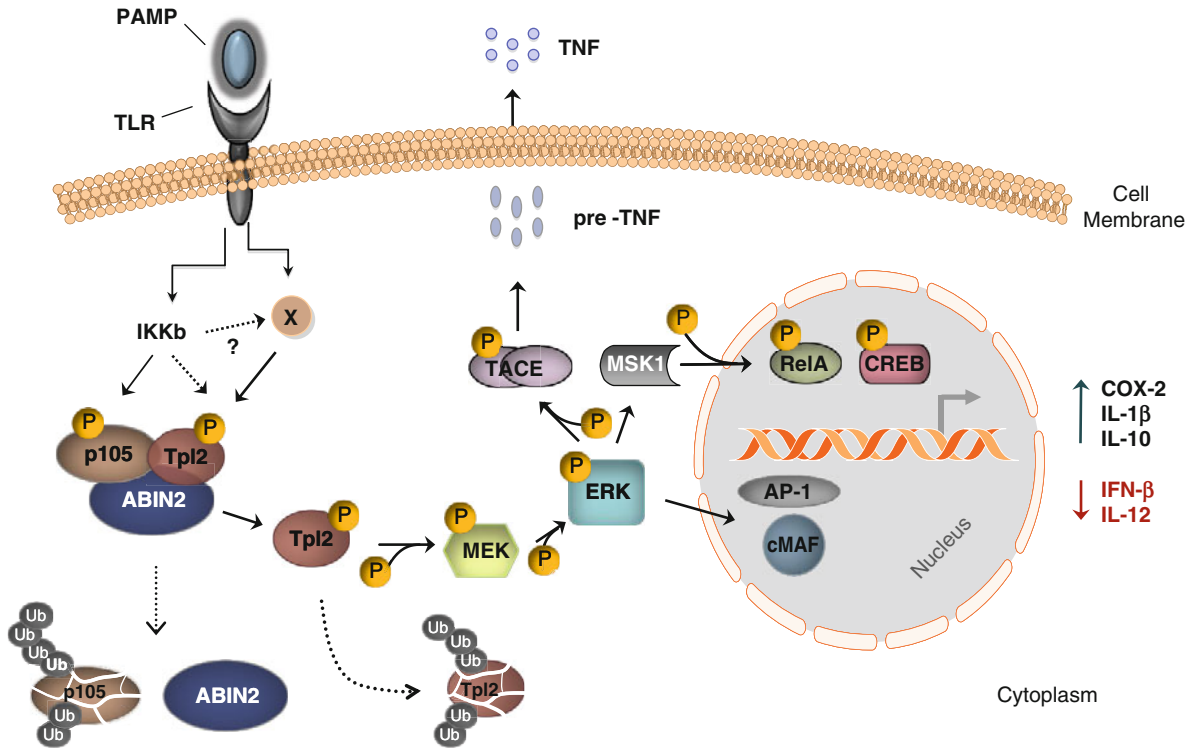
Tpl2 Signal Transduction

When overexpressed, Tpl2 activates several mitogen-activated protein kinases (MAPKs), including the extracellular-signal-regulated kinase (ERK1/2) which is its predominant target, c-Jun N-terminal kinase (JNK), and to a lesser extent p38 and ERK5 by directly phosphorylating their upstream kinases MKK4, MKK6, and MEK5, respectively (Ceci et al. 1997; Salmeron et al. 1996; Chiariello et al. 2000). Overexpression of Tpl2 also results in the transduction of ▶ NF-κB, AP-1 and ▶ NF-AT activation signals potentiating TCR-mediated transcriptional responses (Gomez-Casero et al. 2007; Lin et al. 1999; Tsatsanis et al. 1998a; Tsatsanis et al. 1998b) and in the regulation of the transcription factors c-fos and p53 (Fig. 2) (Choi et al. 2008; Khanal et al. 2009).

In comparison to wild-type Tpl2, expression of the oncogenic C-terminus-deleted Tpl2 associates with elevated catalytic activity and signaling capacity (Ceci et al. 1997). Three mechanisms have been proposed to explain this phenomenon. First, the truncated protein lacks an amino acid sequence called "degron" (aa 435-457) that targets wild-type Tpl2 for proteasomal degradation (Fig. 1) (Gandara et al. 2003). Second, the deletion has been suggested to abolish the intermolecular interaction between the C-terminal tail and the kinase domain of Tpl2, thus increasing its catalytic activity (Ceci et al. 1997). Finally, the C-terminus of Tpl2 is important for the efficient interaction with other proteins that regulate its stability and kinase activity such as the p50 ▶ NF-κB precursor NF-κB1/p105, which functions to sequester Tpl2 from its substrates (Beinke et al. 2003; Waterfield et al. 2003).

Studies in $Tpl2^{-/-}$ mice suggest that Tpl2 has an important physiological role in ERK signaling downstream of a plethora of receptors involved in innate and adaptive immunity including TLRs, CD40, TNF, and IL1 receptors (Banerjee et al. 2008; Das et al. 2005; Dumitru et al. 2000; Eliopoulos et al. 2003; Stafford et al. 2006). In addition to ERK, Tpl2 has been shown to contribute to JNK activation in TNF-stimulated fibroblasts and to be required for ▶ NF-κB transactivation by mediating the Msk1-dependent phosphorylation of the RelA (p65) ▶ NF-κB subunit at Ser^{276} (Das et al. 2005).

The precise molecular mechanisms which regulate Tpl2 activity are not fully defined. In steady state conditions, the entire pool of Tpl2 associates with p105 NF-κB1 (Beinke et al. 2003; Belich et al. 1999). Through this molecular interaction, NF-κB1 stabilizes Tpl2 by masking the "degron" sequence but also inhibits its kinase activity by preventing access to ▶ MEK1, the ERK kinase. The signal-induced phosphorylation of NF-κB1 at Ser^{927} and Ser^{932} by IKKβ leads to NF-κB1 degradation and release of Tpl2 from the complex. Liberated Tpl2 is active toward MEK1 but unstable and consequently undergoes rapid degradation via the proteasome, thus restricting prolonged activation of



TPL2, Fig. 2 *Intracellular signaling cascade involved in Tpl2 activation downstream of Toll-like receptors* (*TLRs*). The main cell signaling events involved in Tpl2 activation from ligation of TLRs by certain pathogen-associated molecular patterns (PAMPs) to translocation and/or activation of key transcription factors. At steady state, Tpl2 associates with the cytoplasmic NF-κB inhibitory protein p105 and ABIN-2. The p105-Tpl2 complex is functionally inactive and its activation involves signal-induced (PAMP) p105 degradation and the dissociation of Tpl2 from the complex. The signal-induced Tpl2 activation requires IKKβ, which phosphorylates p105 at Ser^{927} and Ser^{932} and triggers its proteolysis. Tpl2 is phosphorylated at Thr^{290} and Ser^{400} by as yet unknown kinase(s) (labeled as X) or through autophosphorylation at Ser^{62} while in complex with NF-κB1. Both Thr^{290} and Ser^{400} phosphorylations take place prior to Tpl2 dissociation from NF-κB1/ABIN2. Liberated Tpl2 is active toward MEK1 but unstable and consequently undergoes rapid degradation via the proteasome, restricting prolonged activation of MEK/ERK signaling. MEK phosphorylation of ERK results in activation of various transcription factors that may force the transcription of inflammation-related genes. ERK also mediates the phosphorylation of other kinases, such as Msk1, which contributes to the activation of the transcription factors RelA (p65 NF-κB) and CREB by phosphorylation at Ser^{276} and Ser^{133}, respectively. In addition, ERK is responsible for the post-transcriptional regulation of TNF synthesis. Pro-inflammatory cytokines, such as TNF and IL-1β, activate Tpl2 also through the IKKβ-mediated signals

ERK signaling (Waterfield et al. 2003). The A20 binding inhibitor of NFκB-2 (ABIN-2) is also important for stabilizing Tpl2 but is not required for its activation (Fig. 2) (Beinke et al. 2003; Papoutsopoulou et al. 2006).

Tpl2 is subject to phosphorylation at multiple sites including Thr^{290}, Ser^{400}, and Ser^{62} (Fig. 1) (Cho et al. 2005; Robinson et al. 2007; Stafford et al. 2006). Phosphorylations at Thr^{290} and Ser^{400} are stimulus-induced and are necessary although not sufficient for Tpl2 activation (Cho et al. 2005; Robinson et al. 2007). Both Thr^{290} and Ser^{400} phosphorylations take place prior to the dissociation of the catalytic subunit from NF-κB1/ABIN2. The phosphorylation at Thr^{290} is required for its release from p105 NF-κB1 and has been proposed to be mediated by IKKβ-dependent signals (Cho et al. 2005) or that it is an autophosphorylation event (Mieulet et al. 2010; Rousseau et al. 2008). The phosphorylation of Ser^{400} is believed to contribute to kinase activation through the induction of a conformational change which releases the inhibitory intermolecular interaction between the C-terminal tail and the kinase domain of Tpl2 (Robinson et al. 2007). Ser^{62} becomes autophosphorylated following IL-1 stimulation and has been proposed to contribute to maximal Tpl2 activation (Stafford et al. 2006).

The Functional Role of Tpl2 Kinase in Inflammation and Immunity

The establishment of Tpl2 knockout mice (Dumitru et al. 2000) allowed the analysis of the physiological role of Tpl2 kinase in inflammatory and immune responses. Tpl2-deficient mice were found to be resistant to LPS-induced endotoxic shock because of a defect in ERK activation, resulting in lower TNF-α and COX-2/prostaglandin E2 production by macrophages (Dumitru et al. 2000; Eliopoulos et al. 2002). Tpl2-deficient mice have also been studied in the context of an inflammatory bowel disease (IBD) mouse model ($TNF^{\Delta ARE}$). These mice exhibited attenuated progression of the disease, ablated TNFα-induced ERK activation in macrophages and low numbers of memory $CD4^+$ and peripheral $CD8^+$ lymphocytes showing that Tpl2 kinase regulates the lymphocytic response during progression of IBD (Kontoyiannis et al. 2002). Furthermore, Tpl2 kinase is a critical regulator of acute pancreatitis. In this case, non-myeloid expression of Tpl2 regulates pancreatic inflammation by mediating pro-inflammatory signals and the generation of neutrophil chemoattracting factors (MCP-1, MIP-2, and IL-6). The impact of Tpl2 was also shown in ligature-induced periodontitis (Ohnishi et al. 2010). Tpl2 is involved in the induction of RANKL, TNFα, COX-2, prostaglandin E2, and IL-1β and is essential for the progression of alveolar bone loss and osteoglastogenesis in periodontal tissue during experimental periodontitis (Ohnishi et al. 2010). In addition, Tpl2 kinase regulates obesity-associated metabolic dysfunction as Tpl2-defective mice exhibited improved insulin sensitivity with enhanced glucose uptake in skeletal muscle and increased suppression of glucose and lipid output in liver (Jager et al. 2009).

However, in certain disease models Tpl2 deficiency exacerbates the inflammatory response. Thus, in a mouse model of allergen (ovalbumin)-induced inflammation, $Tpl2^{-/-}$ mice produce considerably higher levels of both OVA-specific and total IgE than $Tpl2^{+/+}$ mice. It was proposed that the upregulation of IgE is correlated with increased secretion of Th2 cytokines such as IL-4 and IL-5 and decreased secretion of IFN-γ by $Tpl2^{-/-}$ T cells exposed to ovalbumin (Watford et al. 2010). The shift toward Th2 polarization of the T cell response to OVA in $Tpl2^{-/-}$ mice is in agreement with studies showing that the defense of Tpl2 knockout mice to *Toxoplama gondii* is impaired because of a T cell autonomous Th2 shift of the T cell response (Watford et al. 2008). Moreover, *Sugimoto* et al. showed that bone marrow–derived DCs from $Tpl2^{-/-}$ mice produce significantly more IL-12 in response to CpG-DNA than those from WT mice and showed Th1-skewed antigen-specific immune responses upon *Leishmania major* infection in vivo (Sugimoto et al. 2004).

Tpl2 may also participate in adaptive immune responses. Thus, it is required for the transduction of ERK activation signals initiated by CD40 engagement and contributes to IgE synthesis in response to IL-4 and anti-CD40 mAb stimulation in B lymphocytes (Eliopoulos et al. 2003). Several published reports suggest that Tpl2 also plays an important role in T cell activation and interleukin-2 production by inducing the transcription of related genes (Tsatsanis et al. 1998a, b).

The Impact of Tpl2 Kinase in Cancer

Early studies identified Tpl2 as a proto-oncogene activated by C-terminal truncation in mouse and rat. Indeed, transgenic mice expressing the truncated Tpl2 under the control of a T-cell-specific promoter develop T cell lymphoblastic lymphomas by the age of 3 months whereas expression of wild-type Tpl2 has no effect (Ceci et al. 1997). However, the role of Tpl2 in human malignancy remains enigmatic (Vougioukalaki et al. 2011). Elevated Tpl2 expression has been reported in human breast cancer, large granular lymphocyte proliferative disorders, natural killer (NK) cell lymphocytosis, gastric colon adenocarcinomas, Epstein-Barr virus (EBV)-related nasopharyngeal carcinoma, Hodgkin's disease, and other virus-related malignancies. Furthermore, increased expression and catalytic activity of Tpl2 promotes androgen depletion-independent (ADI) prostate cancer growth through the activation of the MEK/ERK signaling pathway and ▶ NF-κB (Jeong et al. 2011). In contrast, other studies suggest that Tpl2 may function as an inhibitor of tumor growth in certain cancer types. $Tpl2^{-/-}$ mice bred onto an MHC Class I-restricted T-cell antigen receptor transgenic (TCR2C) background develop $CD8^+$ T-cell lymphomas because of a defect in ERK-dependent CTLA4 induction that renders $CD8^+$ T lymphocytes to hyperproliferate in response to TCR signals. Thus, Tpl2 is required for full activation of ERK in response to TCR signals

(Tsatsanis et al. 20088). Moreover, Tpl2$^{-/-}$ mice exhibit a significantly higher incidence of tumors as well as increased signs of inflammation in chemically induced skin carcinogenesis model (Decicco-Skinner et al. 2011). In line with these findings, human melanomas carrying activating B-Raf mutations express reduced level of Tpl2 and Tpl2 copy number gains associate with resistance to Raf inhibitors (Johannessen et al. 2010). Therefore, Tpl2 may positively or negatively impact on oncogenesis depending on cell/organ type.

Summary

Tpl2 is a serine/threonine protein kinase with an obligatory role in the transduction of Toll-like receptor, death receptor, and G-protein–coupled receptor-mediated ERK MAPK signaling. Tpl2 catalytic activity and stability are closely intertwined and regulated through the interaction of Tpl2 with p105 NF-κB1 and ABIN2. The phosphorylation of Tpl2 is mainly dependent on its association with NF-κB1 and the presence of active IKKβ, an obligatory component of the canonical ▶ NF-κB pathway. The generation of Tpl2 knockout mice has contributed considerably to the delineation of the physiological role of Tpl2 in innate and adaptive immune responses. These studies have demonstrated both positive and negative effects of Tpl2 in inflammatory pathologies and tumorigenesis and warrant further analyses of the molecular mechanisms that govern its regulation and function.

References

Aoki M, Hamada F, Sugimoto T, Sumida S, Akiyama T, Toyoshima K. The human cot proto-oncogene encodes two protein serine/threonine kinases with different transforming activities by alternative initiation of translation. J Biol Chem. 1993;268:22723–32.

Banerjee A, Grumont R, Gugasyan R, White C, Strasser A, Gerondakis S. NF-kappaB1 and c-Rel cooperate to promote the survival of TLR4-activated B cells by neutralizing Bim via distinct mechanisms. Blood. 2008;112:5063–73.

Beinke S, Deka J, Lang V, Belich MP, Walker PA, Howell S, Smerdon SJ, Gamblin SJ, Ley SC. NF-kappaB1 p105 negatively regulates TPL-2 MEK kinase activity. Mol Cell Biol. 2003;23:4739–52.

Belich MP, Salmeron A, Johnston LH, Ley SC. Tpl-2 kinase regulates the proteolysis of the NF-kB-inhibitory protein NF-kB1 p105. Nature. 1999;397:363–8.

Ceci JD, Patriotis CP, Tsatsanis C, Makris AM, Kovatch R, Swing DA, Jenkins NA, Tsichlis PN, Copeland NG. Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation. Genes Dev. 1997;11:688–700.

Chiariello M, Marinissen MJ, Gutkind JS. Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation. Mol Cell Biol. 2000;20:1747–58.

Cho J, Melnick M, Solidakis GP, Tsichlis PN. Tpl2 (tumor progression locus 2) phosphorylation at Thr290 is induced by lipopolysaccharide via an Ikappa-B Kinase-beta-dependent pathway and is required for Tpl2 activation by external signals. J Biol Chem. 2005;280:20442–8.

Choi HS, Kang BS, Shim JH, Cho YY, Choi BY, Bode AM, Dong Z. Cot, a novel kinase of histone H3, induces cellular transformation through up-regulation of c-fos transcriptional activity. FASEB J. 2008;22:113–26.

Das S, Cho J, Lambertz I, Kelliher MA, Eliopoulos AG, Du K, Tsichlis PN. Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner. J Biol Chem. 2005;280:23748–57.

Decicco-Skinner KL, Trovato EL, Simmons JK, Lepage PK, Wiest JS. Loss of tumor progression locus 2 (tpl2) enhances tumorigenesis and inflammation in two-stage skin carcinogenesis. Oncogene. 2011;30:389–97.

Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell. 2000;103:1071–83.

Eliopoulos AG, Dumitru CD, Wang C-C, Cho J, Tsichlis PN. Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. EMBO J. 2002;21:4831–40.

Eliopoulos AG, Wang CC, Dumitru CD, Tsichlis PN. Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40. EMBO J. 2003;22:3855–64.

Erny KM, Peli J, Lambert JF, Muller V, Diggelmann H. Involvement of the Tpl-2/cot oncogene in MMTV tumorigenesis. Oncogene. 1996;13:2015–20.

Gandara ML, Lopez P, Hernando R, Castano JG, Alemany S. The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity. Mol Cell Biol. 2003;23:7377–90.

Gomez-Casero E, San-Antonio B, Iniguez MA, Fresno M. Cot/Tpl2 and PKCzeta cooperate in the regulation of the transcriptional activity of NFATc2 through the phosphorylation of its amino-terminal domain. Cell Signal. 2007;19:1652–61.

Jager J, Gremeaux T, Gonzalez T, Bonnafous S, Debard C, Laville M, Vidal H, Tran A, Gual P, Le Marchand-Brustel Y, Cormont M, Tanti JF. Tpl2 kinase is upregulated in adipose tissue in obesity and may mediate interleukin-1beta and tumor necrosis factor-{alpha} effects on extracellular signal-regulated kinase activation and lipolysis. Diabetes. 2009;59:61–70.

Jeong JH, Bhatia A, Z Z, Oh S, Inn KS, Liao CP, Roy-Burman P, et al. TPL2/COT/MAP3K8 (TPL2) activation promotes androgen depletion-independent (ADI) prostate cancer growth. PLoS One. 2011;6:e16205.

Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, Emery CM, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. Nature. 2010;468:968–72.

Khanal P, Lee KY, Kang KW, Kang BS, Choi HS. Tpl-2 kinase downregulates the activity of p53 and enhances signaling pathways leading to activation of activator protein 1 induced by EGF. Carcinogenesis. 2009;30:682–9.
Kontoyiannis D, Boulougouris G, Manoloukos M, Armaka M, Apostolaki M, Pizarro T, Kotlyarov A, Forster I, Flavell R, Gaestel M, Tsichlis P, Cominelli F, Kollias G. Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. J Exp Med. 2002;196:1563–74.
Lin X, Cunningham ET, Mu Y, Geleziunas R, Greene WC. The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-kB acting through the NF-kB-inducing kinase and IkB kinases. Immunity. 1999;10:271–80.
Mieulet V, Yan L, Choisy C, Sully K, Procter J, Kouroumalis A, Krywawych S, Pende M, Ley SC, Moinard C, Lamb RF. TPL-2-mediated activation of MAPK downstream of TLR4 signaling is coupled to arginine availability. Sci Signal. 2010;3:ra61.
Miyoshi J, Higashi T, Mukai H, Ohuchi T, Kakunaga T. Structure and transforming potential of the human Cot oncogene encoding a putative protein kinase. Mol Cell Biol. 1991;11:4088–96.
Ohnishi T, Okamoto A, Kakimoto K, Bandow K, Chiba N, Matsuguchi T. Involvement of Cot/Tp12 in bone loss during periodontitis. J Dent Res. 2010;89:192–7.
Papoutsopoulou S, Symons A, Tharmalingham T, Belich MP, Kaiser F, Kioussis D, O'Garra, et al. ABIN-2 is required for optimal activation of Erk MAP kinase in innate immune responses. Nat Immunol. 2006;7:606–15.
Patriotis C, Makris A, Bear SE, Tsichlis PN. Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. Proc Natl Acad Sci USA. 1993;90:2251–5.
Robinson MJ, Beinke S, Kouroumalis A, Tsichlis PN, Ley SC. Phosphorylation of TPL-2 on serine 400 is essential for lipopolysaccharide activation of extracellular signal-regulated kinase in macrophages. Mol Cell Biol. 2007;27:7355–64.
Rousseau S, Papoutsopoulou M, Symons A, Cook D, Lucocq JM, Prescott AR, O'Garra A, Ley SC, Cohen P. TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. J Cell Sci. 2008;121:149–54.
Salmeron A, Ahmad TB, Carlile GW, Pappin D, Narsimhan RP, Ley SC. Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. EMBO J. 1996;15:817–26.
Stafford MJ, Morrice NA, Peggie MW, Cohen P. Interleukin-1 stimulated activation of the COT catalytic subunit through the phosphorylation of Thr290 and Ser62. FEBS Lett. 2006;580:4010–14.
Sugimoto K, Ohata M, Miyoshi J, Ishizaki H, Tsuboi N, Masuda A, Yoshikai Y, et al. A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA-induced IL-12 production and Th cell differentiation. J Clin Invest. 2004;114:857–66.
Tsatsanis C, Patriotis C, Bear SE, Tsichlis PN. The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cells. Proc Natl Acad Sci USA. 1998a;95:3827–32.
Tsatsanis C, Patriotis C, Tsichlis PN. Tpl-2 induces IL-2 expression in T cell lines by triggering multiple signaling pathways that activate NFAT and NF-kB. Oncogene. 1998b;17:2609–18.
Tsatsanis C, Vaporidi K, Zacharioudaki V, Androulidaki A, Sykulev Y, Margioris AN, Tsichlis PN. Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells. Proc Natl Acad Sci USA. 2008;105:2987–92.
Vougioukalaki M, Kanellis DC, Gkouskou K, Eliopoulos AG. Tpl2 kinase signal transduction in inflammation and cancer. Cancer Lett. 2011;304:80–9.
Waterfield MR, Zhang M, Norman LP, Sun SC. NF-kappaB1/p105 Regulates Lipopolysaccharide-Stimulated MAP Kinase Signaling by Governing the Stability and Function of the Tpl2 Kinase. Mol Cell. 2003;11:685–94.
Watford WT, Hissong BD, Durant LR, Yamane H, Muul LM, Kanno Y, Tato CM, et al. Tpl2 kinase regulates T cell interferon-gamma production and host resistance to Toxoplasma gondii. J Exp Med. 2008;205:2803–12.
Watford WT, Wang CC, Tsatsanis C, Mielke LA, Eliopoulos AG, Daskalakis C, Charles N, Odom S, Rivera J, O'Shea J, Tsichlis PN. Ablation of tumor progression locus 2 promotes a type 2 Th cell response in ovalbumin-immunized mice. J Immunol. 2010;184:105–13.

TRA1

► Grp94 (HSP90B1)

TRAF6

Maréne Landström[1,2] and Reshma Sundar[1]
[1]Department of Medical Biosciences, Department of Pathology, Umeå University, Umeå, Sweden
[2]Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden

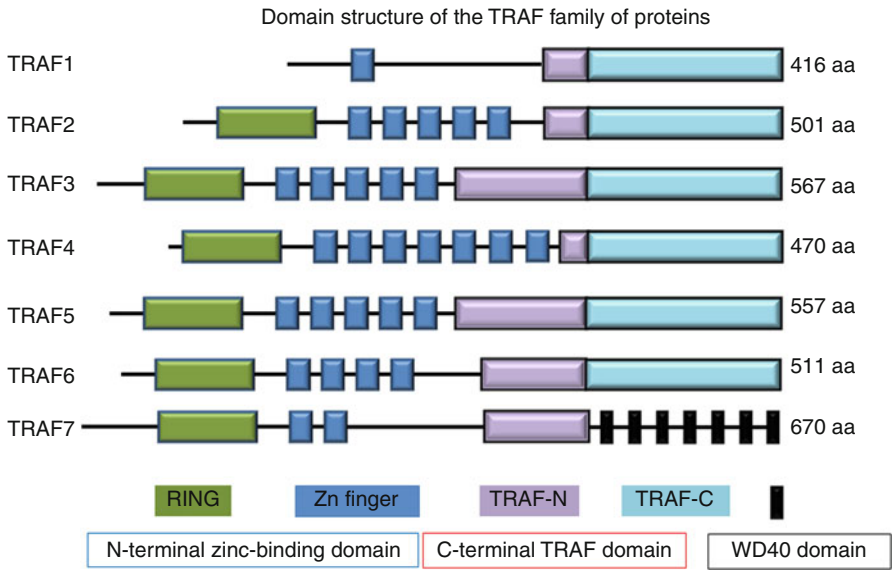
Synonyms

E3 ubiquitin-protein ligase, tumor necrosis factor receptor-associated factor 6 (TRAF6); Interleukin-1 signal transducer; Location chromosome 11p12; MGC:3310; RING finger protein 85 (RNF85)

Historical Background

TRAF Family

Tumor necrosis factor receptor-associated factors 1 and 2 (TRAFs) were initially identified as adaptor proteins that associate with the type-2 tumor necrosis factor



TRAF6, Fig. 1 Domain structures found in members of the tumor necrosis factor receptor-associated factors (TRAFs) family. TRAF1 lack the Really Interesting New Gene (RING) domain which possess ubiquitin ligase activity, while other domains found in TRAF1-TRAF7 are conserved with the exception of the C-terminal TRAF domain. In contrast, TRAF7 harbors seven WD40 domains in its C-terminal part

(TNF) receptor (TNF-R2) (Cao et al. 1996; Ishida et al. 1996; Rothe et al. 1994). The TRAF family members play important roles in the signal transduction cascades that regulate inflammatory responses via nuclear factor kappa-light-chain-enhancer of activated B cells (▶ NF-κB) and mitogen-activated protein kinases (MAPKs) that are initiated by activated cell surface receptors, such as TNF-R, interleukin 1 receptor (IL-1R), and Toll-like receptors (TLRs). The TRAFs have different cellular and physiological functions despite of their conserved C-terminal domain found in TRAF1-6 (Fig. 1). Unlike the other TRAFs, TRAF7 possesses several WD40 repeats in its C-terminal domain (Bouwmeester et al. 2004; Bradley and Pober 2001; Darnay et al. 2007; Xu et al. 2004). The TRAFs are genetically conserved in mammals (Arch et al. 1998), *Dictyostelium discoideum* (Regnier et al. 1995), *Caenorhabditis elegans* (Wajant et al. 1998), and *Drosophila* (Liu et al. 1999; Zapata et al. 2000).

In addition to inflammatory responses, TRAFs proteins regulate cell proliferation and survival. TRAF family members share a stretch of conserved sequence at their C-terminal domains known as the TRAF domain (Rothe et al. 1994). The TRAF domain is divided into a highly conserved carboxyl terminal region (C-domain), the TRAF-C sub-domain, and the coiled-coil amino terminal region and the TRAF-N sub-domain (Inoue et al. 2000). The TRAF domain mediates homo- and hetero-dimerization of TRAF family members, although TRAF4 shows a poor ability to associate with the other family members. The TRAF domain confers also direct or indirect interaction with the intracellular domain of cell surface receptors and signal transducers (Cheng et al. 1995; Kaufman and Choi 1999).

All TRAFs, except TRAF1 contain an amino terminal RING finger motif, in addition to the TRAF domain (Hsu et al. 1996). TRAF4 contains seven zinc finger domains while TRAF2, TRAF3, TRAF5, and TRAF6 have fewer zinc finger domains.

TRAF6

TRAF6 (Gene accession number of TRAF6 NM_004620, http://www.ncbi.nlm.nih.gov/gene/7189) was first identified independently as an adaptor protein, important for ▶ NF-κB activation, initiated by IL-1 and ▶ CD40. It was initially isolated using yeast two hybrid systems, using an EST expression library (Cao et al. 1996; Ishida et al. 1996). TRAF6 is a cytosolic protein although it is predominantly present in membrane bound cellular compartments (Dadgostar and Cheng 2000). TRAF6 contains a C3HC3D-type Really Interesting New Gene (RING) finger followed by five zinc finger regions in its N-terminus (Inoue et al. 2000). TRAF6 plays a specific role in innate and adaptive immune response apart from other diverse range of physiological processes (Naito et al. 1999) and (Lomaga et al. 1999) TRAF6 exists in a trimeric form at very high

concentrations. TRAF6 null mice have an abnormal phenotype of defective bone formation and die at early age (Lomaga et al. 1999; Naito et al. 1999)

TRAF6 functions as an E3 ubiquitin ligase that interact with the E2 conjugating enzyme Msm2, which consists of Ubc13 and Uev1A to synthesize lysine-63 linked polyubiquitin (Deng et al. 2000; Wang et al. 2001). The enzymatic activity of TRAF6 is promoted by its oligomerization and autoubiquitination on Lys124, which has been reported to be the key ubiquitin lysine acceptor site on TRAF6 (Bhoj and Chen 2009; Lamothe et al. 2007). Previously, it has been reported that TRAF6 interacts with tumor members of the TNFR family such as ▶ CD40 and RANK, IL-1R/Toll-like receptors (TLR) family members (Muzio et al. 1997; Suzuki et al. 2002; Wesche et al. 1999). Recent studies have demonstrated that TRAF6 also binds to the type I transforming growth factor β (TGFβ) receptor (TβRI) (Sorrentino et al. 2008).

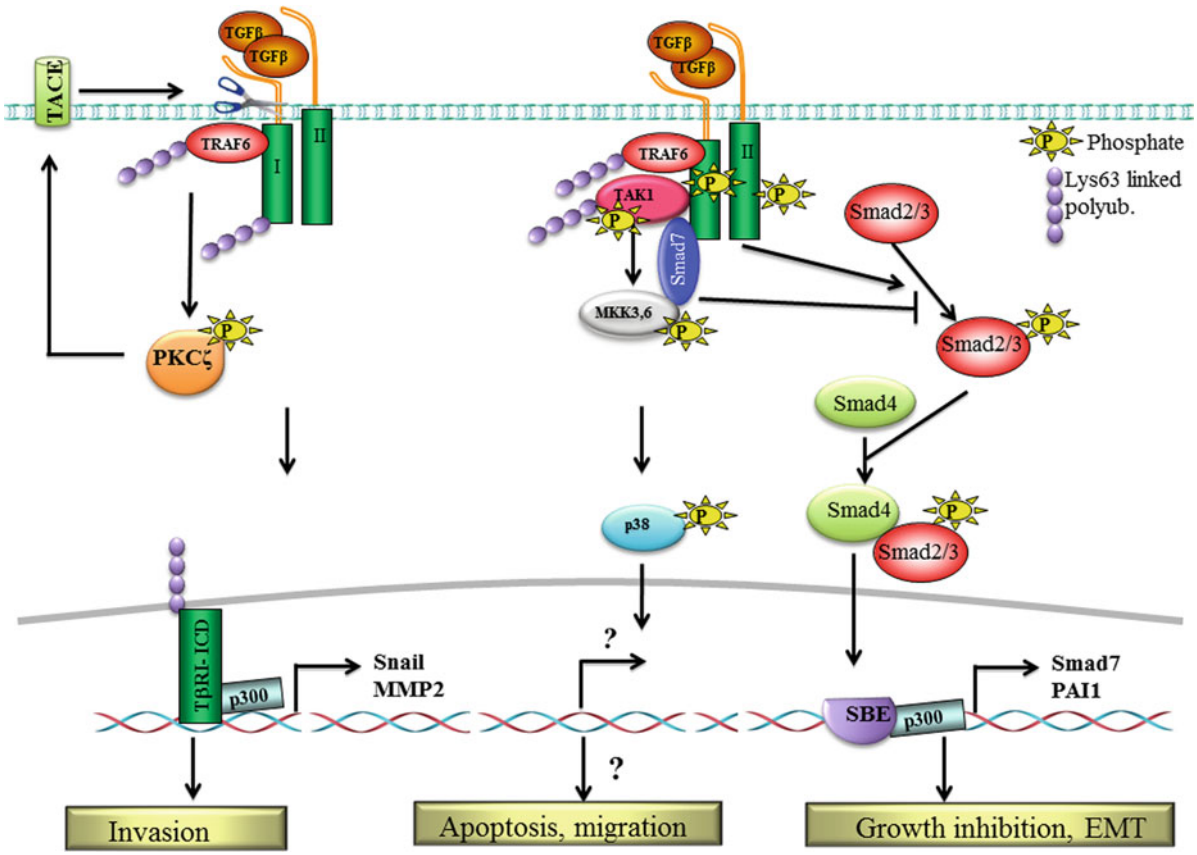
Role of TRAF6 for Smad-Independent TGFβ Signaling

TGFβ is a multifunctional cytokine involved in many critical cellular functions, such as growth arrest, differentiation, and apoptosis, which is a crucial event during embryogenesis, angiogenesis, and epithelial-mesenchymal transition (EMT) (Heldin et al. 2009; Heldin and Moustakas 2011; Massague 2008). TGFβ receptors are transmembrane serine/threonine kinase cell surface glycoproteins, which are expressed ubiquitously. TβRI is a 53 kDa protein and has a high specificity to bind to the TβRII to form a hetero-tetrameric complex. The TGFβ ligand initially binds to the TβRII, this enhances the affinity between TβRII and TβRI and thereby TβRII phosphorylates TβRI at the unique GS domain, which is located in the start of its kinase domain (Groppe et al. 2008; Wrana et al. 1994; Yamashita et al. 1994). When the receptor complex is activated, Smad-dependent and Smad-independent pathways are activated (Heldin and Moustakas 2011; Mu et al. 2011b) (Fig. 2).

TGFβ is known to transduce signals through Smad proteins as well as non-Smad mediators, like extracellular signal-regulated kinases (ERKs), the small GTPases Rho, Rac, Cdc42, c-Jun N-terminal kinases (JNKs), and mitogen-activated protein kinase (MAPK) (Derynck and Zhang 2003; Landstrom 2010; Mu et al. 2011a).(Yamaguchi et al. 1995) originally showed that transforming growth factor-β activated kinase-1 (TAK1), a member of mitogen-activated protein kinase kinase kinase (MAPKKK) family, functions as a mediator in a signaling pathway of TGFβ superfamily members. TAK1 regulates various cellular responses like cell survival through activation of JNK, p38 MAPK and inflammatory responses via IκB kinase (IKK) pathways (Adhikari et al. 2007; Landstrom 2010; Thakur et al. 2009). TAK1 is also crucial for the activation of LKB1 serine/threonine kinase which in turn control cell metabolism, growth, and polarity (Adhikari et al. 2007; Landstrom 2010; Mu et al. 2011a; Shaw 2009; Thakur et al. 2009).

Later, Sorrentino et al. 2008 and Yamashita et al. 2008 reported that TRAF6 is crucial for the activation of downstream targets p38 MAPK pathway in TGFβ signaling cascade, through its enzymatic activity as an E3 ligase. Sorrentino et al. showed that TRAF6 constitutively binds to conserved consensus motif (basic residue-X-P-X-E-X-X-aromatic/acidic acid) TGF-β type 1 receptor (TβR1) and this interaction leads to the autoubiquitination of TRAF6, in response to TGFβ-stimulation of cells which in turn causes Lys63-linked polyubiquitination of TAK1 at Lys-34 (Sorrentino et al. 2008). The kinase activity of TAK1 might be inhibited by N-terminal part of TAK1 itself (Yamaguchi et al. 1995); therefore, it is possible that this cascade of events then leads to the activation of TAK1 either due to a conformational change to open up its structure or recruitment of the TAK1 binding proteins 2 and 3 (TAB2 and 3) (Landstrom 2010; Sorrentino et al. 2008). Kim et al. (2009) reported that TGFβ-induced autoubiquitination of TAK1 recruits the adaptor molecule TAB1.

Recently, Mu et al. (2011b) showed a novel TRAF6-dependent pathway by which TGFβ mediates its oncogenic effects. TRAF6 binds to TβRI via conserved consensus site and upon TGFβ-induced autoubiquitination and activation, TRAF6 causes Lys63-linked polyubiquitination of TβRI. In cancer cells, TβRI undergoes cleavage by TNF-alpha converting enzyme (TACE) in a PKCζ-dependent manner and the intracellular domain (ICD) of cleaved TβRI translocate to the nucleus where it associates with the transcriptional regulator p300 and the snail promoter (Fig. 2). The association of TβRI ICD to p300 and the snail promoter enhances tumor invasion by induction of pro-invasive genes such as *snail* and *MMP2*. The negative regulation of TGFβ signaling



TRAF6, Fig. 2 TGFβ-induced Lys63-linked polyubiquitination and subsequent activation of the ubiquitin ligase TRAF6 causes PKCζ-dependent activation of TNF-alpha converting enzyme (TACE) which results generation of an intracellular fragment of the type I TGFβ receptor (TβRI-ICD) which enters the nucleus where it associates with components of the transcriptional complex, such as p300 and promotes expression of the pro-invasive genes *Snail* and *MMP2*. TRAF6 associates via a conserved consensus site in the TβRI to promote noncanonical and Smad-independent pathways, to cause Lys63-linked polyubiquitination and activation of the TAK1-MKK3/6-p38 MAPK pathway. In contrast, TRAF6 is not required for canonical TGFβ-induced activation of the Smad-signaling pathway, where the activated Smad-complex binds to Smad-binding elements (SBE) in their specific target genes, such as Smad7. Epithelial to mesenchymal transition (EMT) is regulated by the canonical TGFβ-induced Smad-pathway

due to the ectodomain shedding of TβRI by TACE was previously reported by Liu et al. (2009). Their report suggests that the TACE-mediated ectodomain shedding of TβRI also results in reduced TGFβ-mediated growth inhibition and EMT.

Summary

TRAF6 is an important enzyme causing Lys-linked polyubiquitination of its substrates to promote proinflammatory responses downstream of members of the TNF-R, IL-1R and TLR families. Recently, TRAF6 was also identified to be a crucial regulatory enzyme for Lys63-linked activations of TAK1 and TACE-dependent cleavage of TβRI. The identification of TRAF6 as a key regulator of TGFβ-induced tumor invasion will hopefully help to design novel inhibitors to prevent tumorigenic effects.

References

Adhikari A, Xu M, Chen ZJ. Ubiquitin-mediated activation of TAK1 and IKK. Oncogene. 2007;26:3214–26.

Arch RH, Gedrich RW, Thompson CB. Tumor necrosis factor receptor-associated factors (TRAFs)–a family of adapter proteins that regulates life and death. Genes Dev. 1998;12:2821–30.

Bhoj VG, Chen ZJ. Ubiquitylation in innate and adaptive immunity. Nature. 2009;458:430–7.

Bouwmeester T, Bauch A, Ruffner H, Angrand PO, Bergamini G, Croughton K, Cruciat C, Eberhard D, Gagneur J, Ghidelli S, Hopf C, Huhse B, Mangano R, Michon AM, Schirle M, Schlegl J, Schwab M, Stein MA, Bauer A, Casari G, Drewes G, Gavin AC, Jackson DB, Joberty G, Neubauer G, Rick J, Kuster B, Superti-Furga G. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. Nat Cell Biol. 2004;6:97–105.

Bradley JR, Pober JS. Tumor necrosis factor receptor-associated factors (TRAFs). Oncogene. 2001;20:6482–91.

Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV. TRAF6 is a signal transducer for interleukin-1. Nature. 1996;383:443–6.

Cheng G, Cleary AM, Ye ZS, Hong DI, Lederman S, Baltimore D. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. Science. 1995;267:1494–8.

Dadgostar H, Cheng G. Membrane localization of TRAF 3 enables JNK activation. J Biol Chem. 2000;275:2539–44.

Darnay BG, Besse A, Poblenz AT, Lamothe B, Jacoby JJ. TRAFs in RANK signaling. Adv Exp Med Biol. 2007;597:152–9.

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell. 2000;103:351–61.

Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003;425:577–84.

Groppe J, Hinck CS, Samavarchi-Tehrani P, Zubieta C, Schuermann JP, Taylor AB, Schwarz PM, Wrana JL, Hinck AP. Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. Mol Cell. 2008;29:157–68.

Heldin CH, Moustakas A. Role of Smads in TGFbeta signaling. Cell Tissue Res. 2011;12(6):415–27.

Heldin CH, Landstrom M, Moustakas A. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Curr Opin Cell Biol. 2009;21:166–76.

Hsu H, Shu HB, Pan MG, Goeddel DV. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell. 1996;84:299–308.

Inoue J, Ishida T, Tsukamoto N, Kobayashi N, Naito A, Azuma S, Yamamoto T. Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling. Exp Cell Res. 2000;254:14–24.

Ishida T, Mizushima S, Azuma S, Kobayashi N, Tojo T, Suzuki K, Aizawa S, Watanabe T, Mosialos G, Kieff E, Yamamoto T, Inoue J. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. J Biol Chem. 1996;271:28745–8.

Kaufman DR, Choi Y. Signaling by tumor necrosis factor receptors: pathways, paradigms and targets for therapeutic modulation. Int Rev Immunol. 1999;18:405–27.

Kim SI, Kwak JH, Na HJ, Kim JK, Ding Y, Choi ME. Transforming growth factor-beta (TGF-beta1) activates TAK1 via TAB1-mediated autophosphorylation, independent of TGF-beta receptor kinase activity in mesangial cells. J Biol Chem. 2009;284:22285–96.

Lamothe B, Besse A, Campos AD, Webster WK, Wu H, Darnay BG. Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I kappa B kinase activation. J Biol Chem. 2007;282:4102–12.

Landstrom M. The TAK1-TRAF6 signalling pathway. Int J Biochem Cell Biol. 2010;42:585–9.

Liu H, Su YC, Becker E, Treisman J, Skolnik EY. A drosophila TNF-receptor-associated factor (TRAF) binds the ste20 kinase Misshapen and activates Jun kinase. Curr Biol. 1999;9:101–4.

Liu C, Xu P, Lamouille S, Xu J, Derynck R. TACE-mediated ectodomain shedding of the type I TGF-beta receptor downregulates TGF-beta signaling. Mol Cell. 2009;35:26–36.

Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, van der Heiden A, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger JM, Paige CJ, Lacey DL, Dunstan CR, Boyle WJ, Goeddel DV, Mak TW. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev. 1999;13:1015–24.

Massague J. TGFbeta in Cancer. Cell. 2008;134:215–30.

Mu Y, Gudey SK, Landstrom M. Non-Smad signaling pathways. Cell Tissue Res. 2011a

Mu Y, Sundar R, Thakur N, Ekman M, Gudey SK, Yakymovych M, Hermansson A, Dimitriou H, Bengoechea-Alonso MT, Ericsson J, Heldin CH, Landstrom M. TRAF6 ubiquitinates TGFbeta type I receptor to promote its cleavage and nuclear translocation in cancer. Nat Commun. 2011b;2:330.

Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997;278:1612–5.

Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, Nakao K, Nakamura K, Katsuki M, Yamamoto T, Inoue J. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. Genes Cells. 1999;4:353–62.

Regnier CH, Tomasetto C, Moog-Lutz C, Chenard MP, Wendling C, Basset P, Rio MC. Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. J Biol Chem. 1995;270:25715–21.

Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell. 1994;78:681–92.

Shaw RJ. Tumor suppression by LKB1: SIK-ness prevents metastasis. Sci Signal. 2009;2(86):pe55.

Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, Zhang S, Heldin CH, Landstrom M. The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol. 2008;10:1199–207.

Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, Takada H, Wakeham A, Itie A, Li S, Penninger JM, Wesche H, Ohashi PS, Mak TW, Yeh WC. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature. 2002;416:750–6.

Thakur N, Sorrentino A, Heldin CH, Landstrom M. TGF-beta uses the E3-ligase TRAF6 to turn on the kinase TAK1 to kill prostate cancer cells. Future Oncol. 2009;5:1–3.
Wajant H, Muhlenbeck F, Scheurich P. Identification of a TRAF (TNF receptor-associated factor) gene in Caenorhabditis elegans. J Mol Evol. 1998;47:656–62.
Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature. 2001;412:346–51.
Wesche H, Gao X, Li X, Kirschning CJ, Stark GR, Cao Z. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. J Biol Chem. 1999;274:19403–10.
Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. Mechanism of activation of the TGF-beta receptor. Nature. 1994;370:341–7.
Xu LG, Li LY, Shu HB. TRAF7 potentiates MEKK3-induced AP1 and CHOP activation and induces apoptosis. J Biol Chem. 2004;279:17278–82.
Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science. 1995;270:2008–11.
Yamashita H, ten Dijke P, Franzen P, Miyazono K, Heldin CH. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J Biol Chem. 1994;269:20172–8.
Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. Mol Cell. 2008;31:918–24.
Zapata JM, Matsuzawa S, Godzik A, Leo E, Wasserman SA, Reed JC. The Drosophila tumor necrosis factor receptor-associated factor-1 (DTRAF1) interacts with Pelle and regulates NFkappaB activity. J Biol Chem. 2000;275:12102–7.

TRAIL Receptor 1/2 (Death Receptor 4/5, DR4/5)

Eun-Sil Sung and Yong-Sung Kim
Department of Molecular Science and Technology, Ajou University, Yeongtong-Gu, Suwon, South Korea

Synonyms

TRAIL-R1 (tumor necrosis factor-related apoptosis-inducing ligand receptor 1); TRAIL-R2 (tumor necrosis factor-related apoptosis-inducing ligand receptor 2)

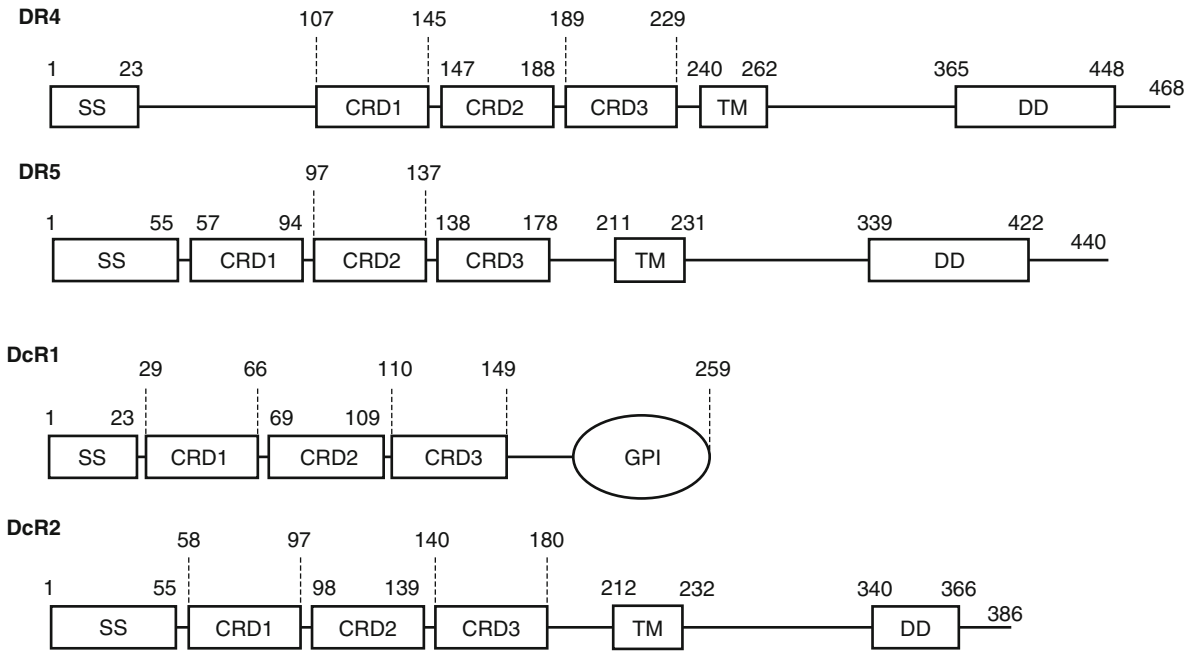
Historical Background

The *tumor necrosis factor* (*TNF*)-related apoptosis inducing ligand (TRAIL, *Apo2L*) identified in the middle of 1990s (Pitti et al. 1996; Wiley et al. 1995) was given its name due to its ability of inducing apoptosis and its high homology to other TNF member ligands. Then, its first receptor, death receptor 4 (DR4, TRAIL-R1), was identified by searching an EST database for sequences related to the *TNF receptor*-1 (*TNFR*1) death domain (DD) (Pan et al. 1997b). Thereafter, by virtue of its sequence homology to the DR4 DD, death receptor 5 (DR5, TRAIL-R2) was also cloned independently by two groups (MacFarlane et al. 1997; Walczak et al. 1997). Both DR4 and DR5 are belonging to members of the *TNF receptor superfamily (TNFRSF)*. However, unlikely with TNFRSF receptors containing the intracellular DD, such as TNFR1 and *CD95*, DR4 and DR5 are capable of transmitting tumor cell-selective apoptosis signaling upon activation by ligation with the cognate ligand of TRAIL (Ashkenazi 2008; Johnstone et al. 2008). DR4 and DR5 share a sequence identity of 58%, and their fucntions are largely redundant. However, recent studies with the receptor-specific monoclonal antibodies (mAbs) and TRAIL mutants have shown that some tumor cells undergo apoptotic cell death through signaling by either pro-apoptotic DR4 or DR5, but not both (Sung et al. 2009).

The two additional membrane receptors, decoy receptor 1 (DcR1/TRAIL-R3/TNFRSF10C/TRID) and decoy receptor 2 (DcR2/TRAIL-R4/TNFRSF10D/TRUNDD), were also identified by sequence homology search with the extracellular domain of DR4 and DR5 (MacFarlane et al. 1997; Marsters et al. 1997; Pan et al. 1997a). DcR1 completely lacks the intracellular DD and is anchored to the membrane via a glycophosphatidyl inositol (GPI) tail, whereas DcR2 is a type I transmembrane protein, but contains a truncated DD (Marsters et al. 1997). Due to the lack of functional DD, the two decoy receptors are not capable of inducing apoptosis. However, they may protect cells against TRAIL-mediated apoptosis by competing with DR4 and DR5 for binding to the ligand (Ashkenazi 2008; Johnstone et al. 2008). In addition to these four cell-surface expressed receptors, a soluble receptor called osteoprotegerin (OPG) was identified to bind to TRAIL with low affinity (Emery et al. 1998).

Structural Features of DR4 and DR5

DR4 and DR5 are single pass type I membrane proteins. The primary structural features of DR4



TRAIL Receptor 1/2 (Death Receptor 4/5, DR4/5), Fig. 1 Schematic representation of the primary structure of membrane-bound TRAIL receptors, DR4, DR5, DcR1, and DcR2 proteins. The secretion signal sequence (SS), cysteine-rich domain (CRD), transmembrane domain (TM) and intracellular death domain (DD) were highlighted in box. Numbers designate amino acids based on the following NCBI reference sequence: DR4 (NP_003835), DR5 (NP_003833), DcR1 (NP_003832), and DcR2 (NP_003831)

(468 residues) and DR5 (440 residues) include a secretion signal, three cysteine-rich domains (CRDs) in the extracellular regions, a transmembrane domain and a death domain in the intracellular regions (Fig. 1). CRD, present from 1 to 6 in all TNFSF receptors in the extracellular regions, is defined by six highly conserved cysteines that form three intrachain disulfide bridges. Both DR4 and DR5 have one partial CRD1 with only one cysteine bond and two complete CRDs, CRD2 and CRD3, with the three cysteine bonds (Cha et al. 2000). Interestingly, DR5 can be expressed in two alternatively spliced isoforms, DR5A/TRICK2A (short) and DR5B/TRICK2B (long): the short isoform of DR5A lacks 29 amino acids by missing the extracellular residues between 185 and 213 of the canonical long isoform of DR5B (Screaton et al. 1997). However, DR5A and DR5B have not shown any functional differences among each other. Secondary structural analysis of the extracellular domain of DR4 and DR5 showed ~15% β-sheet and ~85% unordered secondary structure.

Like the other four TNFRSF receptors containing the DD, the intracellular part of DR4 and DR5 contains an ~80 amino acid long, conserved domain called the DD (MacFarlane et al. 1997; Pan et al. 1997b; Walczak et al. 1997). This structural motif is essential for interaction with a key adaptor protein that is required for transmission of the death signal. Determination of the solution structure of the intracellular DDs of Fas, FADD, and TNFR1 DD by NMR spectroscopy has shown that the DD shares a common secondary structural feature, consisting of six antiparallel amphipathic α-helices packed into a globular structure, despite of low sequence homology below 30% (Fig. 2). Thus the DDs of DR4 and DR5 share less than 30% identity with those of other TNFRSF receptors (Fig. 2), which may be responsible for their different specificity in intracellar signaling through distinct protein–protein interactions. However, the DD of DR4 shares ~63% identity with that of DR5 (Fig. 2), indicative of their redundant functions.

The extracellular domain DR5 protein forms a compact 3:3 complex with TRAIL, where TRAIL forms a central homotrimer around which three DR5 molecules snuggled into long crevices between pairs of

a

```
DR4     365-448  MLFFDKFANIVPFDSWDQLMRQLD-LTKNEIDVVRAGTAG-PGDALYAMLMKWVNKTG-RNASIHTLLDALERMEERHAKEKIQDLL
DR5     339-422  RQCFDDFADLVPFDSWEPLMRKLG-LMDNEIKVAKAEAAG-HRDTLYTMLIKWVNKTG-RDASVHTLLDALETLGERLAKQKIEDHL
TNFR1   356-441  PATLYAVVENVPPLRWKEFVRRLG-LSDHEIDRLELQNGRCLREAQYSMLATWRRRTPRREATLELLGRVLRDMDLLGCLEDIEEAL
DR3     332-413  GPQLYDVMDAVPARRWKEFVRTLG-LREAEIEAVEVEIGR-FRDQQYEMLKRWRQQQP---AGLGAVYAALERMGLDGCVEDLRSRL
DR6     415-498  GIDILKLVAAQVGSQWKDIYQFLCNASEREVAAFSNGYTA-DHERAYAALQHWTIRGP--EASLAQLISALRQHRRNDVVEKIRGLM
FAS     230-314  SKYITTIAGVMTLSQVKGFVRKNG-VNEAKIDEIKNDNVQDTAEQKVQLLRNWHQLHG-KKEAYDTLIKDLKKANLCTLAEKIQTII
```

b

	TNFR1	Fas (CD95)	DR3	DR4	DR5	DR6
TNFR1	-	23.0 %	44.2 %	26.7 %	25.7 %	25.0 %
Fas (CD95)	23.0 %	-	17.9 %	16.5 %	21.7 %	18.1 %
DR3	44.2 %	17.9 %	-	26.7 %	29.4 %	22.2 %
DR4	26.7 %	16.5 %	26.7 %	-	63.1 %	22.7 %
DR5	25.7 %	21.7 %	29.4 %	63.1 %	-	19.3 %
DR6	25.0 %	18.1 %	22.2 %	22.7 %	19.3 %	-

TRAIL Receptor 1/2 (Death Receptor 4/5, DR4/5), Fig. 2 Sequence alignment (**a**) and sequence homology in percentage (**b**) of the intracellular death domain (DD) of the six TNFRSF receptors. The following NCBI reference sequence was used in the analysis : DR4 (NP_003835), DR5 (NP_003833), TNFR1 (NP_001056), Fas (CD95) (NP_000034), DR3 (NP_003781), DR6 (NP_055267). The multiple sequence alignment form and results found in UniProtKB/Swiss-Prot

monomers of the homotrimeric ligand (Cha et al. 2000). There has been no report yet about tertiary structures of DR4 alone or in complex with TRAIL.

Cell and Tissue Expression of DR4 and DR5

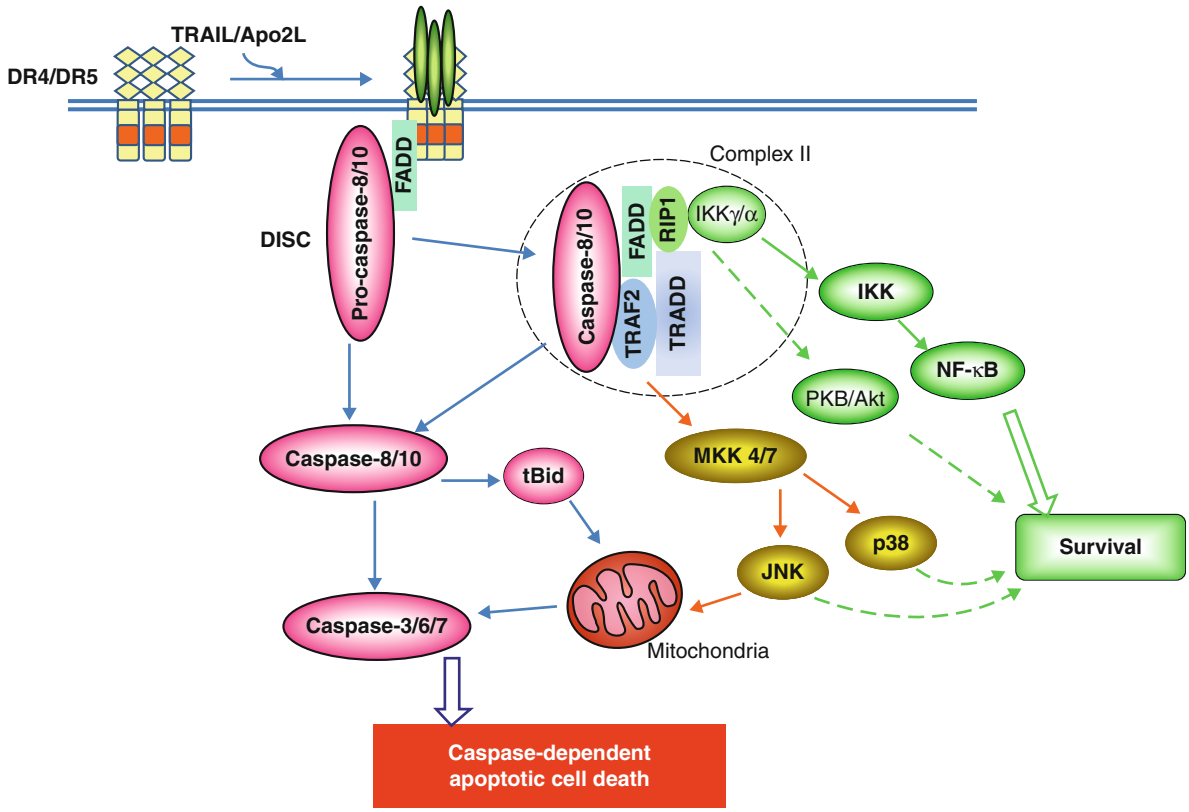
Cellular surface expression of DR4 and DR5 has been reported for a broad spectrum of cancer lines and/or primary tumor cells isolated from tissues, including lung, blood, skin, bone, breast, ovary, thyroid, and the brain (Fox et al. 2010; Johnstone et al. 2008). Weak, but detectable DR4 and/or DR5 expression has been identified by flow cytometry on the surface of normal cell types, including hepatocytes, keratinocytes, astrocytes, and osteoblasts (Fox et al. 2010; Johnstone et al. 2008). Noticeably, however, there has been no definite correlation between expression levels of DR4 and/or DR5 at both the mRNA and protein level, and the susceptibility to apoptosis by the agonist treatments (Ashkenazi 2008; Fox et al. 2010; Johnstone et al. 2008). Although most tumor cells co-express DR4 and DR5 to some degree, DR5 is expressed more widely by both tumor cells and normal tissue than DR4 (Johnstone et al. 2008). A specific physiological role for either DR4 or DR5 has yet to be defined, although the existence of two death receptors for the same ligand might suggest an essential role in tissue homeostasis.

DR4- and DR5-Mediated Signaling

TRAIL has been highlighted as a tumor selective molecule that transmits death signal via ligation to the pro-apoptotic receptors, DR4 and DR5 (Ashkenazi et al. 1999). When stimulated by either TRAIL or agonistic mAbs, DR4 and/or DR5 can mediate apoptotic cell death in most of TRAIL-sensitive cancer cells dominantly through the sequential activation of caspases (Fig. 3) (Ashkenazi 2008; Johnstone et al. 2008). Depending on the external stimuli and specific cell types, however, DR4 and/or DR5 can also transduce multiple cellular signaling pathways in a caspase-dependent or caspase-independent manner, involved in cell survival, differentiation, and death (Figs. 3 and 4) (Pennarun et al. 2010).

Caspase-Dependent Apoptosis Signaling

Binding of trimeric or multimeric TRAIL to the pro-apoptotic receptors results in the receptor clustering to activate the intracellular DD and recruit Fas-associated death domain (FADD) and then the initiator caspases, caspase-8 and/or −10, similarly to what occurs when Fas (CD95) is activated (reviewed in (Gonzalvez and Ashkenazi 2010). This primary signaling complex, consisting of ligand, receptor, FADD, and apical caspase(s), is called the death-inducing signaling complex (DISC) (Gonzalvez and Ashkenazi 2010). Activated caspase-8/10 by the DISC formation triggers



TRAIL Receptor 1/2 (Death Receptor 4/5, DR4/5), Fig. 3 The major signaling pathways of DR4 and DR5. DR4 and DR5 upon activation by the cognate ligand TRAIL multimerize and undergo some conformational change to activate their intracellular death domain to form the primary signaling complex DISC by recruiting FADD and caspase-8 and/or -10. The secondary signaling complex II can be formed, the components of which vary depending on the specific conditions, as described in detail in the text. The primary and secondary signaling complex mainly activate the initiator caspases, caspase-8 and/or -10, which subsequently activate the effector caspases, such as caspase-3, -6, and -7 via the extrinsic and intrinsic pathway, leading to the typical apoptosis. Beside activating caspases, TRAIL may stimulate intracellular kinase cascades such as NF-κB, JNK, p38 MAPK, and PKB/Akt pathways, the final outcomes of which vary depending on the cellular context and specific conditions

the extrinsic pathway by directly activating the downstream effector caspases, such as caspase-3, -6, and -7, which in turn cleave many cellular substrates to exert apoptosis (Fig. 3). The extrinsic pathway can be amplified by cross-talk with the intrinsic pathway, the link of which is Bid cleavage by activated caspase-8 (Ashkenazi 2008; Johnstone et al. 2008). Cleaved Bid (tBid) induces oligomerisation of pro-apoptotic Bax and/or Bak, leading to the release of cytochrome c and Smac/Diablo from mitochondria into cytoplasm and activation of caspase-9 (Ashkenazi 2008; Johnstone et al. 2008). The mitochondrial pathway eventually activates the effector caspases to execute apoptotic cell death. In some cells (designated Type I cells), caspase-8 activation is sufficient to activate the effector caspases to execute apoptosis via the extrinsic pathway, whereas, in other cells (designated Type II cells), amplification of the extrinsic pathway through the intrinsic (mitochondrial) pathway is needed to commit the cells to apoptotic cell death (Pennarun et al. 2010; Sung et al. 2009).

Activation of the initiator caspase-8/10 at DISC can be inhibited, subsequently decreasing the apoptosis signaling through the extrinsic and/or intrinsic pathways. The anti-apoptotic protein of cellular FLICE-inhibitory protein (c-FLIP) can also be recruited to the DISC to replace caspase-8, forming inactive DISC to inhibit the following downstream signaling. Furthermore, DcR1 and DcR2 might form ineffective DISC via heteromeric complex formation with DR4

and/DR5, leading to blocking the following apoptosis signaling (Fox et al. 2010; Pennarun et al. 2010).

In addition to the cognate ligand of recombinant TRAIL, a number of agonistic monoclonal antibodies (mAbs) have been developed by targeting DR4 or DR5, which also induce cell death in various types of tumors in vitro and in vivo (reviewed in Ashkenazi 2008; Fox et al. 2010; Johnstone et al. 2008). More recently, alternative protein scaffold based on human Kringle domain (Lee et al. 2010) and peptides (Pavet et al. 2010) isolated against DR4 or DR5 have been reported to specifically bind to activate DR4 and/or DR5, leading to tumor cell-selective apoptotic cell death. Some of them are now under various phases of clinical trials (Fox et al. 2010; Johnstone et al. 2008). The cell death mechanism of most agonistic mAbs against DR4 or DR5 has been reported to be similar to that of TRAIL, inducing caspase-dependent apoptotic cell death through forming the canonical DISC in the intracellular domain of DR4 and/or DR5 (Ashkenazi 2008).

The receptor-specific agonistic mAb studies demonstrated that, while various solid tumors were more sensitive to DR5-mediated apoptosis than DR4-induced apoptosis, primary chronic lymphocytic leukemia (CLL) cells underwent apoptotic cell death almost exclusively through DR4, not DR5 (MacFarlane et al. 2005), indicative of distinct functions of DR4 and DR5 depending on the specific type.

Kinase Activation Signaling

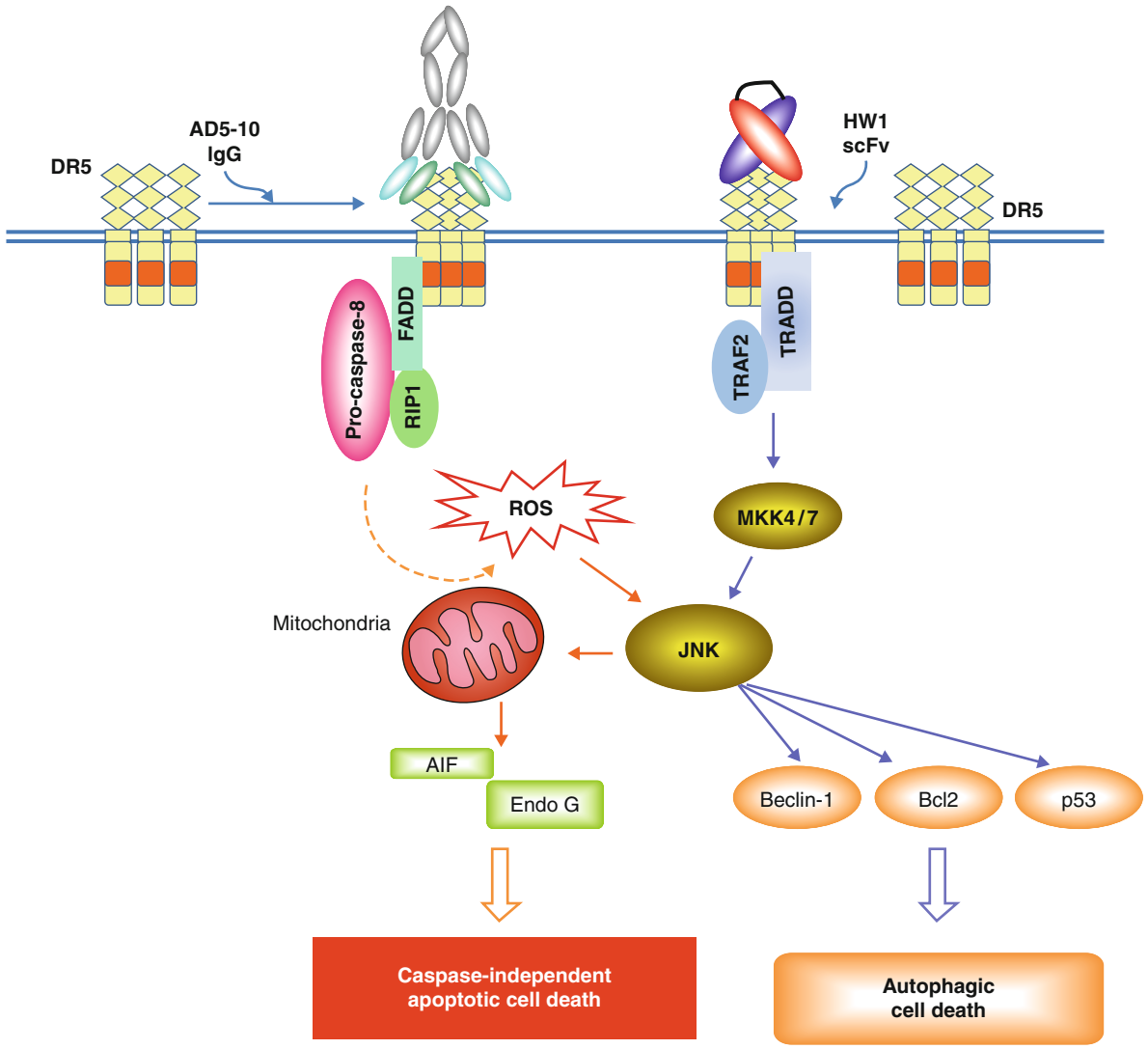
In addition to caspase activation by forming the canonical DISC, TRAIL stimulation of DR4 and/or DR5 can activate several kinase pathways, including nuclear factor (NF)-κB (NF-κB), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK), either by forming a secondary signaling complex in the downstream of DISC or by forming different signaling complex at the intracellular domain (Gonzalvez and Ashkenazi 2010; Pennarun et al. 2010).

Mühlenbeck et al. (2000) showed that TRAIL stimulation can lead to JNK activation, which occurs in a cell type-specific manner. TRAIL-induced JNK activation was dependent on caspase activation in HeLa cells, whereas it was not in Kym-1 cells, indicative of the two independent pathways leading to JNK activation. Further, JNK activation was independent of FADD in HeLa cells, suggesting another adaptor molecule to DR4/DR5 might be involved in the JNK activation. The group also showed that DR4 and DR5 have different capabilities for stimulating the JNK pathway: DR4 can signal NF-κB activation and apoptosis, whereas DR5 can signal NF-κB activation, apoptosis, and JNK activation (Muhlenbeck et al. 2000). However, detailed studies to analyze the molecular components involved in the signaling pathway had not been done.

Lin et al. (2000) had shown that RIP1 was essential for TRAIL-induced NF-κB and JNK activation, but not required for the apoptosis. Askenazi group (Varfolomeev et al. 2005) had shown in detail that TRAIL stimulation in HT1080 human fibrosarcoma cells leads to activations of kinase pathways by promoting the association of a secondary signaling complex II at the downstream of the primary DISC assembly (Fig. 3). The signaling complex II retained the DISC components FADD and caspase-8, but recruited several additional factors, such as Receptor interacting protein 1 (RIP1), TNF receptor-associated factor 2 (TRAF2), and I-κB kinase subunit gamma (IKKγ/NEMO). TRAIL stimulation of JNK and p38 further depended on receptor interacting protein 1 (RIP1) and TRAF2, whereas the inhibitor of κB kinase (IKK) activation required IKKγ/NEMO. More recently, Jin and El-Deiry (2006) showed that TRAIL can induce formation of a complex II containing FADD, RIP, IKKα, and caspase-8 and -10, leading to activation of caspase-8. Thus they showed that TRADD and caspase-10 can be recruited to the signaling complex II.

Secchiero et al. (2003) also showed that TRAIL could activate the protein kinase B (PKB/Akt) and extracellular signal-regulated kinase 1//2 (ERK1/2), without NF-κB, p38 and JNK activations in primary human umbilical vein endothelial cells (HUVECs) and aortic endothelial cells, thereby promoting the cell survival. However, the detailed molecular mechanisms are not completely understood.

Ohtsuka et al. (2003) also reported that, using agonistic anti-DR4 2E12 and anti-DR5 TRA-8 mAbs, both DR4 and DR5 have a capability to activate JNK and p38, which was mediated by MAPK kinase 4 (MKK4). The JNK/p38 signaling activated the mitochondrial apoptosis pathways, leading to caspase-dependent apoptotic cell death of breast MDA-MB-231 cell lines. However, they did not examine the signaling complex recruited to each receptor after mAb



TRAIL Receptor 1/2 (Death Receptor 4/5, DR4/5), Fig. 4 A noncanonical signaling of DR5 triggered by the agonistic antibodies. Recent studies with anti-DR5 agonistic antibodies have shown that the intracellular domain of DR5 has more potential to provide a platform for the formation of multiprotein signaling complexes, which initiating JNK-activation dependent apoptosis and autophagy. For example, mouse anti-DR5 AD5-10 mAb induced apoptotic cell death in Jurkat cells via ROS generation, sustained JNK activation, loss of mitochondrial membrane potential, and Endo G release from mitochondria to the cytosol. A human anti-DR5 scFv, HW1, activated JNK via forming a signaling complex composed of TRADD and TRAF2 at the intracellular domain of DR5 and sequentially upregulated Beclin-1 expression and induced the phosphorylation of Bcl-2 and p53, leading to DR5-mediated autophagic cell death

stimulation. Sah et al. (2003) reported that JNK activation is required for sensitization of prostate PC3 cells to TRAIL-induced apoptosis by translation inhibitors in cells that are otherwise TRAIL-resistant.

Zheng group (Chen et al. 2009) showed that, while TRAIL induces only caspase-dependent cell death, an anti-DR5 agonistic mAb, AD5-10, induces both caspase-dependent and caspase-independent cell death in Jurkat cells. AD5-10-mediated stimulation of DR5 generated reactive oxygen species (ROS) accumulation, which subsequently evoked sustained activation of JNK, loss of mitochondrial membrane potential, and release of apoptosis-inducing factor (AIF) and endonuclease G (Endo G) from mitochondria into the cytosol and then nucleus in Jurkat cells (Fig. 4) (Chen et al. 2009). Immunoprecipitation

experiments showed that AD5-10 induced assembly of DISCs containing DR5, FADD, caspase-8, and RIP in wild-type Jurkat cells. Moreover, a dominant-negative form of JNK enhanced NF-κB activation, suppressed caspase-8 recruitment in DISCs. However, how DR5 stimulation by AD5-10 induced ROS accumulation and how JNK activation impairs the function of mitochondria remain to be investigated.

Caspase-Independent Autophagy Signaling

Autophagic cell death has been involved in physiological cell death during development and reported in cancer cells treated with chemicals or irradiation. TRAIL also can induce caspase-independent autophagic cell death in normal epithelial cells and the breast cancer cell line MCF-10A (Mills et al. 2004), implicating that DR4 and/or DR5 are involved in the autophagic cell death as well as apoptosis. Park et al. (2007) recently reported that an agonistic antibody, single chain variable fragment (scFv) HW1, which specifically binds to DR5, triggered autophagic cell death of cancer cells dominantly through JNK pathway in a caspase-independent manner (Fig. 4). Analysis of the signaling complex induced by HW1 binding to DR5 exhibits the recruitment of TNF receptor (TNFR)-associated death domain (TRADD) and TRAF2 to the receptor, but not FADD, caspase-8, or RIP, which is distinct from the canonical DISC induced by TRAIL. Analyses of upstream kinase(s) for JNK activation upon HW1 binding to DR5 revealed that JNK activation was most likely mediated by TRAF2-MKK4/MKK7-dependent signaling cascade (Park et al. 2009). DR5-stimulated JNK activation by HW1 resulted in upregulation of Beclin-1 expression, Bcl-2 phosphorylation, and p53 phosphorylation, suggesting that these pro-autophagic signaling pathways are involved in the autophagic cell death (Park et al. 2009). The DR5-mediated autophagy signaling sensitized both TRAIL-sensitive and TRAIL-resistant tumor cells with much less cytotoxicity on normal cells (Park et al. 2007, 2009), providing a new strategy for the elimination of cancer cells through the nonapoptotic cell death.

Resistance to DR4- and DR5-Mediated Apoptotic Cell Death Signaling

Many highly malignant tumor cells (>50%) even expressing DR4 and/or DR5 remain resistant to TRAIL-induced and/or anti-DR4/DR5 agonistic mAb-mediated apoptosis, the underlying mechanism of which has been poorly understood and varies with the cellular context (Ashkenazi 2008; Fox et al. 2010; Johnstone et al. 2008). Potential resistance mechanisms are numerous and include loss/mutation of death receptors, overexpression of decoy receptors, overexpression of cellular FLICE inhibitory protein (c-FLIP), absence of proper *O*-glycosylation in the receptors, and/or complex downstream regulation of the extrinsic and intrinsic apoptotic pathways (Gonzalvez and Ashkenazi 2010).

Summary

The agonists against DR4 and/or DR5, including recombinant TRAIL and mAbs, have demonstrated anti-cancer activities both as monotherapy and in combination with anti-cancer agents in preclinical and clinical studies with no apparent systemic toxicity (Ashkenazi 2008; Fox et al. 2010; Johnstone et al. 2008). All of the receptor agonists in clinic have been reported to induce tumor cell death through the caspase-dependent apoptosis (Ashkenazi 2008; Johnstone et al. 2008). Depending on the external stimuli and specific cell types, however, DR4 and/or DR5 can also transduce multiple cellular signaling pathways in a caspase-dependent or caspase-independent manner, including JNK, p38, and NF-κB signaling. These distinct signaling can exert ROS-mediated apoptosis or autophagic cell death, although the components of which remain largely undefined. Recent clinical data of TRAIL and agonistic mAbs have shown much less tumor killing activities in patients than those observed in preclinical studies. Accordingly, deep understanding of the diverse molecular signaling mechanisms mediated by DR4 and/or DR5 and their cross-talks leading to a final outcome of the stimulation will be essential to design next-generation agonists targeting DR4 and/or DR5.

References

Ashkenazi A. Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat Rev Drug Discov. 2008;7:1001–12.

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest. 1999;104:155–62.

Cha SS, Sung BJ, Kim YA, Song YL, Kim HJ, Kim S, et al. Crystal structure of TRAIL-DR5 complex identifies a critical role of the unique frame insertion in conferring recognition specificity. J Biol Chem. 2000;275:31171–7.

Chen C, Liu Y, Zheng D. An agonistic monoclonal antibody against DR5 induces ROS production, sustained JNK activation and Endo G release in Jurkat leukemia cells. Cell Res. 2009;19:984–95.

Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, et al. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem. 1998;273:14363–7.

Fox NL, Humphreys R, Luster TA, Klein J, Gallant G. Tumor Necrosis Factor-related apoptosis-inducing ligand (TRAIL) Receptor-1 and Receptor-2 agonists for cancer therapy. Expert Opin Biol Ther. 2010;10:1–18.

Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene. 2010;29:4752–65.

Jin Z, El-Deiry WS. Distinct signaling pathways in TRAIL- versus tumor necrosis factor-induced apoptosis. Mol Cell Biol. 2006;26:8136–48.

Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer. 2008;8:782–98.

Lee CH, Park KJ, Sung ES, Kim A, Choi JD, Kim JS, et al. Engineering of a human kringle domain into agonistic and antagonistic binding proteins functioning in vitro and in vivo. Proc Natl Acad Sci USA. 2010;107:9567–71.

Lin Y, Devin A, Cook A, Keane MM, Kelliher M, Lipkowitz S, et al. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol. 2000;20:6638–45.

MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J Biol Chem. 1997;272:25417–20.

MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ, Cohen GM. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. Cancer Res. 2005;65:11265–70.

Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, et al. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol. 1997;7:1003–6.

Mills KR, Reginato M, Debnath J, Queenan B, Brugge JS. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation in vitro. Proc Natl Acad Sci USA. 2004;101:3438–43.

Muhlenbeck F, Schneider P, Bodmer JL, Schwenzer R, Hauser A, Schubert G, et al. The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. J Biol Chem. 2000;275:32208–13.

Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science. 1997a;277:815–8.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, et al. The receptor for the cytotoxic ligand TRAIL. Science. 1997b;276:111–3.

Park KJ, Lee SH, Kim TI, Lee HW, Lee CH, Kim EH, et al. A human scFv antibody against TRAIL receptor 2 induces autophagic cell death in both TRAIL-sensitive and TRAIL-resistant cancer cells. Cancer Res. 2007;67:7327–34.

Park KJ, Lee SH, Lee CH, Jang JY, Chung J, Kwon MH, et al. Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death. Biochem Biophys Res Commun. 2009;382:726–9.

Pavet V, Beyrath J, Pardin C, Morizot A, Lechner MC, Briand JP, et al. Multivalent DR5 peptides activate the TRAIL death pathway and exert tumoricidal activity. Cancer Res. 2010;70:1101–10.

Pennarun B, Meijer A, de Vries EG, Kleibeuker JH, Kruyt F, de Jong S. Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. Biochim Biophys Acta. 2010;1805:123–40.

Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem. 1996;271:12687–90.

Sah NK, Munshi A, Kurland JF, McDonnell TJ, Su B, Meyn RE. Translation inhibitors sensitize prostate cancer cells to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by activating c-Jun N-terminal kinase. J Biol Chem. 2003;278:20593–602.

Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI. TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr Biol. 1997;7:693–6.

Secchiero P, Gonelli A, Carnevale E, Milani D, Pandolfi A, Zella D, et al. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. Circulation. 2003;107:2250–6.

Sung ES, Park KJ, Lee SH, Jang YS, Park SK, Park YH, et al. A novel agonistic antibody to human death receptor 4 induces apoptotic cell death in various tumor cells without cytotoxicity in hepatocytes. Mol Cancer Ther. 2009;8:2276–85.

Varfolomeev E, Maecker H, Sharp D, Lawrence D, Renz M, Vucic D, et al. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem. 2005;280:40599–608.

Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. 1997;16:5386–97.

Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. 1995;3:673–82.

TRAIL-R1 (Tumor Necrosis Factor-Related Apoptosis-Inducing ligand Receptor 1)

► TRAIL Receptor 1/2 (Death Receptor 4/5, DR4/5)

TRAIL-R2 (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 2)

▶ TRAIL Receptor 1/2 (Death Receptor 4/5, DR4/5)

TRAM

▶ Toll-like Receptor Adaptor Protein Family Members

TRAM-1

▶ Steroid Receptor Coactivator Family

Translocation-Associated Notch Homolog

▶ Notch (Notch1, Notch2, Notch3, Notch4)

TRB

▶ Tribbles

TRIB

▶ Tribbles

Tribbles

Adrienn Angyal and Endre Kiss-Toth
Department of Cardiovascular Science, University of Sheffield, Sheffield, South Yorkshire, UK

Synonyms

NIPK; SKIP; TRB; TRIB

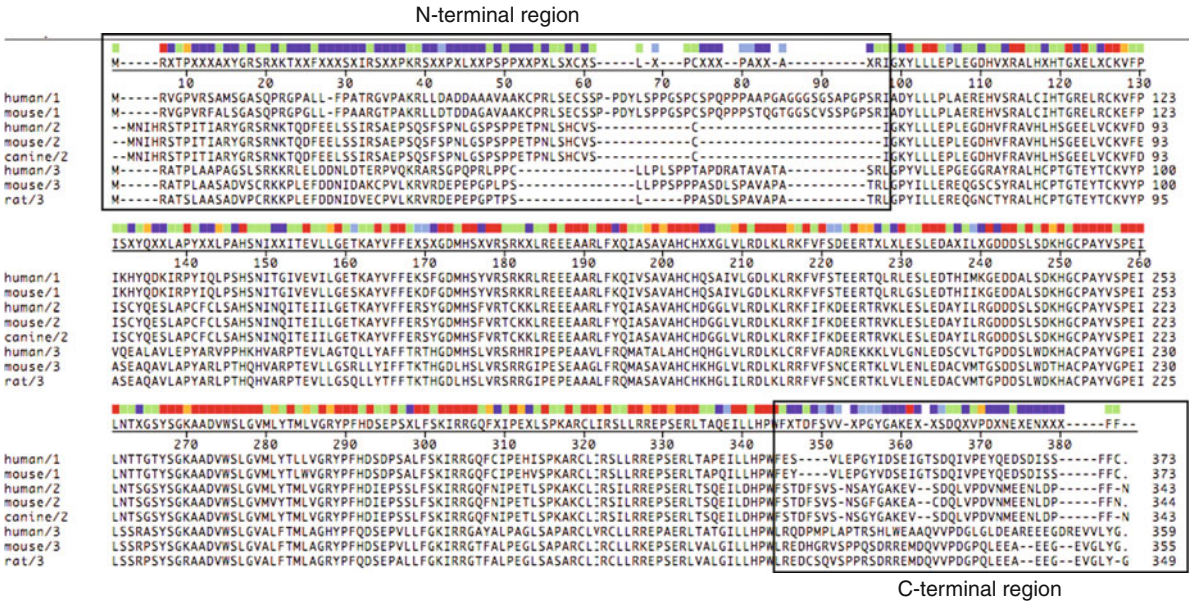
Historical Background

Discovery of Tribbles Genes

Advances in large scale sequencing projects (both cDNA and genome sequencing) combined with the advent of high throughput analytical approaches to detect the targets of certain genes and the molecular consequences of cellular stimulation led to the identification of a number of genes previously unknown to science. A good example of such genes is the tribbles family, where a number of independent strands of research recognized these genes as important regulators and/or targets of cellular function in a range of biological systems.

The name tribbles (TRIB) dates back to a string of papers that published the identification of a novel gene, controlling cell division in early *Drosophila* development. These reports defined an important regulatory role for tribbles in cell cycle progression during the closure of the ventral furrow (Grosshans and Wieschaus 2000), gastrulation (Seher and Leptin 2000), and oogenesis (Rorth et al. 2000). In these studies, absence of trb allowed the premature mitosis of mesodermal cells thus disrupting gastrulation. Tribbles was also identified as a gene that when overexpressed yielded abnormal numbers of germ cells. These experiments defined a molecular role for trb in cell cycle progression by promoting proteolysis of the *Drosophila* cdc25 homologues, *string* and *twine* which dephosphorylate tyrosine 15 of the mitotic cyclin-dependent kinase cdc2 to promote its activation. Thus, trb coordinates mitosis with cell fate determination, and in the absence of this protein mitosis occurs prematurely. Overexpression of trb in *Drosophila* caused oocytes to divide more slowly than normal and become abnormally large. Many subsequent research published in mammalian systems is compatible with these early findings and suggests that tribbles proteins represent an important control point of fundamental cellular processes, such as apoptosis, cell division, and cellular activation.

In parallel to the above studies, mammalian tribbles orthologues have been reported, under a variety of names. Tribbles-2 (C5FW) was described as one of the up-regulated genes in dog thyroid in response to mitogens (Wilkin et al. 1996, 1997). Tribbles-1 (C8FW) was then subsequently reported by this group as a close homologue of tribbles-2 (Wilkin et al. 1997). The first study reporting tribbles-3



Tribbles, Fig. 1 Alignment of vertebrae tribbles-1/2/3 proteins by using Clustal W method. *Red* indicates the strongest and *black* the weakest conservation across the sequences. The N- and C-terminal regions are highlighted in boxes

(Neuronal cell death Inducible Putative Kinase, NIPK) was published by Mayumi-Matsuda et al., demonstrating an increased expression for this gene during neuronal cell death induced by NGF-depletion (Mayumi-Matsuda et al. 1999). These reports were shortly followed by a rapidly increasing body of studies reporting on the functional importance of tribbles, identifying tribbles interacting proteins, and proposing models for their molecular mode of action. This literature has been reviewed systematically in (Docherty and Kiss-Toth 2010; Hegedus et al. 2006, 2007; Kiss-Toth 2005, 2007, 2011).

Below, our current understanding of key aspects of tribbles function will be summarized, as well as some of the key outstanding questions.

Evolution of Tribbles

A comprehensive bioinformatics analysis was performed to identify tribbles orthologues in a range of species and track the origins of this gene family (Hegedus et al. 2006). Systematic analysis of the available EST information in the NCBI database revealed that tribbles-like proteins can already be identified in protozoan species, suggesting that this is an evolutionally ancient, conserved gene. These findings are compatible with the notion that tribbles control fundamental aspects of cellular function. Results of this analysis have also shown that (almost invariably) there is a single tribbles gene per organism in invertebrates. However, all vertebrate species genomes seem to encode for a number of tribbles paralogues. Whilst fish and mammalian species contain three tribbles-like genes, amphibians and birds are missing tribbles-3. Analysis of syntenic regions across vertebrate species suggests that this is due to the loss of a chromosomal segment encoding trb-3 and several other neighboring genes between fish and "modern" amphibians.

Putative Structure of Tribbles Proteins

Tribbles proteins are comprised of three distinct domains (Fig. 1). The N-terminal region of the protein is particularly rich in serine and proline residues, suggesting that this region might be important in protein–protein interactions. It has been shown that this domain is necessary for the nuclear localization of tribbles-1 and -3. The central region of the protein contains an amino acid sequence, which is highly similar to the catalytic domain of serine/threonine kinases. However, some motifs, believed to be crucial to the kinase activity, are missing from tribbles. In particular, kinase sub-domains I, VIB, and VII are absent in tribbles, which suggests that this domain is catalytically inactive and implies evolutionary conservation for structural reasons, necessary for the biological activity of these proteins.

Tribbles, Table 1 Summary of tribbles-1/2/3 interacting molecules

Tribbles	Interacting partner	Tissue/cell type	Reference
1	12-lipoxygenase (12-LOX)	Yeast two-hybrid	Tang et al. (2000)
	MEK-1	HeLa	Kiss-Toth et al. (2004)
	MKK4	HeLa	Kiss-Toth et al. (2004)
	C/EBPβ (NK-IL6)	HEK293	Yamamoto et al. (2007)
2	Akt	Liver	Du et al. (2003)
	C/EBP	U937	Keeshan et al. (2006)
3	Akt	Liver	Du et al. (2003)
	MEK1	HeLa	Kiss-Toth et al. (2004)
	MKK7	HeLa	Kiss-Toth et al. (2004)
	ATF4	Yeast two-hybrid	Bowers et al. (2003)
	C/EBP homologous protein (CHOP)	HEK293	Ohoka et al. (2005)
	Bone morphogenic Protein Type II receptor (BMPRII)	Osteoblast, pulmonary aortic smooth muscle cells	Chan et al. (2007)
	Bone morphogenic regulatory factor 1(Smurf1)	Cos7	Chan et al. (2007)
	p65/Rel A	HEK293	Wu et al. (2003)
	PPARγ	HEK293	Takahashi et al. (2008)
	SIAH1	HEK293T	Zhou et al. (2008)
	CtIP	HeLa	Xu et al. (2007)
	Constitutive photomorphogenic protein 1 (COP1)	Adipose tissue	Qi et al. (2006)
	p300/CREB-binding protein- associated factor (PCAF)	Liver	Yao and Nyomba (2008)

A single study, using cells under non-stimulated conditions, has addressed the issue of catalytic activity of tribbles, with negative results. However, it remains plausible that tribbles only have kinase activity when activated, as is the case for many serine-threonine kinases.

Intracellular Expression

Most published data on intracellular distribution of tribbles to-date is based on experiments using overexpression systems. Based on these data, tribbles-1 and -3 appear to be localized in the nucleus, whilst tribbles-2 being expressed mostly in the cytoplasm. However, immunostaining of tissues for endogenous tribbles suggests that all three human tribbles orthologues can localize in cytoplasm as well as in the nucleus.

Of note, tribbles expression has been reported to be highly regulated. On one hand, this takes place in a tissue specific manner; thus, expression levels of tribbles family members are tightly controlled during differentiation. Further, cellular stimulation also leads to rapid and often transient changes in tribbles levels. In addition to the control of transcription, tribbles proteins are also thought to be highly unstable, as putative motifs responsible for protein degradation have been predicted in many tribbles orthologues (Hegedus et al. 2007). However, experimental evidence for the stability of tribbles is still somewhat scarce.

Molecular Mechanisms of Tribbles Action

Tribbles Interacting Proteins

A large number of proteins have been reported as tribbles binding partners. These interactions are summarized on Table 1. The relevance of most of these findings is discussed in the appropriate sections below.

Tribbles Mediated Control of Signal Transduction

Control of signal transduction networks has been suggested as the main mode of action for tribbles. So far, three main concepts have been established.

A number of studies reported that specific pathways of the mitogen activated protein kinase (MAPK) signaling system are controlled by tribbles, much of this specificity is thought to be due to the tissue specific differences in tribbles expression and/or the altered

expression of tribbles binding partners. In general, tribbles proteins are thought to bind to the middle layer of the three MAPK cascades and thus control the activity of MAPKK proteins (Eder et al. 2008; Gilby et al. 2010; Sung et al. 2007). These interactions and their biological consequences have mainly been investigated in the context of regulating inflammatory responses of vascular and immune cells.

The second major concept by which tribbles are proposed to regulate cell function is via their interaction with Akt/PKB serine-threonine kinases. It has been suggested that the insulin activated PI3K/PKD/Akt signaling pathway is controlled by this interaction, namely, by a polymorphic variant (Q84R) of tribbles-3 that can affect Akt binding. However, the molecular data on the functional importance of this interaction are somewhat conflicting, some studies demonstrating that trb-3 inhibits Akt activity, whilst others found no modulation of Akt-dependent pathways. It is likely that these discrepancies have arisen due to differences in the cell types and/or stimuli used in the various studies, which would also reinforce the notion that intracellular signals are transmitted via large multi-protein complexes and the exact composition of these may be cell type specific. Thus, trb-3/Akt interaction may have different functional consequences, depending on the microenvironment.

Finally, tribbles are thought to control the turnover of other proteins via their interaction with specific ubiquitin ligases. The first line of evidence for such a regulatory mechanism came from *Drosophila* studies, published by Roth et al., demonstrating that the turnover of fly C/EBP (*slbo*) protein is controlled by the Ubp64 ubiquitin ligase, via a tribbles-dependent mechanism (Rorth et al. 2000). Recently, a similar model was put forward in a mammalian setting, reporting that tribbles-1 and -2 but not tribbles-3 are able to promote degradation of C/EBP (Dedhia et al. 2010).

Tribbles Mediated Control of Gene Expression

In addition to interacting with signaling proteins, tribbles have also been reported to bind to several transcription factors and control their transcriptional activity. The first and so far best characterized example of such studies was published by Ord, where they showed that tribbles-3 binds to ATF4 and inhibits the transcriptional activity of this protein (Ord and Ord 2003). Interestingly, whilst tribbles -1 and -2 appear to promote degradation of C/EBP proteins, and tribbles-3 controls ATF4, these two transcription factors seem to cooperate and control the expression of tribbles-3 itself (Ohoka et al. 2005). Putting these findings together, it is likely that complex regulatory feedback loops exist whereby members of the tribbles protein family regulate each other's expression.

Tribbles and Disease

Since their discovery, tribbles have been associated with a number of human diseases, ranging from diabetes, lipid disorders, cardiovascular disease, cancer as well as neurological disorders, such as narcolepsy. Many of these observations were initially correlative and have later been backed-up by functional studies, as summarized below.

Type 2 diabetes: Shortly after the initial characterization of *Drosophila* tribbles, a paper published by Marc Montminy's group have shown that tribbles-3 interacts with unphosphorylated Akt and reduces its activity (Du et al. 2003). Thereby, tribbles-3 was suggested as a novel, stress induced negative regulator of insulin signaling. A follow-on work from this group reported that trb-3 levels were reduced in liver via PPARα and that this is a mechanism to improve glucose tolerance, thus maintain glucose homeostasis. In line with these studies, a polymorphic variant (Q84R) of human tribbles-3 was subsequently identified as a risk factor for the development of type 2 diabetes (Prudente et al. 2009). However, there are also published reports, which suggest that the physiological action of tribbles-3 might be more complex than suggested by these models. A study using primary rat hepatocytes found no role for tribbles-3 in insulin-mediated control of glucose homeostasis (Iynedjian 2005). Further, tribbles-3 deficient mice appear to have a normal glucose tolerance and insulin signaling (Okamoto et al. 2007). Therefore, whist there is strong evidence that tribbles-3 might play a role in the control of insulin signaling and thus contribute to the development of type 2 diabetes, its action is likely to be influenced by other, yet undefined factors.

Lipid metabolism, hyperlipidemia: A molecular study of spontaneous mutations in mice revealed a disorder called fatty liver dystrophy, which is characterized by transient hypertriglyceridemia and fatty liver during the neonatal period, followed by development of a peripheral neuropathy. Dysregulation of tribbles-3 (*fld2*) has been identified in this mouse strain (Klingenspor et al. 1999), which raised the possibility

that elevated trb-3 levels may contribute to the development of this syndrome. However, there is a clear need for further studies to explore the validity of this hypothesis.

Recent genome-wide association studies (GWAS) have identified a number of genes, not previously associated with dysregulated lipid levels in humans. One of these novel candidates is tribbles-1, which was first associated with serum triglyceride levels (Kathiresan et al. 2008) and subsequently with elevated total- and LDL-cholesterol, as well as with an increased risk of coronary heart disease (Tai et al. 2009).

Cancer: An increasing body of evidence has accumulated in recent years to suggest an important role for tribbles in the development of cancer. This is not entirely surprising, given the original observations in *Drosophila*, where tribbles was shown to inhibit cell cycle progression. However, the role tribbles play in the mammalian setting is still somewhat controversial, as discussed below.

A number of cancer types (including colon cancer, acute myeloid leukemia (AML), gliomas, mammary tumors, etc.) have been reported, in which the expression of tribbles proteins appears to be dysregulated. However, the best-studied example is one of AML. Nakamura and his team have suggested that tribbles-1 cooperates with *Hoxa* and *Meis1* in promoting AML. This group as well as work published by Warren Pear and his colleagues suggested a similar model for tribbles-2 and proposed the tribbles-dependent degradation of C/EBP as the molecular mechanism for this. However, a recent analysis of a large microarray dataset over 300 of human AML samples revealed that tribbles-1 and -2 are expressed at particularly low levels in groups where C/EBP levels were reduced. Thus, further work is clearly needed to address the molecular contribution of tribbles in the development of AML.

Narcolepsy: The latest in the list of diseases associated with tribbles is a chronic sleep disorder, called narcolepsy. It has been long suggested that autoimmunity may contribute to the onset of this disease but thus far no autoantigens were identified. However, recent reports from three independent groups have identified that the presence of anti-tribbles-2 antibodies is associated with this disease. However, the mechanism by which this contributes to disease development is currently unclear.

Summary

Tribbles proteins have become of substantial interest for biomedical research over the last decade, as they appear to control the action of key signal transduction pathways, regulate the turnover as well as the action of transcription factors. As they are believed to be pseudokinases, tribbles are most probably exerting their function via interacting with other proteins and modulating their activity. Whist there is a rapidly increasing body of literature to underline their importance in the development of human disease, many basic aspects of their action and the mechanism by which they contribute to the normal physiology and pathology of these diseases is currently unclear and will require further research.

References

Bowers AJ, Scully S, Boylan JF. SKIP3, a novel *Drosophila* tribbles ortholog, is overexpressed in human tumors and is regulated by hypoxia. Oncogene. 2003;22:2823–35.

Chan MC, Nguyen PH, Davis BN, Ohoka N, Hayashi H, Du K, et al. A novel regulatory mechanism of the Bone Morphogenetic Protein (BMP) signaling pathway involving the carboxylterminal tail domain of BMP type II receptor. Mol Cell Biol. 2007;27:5776–89.

Dedhia PH, Keeshan K, Uljon S, Xu L, Vega ME, Shestova O, Zaks-Zilberman M, Romany C, Blacklow SC, Pear WS. Differential ability of Tribbles family members to promote degradation of C/EBPalpha and induce acute myelogenous leukemia. Blood. 2010;116:1321–8.

Docherty L, Kiss-Toth E. SKIP-2/Tribbles-2. AfCS-Nat Mol Pages. 2010. doi:10.1038/mp.a003643.01.

Du K, Herzig S, Kulkarni RN, Montminy M. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science. 2003;300:1574–7.

Eder K, Guan H, Sung HY, Ward J, Angyal A, Janas M, et al. Tribbles-2 is a novel regulator of inflammatory activation of monocytes. Int Immunol. 2008;20:1543–50.

Gilby DC, Sung HY, Winship PR, Goodeve AC, Reilly JT, Kiss-Toth E. Tribbles-1 and -2 are tumour suppressors, down-regulated in human acute myeloid leukaemia. Immunol Lett. 2010;130:115–24.

Grosshans J, Wieschaus E. A genetic link between morphogenesis and cell division during formation of the ventral furrow in Drosophila. Cell. 2000;101:523–31.

Hegedus Z, Czibula A, Kiss-Toth E. Tribbles: novel regulators of cell function; evolutionary aspects. Cell Mol Life Sci. 2006;63:1632–41.

Hegedus Z, Czibula A, Kiss-Toth E. Tribbles: a family of kinase-like proteins with potent signalling regulatory function. Cell Signal. 2007;19:238–50.

Iynedjian PB. Lack of evidence for a role of TRB3/NIPK as an inhibitor of PKB-mediated insulin signalling in primary hepatocytes. Biochem J. 2005;386:113–8.

Kathiresan S, Melander O, Guiducci C, Surti A, Burtt NP, Rieder MJ, Cooper GM, Roos C, Voight BF, Havulinna AS, Wahlstrand B, Hedner T, Corella D, Tai ES, Ordovas JM, Berglund G, Vartiainen E, Jousilahti P, Hedblad B, Taskinen MR, Newton-Cheh C, Salomaa V, Peltonen L, Groop L, Altshuler DM, Orho-Melander M. Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. Nat Genet. 2008;40:189–97.

Keeshan K, He Y, Wouters BJ, Shestova O, Xu L, Sai H, et al. Tribbles homolog 2 inactivates C/EBPalpha and causes acute myelogenous leukemia. Cancer Cell. 2006;10:401–11.

Kiss-Toth E, Bagstaff SM, Sung HY, Jozsa V, Dempsey C, Caunt JC, et al. Human tribbles, a protein family controlling mitogen-activated protein kinase cascades. J Biol Chem. 2004;279:42703–8.

Kiss-Toth E. Tribbles: 'puzzling' regulators of cell signalling. Biochem Soc Trans. 2011;39:684–7.

Kiss-Toth E. SKIP-3/Tribbles-3. AfCS-Nat Mol Pages. 2005. doi:10.1038/mp.a003644.01.

Kiss-Toth E. SKIP-1/Tribbles-1. AfCS-Nat Mol Pages. 2007. doi:10.1038/mp.a003642.01.

Klingenspor M, Xu P, Cohen RD, Welch C, Reue K. Altered gene expression pattern in the fatty liver dystrophy mouse reveals impaired insulin-mediated cytoskeleton dynamics. J Biol Chem. 1999;274:23078–84.

Mayumi-Matsuda K, Kojima S, Suzuki H, Sakata T. Identification of a novel kinase-like gene induced during neuronal cell death. Biochem Biophys Res Commun. 1999;258:260–4.

Ohoka N, Yoshii S, Hattori T, Onozaki K, Hayashi H. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. EMBO J. 2005;24:1243–55.

Okamoto H, Latres E, Liu R, Thabet K, Murphy A, Valenzeula D, Yancopoulos GD, Stitt TN, Glass DJ, Sleeman MW. Genetic deletion of Trb3, the mammalian Drosophila tribbles homolog, displays normal hepatic insulin signaling and glucose homeostasis. Diabetes. 2007;56:1350–6.

Ord D, Ord T. Mouse NIPK interacts with ATF4 and affects its transcriptional activity. Exp Cell Res. 2003;286:308–20.

Prudente S, Morini E, Trischitta V. Insulin signaling regulating genes: effect on T2DM and cardiovascular risk. Nat Rev Endocrinol. 2009;5:682–93.

Qi L, Heredia JE, Altarejos JY, Screaton R, Goebel N, Niessen S, et al. TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism. Science. 2006;312:1763–6.

Rorth P, Szabo K, Texido G. The level of C/EBP protein is critical for cell migration during Drosophila oogenesis and is tightly controlled by regulated degradation. Mol Cell. 2000;6:23–30.

Seher TC, Leptin M. Tribbles, a cell-cycle brake that coordinates proliferation and morphogenesis during Drosophila gastrulation. Curr Biol. 2000;10:623–9.

Sung HY, Guan H, Czibula A, King AR, Eder K, Heath E, et al. Human tribbles-1 controls proliferation and chemotaxis of smooth muscle cells via MAPK signaling pathways. J Biol Chem. 2007;282:18379–87.

Tai ES, Sim XL, Ong TH, Wong TY, Saw SM, Aung T, Kathiresan S, Orho-Melander M, Ordovas JM, Tan JT, Seielstad M. Polymorphisms at newly identified lipid-associated loci are associated with blood lipids and cardiovascular disease in an Asian Malay population. J Lipid Res. 2009;50:514–20.

Takahashi Y, Ohoka N, Hayashi H, Sato R. TRB3 suppresses adipocyte differentiation by negatively regulating PPARgamma transcriptional activity. J Lipid Res. 2008;49:880–92.

Tang K, Finley RLJ, Nie D, Honn KV. Identification of 12-lipoxygenase interaction with cellular proteins by yeast two-hybrid screening. Biochem. 2000;39:3185–91.

Wilkin F, Savonet V, Radulescu A, Petermans J, Dumont JE, Maenhaut C. Identification and characterization of novel genes modulated in the thyroid of dogs treated with methimazole and propylthiouracil. J Biol Chem. 1996;271:28451–7.

Wilkin F, Suarez-Huerta N, Robaye B, Peetermans J, Libert F, Dumont JE, Maenhaut C. Characterization of a phosphoprotein whose mRNA is regulated by the mitogenic pathways in dog thyroid cells. Eur J Biochem. 1997;248:660–8.

Wu M, Xu LG, Zhai Z, Shu HB. SINK is a p65-interacting negative regulator of NF-kappaB-dependent transcription. J Biol Chem. 2003;278:27072–9.

Xu J, Lv S, Qin Y, Shu F, Xu Y, Chen J, et al. TRB3 interacts with CtIP and is overexpressed in certain cancers. Biochim Biophys Acta. 2007;1770:273–8.

Yamamoto M, Uematsu S, Okamoto T, Matsuura Y, Sato S, Kumar H, et al. Enhanced TLR-mediated NF-IL6 dependent gene expression by Trib1 deficiency. J Exp Med. 2007;204:2233–9.

Yao XH, Nyomba BL. Hepatic insulin resistance induced by prenatal alcohol exposure is associated with reduced PTEN and TRB3 acetylation in adult rat offspring. Am J Physiol Regul Integr Comp Physiol. 2008;294:R1797–806.

Zhou Y, Li L, Liu Q, Xing G, Kuai X, Sun J, et al. E3 ubiquitin ligase SIAH1 mediates ubiquitination and degradation of TRB3. Cell Signal. 2008;20:942–8.

TRIF

► Toll-like Receptor Adaptor Protein Family Members

TRIF: TIR Domain–Containing Adaptor-Inducing Interferon β

► TLR5

TRIF-Related Adapter Molecule

► Toll-like Receptor Adaptor Protein Family Members

TRIP13/16E1BP/Thyroid Hormone Receptor Interactor 13 (AGS7)

▶ Activators of G-Protein Signaling (AGS)

Tripartite Motif Protein 23

▶ ARD1/TRIM23

Triple-A (or AAA) is a Family of Proteins Within the AAA+ Superfamily of ATPases Associated with Various Cellular Activities

▶ RPT

TRP

▶ TRP (Transient Receptor Potential Cation Channel)

TRP (Transient Receptor Potential Cation Channel)

Christian Harteneck
Department of Pharmacology and Experimental Therapy, Institute of Experimental and Clinical Pharmacology and Toxicology, Interfaculty Center of Pharmacogenomics and Pharmaceutical Research (ICePhA), Eberhard-Karls-University, Tübingen, Germany

Synonyms

Ankyrine-rich TRP channel, ANKTM1, TRPA1; Classic TRP channels, TRPC1-7; Melastatin-like TRP channels, TRPM1-8; Mucolipins, TRPML1-3; Polycystines, TRPPs; TRP; TRPL; Vanilloid-like TRP channels, TRPV1-6

Historical Background

Calcium is integrated in many physiological processes like muscle contraction, hormone secretion, and intracellular signaling processes (calcium signaling). Prerequisite for this role is the 10,000-fold gradient across the plasma membrane with 2.5 mM extracellular and resting intracellular calcium ion concentration of approximately 100 nM. Calcium ATPases (▶ Plasma membrane calcium-transporting ATPase) and transporters manage the extrusion of calcium whereas calcium-permeable ion channels enable rapid increases in intracellular calcium up to micromolar concentrations. The biological functions of calcium are mediated by direct calcium binding of the modulated proteins or by protein–protein interaction with calcium sensor proteins (calmodulin, CaBP, calcineurin, S100, NCS, etc.). In excitable cells like neurons, heart or skeletal, or smooth muscle cells, the calcium current is mediated by voltage–gated calcium channels. The molecular identity of the ion channels mediating hormone-induced calcium entry in non-excitable cells like endothelial, epithelial, immune cells remained mysterious for a long time. While it was clear that in mammals, receptor-stimulated calcium entry depends on the phospholipase C activity, parallelly in *Drosophila melanogaster*, a protein named Transient Receptor Potential (TRP) was identified and described as a phospholipase C–modulated, calcium-permeable ion channel involved in phototransduction (Montell and Rubin 1989). Upcoming genome and expression profiling projects with expressed sequence tags allowed the cloning of the first mammalian TRP-homologous proteins, the ion channels of the classic TRP family (TRPC). Other approaches identified additional TRP-homologous proteins establishing the melastatin-like and vanilloid-like TRP subfamilies, TRPM and TRPV, respectively (Harteneck et al. 2000; Montell et al. 2002; Wu et al. 2010). With the identification of TRPV1 (vanilloid receptor 1, VR1) as molecular target of capsaicin, another fascinating feature of TRP channels being target of many secondary plant compounds became obvious as well as the involvement of TRP channels in sensory functions (Caterina et al. 1997). Nowadays, the TRP superfamily comprises also the mucolipins (TRPML) and the polycystines (TRPP), calcium-permeable channel proteins with similar transmembrane topology and other

similarities (Montell et al. 2002; Venkatachalam and Montell 2007; Wu et al. 2010). Despite their common structure and sequence identities, the members of the TRP superfamily are involved in many different cellular functions and are integrated in a variety of physiological processes. The basis for the versatility being integrated in so many signaling cascades is founded in the fact that TRP channels as nonselective ion channels are permeable for sodium, calcium, and other divalent cations. The ratio for the main charge carriers, sodium and calcium, varies within the superfamily from highly calcium-selective ion channels (TRPV5 and TRPV6) to calcium-activated, calcium-impermeable, sodium channels (TRPM4 and TRPM5) and represents an important biophysical feature for the characterization of TRP channels in a physiological context.

For the identification, cloning, and biochemical and physiological characterization, common experimental approaches are used, whereas patch clamp as well as calcium imaging techniques provide direct evidence of the channel activity. The patch clamp techniques use small pipettes and directly measure currents carried by the channels studied, whereas calcium imaging approaches depend on intracellular calcium-binding indicator dyes showing differences in fluorescence in the presence and absence of calcium, thereby allowing the measuring of intracellular calcium concentration as a readout of channel activity. The following entry will give an introduction in the broad field of TRP channel research orientated on the TRP channel classification.

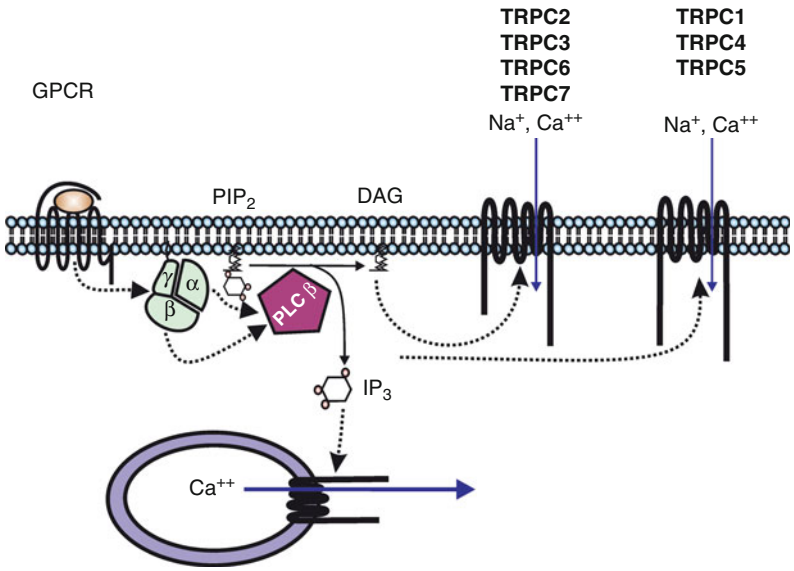
TRPA1 Channel as Signaling Molecule

TRPA1 was initially cloned starting from an up-regulated EST in many tumor cells and described as ANKTM1 based on the most significant structural property of the protein sequence, the abundance of many N-terminal ankyrine-like repeats (Basbaum et al. 2009; Patapoutian et al. 2009). This feature, ANKTM shares with the fly and worm TRP channels of the NOMPC family. The NOMPC channels of *Drosophila melanogaster* and *Caenorhabditis elegans* are involved in mechanotransduction. Based on the similarity, mammalian ANKTM1 was temporarily discussed as putative mechanosensitive ion channels probably involved in hearing (Christensen and Corey 2007). The absence of deafness in ANKTM knockout animals however showed that ANKTM1 is not involved in hearing. TRPA1 plays an important role as cold and chemo sensor in mammalian nociceptive neurons. As protein sensing cold temperatures, TRPA1 is also activated by several cooling compounds like menthol or icilin and on the other hand by many pungent structures like allylisothiocyanate (mustard oil) or allicin or methyl paraben or cinnamaldehyde. The great variety of chemical structures activating TRPA1 and thereby mediating pain and triggering inflammatory processes makes TRPA1 an important target in the pain field.

TRPC Channels as Signaling Molecules

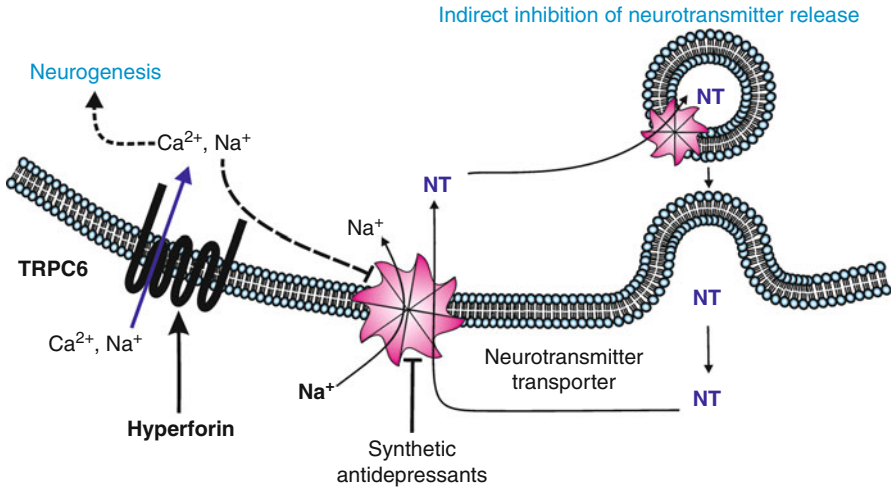
The classic TRP channel family comprises seven different genes with proteins showing the highest sequence similarity to the prototypic *Drosophila* TRP (Harteneck et al. 2000; Montell and Rubin 1989; Venkatachalam and Montell 2007; Wu et al. 2010). Like the prototypic fly channel, the mammalian channel proteins are involved in receptor-regulated calcium entry (Birnbaumer 2009). Stimulation of receptors by hormones, neurotransmitter (GPCR, photoreceptor), and light in *Drosophila* results in the phospholipase C–mediated breakdown of phosphatidylinositides, leading to the formation of inositol 1,4,5-triphosphate and diacylglycerol (Fig. 1). Inositol 1,4,5-triphosphate induces calcium release from intracellular stores via the activation of inositol 1,4,5-triphosphate receptors (▶ IP3 receptor), whereas diacylglycerol directly activates mammalian classic transient receptor potential (TRPC) channels (TRPC2, TRPC3, TRPC6, and TRPC7) in a protein kinase C–independent manner. The prerequisite of phospholipase C stimulation has been shown for TRPC1, TRPC4, and TRPC5 currents; however, the molecular mechanism is still unclear.

TRPC1 is transcribed in nearly all cells, whereas the other TRPC channels show a distinct and characteristic expression profile being adapted to cellular requirements; nevertheless, in many cell types, two and more TRPC channels can be found. On the other hand, the broad expression profile of TRPC channels makes it difficult to assign distinct physiological function to one gene. TRPC2 being expressed in the rodent vomeronasal organ is clearly an exception. In man, TRPC2 is a pseudogene; the transcribed mRNA is functionless due to various stop codons. However, in rodents, the transcription of the TRPC2 gene results in



TRP (Transient Receptor Potential Cation Channel), Fig. 1 Activation pathway of mammalian receptor-activated TRPC channels. G-protein-coupled receptor (GPCR)–mediated activation of phospholipase C isoforms (PLC βγ) result in the breakdown of phosphatidylinositides (PIP_2), leading to the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is the ligand of IP_3 receptors, which are located in the endoplasmic reticulum. Upon activation, IP_3 receptors mediate Ca^{2+} release from intracellular stores, whereas DAG directly activates mammalian TRPC2, TRPC3, TRPC6, and TRPC7 channels. In contrast to the current molecular understanding of the signaling cascade resulting in the stimulation of TRPC2, TRPC3, TRPC6, and TRPC7, the molecular mechanism for the stimulation of TRPC1, TRPC4, and TRPC5 is unclear. The prerequisite of phospholipase C activation in the activation of TRPC1, TRPC4, and TRPC5 is accepted

a functionally active protein involved in sensory response to pheromones. Genetic inactivation of TRPC2 in mice leads to loss of sex discrimination of male mice. Data from TRPC4 knockout mice approaches show that TRPC4 is involved in microvascular permeability. TRPC3 and TRPC5 are discussed to be involved in neuronal differentiation and growth cone guidance. TRPC6 expressed in vascular smooth muscle cells was discussed to be involved in blood pressure regulation. However, the characterization of TRPC6 knockout mice surprisingly showed a slightly increased blood pressure resulting from increased basal activity of TRPC3, up-regulated in TRPC6 knockout mice. The diversity of cellular function of the genes of the TRPC family became visible by reports showing that gain-of-function mutations of TRPC6 resulted in progressive kidney failure and focal segmental glomerulosclerosis (FSGS). The physiological role of TRPC6 in the kidney proposed from the analysis of the genetic disease is that TRPC6 expressed in podocytes is involved in hormone-dependent size regulation of the glomerular slit-diaphragm. Gain-of-function mutation of TRPC6 results in increased calcium load of the podocytes, induction of calcium-dependent apoptosis, progressive loss of podocytes, and glomerulosclerosis responsible for proteinuria found in these patients. The complexity of the different physiological function mediated by TRPC6 became obvious, when TRPC6 was identified as the molecular target of hyperforin, the active principle of St. John Wort extracts, a remedy for the treatment of moderate to mild depression (Fig. 2). The antidepressive action of hyperforin results from the direct activation of TRPC6, leading to enhanced intracellular calcium as well as sodium concentrations. By increasing intracellular sodium, the neurotransmitter uptake is indirectly inhibited by the impairment of the electrochemical gradient of sodium as basis for the reuptake of serotonin, dopamine, and noradrenaline. Like synthetic antidepressive drugs directly blocking the neurotransmitter transporter, hyperforin mediates neurotrophic effects in a TRPC6-dependent manner probably by calcium-induced differentiation processes. Calcium-dependent differentiation processes are regulated in a dual manner. Hyperforin or diacylglycerol concentration regulates TRPC6 activity



TRP (Transient Receptor Potential Cation Channel), Fig. 2 Proposed model of hyperforin-induced, TRPC6-mediated effects in neurons. Synthetic antidepressants block plasma membrane neurotransmitter transporter proteins, thereby increasing concentration and exposure time of neurotransmitters. In contrast to this mode of action, the antidepressive effects of hyperforin are mediated using another pathway. By activation of TRPC6 channels permeating sodium and calcium, the electrochemical gradient of sodium across the plasma membrane prerequisite for the function of the plasma membrane neurotransmitter transporter is reduced, resulting in an indirect inhibition of neurotransmitter uptake. On the other hand, TRPC6-mediated calcium entry may trigger calcium-dependent differentiation processes responsible for changes in neuronal plasticity by still unclear intracellular pathways

and thereby the intracellular calcium concentrations. On the other hand, puromycin treatment of rats leads to increased TRPC6 expression in podocytes and proteinuria, whereas treatment of keratinocytes with betulin or other triterpens also increases TRPC6 expression and enhances keratinocytes differentiation. The duality of wanted as well as unwanted effects induced by the modulation of TRPC6 expression and activity demonstrates that the intracellular calcium concentrations are regulated within a tight interval with serious effects in case of imbalances. These observations transmitted to drug research and therapy show that both blocking as well as stimulating strategies will make sense in particular pathophysiological situations.

TRPM Channels as Signaling Molecules

Melastatin, the founding member of the melastatin-like TRP family, was identified within a screen for proteins differentially regulated in melanocytes and melanoma cells (Harteneck 2005; Venkatachalam and Montell 2007; Wu et al. 2010). Analysis of clinical data showed that the presence of melastatin expression in melanoma patients inversely correlates with the severity and survival. Although melastatin is the first member of the TRPM family, its activation mechanism and physiological role is still unclear. In line with the first description as protein involved in melanocyte physiology, several reports confirmed this view. A completely unexpected function, the integration in retinal signal processing, has recently been discovered by the identification of TRPM1 expression in retinal ON bipolar cells. The critical role of TRPM1 in mammalian phototransduction is also highlighted by several reports describing TRPM1 mutations in patients suffering from congenital stationary night blindness.

From sequence similarity, TRPM3 is phylogenetically the closest neighbor to melastatin. Several stimuli for the activation of TRPM3 have been reported. TRPM3 is activated by hypotonic extracellular solution and represents together with TRPV4, the volume-regulated TRP channels in the kidney. With the help of pharmacological tools, calcium entry induced by the application of hypotonic extracellular solutions can be assigned to TRPV4 and TRPM3 (Harteneck and Reiter 2007; Liedtke 2007; Vriens et al. 2009; Woudenberg-Vrenken et al. 2009). While TRPV4 is activated by 4α-Isomers of phorbolesters and is blocked by ruthenium red, TRPM3 is activated by sphingosine and by pregnenolone-sulfate and blocked by gadolinium ions. TRPM3 is expressed in oligodendrocytes in the brain,

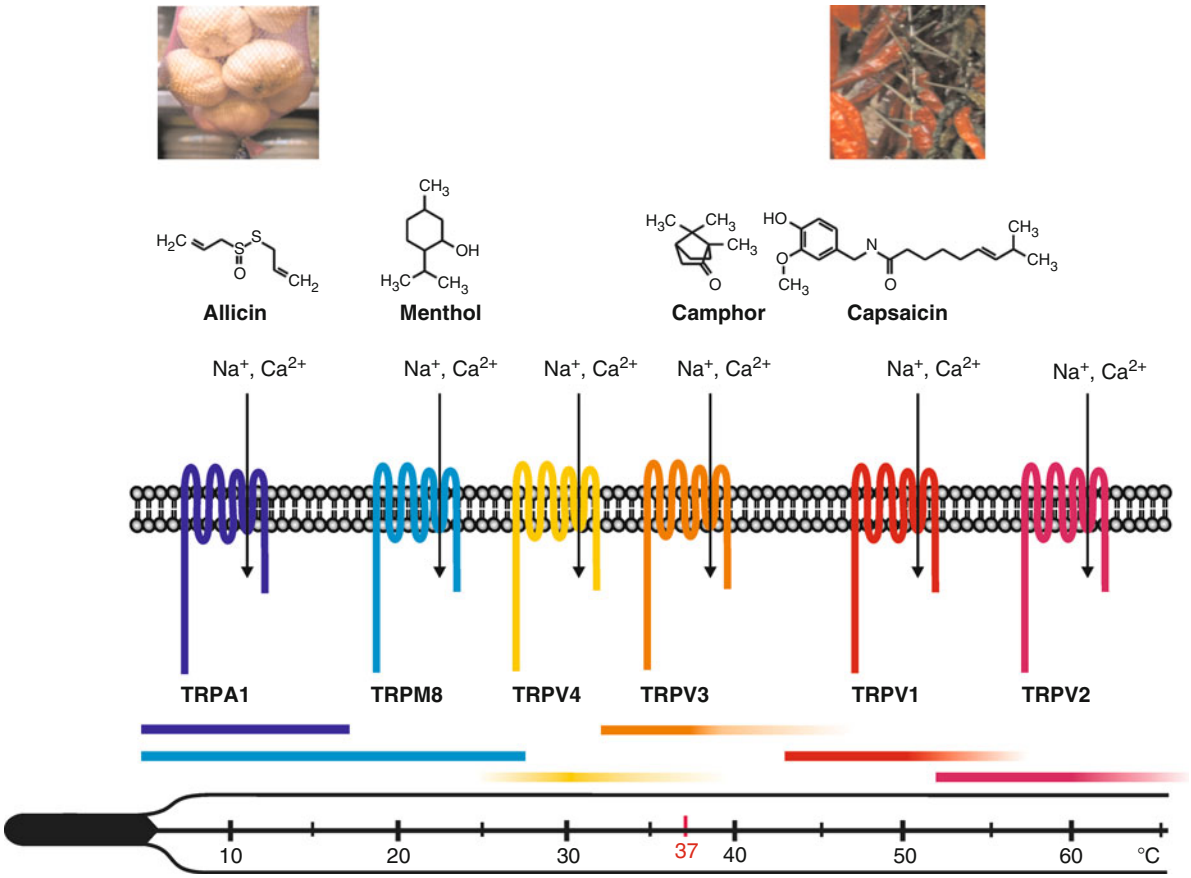
in renal epithelial and pancreatic beta cells. The phylogenetically next neighbors to TRPM1 and TRPM3 are TRPM6 and TRPM7. Latter are involved in the body magnesium homeostasis. While TRPM7 is ubiquitously expressed, TRPM6 is expressed in epithelial cells of the gut and the kidney and responsible for magnesium absorption and reabsorption. Loss-of-function mutations in TRPM6 are linked to autosomal-recessive hypomagnesemia with secondary hypocalcemia. TRPM6 and TRPM7 as well as TRPM2 (see below) share a common structural feature. All three genes code for chimeric proteins combining a hexahelical transmembrane channel forming domain with a C-terminal enzymatic active domain. In the case of TRPM6 and TRPM7, the pore-forming domains are fused to atypical alpha kinase-like structures. The functional role for the enzymatic domain is still under dispute. TRPM6 and TRPM7 are permeable for magnesium and for other essential divalent cations like Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} as well as toxic cations like Ba^{2+}, Sr^{2+}, Ni^{2+}, Cd^{2+} (Hoenderop and Bindels 2008; Woudenberg-Vrenken et al. 2009). While divalent ions are the preferentially carried ion of TRPM6- and TRPM7-mediated currents, TRPM4 and TRPM5 form ion pores impermeable for divalent ions and allow selectively sodium to pass. As sodium channels, TRPM4 and TRPM5 are paradoxically activated by increased intracellular calcium concentrations and represent calcium-activated calcium channels. TRPM4 being ubiquitously expressed is involved in the mast cell activation and the release of inflammatory mediators. Furthermore, TRPM4 contributes to the neurogenic regulation of blood pressure by modulating central catecholamine release. In contrast to TRPM4, the expression of TRPM5 is restricted to a few cell types. TRPM5 is expressed in taste buds of the tongue and involved in the sensation of bitter and sweet taste. The remaining two TRPM channels proteins, TRPM2 and TRPM8, can also be discussed in the light of sensory functions. As already mentioned, TRPM2 represents a chimeric protein having an ADP-ribose hydrolase domain C-terminal to the pore-forming transmembrane domains. Simultaneously to the ADP-ribose hydrolysing activity of the C-terminal enzymatic domain, TRPM2 is activated by ADP-ribose and it has been shown that the C-terminal part is essential for the function as pore-forming channel protein. Increased intracellular ADP-ribose concentrations are linked to genotoxic and/or oxidative stress of cells, leading to the activation of the poly (ADP-ribose) polymerase (PARP) modulating protein stability by the mono- and poly-ADP-ribosylation of proteins. This process of protein stability regulation is additionally controlled by an enzyme called poly (ADP-ribose) glucohydrolase (PARG). PARG reduces the posttranslational poly-ADP-ribose modifications to mono-ADP-ribosylation, thereby increasing the intracellular ADP-ribose concentration leading to the activation of TRPM2. In whole cell calcium imaging experiments, the extracellular application of hydrogen peroxide results in the activation of TRPM2 validating its function as redox sensor. While TRPM2 is highly expressed in immune cells and at low levels in some neurons making the discussion of TRPM2 as redox sensor difficult, TRPM8, the cold sensor, is mainly expressed in sensory neurons. TRPM8 is activated at temperatures between 8°C and 28°C as well as secondary plant compound menthol and synthetic cooling compounds. Together with TRPA1, TRPM8 represents the cold sensors in human. Noxious cold is mediated by TRPA1 (Fig. 3).

TRPML Channels as Signaling Molecules

Mucolipidosis Type IV is an autosomal-recessive neurodegenerative lysosomal storage disorder caused by mutations in TRPML 1 (Montell et al. 2002; Puertollano and Kiselyov 2009). TRPML1 like the other members of the mucolipin family (TRPML1-3) is described to be located as integral membrane proteins of intracellular vesicles especially the lysosomes and probably activated by protons. The lysosomes in mucolipidosis IV patients are enlarged arguing for defects of the intracellular turnover and storage of lipids such as phospholipids, sphingolipids, gangliosides, and mucopolysaccharides. Based on this observation, a contribution of mucolipin channels in the intracellular trafficking and handling of lysosomes is discussed and provides hypotheses for further research.

TRPP Channels as Signaling Molecules

The autosomal-dominant polycystic kidney disease (ADPKD) is characterized by cysts formation in the kidney, a malformation of the renal tubular structure



TRP (Transient Receptor Potential Cation Channel), Fig. 3 TRP channels as thermo sensors. The diagram summarizes the secondary plant compounds and temperature ranges activating temperature-sensitive TRP channels. TRPA1 is activated by pungent and cooling compounds and is activated by temperatures below 17 °C, whereas TRPM8 is activated at temperatures below 27 °C and cooling compounds like menthol. TRPV4 is activated at intermediate temperatures and extracellular hypotonic solutions (see volume-regulated TRP channels TRPV4 and TRPM3). TRPV3 mainly expressed in keratinocytes is activated by temperature from 31 °C to 39 °C and camphor. TRPV1, also named vanilloid receptor 1 or capsaicin receptor 1, is activated by capsaicin and temperature above 42 °C, whereas TRPV2 is activated by heat above 51 °C

(Montell et al. 2002; Zhou 2009). The cyst formation is linked to the disruption of the functional TRPP1/TRPP2 complex. TRPP1, a huge protein consisting of a complex N-terminal extracellular ligand binding and 11 transmembrane domains, and TRPP2, a calcium-permeable ion channel, form a receptor–effector complex implicated in various biological functions, such as cell proliferation, sperm fertilization, and mechanosensation.

TRPV Channels as Signaling Molecules

Vanilloid structures, derivates of vanillin comprising eugenol, zingerone, and capsaicin, are found in many splice plants and known for their individual characteristic flavor. Beside the use as spice, vanilloid-containing plant extracts are used as remedy in the various traditions of folk medicine. The therapeutic and experimental uses of capsaicin in pain treatment inspired research resulting in the unraveling of the molecular target of capsaicin. The molecular target, an ion channel related to *Drosophila* TRP, was named capsaicin or vanilloid receptor and became eponym of the subgroup or structurally related ion channels of the TRP channel superfamily (Caterina et al. 1997). The vanilloid-like TRP channels comprise six members, four proteins (TRPV1 to TRPV4) like TRPV1 are nonselective ion channels involved in thermosensation (Basbaum et al. 2009; Liedtke 2007;

Talavera et al. 2008; Vriens et al. 2009; Wu et al. 2010), while two ion channels (TRPV5 and TRPV6) represent highly calcium-selective ion channels (Hoenderop and Bindels 2008; Woudenberg-Vrenken et al. 2009). Initially described as calcium transporter, TRPV5 and TRPV6 are responsible for the uptake and reuptake of calcium in the gut and kidney, respectively. Expressed in the epithelial cell layers of the gut and kidney, the activity is transcriptionally controlled in a vitamine-D3-dependent manner.

Capsaicin, the ingredient in chili pepper, causing burning and pain activates TRPV1, the heat sensor, activated by increased temperatures under physiological conditions. The capsaicin receptor being expressed in dorsal root ganglia senses increasing temperatures with a threshold of around 42°C. The activation of TRPV1 is realized as heat and burning, the same sensation caused by capsaicin. Illustrative for the modulation of the 42°C threshold of TRPV1 is the burning pain after the application of ethanol or acids in wounds as TRPV1 activity is dramatically enhanced at normal body temperature in the presence of increased proton concentration or in the presence of around 3% ethanol. Since the first description of TRPV1 as the molecular target of capsaicins, many pain-inducing chemicals and toxins have been described as TRPV1 ligands. The warm and heat sensors (TRPV1 to TRPV4) and the cold sensors (TRPM8 and TRPA1) represent the thermosensors of the human body and cover the complete temperature range necessary for human life (Fig. 3). As warning sensors expressed in dorsal root ganglia, the thermo-TRPs are also involved in sensation and modulation of pain and therefore interesting as molecular targets for new pain-treating drugs.

Summary

Transient receptor potential (TRP) channels are a large family of nonselective, calcium-permeable channel proteins activated and regulated by a diversity of mechanisms. TRP channels respond to intracellular stimuli such as calcium, metabolites of the arachidonic acid, or phosphatidylinositol signal transduction pathways. TRP channels sense environmental stimuli such as changes in temperature, osmolarity, and pH and represent the molecular target of pheromones, taste, and secondary plant compounds. The broad function in physiology proposed by the expression profiles of TRP channels becomes visible by the association of TRP channel mutations with hereditary diseases or the analysis of TRP channel knockout mice. The diversity of the chemical structures and the selectivity of the natural occurring compounds modulating TRP channels show the possibility for the pharmacological modulation of TRP channels and inspire to develop new synthetic structures for TRP channel interference at bench and bedside.

References

Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. Cell. 2009;139:267–84.

Birnbaumer L. The TRPC class of ion channels: a critical review of their roles in slow, sustained increases in intracellular Ca^{2+} concentrations. Annu Rev Pharmacol Toxicol. 2009;49:395–426.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature. 1997;389:816–24.

Christensen AP, Corey DP. TRP channels in mechanosensation: direct or indirect activation? Nat Rev Neurosci. 2007;8:510–21.

Harteneck C. Function and pharmacology of TRPM cation channels. Naunyn Schmiedebergs Arch Pharmacol. 2005;371:307–14.

Harteneck C, Reiter B. TRP channels activated by extracellular hypo-osmoticity in epithelia. Biochem Soc Trans. 2007;35:91–5.

Harteneck C, Plant TD, Schultz G. From worm to man: three subfamilies of TRP channels. Trends Neurosci. 2000;23:159–66.

Hoenderop JG, Bindels RJ. Calciotropic and magnesiotropic TRP channels. Physiol Bethesda. 2008;23:32–40.

Liedtke W. Role of TRPV ion channels in sensory transduction of osmotic stimuli in mammals. Exp Physiol. 2007;92:507–12.

Montell C, Rubin GM. Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. Neuron. 1989;2:1313–23.

Montell C, Birnbaumer L, Flockerzi V, Bindels RJ, Bruford EA, Caterina MJ, et al. A unified nomenclature for the superfamily of TRP cation channels. Mol Cell. 2002;9:229–31.

Patapoutian A, Tate S, Woolf CJ. Transient receptor potential channels: targeting pain at the source. Nat Rev Drug Discov. 2009;8:55–68.

Puertollano R, Kiselyov K. TRPMLs: in sickness and in health. Am J Physiol Ren Physiol. 2009;296:F1245–54.

Talavera K, Nilius B, Voets T. Neuronal TRP channels: thermometers, pathfinders and life-savers. Trends Neurosci. 2008;31:287–95.

Venkatachalam K, Montell C. TRP channels. Annu Rev Biochem. 2007;76:387–417.

Vriens J, Appendino G, Nilius B. Pharmacology of vanilloid transient receptor potential cation channels. Mol Pharmacol. 2009;75:1262–79.

Woudenberg-Vrenken TE, Bindels RJ, Hoenderop JG. The role of transient receptor potential channels in kidney disease. Nat Rev Nephrol. 2009;5:441–9.
Wu LJ, Sweet TB, Clapham DE. International Union of Basic and Clinical Pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. Pharmacol Rev. 2010;62:381–404.
Zhou J. Polycystins and primary cilia: primers for cell cycle progression. Annu Rev Physiol. 2009;71:83–113.

TRP53

► p53

TRPL

► TRP (Transient Receptor Potential Cation Channel)

TRPP1

► Polycystin-2

TSAd

► SH2D2A

TSP1

► Thrombospondin-1

TSPAN 28

► CD81

Tspan-25

► CD53

Tspan25l

► CD53

T-Type Channels

► Voltage-Gated Calcium Channels: Structure and Function (CACNA)

Tumor Progression Locus 2

► TPL2

Tumor Rejection Antigen gp96

► Grp94 (HSP90B1)

TWEAK Receptor

► Fn14

Type A Endothelin Receptor

► Endothelin A Receptor (ETAR)

Type II Interferon

► Interferon-Gamma

Type II PIP Kinase

► Phosphatidylinositol 5-phosphate 4-kinase

Type II PIPK

► Phosphatidylinositol 5-phosphate 4-kinase

Type IV cAMP Phosphodiesterase

► PDE4

Type-I Cadherins

► Cadherins

Tyro4 (Rat)

► EphA3, Erythropoietin-Producing Hepatocellular Carcinoma Cell Receptor A3

TZK

► ZAP-70

U

UP50

▶ Fibulins

uPA Receptor

▶ Structure and Functions of the Urokinase Receptor

uPAR

▶ Structure and Functions of the Urokinase Receptor

UPH1

▶ Fibulins

Urea Transporter

▶ UT (Urea Transporter)

Urokinase-Type Plasminogen Activator Receptor

▶ Structure and Functions of the Urokinase Receptor

Urokinase-Type Plasminogen Activator Receptor-Associated Protein (uPARAP)

▶ MRC2

UT (Urea Transporter)

Mitsi A. Blount, Janet D. Klein and Jeff M. Sands
Renal Division, Emory University Woodruff Memorial Research Building, Atlanta, GA, USA

Synonyms

SLC14A1; SLC14A2; Urea transporter; UT-A; UT-B

Historical Background

Since 1934, it has been understood that urea plays a key role in the generation of concentrated urine (Gamble et al. 1934). Functional measurements of urea transport in the kidney inner medullary collecting duct (IMCD), performed in the 1980s, provided evidence for the existence of urea transporter proteins (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). Currently, two families of urea transporters have been cloned: *SLC14A1*, the UT-B urea transporter originally isolated from erythrocytes; and *SLC14A2*, the UT-A group with six distinct isoforms described to date (reviewed in [Sands and Layton 2009; Sands and Layton 2008]) (Table 1). In the

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

UT (Urea Transporter), Table 1 Regulation and location of the known urea transporters. Original citations are reviewed in (Sands and Layton 2008, 2009)

Gene	Isoform	RNA (kb)	Protein (kDa)	AVP	Location
Slc14a1	UT-B1	3.8	43		DVR, RBC[f]
	UT-B2	3.7	43–54	No	Bovine rumen
Slc14a2	UT-A1 (UT-A1b[a])	4.0 (3.5)	117, 97	Yes	IMCD (medulla[b])
	UT-A2 (UT-A2b[a])	2.9 (2.5)	55	No	tDL, liver (medulla[b], heart)
	UT-A3 (UT-A3b[a])	2.1 (3.7)	67, 44	Yes	IMCD (medulla[b])
	UT-A4[c]	2.5	43	Yes	medulla[b]
	UT-A5[d]	1.4			testis
	UT-A6[e]	1.8			colon

AVP, urea flux is stimulated by vasopressin
[a]human RNA form with additional 3′ untranslated regions
[b]exact tubular location unknown
[c]cloned from rat only
[d]cloned from mouse only
[e]cloned from human only
[f]also expressed in several other tissues and endothelial cells

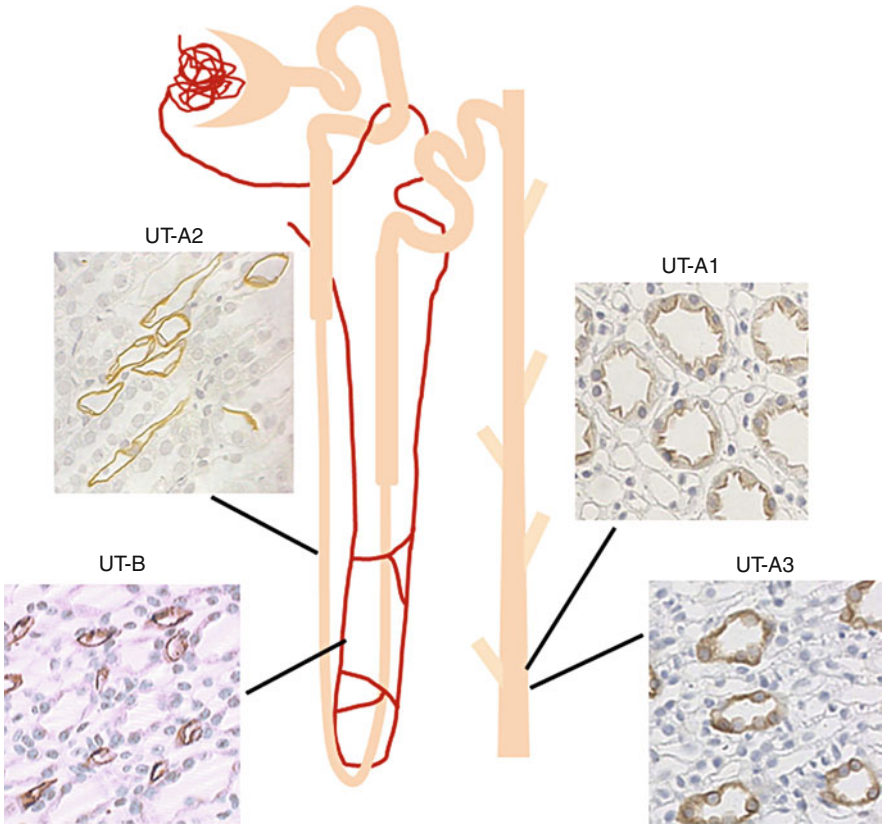
kidney, UT-A1 and UT-A3 are expressed in the IMCD; UT-A2 is expressed in the thin descending limb (tDL); and UT-B is located primarily in the descending vasa recta and red blood cells (Fig. 1). Although the exact location has yet to be determined, UT-A4 is expressed in rat kidney medulla but not in the mouse medulla (reviewed in [Sands and Layton 2009; Sands and Layton 2008]).

The UT-A family of urea transporters currently consists of six protein isoforms. The four renal isoforms are shown in Fig. 2. UT-A1 is the largest protein containing 11 transmembrane helices. Helices 6 and 7 are connected by a large intracellular loop that recent studies show is crucial to the functional properties of UT-A1 (Blount et al. 2008; Mistry et al. 2010; Klein et al. 2010). UT-A3 is the N-terminal half of UT-A1 while UT-A2 is the C-terminal half of UT-A1. UT-A4 is the N-terminal quarter of UT-A1 spliced to the C-terminal quarter. Although the need for the multiple isoforms of UT-A is not completely understood, a recent crystal structure of a bacterial urea-conducting channel, dvUT, suggests that these transporters require some sort of oligomeric organization to function (Levin et al. 2009).

The UT-B gene can produce two isoforms, UT-B1 and UT-B2, each of which are tissue specific. Despite the expression of two distinct transcripts (4.4 and 2 kb) cloned for the human UT-B1 transporter, both appear to encode for a single 43 kDa protein. Sequence alignment with other urea transporters shows that UT-B1 shares more than 60% identity with UT-A2. Additionally, hydrophilicity profiles indicate that UT-B1 and UT-A2 proteins show similar topology (reviewed in [Bagnasco 2006]). Another isoform of UT-B, UT-B2 is expressed in the bovine rumen but has not yet been detected in humans. Interestingly, UT-B2 is not regulated by vasopressin, cAMP, calcium, or protein kinases.

Acute Regulation of Urea Transport by Vasopressin

When the body is dehydrated, vasopressin (also known as antidiuretic hormone) is released (Fig. 3). When vasopressin is increased, the hormone binds to the V_2 vasopressin receptor, located on the basolateral membrane of the IMCD, and activates the heterotrimeric G protein, Gs_{α}. Activation of the G protein stimulates ▶ adenylyl cyclase to synthesize cAMP. The increase of intracellular cAMP stimulates several downstream proteins including protein kinase A (PKA) and Epac (exchange protein directly activated by cAMP). Both are involved in the vasopressin-mediated increase in urea permeability (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). Vasopressin also increases urea flux in freshly isolated IMCD cell suspensions (Zhang et al. 2002), UT-A1-MDCK cells (Fröhlich et al. 2006), UT-A1-mIMCD3 cells (Klein et al. 2010), and mUT-A3-MDCK cells



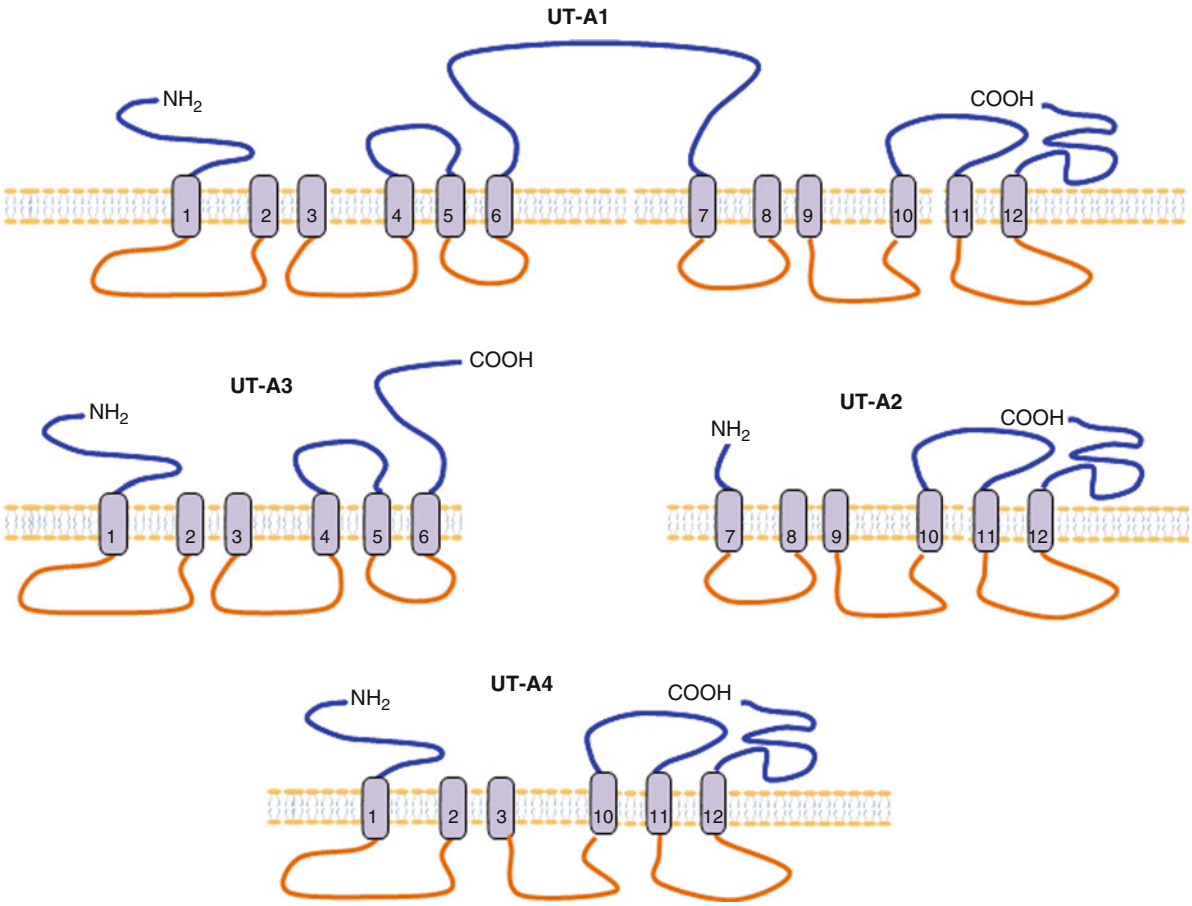
UT (Urea Transporter), Fig. 1 Location of the Renal Urea Transporters. UT-A2 is found in the thin descending limbs of Henle's loop in the inner stripe of the outer medulla. UT-A1 and UT-A3 are expressed in the IMCD. UT-A1 is primarily found in the cytosol and the apical membrane of the cell whereas UT-A3 is located in the cytosol and the basolateral membrane of the IMCD. UT-B is located primarily in the descending vasa recta and red blood cells

(Stewart et al. 2009). The acute effect of vasopressin on UT-A2 is still not understood. One group of studies showed that vasopressin and cAMP increased urea flux in mUT-A2-MDCK cells (Potter et al. 2006); however, other work found that treatment with cAMP or forskolin did not increase urea transport via UT-A2 (Fenton et al. 2002). It was recently demonstrated that cAMP-treated oocytes that have been injected with UT-A2 fail to display increased urea flux (Mistry et al. 2010). Further work will be needed to address the acute regulation of UT-A2 by vasopressin. The acute regulation of UT-A1 and UT-A3 has been studied in great detail, which is further discussed below.

Phosphorylation

An in silico analysis of the amino acid sequence of the urea transporter reveals a large number of consensus phosphorylation sites for variety of kinases including several PKA consensus phosphorylation sites for both UT-A1 and UT-A3 (Fig. 4). In rat terminal IMCDs, vasopressin increases the phosphorylation of both UT-A1 and UT-A3, with a similar time-course and dose-response for that observed in the vasopressin-stimulated increase in urea permeability in perfused rat terminal IMCDs (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). Vasopressin, acting through cAMP, stimulates Epac, which also increases urea permeability and UT-A1 phosphorylation in rat IMCDs (Wang et al. 2009).

Vasopressin stimulation results in the direct phosphorylation of UT-A1 at S486 and S499 in rat kidney (corresponding to S477 and S490 in the human UT-A1 sequence) (Blount et al. 2008). Both of these residues are located in the large intracellular loop that is unique to UT-A1. Studies show that attaching the loop region of UT-A1 (aa 460–532) that contains S486 and S499 to UT-A2 confers vasopressin sensitivity to UT-A2 (Mistry et al. 2010) confirming the importance of these phosphorylation sites. Site directed mutagenesis revealed that neither of the two PKA consensus sites



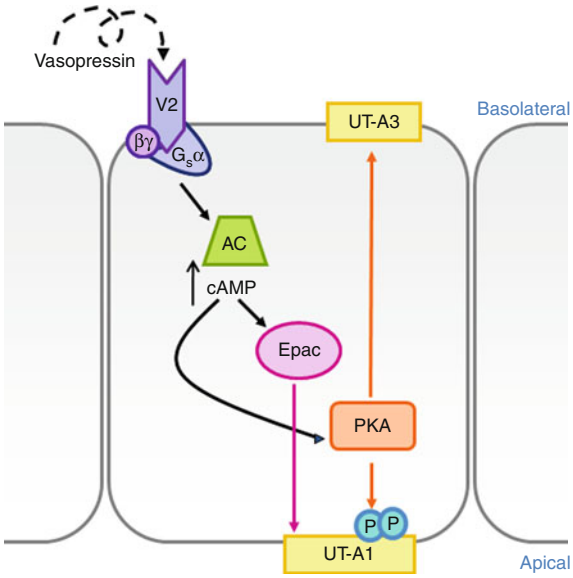
UT (Urea Transporter), Fig. 2 Four Renal Isoforms of UT-A. This schematic demonstrates that UT-A1 is the largest protein transcribed from the UT-A gene containing 11 transmembrane helices. UT-A3 is the N-terminal half of UT-A1 and UT-A2 is the C-terminal half of UT-A1. UT-A4 is the N-terminal quarter of UT-A1 spliced to the C-terminal quarter. Loop regions shown in blue are intracellular whereas those highlighted in orange are extracellular. Transmembrane structure prediction was based on the GES-scale (Goldman Engelman Steitz) analysis of the primary structure which employees hydropathy calculations in combinations with helical probabilities

(S85 or S92) in mouse is involved in vasopressin-induced phosphorylation (reviewed in [Sands and Layton 2009; Sands and Layton 2008]); however, vasopressin does increase phosphorylation at S84 in rat UT-A1 and UT-A3 (Hwang et al. 2010). The vasopressin-stimulated phosphorylation site in human UT-A3 has not been determined as S84 is not conserved in the human sequence.

Plasma Membrane Accumulation

Vasopressin increases the plasma membrane accumulation of UT-A1 and UT-A3 in IMCDs from normal rats but not from either vasopressin-deficit Brattleboro rats or water diuretic rats (Blount et al. 2007; Klein et al. 2006). When forskolin is used to stimulate cAMP instead of vasopressin, the plasma membrane accumulation of UT-A1 is increased in IMCDs from normal and diuretic rats (Klein et al. 2006). Activating Epac also increases UT-A1 plasma membrane accumulation in rat IMCDs (Wang et al. 2009).

UT-A1 apical plasma membrane accumulation is increased by vasopressin or forskolin in UT-A1-MDCK cells (Chen et al. 2006; Klein et al. 2006) and UT-A1-mIMCD3 cells (Klein et al. 2010). Mutation of both of the PKA sites in UT-A1, S486 or S499, eliminates vasopressin stimulation of UT-A1 apical plasma membrane accumulation and urea transport. Interestingly, increased membrane insertion of UT-A1 requires that both PKA sites are functional as mutation of either S486 or S499 individually has



UT (Urea Transporter), Fig. 3 Vasopressin Signaling in the IMCD. When increased, vasopressin binds to the vasopressin receptor, V_2 located on the basolateral cell membrane and activates the heterotrimeric G protein, Gs_α. Activation of the G protein stimulates adenylyl cyclase (AC) to synthesize cAMP. Increased cAMP can activate two downstream targets, Epac and PKA; both have shown to affect urea transporter function. Increased levels of cAMP lead to direct phosphorylation of UT-A1 and UT-A3. Direct phosphorylation of UT-A1 is required for apical membrane insertion. UT-A3 membrane accumulation is also increased in the presence of high cAMP levels

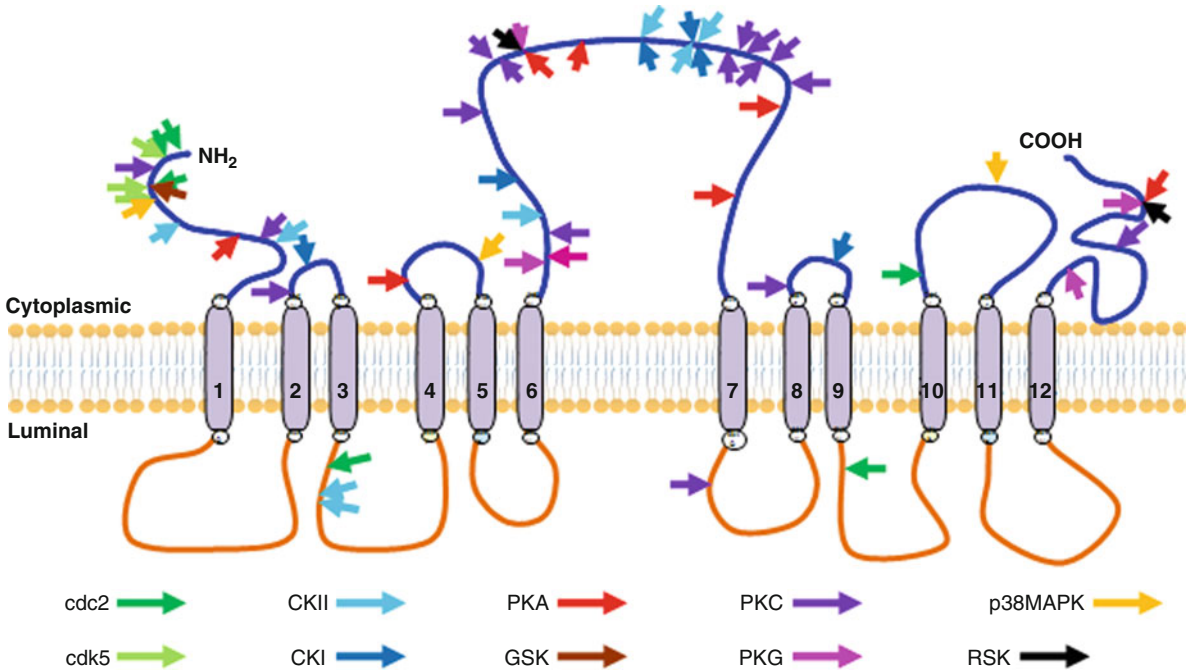
no effect on cAMP-mediated UT-A1 trafficking (Blount et al. 2008). An antibody that specifically detects UT-A1 phosphorylated at the S486 site was used to confirm that vasopressin increases UT-A1 accumulation in the apical plasma membrane and that the S486-phospho-UT-A1 form is primarily detected in the apical plasma membrane (Klein et al. 2010).

Acute Regulation of Urea Transport by Hyperosmolality

Depending upon the hydration status of an animal, the osmolality of the renal medulla varies over a wide range. Increasing osmolality rapidly increases urea permeability in rat terminal IMCDs, even in the absence of vasopressin, suggesting that hyperosmolality is an independent regulator of urea transport. Increasing osmolality has an additive stimulatory effect on urea permeability when vasopressin is present. Both phloretin and thiourea inhibit hyperosmolality-stimulated urea permeability. Hyperosmolality, like vasopressin, increases urea permeability by increasing the V_{max} rather than the K_m. However, these two agonists stimulated urea permeability through different signaling pathways: hyperosmolality via increases in intracellular calcium and PKC; vasopressin via increases in cAMP and PKA activity. These various aspects of hyperosmolar influences on urea permeability are reviewed in (Sands and Layton 2009; Sands and Layton 2008). Hypersomolality, like vasopressin, increases the phosphorylation and the plasma membrane accumulation of both UT-A1 and UT-A3 (Blessing et al. 2008; Blount et al. 2007; Klein et al. 2006). Thus, both hyperosmolality and vasopressin rapidly increase urea permeability but they do so via different signaling pathways. The effects of vasopressin and tonicity are compared in Table 2.

Acute Regulation of Urea Transport by Other Factors

In addition to vasopressin regulation of urea permeability, several other hormones have been shown to affect urea transporter function. Angiotensin II increases both vasopressin-stimulated urea permeability and UT-A1 phosphorylation in rat terminal IMCDs via a PKC-mediated effect. Angiotensin II does not affect urea permeability in the absence of vasopressin (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). Increased levels of glucagon, another hormone that activates the production of cAMP, was shown to have varied effects on urea transport between studies. In one study, glucagon decreased urea permeability in perfused rat terminal IMCDs and UT-A1 protein abundance by stimulating a PKC signaling pathway (Yano et al. 2008). However, in other studies, glucagon did not alter basal or vasopressin-stimulated urea permeability, nor cAMP production, in either the initial or terminal IMCD (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). Oxytocin stimulates urea permeability by binding to V_2-receptors and increasing cAMP production. Alpha-2 adrenergic agonists, such as clonidine and epinephrine, inhibit vasopressin-stimulated urea permeability in the rat terminal IMCD. Likewise, furosemide and amphotericin B also inhibit vasopressin-stimulated urea permeability; however, neither atrial natriuretic peptide nor insulin alters urea permeability in perfused rat terminal IMCDs (reviewed in [Sands



UT (Urea Transporter), Fig. 4 Consensus Phosphorylation Sites in Human UT-A1. The human UT-A1 sequence was analyzed using the NetPhosK 1.0 Server. Arrows indicate predicted sites with a greater than 50% of phosphorylation by the indicated kinase

UT (Urea Transporter), Table 2 Comparison of vasopressin and hyperosmolality in the IMCD. Original citations are reviewed in Sands and Layton 2008, 2009

Property	Vasopressin	Hyperosmolality
Signaling pathway	cAMP and adenylyl cyclase	Intracellular calcium and PKC
Inhibitors	Phloretin, thiourea	Phloretin, thiourea
V_{max}	Increases	Increases
K_m	Unchanged	Unchanged
Phosphorylation	Increases UT-A1 and UT-A3	Increases UT-A1 and UT-A3
Plasma membrane accumulation	Increases UT-A1 and UT-A3	Increases UT-A1 and UT-A3
UT-A Promoter I activity	No effect of cAMP (and no consensus CRE)	Increases via TonE and TonEBP

and Layton 2009; Sands and Layton 2008]). These collective findings suggest that there are many other complex signaling cascades involved in urea transporter function that remain to be elucidated.

Long-Term Regulation of Urea Transport by Vasopressin

Vasopressin may also exert long-term regulation of UT-A1 and UT-A3 through changes in protein abundance. Two approaches that have been used to vary vasopressin levels in animals are: alter endogenous vasopressin levels by altering hydration status; and administering exogenous vasopressin, or dDAVP, a V_2-selective vasopressin receptor agonist, to induce a constant high level of vasopressin.

UT-A1

Treating Brattleboro rats (which lack endogenous vasopressin) with vasopressin for 5 days decreases UT-A1 protein abundance in the inner medulla.

Similarly, terminal IMCDs from water-loaded (3 days) rats have higher basal and higher vasopressin-stimulated urea permeabilities and increased UT-A1 expression by immunohistochemistry than from rats given water ad libitum. The changes in UT-A1 protein abundance in response to changes in vasopressin levels or hydration state do not appear to be regulated by transcription (reviewed in [Sands and Layton 2009; Sands and Layton 2008]).

When vasopressin is administered to Brattleboro rats for a longer period of time (12 days), UT-A1 protein abundance is significantly increased. Similarly, water-loaded (2 weeks) rats have significant decreases in UT-A1 protein abundance. The delayed increase in UT-A1 protein abundance following vasopressin administration to Brattleboro rats is consistent with the time course for the increase in inner medullary urea concentration (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). Analysis of the UT-A promoter I may explain this time-course since it does not contain a cAMP response element (CRE) in the 1.3 kb that has been cloned and thus cAMP does not increase promoter I activity. However, a tonicity enhancer element (TonE) is present in UT-A promoter I and hyperosmolality increases promoter activity. Thus, increasing vasopressin may initially increase the transcription of the Na-K-2Cl co-transporter, NKCC2/BSC1, in the thick ascending limb; the increase in NaCl reabsorption will in turn increase inner medullary osmolality, which will in turn increase UT-A1 transcription through TonE (reviewed in [Sands and Layton 2009; Sands and Layton 2008]).

UT-A2 and UT-A3

The mRNA abundances of UT-A2, UT-A2b, UT-A3, and UT-A3b are decreased in the inner medulla of 3 day water-loaded rats and increased in: (1) 3 day water restricted rats and mice, (2) rats receiving dDAVP for 3 weeks, and (3) Brattleboro rats treated with vasopressin for 1 week (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). UT-A2 and UT-A3 protein expression are decreased in 3 day water-loaded rats without a change in the subcellular distribution of either urea transporter. UT-A2 protein abundance is increased by 7 days of dDAVP administration to Brattleboro rats. UT-A2 regulation could involve transcriptional mechanisms stimulated by vasopressin since UT-A2 is under the control of UT-A promoter II, which contains a CRE element and its promoter activity is increased by cAMP (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). UT-A3, like UT-A1, is under the control of UT-A promoter I, which contains TonE. Tonicity-responsive transcription of UT-A3 would explain the increased UT-A3 protein abundance observed in water deprived rats (reviewed in [Sands and Layton 2009; Sands and Layton 2008]).

Urea Transporter Knockout Mice

Within the last decade, several animal models have been developed to examine the role or the urea transporters in vivo. Each was shown to have urine concentrating defects supporting the hypothesis that urea transporters must be present for proper urine concentration (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). The significant findings are discussed in detail below.

UT-A1/UT-A3 Knockout Mice

Mice lacking both UT-A1 and UT-A3 have reduced urine concentrating ability, reduced inner medullary interstitial urea content, and lack vasopressin-stimulated urea transport in their IMCD (reviewed in [Fenton and Knepper 2007]). These mice are able to concentrate their urine almost as well as wild-type mice when both are fed a low-protein diet, supporting the hypothesis that IMCD urea transport contributes to urine concentrating ability by preventing urea-induced osmotic diuresis (reviewed in [Fenton and Knepper 2007]). Inner medullary tissue urea content was markedly reduced following water restriction, but there was no measurable difference in NaCl content between UT-A1/UT-A3 knockout mice and wild-type mice (reviewed in [Fenton and Knepper 2007]). This finding has been interpreted both as being inconsistent with the predictions of the passive mechanism (Fenton and Knepper 2007) as well as supporting it (Pannabecker et al. 2008).

UT-A2 Knockout Mice

Mice lacking UT-A2 mice have a reduced urine concentrating ability (Uchida et al. 2005). The reduction in urine concentrating ability is less severe than in the UT-A1/UT-A3 knockout mice and likely results from impairment of urea recycling (Uchida et al. 2005).

UT-B1 Knockout Mice

Mice lacking UT-B1 mice have impaired urine concentrating ability, achieving a maximal urine osmolality of 2,400 mOsm/kg H_2O as compared to 3,400 in a wild-type mouse (Yang et al. 2002). This phenotype is similar to humans lacking UT-B1, the Kidd antigen, who are unable to concentrate their urine above 800 mOsm/kg H_2O, even following overnight water deprivation and exogenous vasopressin administration (reviewed in [Sands and Layton 2009; Sands and Layton 2008]). These findings support the hypothesis that urea transport in red blood cells is necessary to preserve the efficiency of countercurrent exchange (reviewed in [Fenton and Knepper 2007; Sands and Layton 2009; Sands and Layton 2008]).

Summary

Urea plays a critical role in the urinary concentrating mechanism, and therefore, in the regulation of the body's water balance. The kidney needs to be able to regulate urea excretion in order to vary urine osmolality and maintain (or restore) plasma osmolality within (to) the normal range. Functional data suggested that urea transport in the IMCD was mediated by specific urea transporter proteins. Subsequent molecular approaches resulted in the cloning of two gene families for facilitated urea transporters, UT-A and UT-B. Since the identification of the four UT-A renal isoforms and UT-B in the vasa recta, great strides have been made toward the understanding of the regulatory signaling mechanisms that control urea permeability. Vasopressin and hypertonicity regulate UT-A1 and UT-A3 by changes in phosphorylation as well as by changes in plasma membrane accumulation. In addition to acute regulation by vasopressin, urea transporter protein abundance is also regulated by chronic vasopressin exposure. Genetically engineered mice that lack one of the urea transporter proteins show that urea transporters play a critical role in the urine concentrating mechanism. Further use of advancing technology including the generation of transgenic animals and proteomic data will continue to elucidate the complex signaling cascades that regulate the urine concentration mechanism.

References

Bagnasco SM. The erythrocyte urea transporter UT-B. J Membr Biol. 2006;212(2):133–8.

Blessing NW, Blount MA, Sands JM, Martin CF, Klein JD. Urea transporters UT-A1 and UT-A3 accumulate in the plasma membrane in response to increased hypertonicity. Am J Physiol Renal Physiol. 2008;295(5):F1336–41.

Blount MA, Klein JD, Martin CF, Tchapyjnikov D, Sands JM. Forskolin stimulates phosphorylation and membrane accumulation of UT-A3. Am J Physiol Renal Physiol. 2007;293(4):F1308–13.

Blount MA, Mistry AC, Fröhlich O, Price SR, Chen G, Sands JM, et al. Phosphorylation of UT-A1 urea transporter at serines 486 and 499 is important for vasopressin-regulated activity and membrane accumulation. Am J Physiol Renal Physiol. 2008;295(1):F295–9.

Chen G, Fröhlich O, Yang Y, Klein JD, Sands JM. Loss of N-linked glycosylation reduces urea transporter UT-A1 response to vasopressin. J Biol Chem. 2006;281(37):27436–42.

Fenton RA, Knepper MA. Urea and renal function in the 21st century: insights from knockout mice. J Am Soc Nephrol. 2007;18(3):679–88.

Fenton RA, Stewart GS, Carpenter B, Howorth A, Potter EA, Cooper GJ, et al. Characterization of the mouse urea transporters UT-A1 and UT-A2. Am J Physiol Renal Physiol. 2002;283(4):F817–25.

Fröhlich O, Klein JD, Smith PM, Sands JM, Gunn RB. Regulation of UT-A1-mediated transepithelial urea flux in MDCK cells. Am J Physiol Cell Physiol. 2006;291:C600–6.

Gamble JL, McKhann CF, Butler AM, Tuthill E. An economy of water in renal function referable to urea. Am J Physiol. 1934;109:139–54.

Hwang S, Gunaratne R, Rinschen MM, Yu M-J, Pisitkun T, Hoffert JD, et al. Vasporessin increases phosphorylation of ser84 and ser486 in Slc14a2 collecting duct urea transporters. Am J Physiol Renal Physiol. 2010;299:F559–67.

Klein JD, Fröhlich O, Blount MA, Martin CF, Smith TD, Sands JM. Vasopressin increases plasma membrane accumulation of urea transporter UT-A1 in rat inner medullary collecting ducts. J Am Soc Nephrol. 2006;17:2680–6.

Klein JD, Blount MA, Fröhlich O, Denson C, Tan X, Sim J, et al. Phosphorylation of UT-A1 on serine 486 correlates with membrane accumulation and urea transport activity in both rat IMCDs and cultured cells. Am J Physiol Renal Physiol. 2010;298(4):F935–40.

Levin EJ, Quick M, Zhou M. Crystal structure of a bacterial homologue of the kidney urea transporter. Nature. 2009;462:757–62.

Mistry AC, Mallick R, Klein JD, Sands JM, Froehlich O. Functional characterization of the central hydrophilic linker region of the urea transporter UT-A1: cAMP activation and snapin binding. Am J Physiol Cell Physiol. 2010;298:C1431–7.

Pannabecker TL, Dantzler WH, Layton HE, Layton AT. Role of three-dimensional architecture in the urine concentrating mechanism of the rat renal inner medulla. Am J Physiol Renal Physiol. 2008;295(5):F1271–85.

Potter EA, Stewart G, Smith CP. Urea flux across MDCK-mUT-A2 monolayers is acutely sensitive to AVP, cAMP, and $[Ca^{2+}]_i$. Am J Physiol Renal Physiol. 2006;291(1):F122–8.

Sands JM, Layton HE. The urine concentrating mechanism and urea transporters. In: Alpern RJ, Hebert SC, editors. The Kidney: Physiology and Pathophysiology. 4th ed. San Diego: Academic; 2008. p. 1143–78.

Sands JM, Layton HE. The physiology of urinary concentration: an update. Semin Nephrol. 2009;29(3):178–95.

Stewart GS, Thistlethwaite A, Lees H, Cooper GJ, Smith C. Vasopressin regulation of the renal UT-A3 urea transporter. Am J Physiol Renal Physiol. 2009;296:F642–9.

Uchida S, Sohara E, Rai T, Ikawa M, Okabe M, Sasaki S. Impaired urea accumulation in the inner medulla of mice lacking the urea transporter UT-A2. Mol Cell Biol. 2005;25(16):7357–63.

Wang Y, Klein JD, Blount MA, Martin CF, Kent KJ, Pech V, et al. Epac regulation of the UT-A1 urea transporter in rat IMCDs. J Am Soc Nephrol. 2009;20(3):2018–24.

Yang B, Bankir L, Gillespie A, Epstein CJ, Verkman AS. Urea-selective concentrating defect in transgenic mice lacking urea transporter UT-B. J Biol Chem. 2002;277:10633–7.

Yano Y, Rodrigues Jr AC, de Braganca AC, Andrade LC, Magaldi AJ. PKC stimulated by glucagon decreases UT-A1 urea transporter expression in rat IMCD. Pflugers Arch. 2008;456:1229–37.

Zhang C, Sands JM, Klein JD. Vasopressin rapidly increases the phosphorylation of the UT-A1 urea transporter activity in rat IMCDs through PKA. Am J Physiol Renal Physiol. 2002;282(1):F85–90.

UT-A

► UT (Urea Transporter)

UT-B

► UT (Urea Transporter)

V

Vaccinia Virus B1R-Related Kinase 1

► VRK1

Vaccinia Virus B1R-Related kinase 2

► VRK2

Vaccinia Virus B1R-Related Kinase 3

► Vaccinia-Related Kinase-3 (VRK3)

Vaccinia-Related Kinase 1

► VRK1

Vaccinia-Related Kinase 2

► VRK2

Vaccinia-Related Kinase 3

► Vaccinia-Related Kinase-3 (VRK3)

Vaccinia-Related Kinase-3 (VRK3)

Marta Vázquez-Cedeira, Diana M. Monsalve, Marta Sanz-García and Pedro A. Lazo
Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Synonyms

Vaccinia-related kinase 3; Vaccinia virus B1R-related kinase 3; VRK-3

Historical Background

VRK3 is a member of the vaccinia-related kinase family (Manning et al. 2002), which comprises a group of three proteins, of which two, VRK1 and VRK2, are catalytically active. VRK3 has substitutions in key residues within its catalytic domain and is therefore a pseudokinase without kinase activity (Nichols and Traktman 2004). The biological effects of VRK3 are mediated by protein–protein interactions. VRK3 can de-activate mitogen-activated protein (MAP) kinase signaling in the nucleus by activating phosphatases such as VHR, which dephosphorylates extracellular signal-regulated protein kinase (ERK), and reducing its ability to activate transcription.

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

VRK3 Gene Expression

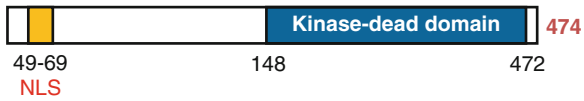
The human *VRK3* gene is located in chromosome 19q13.33, has 15 exons, and its mRNA is 1.9 kb in length (Nichols and Traktman 2004); and although there are several alternative messages, only one protein has been identified.

Murine *VRK3* gene is expressed throughout mouse embryonic hematopoietic development. Peak expression occurs in liver at days E11.5 to E12.5, followed by an order of magnitude drop at day E13.5 to E15.5. Expression levels recover again by day E17.5 in embryogenesis. In fetal peripheral blood lymphocytes expression is high at days E10.5 to E11.5, which is followed by a two- to threefold reduction. Expression levels then remain constant until the end of fetal development (Vega et al. 2003). In the adult mouse, VRK3 mRNA is expressed at similar levels in the liver, kidney, muscle, thymus, and bone marrow. Expression in the adult spleen is one order of magnitude lower (Vega et al. 2003). In an independent study, mouse VRK3 mRNA expression was detected at very high levels in kidney, testis, and small intestine. VRK3 protein was also detectable at significant levels in heart, kidney, and testis, and at lower levels in lung, stomach, liver, spleen, and skeletal muscle. However, the variations in protein expression were much less significant and do not correspond with the large variations in mRNA expression (Kang and Kim 2008).

In rat embryos, the highest VRK3 expression was detected between E15 to E17. However, this study examined whole-embryo expression only during days E13 through E17 (Kang and Kim 2008). VRK3 is highly upregulated in some rat tissues including kidney and testis, which correlates with reduced levels of phospho-ERK (Kang and Kim 2008). Intriguingly, it has been reported that ERK activity promotes transcription of VRK3, suggesting the existence of a feedback inhibition mechanism (Kang and Kim 2006, 2008).

VRK3 Protein Structure

The VRK3 protein has 474 aminoacids and 52.8 kDa (Fig. 1). The crystal structure of VRK3 reveals that despite conservation of the kinase domain fold (PDB: 2JII), mainly in its surface, it presents several changes that render it catalytically inactive, but may function as a scaffold protein and it maintains the overall structure of the kinase domain (Scheeff et al. 2009). The inactivating substitutions occurred early in evolution, and they hinder binding to ATP. VRK3 also has differences in the activation loop, in which a helix is replaced by a kink (Scheeff et al. 2009). Human VRK3 is a pseudokinase and plays a scaffolding role in the nucleus where it recruits the VHR phosphatase (Kang and Kim 2006, 2008). However, the mechanism through which its interaction with VHR is regulated has not been identified. VRK3 is a nuclear protein and has a nuclear localization signal in its N-terminal region (Nichols and Traktman 2004).



Vaccinia-Related Kinase-3 (VRK3), Fig. 1 Structure of human VRK3. NLS: nuclear localization signal

Studies with GFP-hVRK3 fusion proteins revealed that the protein is located in the nucleus. A bipartite nuclear localization signal is found within the first 149 amino acids of VRK3 (Nichols and Traktman 2004). No variant proteins have been identified in vivo (Nichols and Traktman 2004).

VRK3 Signaling

Unlike other vaccinia-related kinases, VRK3 does not have kinase activity. Although it contains a weakly conserved catalytic domain that is related to casein kinases, this domain has several substitutions in residues that are crucial for catalytic activity (Nichols and Traktman 2004). Indeed, its structure reveals that it has a poorly conserved catalytic site, but a highly conserved kinase fold (Scheeff et al. 2009). The human (h)VRK3 and mouse (m)VRK3 proteins are catalytically inactive (Nichols and Traktman 2004) and VRK3 does not bind ATP (Scheeff et al. 2009).

However, VRK3 plays a scaffolding role in the nucleus (Kang and Kim 2006). Human VRK3 is able to directly interact with, and enhance, the activity of the tyrosine phosphatase VHR. This interaction results in dephosphorylation of phospho-ERK1/2 and subsequent downregulation of ERK signaling in the nucleus (Kang and Kim 2006, 2008). Furthermore, in rat neurons treated with cisplatin, the expression levels of

VRK3 and the DUSP6 phosphatase are selectively reduced, resulting in an increased and sustained accumulation of phospho-ERK in these cells (Gozdz et al. 2008). Activation of ERK by phorbol ester (PMA: phorbol-12-mirystate 13-acetate), an analog of diacylglycerol, induces an increase in VRK3 levels, thus establishing a negative feedback in MAPK signaling mediated by VRK3 (Kang and Kim 2006, 2008).

In an analysis of the kinome in response to EGFR signaling, VRK3 and ▶ VRK2 correlated with low levels of p-ERK (Komurov et al. 2010), which is consistent with its role in recruiting phosphatases and dephosphorylating associated proteins.

Human VRK3 also preferentially interacts with the GDP-bound form of the ▶ Ran small GTPase, but the functional significance of this interaction is not known (Sanz-Garcia et al. 2008).

Summary

VRK3 is the most divergent member of the VRK family. It has no kinase activity due to several substitutions in key residues within its catalytic domain. VRK3 is mostly located within the nucleus and appears to play a scaffold role, where it downregulates MAPK signaling and reduces the level of p-ERK by recruiting the VHR phosphatase.

References

Gozdz A, Vashishta A, Kalita K, Szatmari E, Zheng JJ, Tamiya S, Delamere NA, Hetman M. Cisplatin-mediated activation of extracellular signal-regulated kinases 1/2 (ERK1/2) by inhibition of ERK1/2 phosphatases. J Neurochem. 2008;106:2056–67.

Kang TH, Kim KT. Negative regulation of ERK activity by VRK3-mediated activation of VHR phosphatase. Nat Cell Biol. 2006;8:863–9.

Kang TH, Kim KT. VRK3-mediated inactivation of ERK signaling in adult and embryonic rodent tissues. Biochim Biophys Acta. 2008;1783:49–58.

Komurov K, Padron D, Cheng T, Roth M, Rosenblatt KP, White MA. Comprehensive mapping of the human kinome to epidermal growth factor receptor signaling. J Biol Chem. 2010;285:21134–42.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298:1912–34.

Nichols RJ, Traktman P. Characterization of three paralogous members of the mammalian vaccinia related kinase family. J Biol Chem. 2004;279:7934–46.

Sanz-Garcia M, Lopez-Sanchez I, Lazo PA. Proteomics identification of nuclear Ran GTPase as an inhibitor of human VRK1 and VRK2 (vaccinia-related kinase) activities. Mol Cell Proteomics. 2008;7:2199–214.

Scheeff ED, Eswaran J, Bunkoczi G, Knapp S, Manning G. Structure of the pseudokinase VRK3 reveals a degraded catalytic site, a highly conserved kinase fold, and a putative regulatory binding site. Structure. 2009;17:128–38.

Vega FM, Gonzalo P, Gaspar ML, Lazo PA. Expression of the VRK (vaccinia-related kinase) gene family of p53 regulators in murine hematopoietic development. FEBS Lett. 2003; 544:176–80.

VAMP1/2/3/7

Thierry Galli[1,2,3] and Véronique Proux-Gillardeaux[1,2,3,4]

[1]Membrane Traffic in Neuronal & Epithelial Morphogenesis, INSERM ERL U950, Paris, France

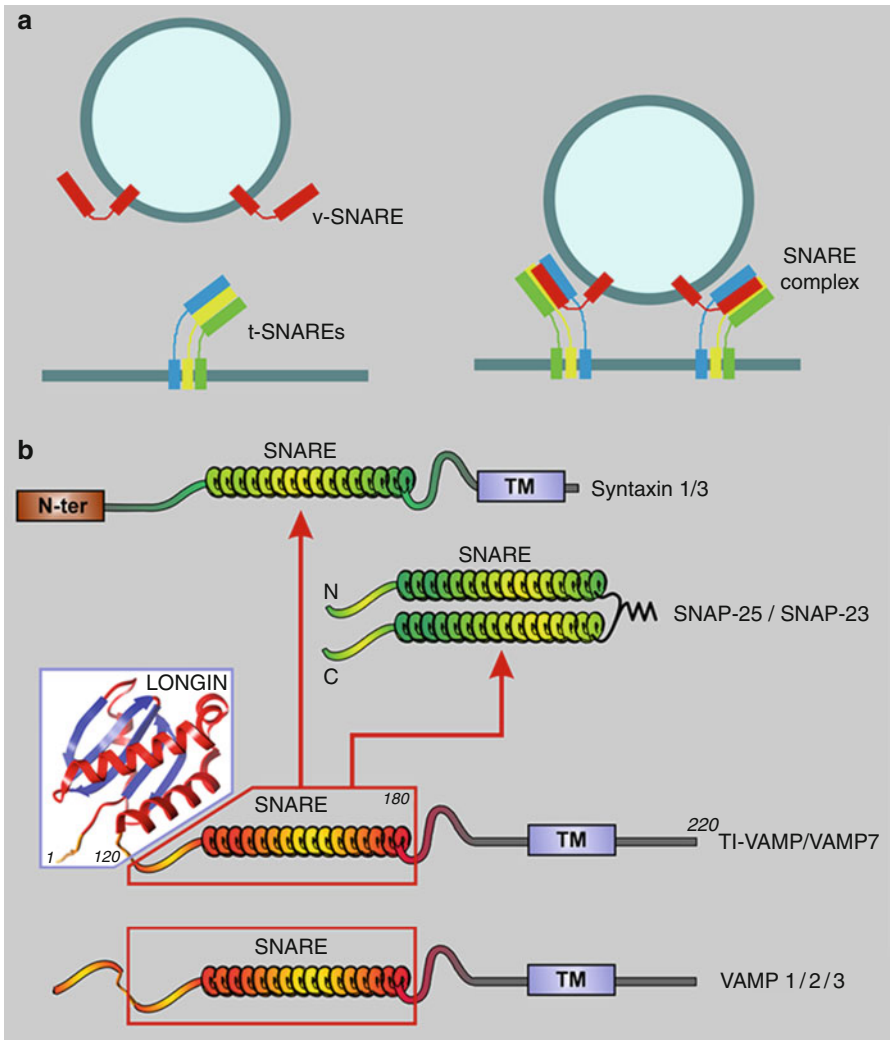
[2]Program in Development & Neurobiology, Jacques Monod Institute, CNRS UMR7592, Paris, France

[3]University Denis Diderot–Paris 7, Paris, France

[4]Membrane Traffic in Neuronal and Epithelial Morphogenesis; Program in Development, Jacques Monod Institute CNRS UMR7592, Equipe INSERM ERL U950, CNRS & Univ. Denis Diderot/Paris 7, Paris, Cedex 13, France

Overview

Intracellular membrane trafficking in eukaryotes is a multiple-step process that can be artificially divided in the budding of vesicles from a donor compartment, their translocation into the cytoplasm along cytoskeletal elements, their tethering and subsequent fusion with the membrane of the target compartment. Membrane fusion involves SNARE proteins, classified into two categories, vesicular (v)-SNAREs and target (t)-SNAREs present on the acceptor membrane. It is the specific pairing of v-SNAREs with their cognate t-SNAREs *in trans* that is responsible for bringing the lipid bilayers together for membrane fusion and the zippering of SNAREs provides the required energy (Fig. 1). This review focus on the discovery of SNAREs and then on four of the nine v-SNAREs: the clostridial neurotoxin sensitive VAMPs 1, 2, and 3 and on Tetanus neurotoxin-Insensitive Vesicle-Associated Membrane Protein, TI-VAMP/VAMP7.



VAMP1/2/3/7,
Fig. 1 Functions and structures of SNARE proteins. (**a**) Membrane fusion involves two types of SNARE proteins, vesicular (v)-SNAREs (in *red*) and target (t)-SNAREs (in *yellow*, *green* and *blue*) present on the acceptor membrane. It is the specific pairing of v-SNAREs with their cognate t-SNAREs *in trans* that form the *SNARE complex* (*right panel*) responsible for bringing the lipid bilayers together for membrane fusion. (**b**) The different structures of SNAREs are represented. t-SNAREs (*top* in *yellow* and *green*) and v-SNAREs (*bottom* in *red*). All SNAREs (v-and t-) share a segment in their cytosolic domain called the *SNARE motif* (boxed in *red* for the v-SNAREs). These domains are responsible for SNAREs interactions by their capacity to assembly into four-helix bundles when forming *SNARE complexes* represented in A. In comparison to VAMP 1/2/3 (100–116aa in human), TI-VAMP (220aa) possesses a long amino-terminal domain (Longin domain) which adopts a "profilin-like" structure, represented in the *blue* box

VAMP7, unlike the first ones, possess a long amino-terminal domain called the Longin domain and thus defining the Longin subfamily of v-SNAREs. Here are reviewed the current knowledge on the functions of four Vesicle Associated Membrane Proteins (VAMPs), VAMPs 1, 2, 3, and 7. VAMPs 1–3 play major role in endosomal vesicular fusion including the recycling of synaptic vesicles and receptors whereas VAMP7 plays multiple functions in post-Golgi trafficking pathways regulating epithelial and neuronal morphogenesis. The role of SNAREs in membrane fusion is now well established, nevertheless, the high homology of v-SNAREs and their apparent redundancy now raise the question of their specificities that still have to be investigated. Also little is known on SNARE mediated fusion regulation. This will certainly be addressed in the next few years.

VAMP Family Members

VAMP1: Vesicle-Associated Membrane Protein 1, Synaptobrevin 1, Syb1, Syb-1, VAMP-1, DKFZp686H12131
VAMP2: Vesicle-Associated Membrane Protein 2, Synaptobrevin 2, Syb2, Syb-2, VAMP-2, FLJ11460
VAMP3: Vesicle-Associated Membrane Protein 3, Cellubrevin, Cb, Ceb, VAMP-3, Synaptobrevin 3, Syb3

VAMP7: Vesicle-Associated Membrane Protein 7, Tetanus neurotoxin Insensitive Vesicle-Associated Membrane Protein (TI-VAMP, TIVAMP, Ti-VAMP), Synaptobrevin-like protein1, Synaptobrevin like 1, Sybl1, VAMP-7, FLJ53045, FLJ53762, FLJ54296

Historical Background: The SNAREs Discovery

VAMP stands for Vesicle-Associated Membrane Protein (For reviews see, Jahn and Scheller 2006). Seven VAMPs have been described: 1, 2, 3, 4, 5, 7, and 8. The first discovered were VAMPs 1 and 2 initially identified as synaptic vesicle proteins thus also called Synaptobrevins 1 and 2. They were then found to be the target of clostridial neurotoxins: botulinum neurotoxins B, D, F, and G and tetanus neurotoxin. Major breakthrough to define VAMPs function originated down the line of in vitro experiments aimed at characterizing transport between Golgi membranes (Balch et al. 1984). Indeed, fusion of Golgi vesicles with acceptor membranes is inhibited by pretreatment of cytosol with the cysteine alkylating agent NEM (N-ethylmaleimide) (Orci et al. 1989). The NEM-sensitive cytosolic component, essential for intra Golgi transport after coat recruitment and vesicle formation, is NSF protein (for *N*EM *S*ensitive *F*actor), the mammalian homologue of Sec18p identified by Novick and Schekman (Block and Rothman 1992). Whereas NSF could be found associated with membrane, purified NSF would not bind to Golgi membranes unless cytosol was added. This triggered the search and purification of the *S*oluble *N*SF *A*ttachment *P*roteins or SNAPs. The homologous proteins α-, β-, and γ-SNAP were purified from bovine brain based on their ability to attach NSF to Golgi membranes and thus restore transport in the in vitro assay. As with NSF, a yeast mutant (Sec17) also identified in the screen of Novick and Schekman failed to restore SNAP activity, but could be rescued by addition of purified mammalian SNAP further strengthening the view that membrane fusion depended on a conserved fusion machinery. Consequently, three proteins were isolated from bovine brain as SNAP receptors which had been previously cloned and found to be associated with the presynaptic terminal: VAMP/Synaptobrevin, Syntaxin 1, and SNAP25. Further characterization of the complex formed by VAMP/Synaptobrevin, Syntaxin 1, and SNAP25 revealed that all three SNAP receptors or "SNARE" proteins are present in a single 20S particle together with NSF and SNAP, and that the ATPase activity of NSF dissociates the complex. An essential role of Synaptobrevin in neurotransmitter release was proposed simultaneously by demonstrating that Synaptobrevin is the target of several neurotoxins. Indeed, clostridial neurotoxins, the most potent blockers of neurotransmitter release, are proteases which cleave the synaptic SNARE proteins Synaptobrevin, SNAP25 or Syntaxin 1. Taking all these observations into consideration, Rothman and colleagues suggested a molecular mechanism, which would explain vesicle docking and fusion in molecular terms, the so-called *SNARE hypothesis* (Fig. 1a) (Söllner et al. 1993a, b; Rothman and Warren 1994): It is now well known that each transport step within the eukaryotic cell is mediated by an original SNARE complex composed of one protein in the vesicle (the v-SNARE also called VAMP) and several in the target membrane (the t-SNAREs). These now historical findings suggested an important role for SNARE proteins at a late step in membrane fusion, a conclusion which has now been largely validated by a large series of experiments in vitro and in vivo. Indeed, SNARE-mediated lipid mixing can be efficiently recapitulated in vitro assays reconstituting fusion with v-SNARE and t-SNARE proteins into separate liposome populations. These assays allowed for identification of lipidic and proteic regulations such as Sec1/Munc18 family of proteins (for a summary of some important results obtained with these in vitro experiments see Ji et al. 2010).

Structure and Function

All SNAREs (v-and t-) share a segment in their cytosolic domain called the *SNARE motif* which can be considered the signature of these proteins (For reviews see, Proux-Gillardeaux et al. 2005a; Jahn and Scheller 2006). It consists of a domain of 60–70 amino acids (Fig. 1b) that is unstructured in solution but yet capable to adopt an α-helicoidal structure upon assembly into four-helix bundles when forming *SNARE complexes* with t-SNAREs.

Membrane fusion requires the assembly of few SNARE complexes (Mohrmann et al. 2010; van den Bogaart et al. 2010), which are composed of one SNARE motif provided by the donor membrane v-SNARE and three SNARE motifs provided by two or three acceptor membrane t-SNAREs. In the best so far characterized SNARE complex, one α-helix is contributed by the v-SNARE VAMP2, one by Syntaxin 1 and two are contributed by SNAP25. Hence, the SNARE core complex consists of four entwisted alpha-helices in which the 'a' and 'd' positions of the SNARE heptad repeats form a 16-layered hydrophobic core with a conserved ionic layer present at the center. This layer, also called the 'zero' layer, is composed of one arginine residue(R), contributed by the v-SNARE, forming hydrogen bonds with three glutamines (Q), each provided from the t-SNAREs. This results in another classification of the v- and t-SNAREs as R-and Q-SNAREs, respectively.

The assembly of v- and t-SNAREs into "trans" complexes likely bridges the opposing lipid bilayers of membranes belonging to donor and acceptor compartments, bringing them in proximity by "zippering" of the coiled–coil domains and inducing their fusion (Li et al. 2007).

Nine v-SNAREs/R-SNAREs have been identified in the human genome, VAMP1, 2, 3, 4, 5, 7, 8, Sec22 and Ykt6. v-SNAREs are small abundant C-terminally anchored type II membrane proteins (Fig. 1b). They vary in their structure and size extension in their amino-terminus part as detailed afterward in the VAMP7 paragraph. The membrane anchor of VAMPs is a transmembrane domain. Although a high level of homology between these proteins and the fact that common properties have been pointed out from structural and functional studies of SNAREs, the different v-SNAREs also have specific characteristics. Notably, the different VAMPs show different membrane fusion efficiency in cell fusion assay. VAMPs 1 and 3 being the most efficient, whereas VAMPs 4, 7, and 8 exhibit 30–50% lower fusion activities (Hasan et al. 2010).

VAMP1-3

VAMPs 1 and 2, also known as Synaptobrevins 1 and 2, are preferentially expressed in the brain where they are associated with synaptic vesicles and are implicated in neurotransmitter release (For reviews see, (Proux-Gillardeaux et al. 2005a; Montecucco et al. 2005). Despite very high homology, they show a differential distribution in the rat brain, with partial overlap. VAMP2 is the most abundant isoform and is widely distributed whereas VAMP1 is less abundant and the main isoform in certain areas (e.g., zona incerta of the subthalamus or nerve terminals surrounding thalamic neurons) (Raptis et al. 2005). VAMP1 plays also an important role implicated in neurotransmitter release at the neuromuscular junction, the synaptic connection between nerve and muscle (Liu et al. 2011).

VAMP3 is the non-neuronal isoform of VAMP2, with a widespread tissue distribution, and participates to regulated and constitutive exocytosis. It has been localized to early endosomes. VAMP3 is also detected in the nervous system, but it is expressed at significant levels only by glia and vascular cells. It has been implicated for instance in the CNS myelination by oligodendrocytes (Feldmann et al. 2011). VAMPs1/2, albeit primarily neuronal, are also expressed by certain non-neuronal cells in which they are involved in various regulated secretory processes. For example, VAMP2 is associated with insulin secretory granules in pancreatic beta cells in the Islets of Langerhans and mediates insulin release (Hou et al. 2009). Interestingly, as for neurotransmitter release, insulin secretion is directly triggered by Ca2+ dependent Insulin granule fusion with the plasma membrane, a process which is lost in Type 1 diabetes with the destruction of beta cells.

Like VAMPs 1 and 2 in neurons, VAMP3 is sensitive to clostridial neurotoxins. Two approaches were undertaken to unravel the function of VAMP2/3: first, a pharmacological approach with clostridial neurotoxins that cleave VAMPs1, 2 and 3, antibodies directed against VAMP 2 or 3, or recombinant VAMPs deleted of their C-terminal transmembrane domains, and second, a genetic approach by mutating the gene in fly and mouse or RNA-mediated interference (RNAi) in cultured cells.

1. *Clostridial neurotoxins* (tetanus neurotoxin (TeNT) and botulinum neurotoxins B, D, F, and G) cleave VAMPs 1, 2 and 3. Clostridial neurotoxins block neurotransmitter release in vivo and in cultured neurons. TeNT-expressing bipolar cell axons

further show synapse formation defects in the retina (Kerschensteiner et al. 2009). In non-neuronal cells, TeNT inhibits the recycling of plasma-membrane receptors including: transferrin receptor, T-cell receptors to the immune synapse, and integrin β_1 in epithelial cells (Proux-Gillardeaux et al. 2005b; Skalski and Coppolino 2005; Tayeb et al. 2005; Hager et al. 2010; Skalski et al. 2010); it inhibits retrograde transport from early endosomes to the Golgi apparatus, apical transport of H^+-ATPase in kidney epithelial cells, and cytokine secretion (Murray et al. 2005); and it decreases the efficiency of phagocytosis by inhibiting focal exocytosis at sites of phagocytosis in macrophages, as does botulinum neurotoxin B. TeNT also impairs cell adhesion and cell migration (Proux-Gillardeaux et al. 2005b; Tayeb et al. 2005; Luftman et al. 2009; Skalski et al. 2010; Veale et al. 2010) and the release of retroviruses. In polarized MDCK cells, TeNT inhibits basolateral sorting of AP-1B dependant cargos (Fields et al. 2007). VAMP3 also plays a role in autophagocytosis in the fusion between MVBs with autophagosomes to generate the amphisome, allowing the maturation of the autophagosome (Fader et al. 2009). Antibodies directed against VAMP3 inhibit α-granule secretion in permeabilized platelets (Flaumenhaft 2003). The cytoplasmic domain of VAMP3 is also a potent inhibitor of both α-granule and dense granule secretion in permeabilized platelets (Polgar et al. 2002).

2. *VAMP2-null mice* show an almost complete block of neurotransmitter release leading to death at birth and a strong inhibition of synaptic vesicle endocytosis. It is unclear if neuronal synapses are fully normal in this mutant in vivo. *VAMP3-null mice* do not show a strong phenotypic defect except for a decrease in phagocytosis of zymosan in macrophages. The *VAMP2–VAMP3 double-knockout mice* show that VAMP3 contributes to secretion in chromaffin cells (Borisovska et al. 2005) but the lethality at birth of these mice prevents further analysis in vivo. Nevertheless, in neurons, the phenotype of VAMP2–VAMP3 double-knockout mice is identical with the single VAMP2 knockout (Deak et al. 2006), thus confirming that VAMP3 is not expressed in neurons as suggested previously. Silencing of VAMP3 by RNAi inhibits the delivery of tumor necrosis factor-α to the plasma membrane in macrophages (Murray et al. 2005; Kay et al. 2006). Functional at least partial redundancy with VAMP4, VAMP7, or VAMP8 may explain the fact that impairment of phagocytosis of zymosan in macrophages was the only clear defect observed in *VAMP3*-null mice. The ortholog of VAMP3 in *Drosophila melanogaster* is the ubiquitously expressed Syb, N-Syb being restricted to neuronal cells. Syb and N-Syb are functionally interchangeable. The loss of Syb in a somatic clone of cells is lethal (Bhattacharya et al. 2002).

VAMP7

In contrast to VAMPs 1, 2, and 3, VAMP7 is resistant to clostridial neurotoxins (tetanus and botulinum neurotoxins B, D, F, and G) (For reviews see, Proux-Gillardeaux et al. 2005a; Chaineau et al. 2009; Pryor and Luzio 2009). For that reason, VAMP7 was also called Tetanus neurotoxin-Insensitive Vesicle-Associated Membrane Protein (TI-VAMP). VAMP7 is also called Synaptobrevin-like 1 (Sybl1), it is encoded by the first pseudoautosomal gene found to be X and Y inactivated *Sybl1* gene is ubiquitously expressed and is extremely conserved. In adult rat brain, VAMP7 is localized in the somatodendritic compartment of neurons and also in subsets of axon terminals including, the hippocampal mossy fibers of the dentate gyrus and of CA3, the striatal peridendritic terminal plexuses in the globus pallidus, substantia nigra pars reticulata, peridendritic plexuses in the central nucleus of the amygdala, and the primary sensory afferents in the dorsal horn of the spinal cord. Unlike the brevins (VAMPs 1, 2, 3 and 8), but like Syntaxins, VAMP7 possess a long amino-terminal extension of about 100 amino acids, also called the "Longin" domain (Fig. 1). Thus VAMP7 is seen as the prototype of the family of 'Longin' v-SNAREs also containing Sec22 and Ykt6. Their Longin domains adopt a conserved globular "profilin-like" structure, similar to the profilin domain of the human SEDL protein and of domain present in the sigma and mu subunits of the AP-2 clathrin adaptor complex. This domain adopts a closed conformation onto their SNARE domain to prevent the formation of SNARE complexes (Vivona et al. 2010). In VAMP7, the Longin domain has two functions: it inhibits the formation of SNARE

complexes and it binds to the δ subunit of the molecular coat AP-3 protein to target VAMP7 to late endosomes or synaptic vesicles. Interestingly, the homologous of VAMP7 in Dictostelium discoideum has been shown to interact with all four Adaptor Protein complexes (AP-1, 2, 3, and 4) and at least AP-2 and AP-3 participate in the sorting of VAMP7 to the endocytic pathways. The endocytosis of VAMP7 depends on interaction with Hrb, a sorting mechanism which depends on the Longin domain. VAMP7 interacts with plasma membrane and endosomal target SNAREs. VAMP7 localizes to the trans-Golgi network (TGN) (Burgo et al. 2009; Danglot et al. 2010) and to late endosomes and lysosomes and synaptic vesicles in certain neurons. VAMP7 allows for heterotypic fusion between endosomes and lysosomes. VAMP7 is also involved in fusion of secretory vesicles with the plasma membrane, which is necessary for phagocytosis, neurite growth, axonogenesis, lysosomal and granule secretion in polarized, migrating, and invading cells. VAMP7 mediates the transport of rafts to the cell surface and rafts are involved in immune and neuronal synapse formation (Bunnell et al. 2002; Francesconi et al. 2009). A function of VAMP7 has been linked to immune responses through focal secretion in plants (Kwon et al. 2008).

Acknowledgments We apologize to all the authors that are not cited in the text due to strict reference limitation, we have cited very few reviews on the topic and only the most recent papers that were not cited in these. Our work is supported in part by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Centre National de la Recherche Scientifique (CNRS), the Association pour la Recherche sur le Cancer (ARC), the Association Française contre les Myopathies (AFM), the Fondation pour la Recherche Médicale (FRM), the Mairie de Paris Medical Research and Health Program, Fédération pour la Recherche sur le Cerveau (FRC), and the Ecole des Neurosciences de Paris-Ile de France (ENP).

References

Balch WE, Dunphy WG, et al. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. Cell. 1984;39(2 Pt 1):405–16.

Bhattacharya S, Stewart BA, et al. Members of the synaptobrevin/vesicle-associated membrane protein (VAMP) family in Drosophila are functionally interchangeable in vivo for neurotransmitter release and cell viability. Proc Natl Acad Sci USA. 2002;99(21):13867–72.

Block MR, Rothman JE. Purification of N-ethylmaleimide-sensitive fusion protein. Methods Enzymol. 1992;219:300–9.

Borisovska M, Zhao Y, et al. v-SNAREs control exocytosis of vesicles from priming to fusion. EMBO J. 2005;24(12):2114–26.

Bunnell SC, Hong DI, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. J Cell Biol. 2002;158(7):1263–75.

Burgo A, Sotirakis E, et al. Role of Varp, a Rab21 exchange factor and TI-VAMP/VAMP7 partner, in neurite growth. EMBO Rep. 2009;10(10):1117–24.

Chaineau M, Danglot L, et al. Multiple roles of the vesicular-SNARE TI-VAMP in post-Golgi and endosomal trafficking. FEBS Lett. 2009;583(23):3817–26.

Danglot L, Chaineau M, et al. Role of TI-VAMP and CD82 in EGFR cell-surface dynamics and signaling. J Cell Sci. 2010;123(5):723–35.

Deak F, Shin OH, et al. Structural determinants of synaptobrevin 2 function in synaptic vesicle fusion. J Neurosci. 2006;26(25):6668–76.

Fader CM, Sanchez DG, et al. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochim Biophys Acta. 2009;1793(12):1901–16.

Feldmann A, Amphornrat J, et al. Transport of the major myelin proteolipid protein is directed by VAMP3 and VAMP7. J Neurosci. 2011;31(15):5659–72.

Fields IC, Shteyn E, et al. v-SNARE cellubrevin is required for basolateral sorting of AP-1B-dependent cargo in polarized epithelial cells. J Cell Biol. 2007;177(3):477–88.

Flaumenhaft R. Molecular basis of platelet granule secretion. Arterioscler Thromb Vasc Biol. 2003;23(7):1152–60.

Francesconi A, Kumari R, et al. Regulation of group I metabotropic glutamate receptor trafficking and signaling by the caveolar/lipid raft pathway. J Neurosci. 2009;29(11):3590–602.

Hager HA, Roberts RJ, et al. Identification of a novel Bves function: regulation of vesicular transport. EMBO J. 2010;29(3):532–45.

Hasan N, Corbin D, et al. Fusogenic pairings of vesicle-associated membrane proteins (VAMPs) and plasma membrane t-SNAREs–VAMP5 as the exception. PLoS One. 2010;5(12):e14238.

Hou JC, Min L, et al. Insulin granule biogenesis, trafficking and exocytosis. Vitam Horm. 2009;80:473–506.

Jahn R, Scheller RH. SNAREs – engines for membrane fusion. Nat Rev Mol Cell Biol. 2006;7(9):631–43.

Ji H, Coleman J, et al. Protein determinants of SNARE-mediated lipid mixing. Biophys J. 2010;99(2):553–60.

Kay JG, Murray RZ, et al. Cytokine secretion via cholesterol-rich lipid raft-associated SNAREs at the phagocytic cup. J Biol Chem. 2006;281(17):11949–54.

Kerschensteiner D, Morgan JL, et al. Neurotransmission selectively regulates synapse formation in parallel circuits in vivo. Nature. 2009;460(7258):1016–20.

Kwon C, Neu C, et al. Co-option of a default secretory pathway for plant immune responses. Nature. 2008;451(7180):835–40.

Li F, Pincet F, et al. Energetics and dynamics of SNAREpin folding across lipid bilayers. Nat Struct Mol Biol. 2007;14(10):890–6.

Liu Y, Sugiura Y, et al. The role of Synaptobrevin1/VAMP1 in Ca2+ – triggered neurotransmitter release at the mouse neuromuscular junction. J Physiol. 2011;589(Pt 7):1603–18.
Luftman K, Hasan N, et al. Silencing of VAMP3 inhibits cell migration and integrin-mediated adhesion. Biochem Biophys Res Commun. 2009;380(1):65–70.
Mohrmann R, de Wit H, et al. Fast vesicle fusion in living cells requires at least three SNARE complexes. Science. 2010;330(6003):502–5.
Montecucco C, Schiavo G, et al. SNARE complexes and neuroexocytosis: how many, how close? Trends Biochem Sci. 2005;30(7):367–72.
Murray RZ, Kay JG, et al. A role for the phagosome in cytokine secretion. Science. 2005;310(5753):1492–5.
Orci L, Malhotra V, et al. Dissection of a single round of vesicular transport: sequential intermediates for intercisternal movement in the Golgi stack. Cell. 1989;56:357–68.
Polgar J, Chung SH, et al. Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. Blood. 2002;100(3):1081–3.
Proux-Gillardeaux V, Gavard J, et al. Tetanus neurotoxin-mediated cleavage of cellubrevin impairs epithelial cell migration and integrin-dependent cell adhesion. Proc Natl Acad Sci USA. 2005a;102(18):6362–7.
Proux-Gillardeaux V, Rudge R, et al. The tetanus neurotoxin-sensitive and insensitive routes to and from the plasma membrane: fast and slow pathways? Traffic. 2005b;6(5):366– 73.
Pryor PR, Luzio JP. Delivery of endocytosed membrane proteins to the lysosome. Biochim Biophys Acta. 2009;1793(4):615–24.
Raptis A, Torrejon-Escribano B, et al. Distribution of synaptobrevin/VAMP 1 and 2 in rat brain. J Chem Neuroanat. 2005;30(4):201–11.
Rothman JE, Warren G. Implication of the SNARE hypothesis for intracellular membrane topology and dynamics. Curr Biol. 1994;4:220–33.
Skalski M, Coppolino MG. SNARE-mediated trafficking of alpha5beta1 integrin is required for spreading in CHO cells. Biochem Biophys Res Commun. 2005;335(4):1199–210.
Skalski M, Yi Q, et al. Lamellipodium extension and membrane ruffling require different SNARE-mediated trafficking pathways. BMC Cell Biol. 2010;11:62.
Söllner T, Bennett MK, et al. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell. 1993a;75:409–18.
Söllner T, Whiteheart SW, et al. SNAP receptors implicated in vesicle targeting and fusion. Nature. 1993b;362:318–24.
Tayeb MA, Skalski M, et al. Inhibition of SNARE-mediated membrane traffic impairs cell migration. Exp Cell Res. 2005;305(1):63–73.
van den Bogaart G, Holt MG, et al. One SNARE complex is sufficient for membrane fusion. Nat Struct Mol Biol. 2010;17(3):358–64.
Veale KJ, Offenhauser C, et al. Recycling endosome membrane incorporation into the leading edge regulates l amellipodia formation and macrophage migration.Traffic. 2010;11(10):1370–9.
Vivona S, Liu CW, et al. The longin SNARE VAMP7/TI-VAMP adopts a closed conformation. J Biol Chem. 2010;285(23):17965–73.

Vanilloid-Like TRP Channels, TRPV1-6

► TRP (Transient Receptor Potential Cation Channel)

Vav

► Vav Family

Vav Family

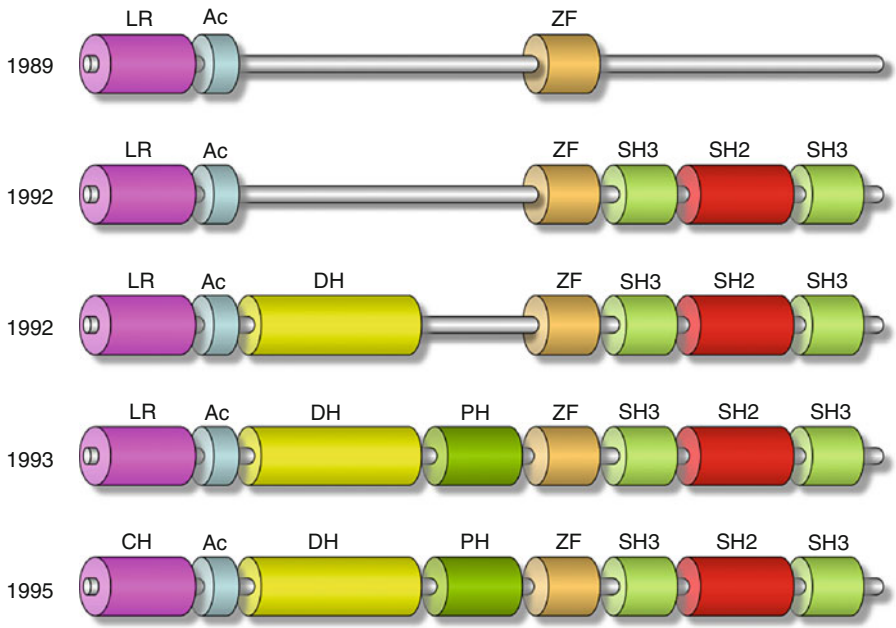
Xosé R. Bustelo
Centro de Investigación del Cáncer/Cancer Research Center, CSIC-University of Salamanca, Salamanca, Spain

Synonyms

Vav1 (Vav; Vav-1 oncogene; Vav oncogene), Vav2 (Vav-2 oncogene), Vav3 (Vav-3 oncogene)

Historical Background

The Vav family is a group of signal transduction molecules that work primarily as phosphorylation-dependent GDP/GTP exchange factors (GEFs) for GTPases of the Rho/Rac subfamily. This family is composed of three members in vertebrates (Vav1, Vav2, and Vav3) and single representatives in invertebrate species (known generically as Vav). Instead, Vav proteins are not present in unicellular organisms or plants. The first member of this family was discovered in 1989 due to the spurious stimulation of its transforming activity during transfections of a human tumor–derived genomic DNA in rodent fibroblasts (Katzav et al. 1989). Since that oncogene was the sixth one isolated in Mariano Barbacid's lab, it received the name of the sixth letter of the Hebrew alphabet (*Vav*). The product encoded by the proto-oncogene was designated as Vav or, taking into consideration its molecular weight and the notation for oncogenes at that time, as $p95^{vav}$. The Vav2 and Vav3 proteins were subsequently identified in human



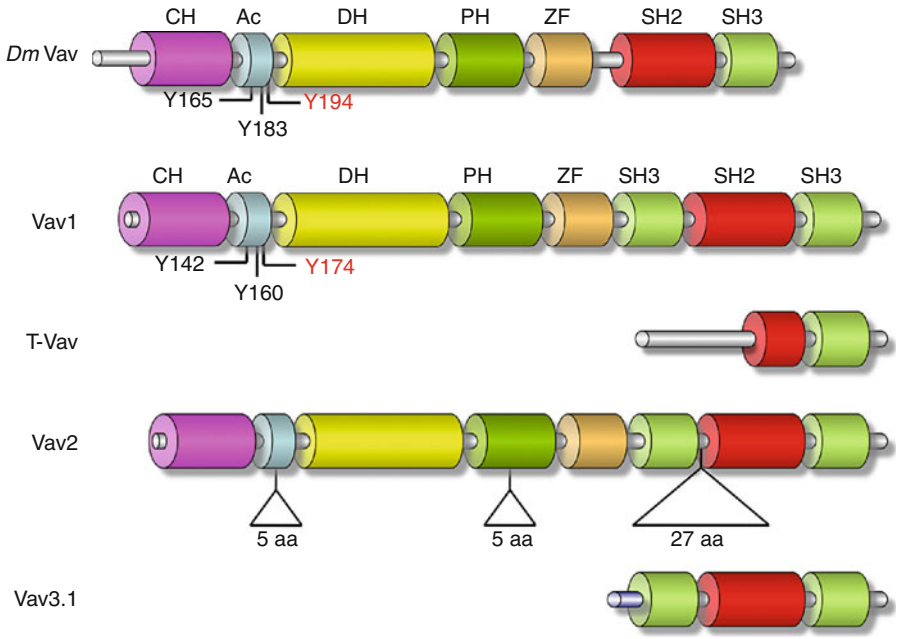
Vav Family,
Fig. 1 Evolution of the structural composition of Vav family proteins. The years in which the domains were discovered are indicated on the left. LR, leucine-rich/helix-loop-helix domain. The rest of abbreviations are described in the main text

and rodent species between 1995 and 2000. Since then, $p95^{vav}$ was re-baptized as Vav1 to maintain a consistent nomenclature within the family. The explosion of gene data derived from current genome sequencing efforts has led to the discovery of the rest of the Vav family members present in vertebrate and invertebrate species in the last decade.

The function of Vav1 could not be easily inferred upon its isolation, since many of the currently known structural domains had not been characterized at that time (Fig. 1). Due to this, the initial structure described for Vav1 only included the Ac, the ZF, and a putative N-terminal leucine-rich region/helix-loop-helix domain (Fig. 1) (Katzav et al. 1989). The presence of the latter domain, which is commonly found in transcriptional factors, led to the idea that Vav1 was perhaps a transcriptional factor. The concept that Vav1 was a signal transduction molecule implicated in protein tyrosine kinase–dependent routes crystallized in 1992 upon the discovery that this protein contained ▶ Src-homology (SH) regions and that it could become tyrosine phosphorylated upon stimulation of both mitogenic and antigenic receptors (Bustelo and Barbacid 1992; Bustelo et al. 1992). The discovery that Vav1 was a phosphorylation-dependent Rho/Rac GEF was reported 5 years later (Crespo et al. 1997). This final step allowed to obtain an integrated view of the mechanisms mediating the activation of Vav proteins during physiological and oncogenic conditions. However, a complete view of the mechanism by which the catalytic activity of these proteins was regulated by tyrosine phosphorylation could not be achieved until 2010 (Yu et al. 2010). Since 1994, extensive work using model organisms demonstrated that these proteins, either alone or acting together in a concerted manner, modulate important biological processes in vivo in species ranging from *C. elegans* to mice. This chapter will provide an overall view of the current knowledge about the structure, biological function, and regulatory mechanisms of these proteins. Readers can find further information in published reviews (Bustelo 2000, 2001; Turner and Billadeau 2002; Tybulewicz et al. 2003) and in the UCSD-Nature Molecular Pages initiative available online (Bustelo 2008; Bustelo and Couceiro 2008).

Structure

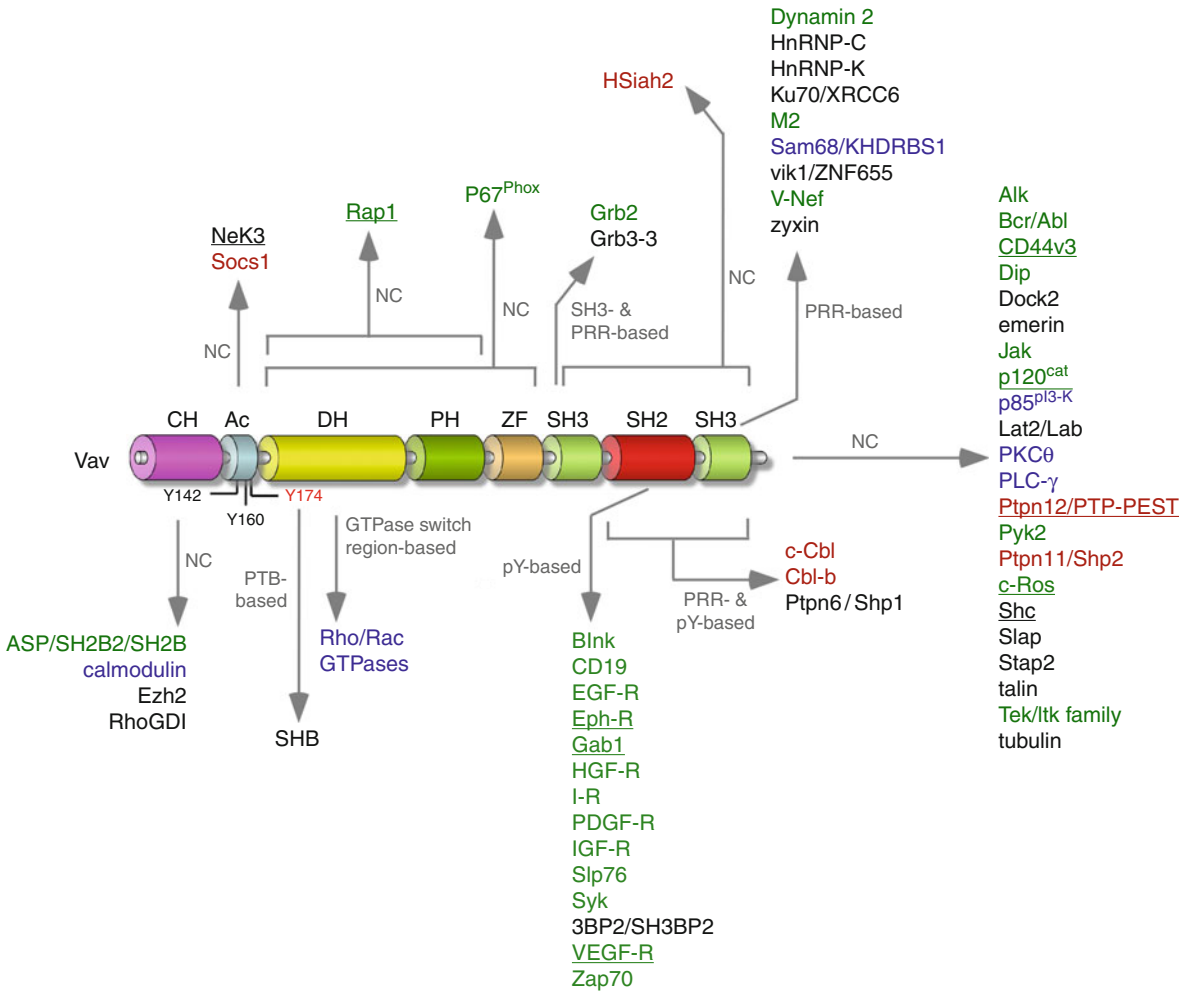
All vertebrate and prochordate Vav family members contain eight different structural domains: a calponin-homology (CH) region, an acidic (Ac) domain, a Dbl-homology region (DH), a pleckstrin-homology (PH) domain, a zinc finger (ZF) region, two SH3 domains, and a SH2 region (Figs. 1 and 2). Vav family proteins from invertebrate species lack the most N-terminal SH3 region (Fig. 2). *Vav* family genes can generate a number of Vav isoforms by differential splicing



Vav Family, Fig. 2 Structure of Vav family members and main isoforms. The structure of *D. melanogaster* (*Dm*) and mammalian Vav family members and isoforms are depicted. In addition to the structural domains present in those proteins, the three regulatory phosphorylation sites present in the Ac domains of Vav family proteins are shown. The main phosphosite is indicated in red. The lengths of the insertions that can be found in Vav2 isoforms are also indicated. aa, amino acid

(Fig. 2). The functional significance of these alternative isoforms is currently unknown. Although all the domains present in Vav proteins are commonly found in many signaling molecules, Vav proteins are the only ones combining on the same molecule a DH-PH-ZF cassette and a SH2 region (Bustelo 2000). This is due to their quite specific requirements for catalysis (see section Biological Activity) and activation (see section Regulation of Biological Activity), respectively.

Current evidence suggests that each structural domain has multifunctional roles, participating in intramolecular regulatory actions, protein–protein interactions, and/or effector roles (Bustelo 2000, 2008; Bustelo and Couceiro 2008). The N-terminal CH and Ac regions participate in phosphorylation-dependent intramolecular interactions that regulate the catalytic activity of Vav proteins (see below, section Regulation of Biological Activity) and, at the same time, establish physical interactions with other proteins (Fig. 3). Although the functional significance of most of those interactions is still unclear, some of them appear to be important for either the activation of specific Vav family members (i.e., APS contributes to Vav3 activation; Fig. 3) or the assembly of downstream, T-cell-specific functions (i.e., calmodulin, Fig. 3) (Bustelo 2000, 2008; Bustelo and Couceiro 2008). The Vav family DH regions are the catalytic domains that stimulate GDP/GTP exchange on Rho/Rac proteins and, at the same time, participate in the formation of heteromolecular interactions that contribute to either the activation ($p67^{Phox}$, Fig. 3) or subcellular localization (Rap1, Fig. 3) of specific Vav family members (Bustelo 2000, 2008; Bustelo and Couceiro 2008). The Vav family PH and ZF domains form a structural unit with the DH domain that favors the formation of a catalytic competent conformation of the DH region (Chrencik et al. 2008; Rapley et al. 2008). The most N-terminal SH3 domain and an adjacent upstream proline-rich region (PRR) participate in the formation of protein–protein interactions with Grb2 family members (Grb2, Grb3-3; Fig. 3). The interaction with Grb2 is important to allow the association of Vav proteins with specific transmembrane proteins (Bustelo 2000, 2008; Bustelo and Couceiro 2008). The Vav family SH2 domains are crucial for the activation of Vav family proteins during signal transduction, because they allow the interaction of the inactive Vav proteins with a wide spectrum of activated, tyrosine-phosphorylated transmembrane and cytoplasmic tyrosine kinases (Fig. 3). In addition, it favors the interaction of active Vav proteins with cytoplasmic proteins (Slp76, Blnk) that participate in their signal transduction pathways (Fig. 3) (Bustelo 2000, 2008; Bustelo and Couceiro 2008). The most C-terminal SH3 domain participates in the formation of extensive heteromolecular interactions with PRR-containing



Vav Family, Fig. 3 Vav-binding partners. Proteins playing positive, negative, and effector roles are depicted in green, red, and blue, respectively. Note however that some regulatory proteins play also downstream roles (i.e., Blnk, Slp76). Proteins with unclear function in the Vav route are shown in black. Molecules identified only in either Vav2 or Vav3 are underlined. Proteins that bind to Vav proteins in co-immunoprecipitation experiments through uncharacterized mechanisms are indicated on the right. The structural domains and type of interactions are also indicated. PTB, phosphotyrosine-binding domain; pY, tyrosine phosphorylation. NC, not characterized

proteins (Fig. 3). Some of those SH3 partners facilitate the translocation of Vav proteins (dynamin 2, Fig. 3) or participate in the catalytic-independent effector functions of these proteins (Sam68; Fig. 3). However, the functional significance of many of the Vav family C-terminal SH3–binding partners remains to be determined (Bustelo 2000, 2008; Bustelo and Couceiro 2008). Interestingly, the Vav C-terminal SH3 region is also the docking site for proteins encoded by viral pathogens such as the human immunodeficiency virus (HIV, v-Nef protein) and γ-herpesvirus (M2 protein). The interaction of those two proteins allows the spurious, stimulus-independent activation of some Vav family proteins during viral infections. Some of the C-terminal Vav domains act coordinately in the formation of intermolecular associations with proteins that negatively modulate Vav-dependent pathways such as Cbl family members, hSiah2, and the protein tyrosine phosphatase ▶ Ptpn6/Shp1 (Fig. 3) (Bustelo 2000, 2008; Bustelo and Couceiro 2008). It is likely that the Vav structural domains will participate in additional heteromolecular interactions, because Vav proteins can co-immunoprecipitate with a large collection of signaling proteins (Fig. 3) (Bustelo 2000, 2008;

Bustelo and Couceiro 2008). Although the function of most of those interacting molecules in the context of Vav-dependent routes is still unknown, some of them have been shown to act as upstream kinases (Alk, Bcr/Abl, Jak), facilitators of the translocation of Vav proteins to the plasma membrane (CD44v3, Dip, $p120^{Cat}$, Pyk2, Itk), effector molecules ($p85^{PI3\text{-}K}$, PKCθ, phospholipase-γ (PLC-γ) family members), and as Vav family inhibitors (Ptpn11 and Ptpn12 protein tyrosine phosphatases) (Fig. 3).

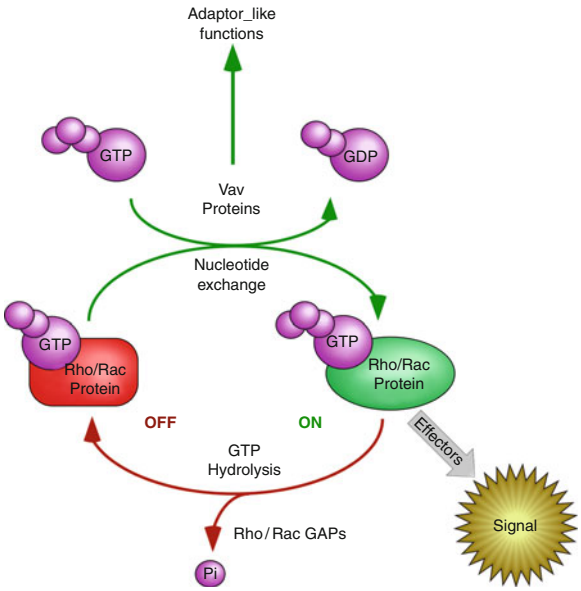
It is conceivable that all Vav family members will share most of those interactions, although current evidence indicates that some selectivity must exist. For example, M2 and v-Nef interact with Vav1 and Vav2 but not with Vav3. Likewise, dynamin 2 binds to Vav1 but not to the other two mammalian Vav family members. A detailed, side-by-side comparison of the signaling properties of all Vav family members is still needed in order to fully understand the level of overlap and phylogenetic conservation of the Vav family interactomes.

Expression

Vav1 is primarily expressed in hematopoietic cells both in the embryonic and adult periods. Vav2 and Vav3 are also expressed in hematopoietic cells but, unlike the case of Vav1, also show high levels of expression in non-hematopoietic tissues (Bustelo 2000). *C. elegans* Vav is detected in contractile organs such as the pharynx, proximal gonad, intestine, body wall muscle, and vulval epithelia (Norman et al. 2005). *D. melanogaster* Vav is ubiquitously expressed during early embryos and accumulates in both the central nervous system and invaginating midgut at later embryonic stages (Malartre et al. 2010). These data indicate that Vav1 is, so far, the Vav family member with a more restricted pattern of expression.

Biological Activity

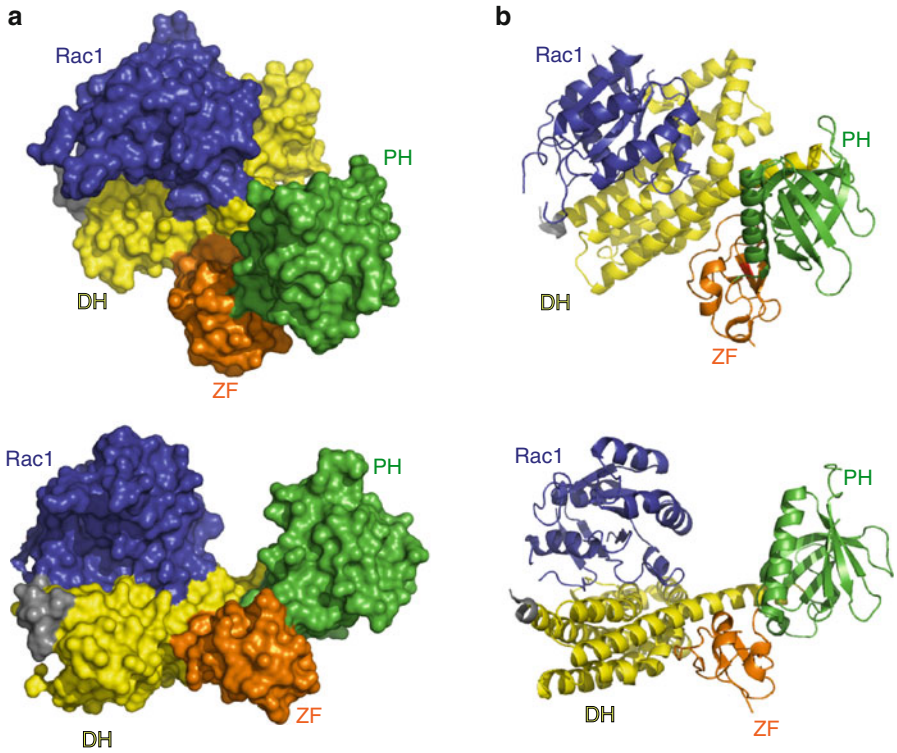
The main known function of Vav family proteins is to act as GEFs for Rho/Rac subfamily GTPases (Crespo et al. 1997; Movilla and Bustelo 1999; Schuebel et al. 1998). This activity favors the rapid shift of those GTP-binding proteins from the inactive (GDP-bound) to the active (GTP-bound) state during signal transduction (Fig. 4). Vav proteins promote the activation of Rac subfamily GTPases (e.g., Rac1, RhoG) and, to a lower extent, of Rho (e.g., RhoA) subfamily proteins in vitro. There is discrepancy about the possible



Vav Family, Fig. 4 Vav family proteins work as Rho/Rac GEFs and adaptor proteins. Inactive and active conformations of the GTPase substrates are indicated in red and green, respectively. GAPs, GTPase-activating proteins that favor the transition of Rho/Rac proteins from the active state to the inactive state; Pi, inorganic phosphate released upon GTP hydrolysis

activation of Cdc42 among different reports (Bustelo 2000, 2008; Bustelo and Couceiro 2008). Unlike other activators of Rho/Rac proteins whose enzyme activity relies exclusively within the catalytic DH region, the catalysis of nucleotide exchange on GTPase substrates requires the entire DH-PH-ZF cassette. The structural details of the catytically competent Vav1 DH-PH-ZF structure and the Vav1/Rac1 complex have been recently described using crystallographic methods (Fig. 5) (Chrencik et al. 2008; Rapley et al. 2008). Vav proteins require prior phosphorylation on three tyrosine residues present in the Ac domain (Y174 and, to a lesser extent, of Y142 and Y160; Figs. 2 and 3) by upstream protein tyrosine kinases to become catalytically active (see below, section Regulation of Biological Activity). Due to this, Vav proteins always work downstream of transmembrane receptors that contain either intrinsic (i.e., transmembrane protein tyrosine kinase receptors such as those for epidermal growth and platelet-derived growth factors) or associated (i.e., antigen receptors) tyrosine kinase activity (Bustelo 2000, 2008; Bustelo and Couceiro 2008).

Consistent with their role as Rho/Rac GEFs, Vav proteins induce Rho-/Rac-dependent biological



Vav Family, Fig. 5 3D structure of the Vav1 DH-PH-ZF cassette bound to Rac1 in surface (**a**) and ribbon (**b**) views. The N-terminal region of the Vav1 DH domain is shown in gray. The structures shown at the bottom have been rotated ≈ 90º in their horizontal axis relative to those shown in the top

responses in tissue culture such as F-actin polymerization, invasiveness, motility, integrin-mediated adhesion, cell cycle transitions, or metastasis. They also stimulate canonical signaling pathways located downstream of Rho/Rac GTPases such as p21-activated kinases (Pak), c-Jun N-terminal kinase (JNK), the nuclear factor kappa-light-chain-enhancer of activated B cells (► NF-κB), activator protein-1 (AP-1) family proteins, and serum responsive factor (SRF) (Bustelo 2000, 2008; Bustelo and Couceiro 2008; Turner and Billadeau 2002). The crucial importance of Vav family–dependent pathways in vivo has been demonstrated by analyzing the phenotypes of single or compound null mutations in model organisms. In agreement with its hematopoietic-specific pattern of expression, it has been shown that mouse Vav1, either alone or in combination with the rest of Vav family members, plays crucial roles in the development, selection, and effector functions of lymphocytes (Table 1) (Bustelo 2008; Bustelo and Couceiro 2008; Turner and Billadeau 2002; Tybulewicz et al. 2003). Signaling defects have been also found in a large variety of hematopoietic cells including platelets, neutrophils, macrophages, mast cells, natural killer cells, and dendritic cells (Table 1) (Bustelo 2008; Bustelo and Couceiro 2008; Turner and Billadeau 2002). In the case of Vav2- and Vav3-deficient mice, defects have been also observed outside the hematopoietic system. Those include dysfunctions in axon guidance, angiogenesis, bone remodeling, cerebellar development, and cardiovascular system homeostasis (Table 1) (Bustelo 2008; Bustelo and Couceiro 2008). The signaling dissection of those defects indicates that Vav proteins are essential for a large number of signaling responses induced by antigen receptors in hematopoietic cells. Those include: (1) T-cell and B-cell receptor responses that contribute to cytoskeletal rearrangement, formation of lymphocyte/antigen-presenting cell contacts, and lymphocyte responsiveness. (2) Signaling diversification events such as the stimulation of phoshatidylinositol-3 kinase (PI3-K)/Akt-, PLC-γ-, Ras/Erk-, PKC-, ► PKD-, and PLD-dependent routes. (3) Activation of downstream transcriptional factors such as the nuclear factor of activated T cells (► NFAT), ► NF-κB, SRF, and AP-1 family proteins. (4) Expression of lymphocyte surface markers and cytokines. (5) Modulation of cell cycle regulators and survival pathways (Bustelo 2008; Bustelo and Couceiro 2008; Turner and Billadeau 2002; Tybulewicz et al. 2003). In non-hematopoietic cells, Vav proteins have been

Vav Family, Table. 1 Phenotypes observed in Vav family knockout mice

Cell type	$Vav1^{-/-}$[a]	$Vav2^{-/-}$	$Vav3^{-/-}$	$Vav1^{-/-};Vav2^{-/-}$	$Vav1^{-/-};Vav3^{-/-}$	$Vav2^{-/-};Vav3^{-/-}$	$Vav1^{-/-};Vav2^{-/-};Vav3^{-/-}$
T cells							
Thymocytes	• Defective developmental transitions • Defective positive and negative selection • T-cell lymphomas at older ages	• Normal	• Normal	• Similar to *Vav1*$^{-/-}$ mice when tested	• Aggravation of *Vav1*$^{-/-}$ phenotype	• Normal	• Aggravation of *Vav1*$^{-/-}$; *Vav3*$^{-/-}$phenotype
Mature	• Reduced numbers • Defective antigenic, BCR responses • Defective recruitment of activated T cells in peripheral tissues	• Normal	• Normal	• Similar to *Vav1*$^{-/-}$ mice when tested	• Aggravation of *Vav1*$^{-/-}$ phenotype	• Normal	• Aggravation of *Vav1*$^{-/-}$; *Vav3*$^{-/-}$phenotype
B cells							
Immature	• Absence of peritoneal B_1 cells	• Normal	• Normal	• Blockage in B-cell maturation	• NA[b]	• NA	• Blockage in B-cell maturation
Mature	• Defective maturation in periphery • Defective T-cell-dependent responses • Defective BCR-dependent proliferative and adhesion responses • Defective CD180-dependent responses	• Defective BRC-dependent proliferation under suboptimal conditions of stimulation		• Reduced numbers • Defective maturation in periphery • Defective CD180- and LPS-dependent responses			• Defects in peripheral B-cell survival • Defective responses to chemokine receptors
Platelets	• Normal	• Normal	• Normal	• NA	• Defects in platelet aggregation and spreading	• NA	• Similar to *Vav1*$^{-/-}$;*Vav3*$^{-/-}$ mice

(*continued*)

Table. 1 (continued)

Cell type	Vav1$^{-/-a}$	Vav2$^{-/-}$	Vav3$^{-/-}$	Vav1$^{-/-}$;Vav2$^{-/-}$	Vav1$^{-/-}$;Vav3$^{-/-}$	Vav2$^{-/-}$;Vav3$^{-/-}$	Vav1$^{-/-}$;Vav2$^{-/-}$;Vav3$^{-/-}$
Neutrophils	• Crawling and migration defects • Defective extravasation in inflamed vasculature	• Normal	• Normal	• NA	• Defects in integrin-dependent adhesion, spreading, and phagocytosis • Suboptimal activation of IgG/FcγR-dependent hemorrhage and edema in lung and skin	• NA	• Lack of oxidative burst • Defective interstitial transit to sites of bacterial infection • Defective responses to nosocomial organisms
Macrophages	• No defects	• No defects	• No defects	• NA	• Defective complement-mediated phagocytosis	• NA	• Similar to *Vav1*$^{-/-}$;*Vav3*$^{-/-}$ mice
Mast cells	• Defective degranulation and cytokine production • Suboptimal anaphylactic responses	• NA	• NA	• NA	• NA	• NA	• NA
Natural killer cells	• Defective natural and antitumoral cytotoxic responses • Defective responses induced by DAP10 receptor	• NA	• NA	• NA	• NA	• Defective DAP12- and FcRy-dependent cytotoxic responses	• Impaired PAP10-, DAP12- and FcRg-dependent killing
Dendritic cells	• Defective integrin-mediated adhesion and podosomal dynamics	• NA	• NA	• NA	• NA	• NA	• Defective antigen presentation due to improper function of DAP12 and FcRg receptors

Cardiovascular system	• Normal	• Hypertension • Renocardiovascular disease • Defective nitric oxide–dependent vasodilatation responses of vascular smooth muscle cells	• Hypertension • Renocardiovascular disease • Defective late maturation steps of osteoclasts	• NA	• NA	• Hypertension • Renocardiovascular disease • Defective nitric oxide–dependent vasodilatation responses of vascular smooth muscle cells • The phenotype is the combination of those found in single knockout animals. There is no aggravation of phenotype	• NA
Nervous system	• Normal	Hydrocephaly	• Hydrocephaly • Cerebellar development and motor coordination defects			• Hydrocephaly • Axon guidance of retinal ganglion cells	• NA
Bone	• Normal	• Normal	• Defective osteoclasts function • Defective bone remodeling	• NA	• NA	• NA	• NA
Endothelial cells	• NA	• NA	• NA	• NA	• NA	• Angiogenesis defects	• NA
Intestine	• NA	• NA	• NA	• NA	• NA	• NA	• Minor defects in cecal enterocytes • Ulcerative lesions in intestine in old animals

[a]The genotypes of animals analyzed are indicated
[b]NA, not analyzed

Vav Family, Table. 2 Phenotypes derived from the alteration of the function of invertebrate species Vav family members

Organism	Phenotype
C. elegans	
Gain-of-function[a]	• Hypercontracted muscles
Loss-of-function[b]	• Feeding problems during early development • Larval lethality • Defective pharyngeal contractility • Defective and arrhythmic ovulation and defecation cycles • Increased activity of Lin-12/Notch, leading to the generation of hermaphrodites with multiple pseudovulvae
D. melanogaster	
Gain-of-function	• Defective dorsal closure • Problems in myoblast fusion • Defective migration of caudal visceral mesodermal cells • Dysfunctional tracheal development
Loss-of-function	• Semilethal embryonic lethality, some of which can be due to improper eclosion of flies from eggs due to locomotor deficits • Flies that escape embryonic lethality display severe locomotor defects and a "shaky" phenotype • Axon guidance defects • In combination with loss of Trio (another Rac1 GEF), embryonic patterning defects and aggravation of the axon guidance defects found in embryos lacking either Vav or Trio. • In combination with loss of Sos1 (a Rac1/Ras GEF), aggravation of the axonal problems found in the absence of Vav

[a]Gain-of-function refers to phenotypes obtained upon the ectopic expression of oncogenic versions of these proteins in the respective species

[b]Loss-of-function refers to phenotype derived from animals in which the *Vav* family genes have been inactivated by genetic techniques

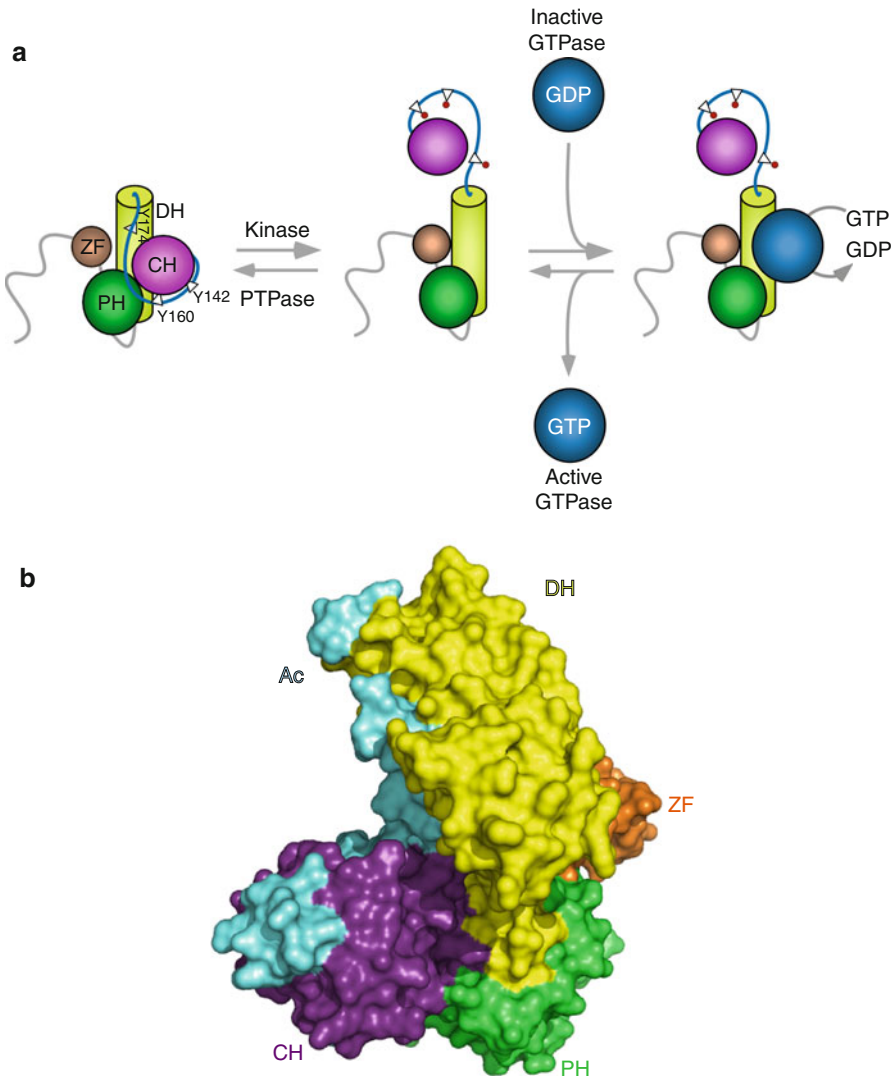
shown to be important for ▶ Eph receptor signaling during angiogenesis and axon guidance as well as for the proper assembly of nitric oxide–dependent signals in vascular smooth muscle cells (Bustelo 2008; Bustelo and Couceiro 2008).

In invertebrates, *C. elegans* Vav is important for the rhythmic behavior of a number of tissues, leading to improper pharyngeal contractility as well as defective ovulation and defecation activities. This leads to larval lethality when its locus is disrupted genetically in this nematode (Table 2) (Norman et al. 2005). *D. melanogaster* Vav is important for neuronal axon guidance and proper locomotor coordination (Table 2) (Malartre et al. 2010). These phenotypes are in agreement with the expression patterns of these two Vav family members in those two species and consistent with their role as Rac-specific GEFs.

Vav proteins can also work as adaptor molecules due to the extensive formation of heteromolecular complexes that they can assemble in vivo (Figs. 3 and 4). Recently, this adaptor role has been formally demonstrated using a knock-in mouse expressing a catalytically inactive Vav1 mutant. In these mice, it has been shown that the activation of PLC-γ-dependent routes in T cells can be fully achieved in a GTPase-independent manner (Saveliev et al. 2009).

Regulation of Biological Activity

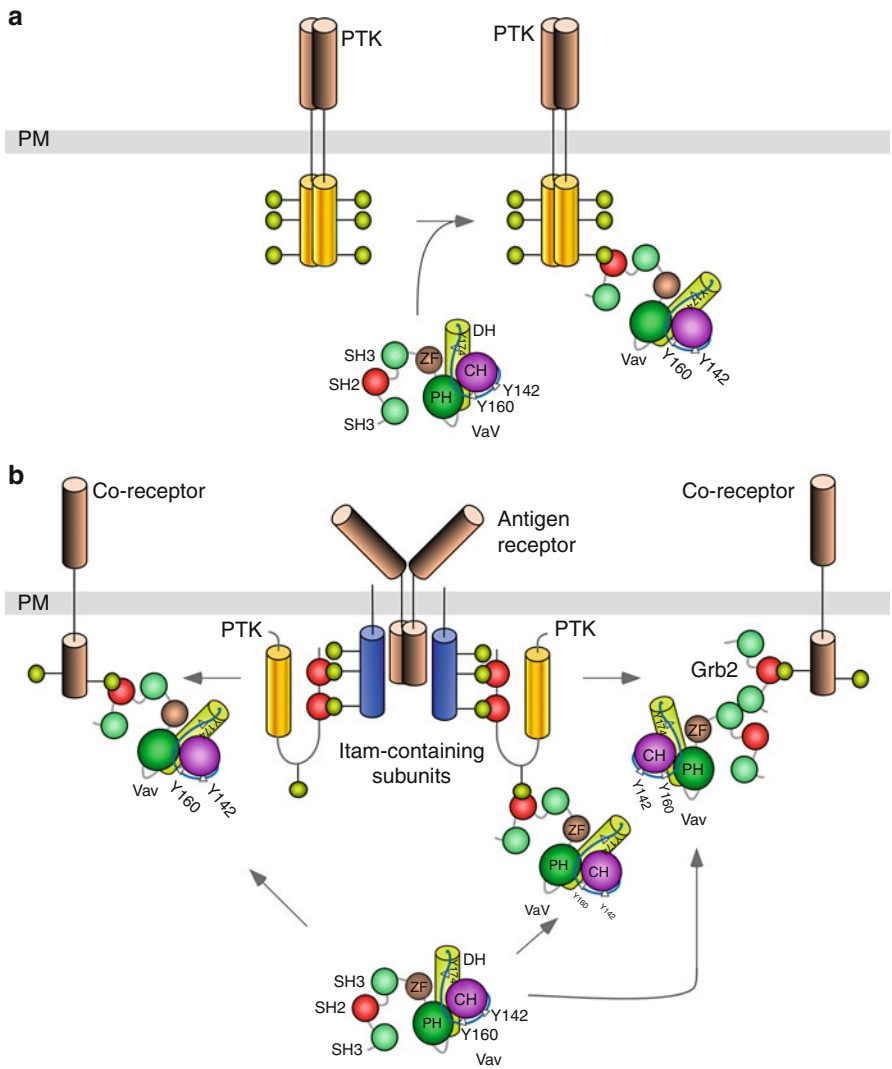
The main regulatory control of Vav family proteins is the modulation of their catalytic activity by direct tyrosine phosphorylation (Crespo et al. 1997; Movilla and Bustelo 1999; Schuebel et al. 1998). Recent crystallographic studies using a Vav1 CH-Ac-DH-PH-ZF fragment have helped in understanding the intramolecular mechanism that regulates this regulatory step (Fig. 6) (Yu et al. 2010). These analyses indicate that the inactive, non-phosphorylated state of Vav1 is maintained through extensive interactions among: (1) The CH domain and the PH, DH, and two tyrosine residues (Y142 and Y160) present in the Ac region. (2) The Ac region with the PH domain. (3) A tyrosine residue of the Vav1 Ac region (Y174) with the GTPase-binding domain of the Vav1 DH region. All these interactions induce cooperatively a "closed" conformation of Vav1 that is totally incompatible with the binding of GTPase substrates to the catalytic region (Fig. 6). The phosphorylation of the three Ac phosphosites leads to the release of those intramolecular inhibitory interactions, the exposure of the GTPase-binding site, and effective catalytic reactions (Fig. 6). Biochemical and signaling evidence indicates that this mechanism is conserved in all Vav family members studies so far, including *C. elegans* and *D. melanogaster* Vav proteins.



Vav Family, Fig. 6 Intramolecular mechanism controlling the enzyme activity of Vav proteins. (**a**) Schematic representation of the phosphorylation-dependent activation of Vav proteins during physiological conditions. In the inactive state (*left*), Vav proteins are in a closed conformation incompatible with GTPase binding. Upon association with protein tyrosine kinases, Vav proteins become phosphorylated (*red balls*) on three phosphosites (*open triangles*) present in the Ac region, leading to the opening of the molecule and optimal catalytic activity toward Rho/Rac proteins. Active forms of Vav proteins return to the inactive conformation by dephosphorylation by protein tyrosine phosphatases (PTPase). (**b**) 3D structure showing the inhibited state of non-phosphorylated Vav1 based on a recent word done with the Vav1 CH-Ac-DH-PH-ZF fragment (Yu et al. 2010). The continuity of the Ac region is broken since it could not be crystallized in some specific areas

Vav proteins are also controlled by other regulatory steps in vivo. The most important is the translocation of the proteins from the cytosol to the plasma membrane, a step required for the interaction with both upstream tyrosine kinases and membrane-localized downstream effectors. In non-hematopoietic cells, the membrane translocation of Vav family proteins usually involves a single event mediated by the direct interaction of the Vav SH2 region with the autophosphorylated tyrosine kinase receptors (Bustelo 2000, 2008; Bustelo and Couceiro 2008). Once bound to them, Vav proteins become transphosphorylated by the associated kinase (Fig. 7a). In the case of lymphocytes, the translocation of Vav proteins usually entails their interaction with membrane-localized proteins. These can be either transmembrane co-receptors (CD28, CD19) or plasma membrane inner leaflet–associated adaptor molecules (linker for activation of T cells or ▶ Lat) (Fig. 7b). The association with co-receptors is either by direct physical contact (i.e., the association of the Vav SH2 domain with a phosphorylated residue located on the cytoplasmic tail of CD19) or carried out indirectly using bridge molecules (i.e., the binding of Gbr2 to the CD28 co-receptor) (Fig. 7b). The association with membrane-anchored adaptors entails the use of signaling adaptors such as the Vav-binding proteins Grb2, Slp76, Blnk, Pyk2, and Itk (Bustelo 2000, 2008; Bustelo and Couceiro 2008).

Current evidence suggest that the biological activity of Vav proteins can be regulated by additional

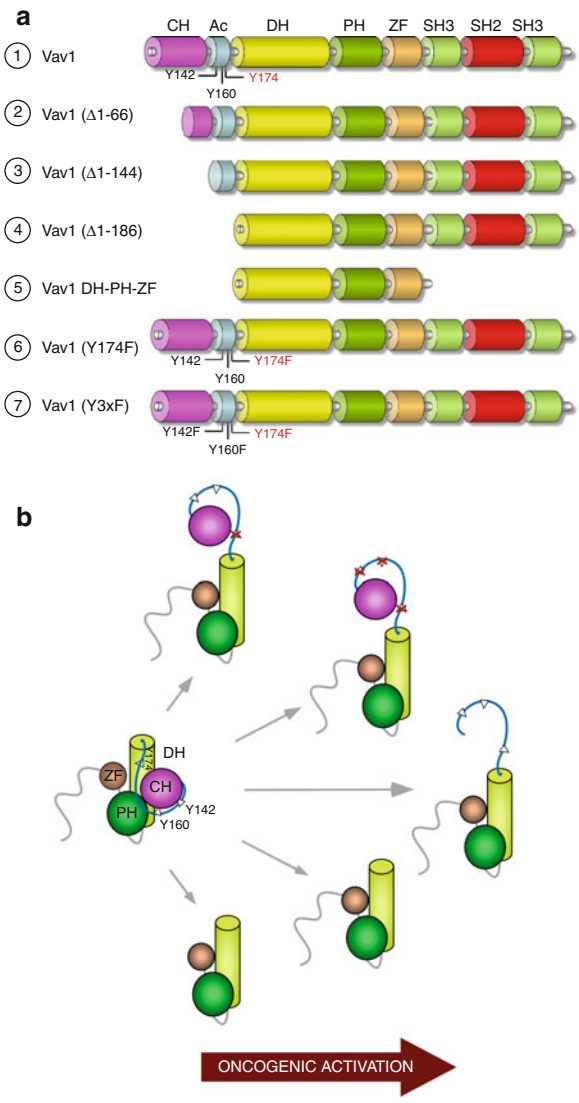


Vav Family, Fig. 7 Examples of the interaction of Vav proteins with transmembrane receptors. (**a**) Direct interaction of Vav proteins with autophosphorylated receptors with intrinsic tyrosine kinase activity. (**b**) Vav proteins can establish direct physical interactions with intracellular tyrosine kinases of Syk/Zap70 family (*center*) and with lymphoid co-receptors (i.e., CD19, *left*). Vav proteins can also interact with other co-receptors (i.e., CD28) via Vav-binding proteins such as Grb2 (*right*). In A and B, kinase domains are shown in dark yellow. Phosphorylation sites are indicated as yellow spheres. Vav and Grb2 SH3 are shown in light green. SH2 domains of Vav, Grb2, and the cytoplasmic tyrosine kinase are shown in red. The rest of Vav domains and residues depicted in this figure have been described in the legend to Fig. 6. Note that for the sake of simplicity, two types of co-receptors have been included together in this figure despite the fact that they are not co-expressed in the same lymphoid cell types. Likewise, the translocation of Vav proteins can be mediated by other molecules as indicated in the main text. ITAM, immunoreceptor tyrosine-based activation motif; PM, plasma membrane; PTK, protein tyrosine kinase

posttranslational mechanisms including caspase-dependent cleavage during anergic T-cell conditions, Cbl family–dependent ubiquitinylation, arginine methylation and, possibly, lysine acetylation (Bustelo 2000, 2008; Bustelo and Couceiro 2008). The physiological significance of all those posttranslational modifications remains to be fully determined. Recent observations indicate that the levels of some *Vav* family mRNAs (human *Vav3*, *C. elegans Vav*) can be regulated by microRNAs.

Implication of Vav Family Proteins in Pathological States

Mutations that eliminate the intramolecular contacts that inhibit the catalytic activity of mammalian Vav proteins lead to the generation of oncogenic proteins (Fig. 8) (Bustelo 2000, 2008; Bustelo and Couceiro 2008). Similar mutations have been also described in *C. elegans* and *D. melanogaster Vav* family genes (Couceiro et al. 2005; Norman et al. 2005), leading to tumorigenesis and developmental problems in the respective species (Table 2). These alterations have only academic interest, because no mutations have been found so far for any *Vav* family gene in either tumors or other pathologies. In vivo, the deregulation of Vav family proteins appears to occur through changes in either the expression or phosphorylation levels of the wild type proteins. For example, Vav1 is detected overexpressed in subsets of pancreatic, lung, and hematopoietic tumors. Higher levels of Vav1 expression, linked to the presence of an intronic single nucleotide polymorphism on its gene, seem to be associated with human cohorts with a higher tendency to develop multiple sclerosis. Hyperphosphorylation of Vav family proteins has been observed in cancer cells expressing oncogenic versions of protein tyrosine kinases (i.e., Alk, Bcr/Abl) or, alternatively, displaying active, mitogen-dependent autocrine loops. Finally, it has been observed that Vav1 and Vav3 can become upregulated in lymphocytes upon infection of viral pathogens encoding Vav family–interacting molecules such as v-Nef and M2. This viral-dependent activation is important for the infectivity cycle and the establishment of the latency stage of HIV and γ-herpesvirus, respectively. On the other side of the coin, a blockage in activation of Vav1 has been detected in anergic T cells via a caspase-dependent proteolysis. The actual significance of those inhibitory events has not been fully studied so far. Taken together, these results suggest that Vav proteins may play important roles in a number of high-incidence illnesses such as cancer, autoimmune disease, and viral pathogenesis.



Vav Family, Fig. 8 Oncogenic activation of Vav proteins. (**a**) Scheme of wild type and oncogenic versions of Vav1. The first oncogenic version described was a truncated Vav1 protein lacking the first 66 N-terminal amino acids (protein 2). Similar mutations have been described in other Vav family members. (**b**) Depiction of the intramolecular changes taking place in Vav family proteins upon oncogenic activation. When present, the C-terminal SH3-SH2-SH3 region is presented as a wavy gray lane for the sake of simplicity

Summary

Despite the progress during made these last 30 years in this field, many challenges lie still ahead in this field. Hence, the role of other posttranslational modifications on Vav activity is not known yet. There is also a lack of information regarding the functional significance of many of the Vav-interacting proteins identified to date or, in a related topic, the relative contribution of both GTPase-dependent and independent routes to the biological responses regulated by those proteins. Another issue of interest is the level of functional

redundancy/signaling specificity existing among the three mammalian Vav family members. In this regard, current data indicate that Vav proteins have both unique and overlapping functions depending on the cell type and/or biological response analyzed. However, these analyses have been done in a limited number of cell types, so a comprehensive view of the level of functional redundancy among Vav family members is still missing. Finally, there is still very scant information about the actual implication of Vav proteins in pathological states and, as consequence, no solid information is available about the possible interest of these proteins as drug targets to treat human disease. The available collection of animal models for this protein family will be an important tool in the near future to address that question. Collectively, this future work will give a holistic and, hopefully a final, picture of the implication of this important protein family in physiological and pathological responses.

References

Bustelo XR. Regulatory and signaling properties of the Vav family. Mol Cell Biol. 2000;20:1461–77.

Bustelo XR. Vav proteins, adaptors and cell signaling. Oncogene. 2001;20:6372–81.

Bustelo XR. Vav2. UCSD Nature Mol Pages. 2008. doi:10.1038/mp.a002361.01.

Bustelo XR, Barbacid M. Tyrosine phosphorylation of the *vav* proto-oncogene product in activated b cells. Science. 1992;256:1196–9.

Bustelo XR, Couceiro JR. Vav3. UCSD Nature Mol Pages. 2008. doi:10.1038/mp.a002362.01.

Bustelo XR, Ledbetter JA, Barbacid M. Product of *vav* proto-oncogene defines a new class of tyrosine protein kinase substrates. Nature. 1992;356:68–71.

Chrencik JE, Broun A, Zhang H, Mathews II, Hura GL, et al. Structural basis of guanine nucleotide exchange mediated by the T-cell essential Vav1. J Mol Biol. 2008;380:828–43.

Couceiro JR, Martin-Bermudo MD, Bustelo XR. Phylogenetic conservation of the regulatory and functional properties of the Vav oncoprotein family. Exp Cell Res. 2005;308:364–80.

Crespo P, Schuebel KE, Ostrom AA, Gutkind JS, Bustelo XR. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the *vav* proto-oncogene product. Nature. 1997;385:169–72.

Katzav S, Martin-Zanca D, Barbacid M. *Vav*, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. EMBO J. 1989;8:2283–90.

Malartre M, Ayaz D, Amador FF, Martin-Bermudo MD. The guanine exchange factor Vav controls axon growth and guidance during drosophila development. J Neurosci. 2010;30:2257–67.

Movilla N, Bustelo XR. Biological and regulatory properties of Vav-3, a new member of the vav family of oncoproteins. Mol Cell Biol. 1999;19:7870–85.

Norman KR, Fazzio RT, Mellem JE, Espelt MV, Strange K, et al. The Rho/Rac-family guanine nucleotide exchange factor Vav-1 regulates rhythmic behaviors in C. elegans. Cell. 2005;123:119–32.

Rapley J, Tybulewicz VL, Rittinger K. Crucial structural role for the PH and C1 domains of the Vav1 exchange factor. EMBO Rep. 2008;9:655–61.

Saveliev A, Vanes L, Ksionda O, Rapley J, Smerdon SJ, et al. Function of the nucleotide exchange activity of Vav1 in T-cell development and activation. Sci Sig. 2009;2:ra83.

Schuebel KE, Movilla N, Rosa JL, Bustelo XR. Phosphorylation-dependent and constitutive activation of Rho proteins by wild-type and oncogenic Vav-2. EMBO J. 1998;17:6608–21.

Turner M, Billadeau DD. Vav proteins as signal integrators for multi-subunit immune-recognition receptors. Nat Rev Immunol. 2002;2:476–86.

Tybulewicz VL, Ardouin L, Prisco A, Reynolds LF. Vav1: a key signal transducer downstream of the tcr. Immunol Rev. 2003;192:42–52.

Yu B, Martins IR, Li P, Amarasinghe GK, Umetani J, et al. Structural and energetic mechanisms of cooperative autoinhibition and activation of Vav1. Cell. 2010;140:246–56.

Vav Oncogene

► Vav Family

Vav1

► Vav Family

Vav-1 Oncogene

► Vav Family

Vav2

► Vav Family

Vav-2 Oncogene

► Vav Family

Vav3

► Vav Family

Vav-3 Oncogene

► Vav Family

VDR, The Vitamin D Receptor

Marina Rode von Essen and Carsten Geisler
Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, DK, Denmark

Synonyms

Calcitriol receptor; Nuclear receptor subfamily 1, group I, member 1 (NR1I1); Vitamin D receptor (VDR)

Historical Background

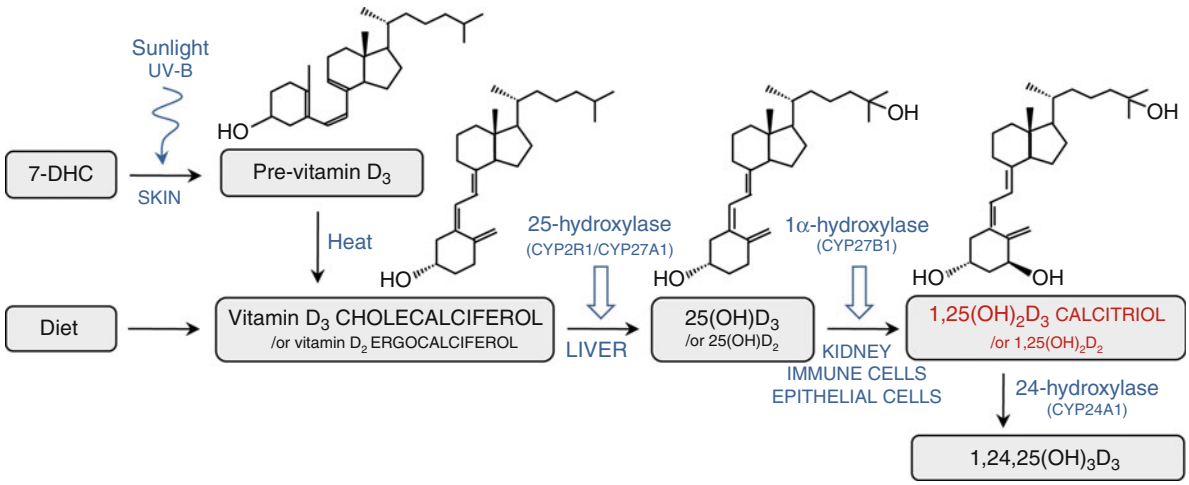
Almost from the time life began, vitamin D has been produced by plants and animals. As the structures of plants and animals became more complex, the sites of vitamin D production, its subsequent metabolism, as well as its sites of action separated. The ability to transport and metabolize vitamin D into more active forms therefore evolved. The metabolic active form $1,25(OH)_2D_3$ of vitamin D exerts its actions through interaction with the vitamin D receptor, VDR (Bikle 2011). Although not as ancient, VDR has been highly conserved between species through evolution (Hochberg and Templeton 2010).VDR is found in almost all cells and tissues of higher-order animals, further emphasizing the importance of the receptor. Evidence for the existence of VDR was first provided in 1969 by Haussler and Norman (Feldman et al. 2005), and since then a substantial amount of data on the structure and function of VDR has been accomplished.

The Source and Metabolism of the Vitamin D Receptor Ligand

Vitamin D is known as "the sunshine vitamin" as humans acquire more than 90% of the vitamin D needed from the sun. During exposure to sunlight, ultraviolet B (UVB) radiation is absorbed by 7-dehydrocholesterol (7-DHC) present in the skin generating previtamin D_3, which quickly is transformed into the stable vitamin D_3 (Fig. 1).Very few foods naturally contain vitamin D, however, egg yolks, cod liver oil, beef liver, some fungi, and in particular oily fish such as salmon, mackerel, and sardines make up a minor source of vitamin D (Holick 2004; Feldman et al. 2005). Two forms of vitamin D can be obtained by nutritional intake: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol), the latter from foods of animal origin. Regardless of the source of vitamin D, it needs to be hydroxylated twice in order to become biologically active (Jones et al. 1998). The first hydroxylation step is catabolized in the liver by the enzyme 25-hydroxylase (CYP2R1/CYP27A1) generating $25(OH)D_3$ (or D_2), and the second by the related 1α-hydroxylase (CYP27B1) mainly in the kidney resulting in the metabolic active calcitriol $1,25(OH)_2D_3$ (or D_2). There is little evidence that the two active forms D_3 and D_2 differ in their mode of action (Feldman et al. 2005). $1,25(OH)_2D_3$ limits its own activity in the target organs by inducing 24-hydroxylase (CYP24A1), transforming $1,25(OH)_2D_3$ to ten times less biologically active $1,24,25(OH)_2D_3$, and thus preventing excessive vitamin D signaling (Fig. 1) (Jones et al. 1998).

Mechanism of Action, the Vitamin D Receptor

$1,25(OH)_2D_3$ exerts its actions by binding to the vitamin D receptor, VDR. VDR is a ligand-dependent transcription regulator molecule belonging to the superfamily of nuclear receptors. In the absence of $1,25(OH)_2D_3$ VDR is distributed largely to the cytoplasm. Upon ligand interaction VDR dimerizes with the retinoid x receptor (RXR) and translocates to the nucleus. Here the VDR-RXR heterodimer binds to vitamin D response elements (VDREs) present in the promoter region of responsive genes (Fig. 2)



VDR, The Vitamin D Receptor, Fig. 1 Production and metabolism of vitamin D. Vitamin D_3 is produced either from conversion of 7-DHC in the skin through sunlight exposure or from precursors in food. In the liver vitamin D_3 is hydroxylated in position 25, generating $25(OH)D_3$. A second hydroxylation in position 1 produces the active metabolite $1,25(OH)_2D_3$. This step mainly takes place in the kidneys, but also in other tissues expressing the enzyme 1α-hydroxylase

(Nagpal et al. 2005; Feldman et al. 2005). Almost 3% of the human genes include a VDRE and hence their expression can be modulated accordingly. Although various noncanonical VDREs have been described, canonical VDREs are defined as a direct repeat of 5′-AGG/TTCA-3′ or a minor variation of this motif separated by three nucleotides (Dusso et al. 2005; Nagpal et al. 2005; Feldman et al. 2005). Depending on the target gene, either co-activators or corepressors are attracted to the complex to induce or repress gene transcription (Jones et al. 1998; Nagpal et al. 2005).

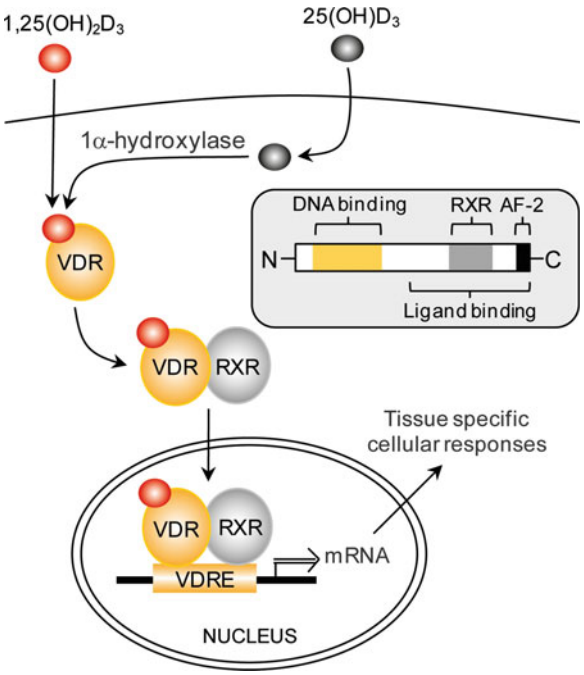
Besides regulation through VDREs of target genes, VDR can inhibit the expression of some genes by antagonizing the action of certain transcription factors, such as ▶ NFAT and NF-κB (Nagpal et al. 2005). An example is the VDR-dependent inhibition of the cytokines IL-2 and GM-CSF which appears more complex than the involvement of positive and negative VDREs. Here, VDR first competes with NFAT1 for binding to the enhancer motif of the NFAT1-activator protein 1 (AP1) and subsequently VDR binds to c-Jun. This co-occupancy of VDR-c-Jun to AP1 leads to inhibition of activated IL-2 and GM-CSF expression (Nagpal et al. 2005).

To fully accomplish its functions, the VDR protein is divided into an N-terminal domain including the DNA-binding region and a multifunctional C-terminal domain harboring the ligand-binding region, the RXR heterodimerization motif, and a ligand-dependent transactivation function (AF-2). The C-terminal domain consequently exerts absolute regulatory control over the DNA-binding properties of the VDR (Fig. 2) (Jones et al. 1998; Feldman et al. 2005).

Regulation of Vitamin D Receptor Activity

Due to the influence of VDR on such a broad array of genes, its activity is ascribed strict control. Besides the obvious limitations of VDR activity due to availability of the VDR ligand, $1,25(OH)_2D_3$ also directly influences the expression of VDR. Cells treated with $1,25(OH)_2D_3$ induce VDR-mRNA and furthermore stabilize the mRNA, increasing the total amount of cellular VDR. VDR expression can also be modulated by numerous other physical stimuli such as dietary composition (calcium and phosphorus), steroid hormones and retinoids, growth factors, and peptide hormones (Feldman et al. 2005).

The various physiological inputs that affect VDR levels involve complex interactions among the intracellular signaling transduction pathways. As an example, cellular response to the parathyroid hormone (PTH) activates protein kinase A (PKA) causing an increase in VDR level, whereas stimuli that induce protein kinase C (PKC) activity lead to a decrease in VDR expression. Given the central role of PKA and PKC in the integration of cellular responses to



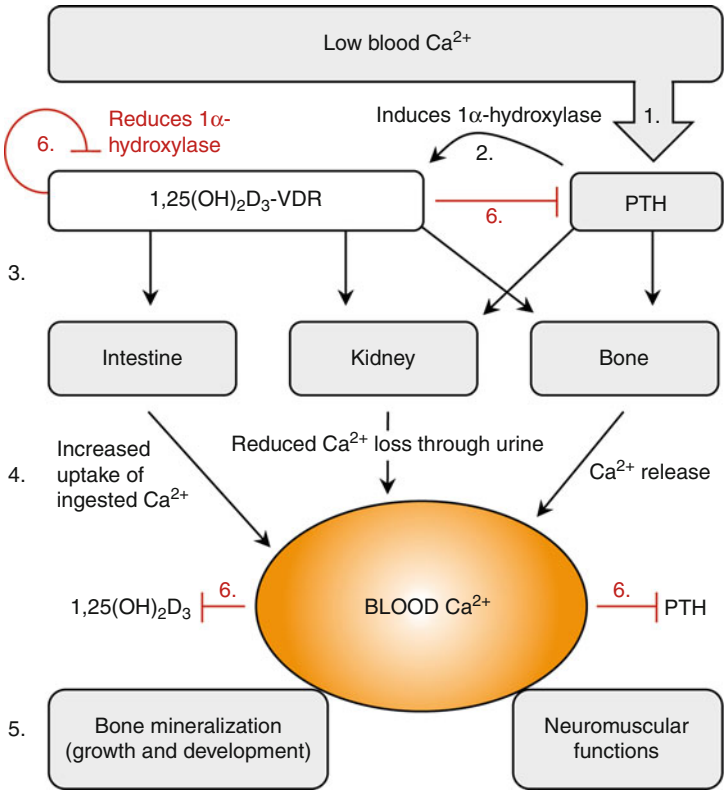
VDR, The Vitamin D Receptor, Fig. 2 The mechanism of VDR action. Binding of VDR to its ligand, $1,25(OH)_2D_3$, enables dimerization of VDR and RXR, allowing nuclear translocation and binding of the VDR-RXR complex to VDREs in the promoter region of responsive genes. Binding to the VDREs is accompanied by formation of a large complex including either co-activators or corepressors leading to induction or inhibition of target genes, respectively. A schematic illustration of the VDR gene is depicted in the *box*

extracellular stimuli, these pathways are central in the mechanisms that regulate VDR levels in target cells (Feldman et al. 2005). A signaling pathway to induce VDR expression not including PKA and PKC has recently been described in human naïve T cells of the immune system. Here, the cellular response to a foreign pathogen induces the expression of VDR through activation of the kinase p38, a process mandatory for the activation of the naive T cells (von Essen et al. 2010). In addition, cellular signaling motivating physical interaction of VDR-$1,25(OH)_2D_3$ with SUG1 of the proteasome complex targets VDR for ubiquitination and subsequent proteolysis (Dusso et al. 2005). Regulation of VDR expression and abundance is therefore an important mechanism for the modulation of cellular responsiveness to $1,25(OH)_2D_3$ and hence for controlling a variety of cellular functions. It is also possible that phosphorylation of VDR, which induces a conformational change that releases corepressors as well as posttranslational VDR modifications, plays a role in regulating the activity of VDRs (Dusso et al. 2005).

Biological Functions of the Vitamin D Receptor

$1,25(OH)_2D_3$ is traditionally viewed as an endocrine hormone whose function is to regulate calcium and phosphorus homeostasis. Without proper maintenance of blood calcium levels, bone is under-mineralized, and if the calcium levels drop further, neuromuscular functions fail and death can occur as a result of tetany (spasms) (Plum and DeLuca 2010). Low blood levels of calcium induce the parathyroid gland to release PTH which results in a rapid mobilization of calcium from bone, an increase in calcium reabsorption from kidneys, and stimulates the transcription of 1α-hydroxylase (in the kidneys) resulting in the production of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ in conjunction with VDR then stimulates intestinal absorption of calcium and phosphorus, and like PTH, mobilizes calcium (and phosphorous) from the bones and reduces the loss of calcium through the urine. To ensure that no excess calcium is released to the blood, $1,25(OH)_2D_3$-VDR together with the elevated serum levels of calcium suppress 1α-hydroxylase transcription and stimulate 24-hydroxylase in the kidneys along with repression of PTH production and release from the parathyroid gland (Fig. 3) (Plum and DeLuca 2010; Feldman et al. 2005). In this way, the vitamin D endocrine system manages to maintain extracellular calcium levels within narrow limits, a process vital for normal cellular function and bone integrity.

Within the last two decades it has been clear that VDRs are not only present in tissues associated with calcium and phosphorus metabolism, but also in "nonclassical" organs like immune cells, brain, eyes, heart, pancreatic islets (β cells), muscle, adipose tissue, thyroid, parathyroid, and adrenal glands, suggesting a broader role for VDR (Baeke et al. 2010; Feldman et al. 2005). In particular, its role in the immune cells has received much attention due to a great potential as a preventive or therapeutic target in a variety of inflammatory and autoimmune diseases as well as in cancer. The immune system is divided into an innate immune system that makes up the critical first line of defense against invading pathogens and an adaptive immune system containing pathogen-specific cells to

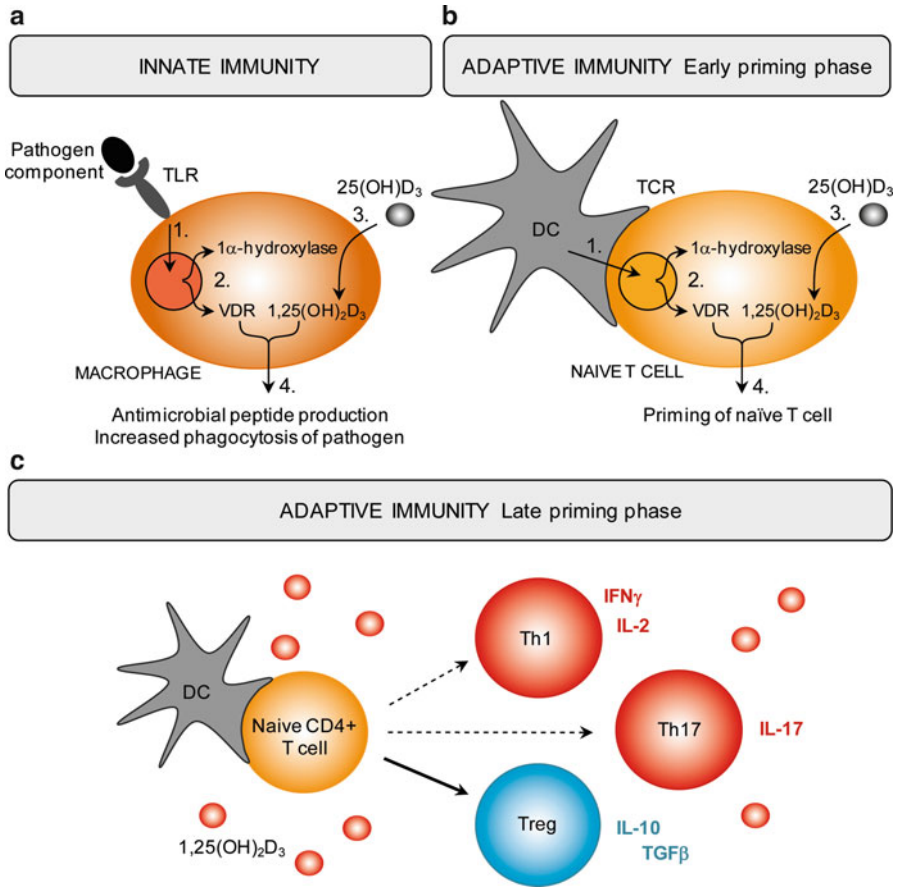


VDR, The Vitamin D Receptor, Fig. 3 The role of $1,25(OH)_2D_3$-VDR in maintaining calcium homeostasis. (1) Low blood calcium levels induce PTH release from the parathyroid gland. (2) PTH induces 1α-hydroxylase transcription generating $1,25(OH)_2D_3$. (3–4) PTH and $1,25(OH)_2D_3$ increases the blood calcium level through different mechanisms, (5) Necessary for maintaining neuromuscular functions and bone strength. (6) The elevated blood calcium levels and $1,25(OH)_2D_3$ suppress the production of PTH, and limit the production of $1,25(OH)_2D_3$, thereby completing the endocrine loop

combat the foreign intruder (Abbas et al. 2007). VDR is widely expressed in most immune cell types and consequently $1,25(OH)_2D_3$ has proven to have potent immunomodulatory effects on cells of both the innate and adaptive immune systems. Notably, several of these cells also express vitamin D activating enzymes such as 1α-hydroxylase, allowing local activation of vitamin D (Bikle 2009; Baeke et al. 2010).

An important mechanism used by macrophages of the innate immune system to clear an infection is the production of antimicrobial peptides that kill the pathogen. When macrophages sense the presence of a foreign pathogen through surface toll-like-receptors(TLRs), VDR and 1α-hydroxylase are upregulated and $1,25(OH)_2D_3$ produced from circulating $25(OH)D_3$. Activation of the VDR subsequently induces the production of antimicrobial peptides, followed by killing of, for example, mycobacterium tuberculosis. In addition, $1,25(OH)_2D_3$ has been shown to enhance the phagocytic capacity of macrophages, which further promotes adaptive immunity (Fig. 4) (Bikle 2009; Baeke et al. 2010).

For adaptive immunity to be encouraged, naïve T lymphocytes (T cells) must receive an activation signal from antigen-presenting cells like macrophages and in particular dendritic cells (DCs). Depending on the nature of the signal provided and the local environment, the lymphocytes can pursue different developmental programs (Abbas et al. 2007). Both DCs and T cells express the VDR and 1α-hydroxylase and have been identified as direct targets of $1,25(OH)_2D_3$ (Baeke et al. 2010). As for innate immunity, $1,25(OH)_2D_3$ has been shown to promote adaptive responses; $1,25(OH)_2D_3$-VDR is required for the early phase of T-cell priming as VDR-signaling induces expression of PLC-γ1, which is mandatory for differentiation of the naïve T cells (von Essen et al. 2010). Still, the most well-known described effect of $1,25(OH)_2D_3$ on adaptive immunity is that of inhibition (Bikle 2009; Baeke et al. 2010; van Etten and Mathieu 2005). DCs under the influence of $1,25(OH)_2D_3$ alter their phenotype to favor differentiation of a regulatory subset of T cells (Treg) with immune suppressive functions, instead of priming the T-cell subset with inflammatory effects (Th1 and Th17). Both Th1 and Th17 cells are commonly accepted to be main players in the development and progression of various inflammatory and autoimmune



VDR, The Vitamin D Receptor, Fig. 4 Proposed model for the immunomodulatory effects of $1,25(OH)_2D_3$-VDR. *Innate immunity*. (**a**) (1) In response to TLR stimulation, (2) macrophages induce expression of 1α-hydroxylase and VDR, (3) leading to production of $1,25(OH)_2D_3$ and activation of VDR, (4) promoting the production of antimicrobial peptides and increased phagocytosis of the pathogen. *Adaptive immunity*. (**b**) (1) In response to DC-induced TCR stimulation, (2) naive T cells induce expression of 1α-hydroxylase and VDR, (3) leading to production of $1,25(OH)_2D_3$ and activation of VDR, (4) encouraging priming of the naïve T cells. (**c**) In the secondary lymphoid organs, where T-cell priming takes place, an increase in $1,25(OH)_2D_3$ is expected. $1,25(OH)_2D_3$ influences the DCs to favor differentiation of Treg cells as opposed to Th1 and Th17 effector T cells. $1,25(OH)_2D_3$ also enhances the function of the Treg cells and inhibits the function of Th1 and Th17 cells. Dotted lines and text rendered in red indicate inhibitory actions of $1,25(OH)_2D_3$ and black lines and text rendered in blue increased actions

diseases. Furthermore, $1,25(OH)_2D_3$ also directly modulates the function of the different T-cell subsets. Treg cells increase their ability to suppress immunity and the effector T cells decreases their ability to proliferate and exert their effector functions, for example, through secretion of cytokines. The net result is downregulation of the effector T-cell response (Fig. 4) (van Etten and Mathieu 2005). The apparent discrepancies of the influence of $1,25(OH)_2D_3$ on adaptive immunity might be explained by the availability of $1,25(OH)_2D_3$ at the different stages of an immune response. Early studies have shown a pro-proliferative effect of low concentrations of $1,25(OH)_2D_3$ on T cells (Lacey et al. 1987), whereas more recent studies using higher concentrations have shown the exact opposite. Although it has to be investigated, one can speculate that the modest quantity of $1,25(OH)_2D_3$ present at the beginning of an immune response is sufficient to induce T-cell priming, whereas the increasing amount supposedly generated during the response exerts a negative feedback to ensure that the immune system does not overreact.

VDR-Mediated Signaling in Health and Disease

Allelic variants (polymorphisms) of the VDR gene which occurs naturally in the human population are associated with various medical conditions such as decreased bone density, tendency to hyperparathyroidism (excess PTH production), and susceptibility to infections, autoimmune diseases, and cancer. This may in part be due to the effect of VDR polymorphism on VDR-mRNA stability and reduced expression of VDR (Feldman et al. 2005). The importance of such changes in VDR expression for disease development is strengthened by the observation that low VDR expression is associated with more invasive and lethal tumors (Hendrickson et al. 2011; Lopez et al. 2010) perhaps due to less proliferative control and apoptosis induction, which are two major processes modulated by VDR-signaling.

In addition to allelic variations of the VDR gene, two rare genetic disorders have been described in which either the CYP27B1 gene or the VDR gene itself contain mutations that render the gene products nonfunctional. In patients with a nonfunctional 1α-hydroxylase (due to a mutation in CYP27B1) $1,25(OH)_2D_3$ is no longer synthesized. Individuals with a nonfunctional VDR suffer from absence of VDR-signaling giving rise to the disease hereditary vitamin D resistant rickets (HVDRR). Both diseases are characterized by hypocalcemia (low blood calcium), hyperparathyroidism, and early onset of rickets (bone deformities in children). Even though the two diseases appear alike, a crucial difference is the blood level of $1,25(OH)_2D_3$: It is almost absent in patients with 1α-hydroxylase deficiency, whereas it is exceedingly high in HVDRR. The apparent consequence is that patients with a defect CYP27B1 gene respond to treatment with $1,25(OH)_2D_3$ and can expect complete remission of the disease, whereas most HVDRR-affected individuals are resistant to vitamin D therapy. In this case, intensive calcium therapy is used, surprisingly reversing all aspects of the disease. As there are only very few cases of HVDRR and as the abnormalities are corrected relatively early in life, long-term effects of defective VDR-signaling such as development of autoimmune diseases and cancer have not been observed (Malloy and Feldman 2010). A promising model system regarding these issues is a mouse model in which the VDR gene has been deleted, or in which severe vitamin D deficiency has been introduced through a vitamin D depleted diet. These mice do show increased sensitivity to certain autoimmune diseases, and are also more prone to oncogene- and chemocarcinogen-induced tumors (Bouillon et al. 2008). Furthermore, it has been demonstrated in a variety of animal disease models that pretreatment with $1,25(OH)_2D_3$ is effective in preventing the onset of both multiple sclerosis, type I diabetes, rheumatoid arthritis, and inflammatory bowel disease (Nagpal et al. 2005; Guillot et al. 2010).

Consistent with the observations from mice models, an inverse association between the vitamin D status (blood level of $25(OH)D_3$) and development of a variety of autoimmune and inflammatory diseases as well as an increased risk for development of certain types of cancers has been described in epidemiological studies in humans (Nagpal et al. 2005; van Etten and Mathieu 2005; Guillot et al. 2010; Luong and Nguyen 2010). Even so, many of the epidemiological studies performed measured the vitamin D status at the time of disease diagnosis. It therefore has to be considered that the low vitamin D status could be a consequence of disease development rather than a direct cause, as daily routines and habits are often changed upon development of these diseases (e.g., more indoor activities). Thus, the usage of the vitamin D status as a "probability marker" for disease development awaits clarification through future studies.

Summary; Future Directions

VDR is a nuclear, ligand-induced transcription factor that in complex with hormonally active vitamin D, $1,25(OH)_2D_3$, regulates the expression of more than 900 genes involved in a wide array of physiological functions, for example, calcium homeostasis, growth control, differentiation, and immune responses.

The ligand for VDR, $1,25(OH)_2D_3$ is attained mainly from 7-DHC in the skin. 7-DHC is transformed to a vitamin D precursor through UVβ irradiation from the sun. This vitamin D precursor can also be obtained in minor amounts through diet. Either way, two hydroxylation steps involving 25-hydrolylase and 1α-hydroxylase transform the precursor into its active metabolite. Once formed $1,25(OH)_2D_3$ binds to VDR in the target cell and enables the receptor to bind to RXR and translocate into the nucleus where it

participates in the regulation of the expression of a great variety of genes. In the target cell, $1,25(OH)_2D_3$ limits its own activity by inducing the enzyme 24-hydroxylase that transforms $1,25(OH)_2D_3$ to a less active metabolite. Other mechanisms important for the regulation of VDR activity include control of VDR expression and possibly phosphorylation and posttranslational VDR modifications. The biological functions of VDR are numerous, with its role as a regulator of blood calcium and phosphorus level as the most commonly known. However, lately the role of VDR in modulating immune responses has been the focus of many studies.

It is generally accepted that vitamin D deficiency is highly prevalent in many populations around the world (Baeke et al. 2010). Therefore, vitamin D supplementation represents an attractive strategy to ensure sufficient $25(OH)D_3$ levels for adequate bone metabolism and immune functions thereby eliminating one of the proposed risk factors that may underlie inflammatory or autoimmune diseases as well as certain types of cancer. In addition to a potential disease-preventive role of $1,25(OH)_2D_3$, the immunomodulating effects of vitamin D suggest that vitamin D holds therapeutic promises in inflammatory and autoimmune diseases. In a variety of animal studies, $1,25(OH)_2D_3$ has been shown either to halt progression or reduce disease severity, counting a mouse model for multiple sclerosis, type I diabetes, rheumatoid arthritis, and inflammatory bowel disease (Nagpal et al. 2005; Plum et al. 2010; Guillot et al. 2010). Unfortunately, the therapeutic application of $1,25(OH)_2D_3$ is obstructed by toxicity issues as the supraphysiological doses needed to exert an immune inhibitory effect elicit unfortunate calcemic side effects. To overcome this limitation, structural analogues of $1,25(OH)_2D_3$ are being designed that have reduced calcemic effects with similar immunoregulatory activity (Yee et al. 2005). Today, such analogues have become first-line therapy for treatment of psoriasis. The vitamin D analogue is applied topically onto the psoriasis plaques where it inhibits the massive keratinocyte proliferation and the underlying inflammation. Due to its short half-life after it enters circulation, it is destroyed before it has an opportunity to affect calcium homeostasis (Yee et al. 2005). A similar "soft-drug-approach" cannot be employed for other inflammatory, autoimmune, and cancer diseases as a systemic effect (not including calcemic effects) would be the goal of therapy. However, the development of orally available noncalcemic and even tissue-specific vitamin D analogues is a working field in progress, and the prospect of vitamin D therapeutics for treating diseases beyond metabolic bone disorders seems promising.

References

Abbas AK, Lichtman AH, Pillai S. Cellular and molecular immunology. 6th ed. Oxford, UK: Elsevier; 2007.

Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D, modulator of the immune system. Curr Opin Pharmacol. 2010;10:482–96.

Bikle DD. Nonclassic actions of vitamin D. J Clin Endocrinol Metab. 2009;94:26–34.

Bikle DD. Vitamin D, an ancient hormone. Exp Dermatol. 2011;20:7–13.

Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, Lieben L, Mathieu C, Demay M. Vitamin D and human health: lessons from vitamin D receptor null mice. Endocr Rev. 2008;29:726–76.

Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. Am J Physiol Renal Physiol. 2005;289:F8–28.

Feldman D, Pike JW, Glorieux FH. Vitamin D. 2nd ed. San Diego, USA: Elsevier; 2005.

Guillot X, Semerano L, Saidenberg-Kermanac'h N, Falgarone G, Boissier MC. Vitamin D and inflammation. Joint Bone Spine. 2010;77:552–7.

Hendrickson WK, Flavin R, Kasperzyk JL, Fiorentino M, Fang F, Lis R, Fiore C, Penney KL, Ma J, Kantoff PW, Stampfer MJ, Loda M, Mucci LA, Giovannucci E. Vitamin D receptor protein expression in tumor tissue and prostate cancer progression. J Clin Oncol. 2011;29:2378–85.

Hochberg Z, Templeton AR. Evolutionary perspective in skin color, vitamin D and its receptor. Hormones. 2010;9:307–11.

Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am J Clin Nutr. 2004;80:1678S–88.

Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. Physiol Rev. 1998;78:1193–231.

Lacey DL, Axelrod J, Chappel JC, Kahn AJ, Teitelbaum SL. Vitamin D affects proliferation of a murine T helper cell clone. J Immunol. 1987;138:1680–6.

Lopez N, Sousa B, Martins D, Gomes M, Vieira D, Veronese LA, Milanezi F, Paredes J, Costa JL, Schmitt F. Alterations in vitamin D signaling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions vitamin D pathways unbalanced in breast lesions. BMC Cancer. 2010;10:483–95.

Luong KVQ, Nguyen LTH. The beneficial role of vitamin D and its analogs in cancer treatment and prevention. Crit Rev Oncol/Hematol. 2010;73:192–201.

Malloy PJ, Feldman D. Genetic disorders and defects in vitamin D action. Endocrinol Metab Clin North Am. 2010;39:333–46.

Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. Endocr Rev. 2005;26:662–87.
Plum LA, DeLuca HF. Vitamin D, disease and therapeutic opportunities. Nat Rev Drug Discov. 2010;9:941–55.
van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D2:basic concepts. J Steroid Biochem Mol Biol. 2005;97:93–101.
von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Odum N, Geisler C. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. Nat Immunol. 2010;11:344–9.
Yee YK, Chintalacharuvu SR, Lu J, Nagpal S. Vitamin D receptor modulators for inflammation and cancer. Mini Rev Med Chem. 2005;5:761–78.

Very Late Activation Antigen 1 (VLA-1)

▶ Integrin α1 (ITGA1)

Very Late Activation Antigen 2 (VLA-2)

▶ Integrin α2 (ITGA2)

Vesl

▶ Homer

v-H-Ras

▶ RAS (H-, K-, N-RAS)

Vitamin D receptor (VDR)

▶ VDR, The Vitamin D Receptor

Vitronectin Receptor Alpha (VNRA)

▶ Integrin Alpha V (ITGAV)

Vitronectin Receptor, Alpha Polypeptide

▶ Integrin Alpha V (ITGAV)

v-K-Ras

▶ RAS (H-, K-, N-RAS)

V-Myb Myeloblastosis Viral Oncogene Homolog

▶ c-Myb

v-myc Myelocytomatosis Viral Oncogene Homolog

▶ Myc

v-N-Ras

▶ RAS (H-, K-, N-RAS)

Voltage-Gated Calcium Channels: Structure and Function (CACNA)

Thomas L. Pallone[1], Sandeep Khurana[2] and Chunhua Cao[1]
[1]Department of Medicine, Division of Nephrology, University of Maryland, Baltimore, Baltimore, MD, USA
[2]Department of Medicine, Division of Gastroenterology, University of Maryland, Baltimore, Baltimore, MD, USA

Synonyms

L-type; N-type; P/Q-type; R-type; T-type channels

Historical Background

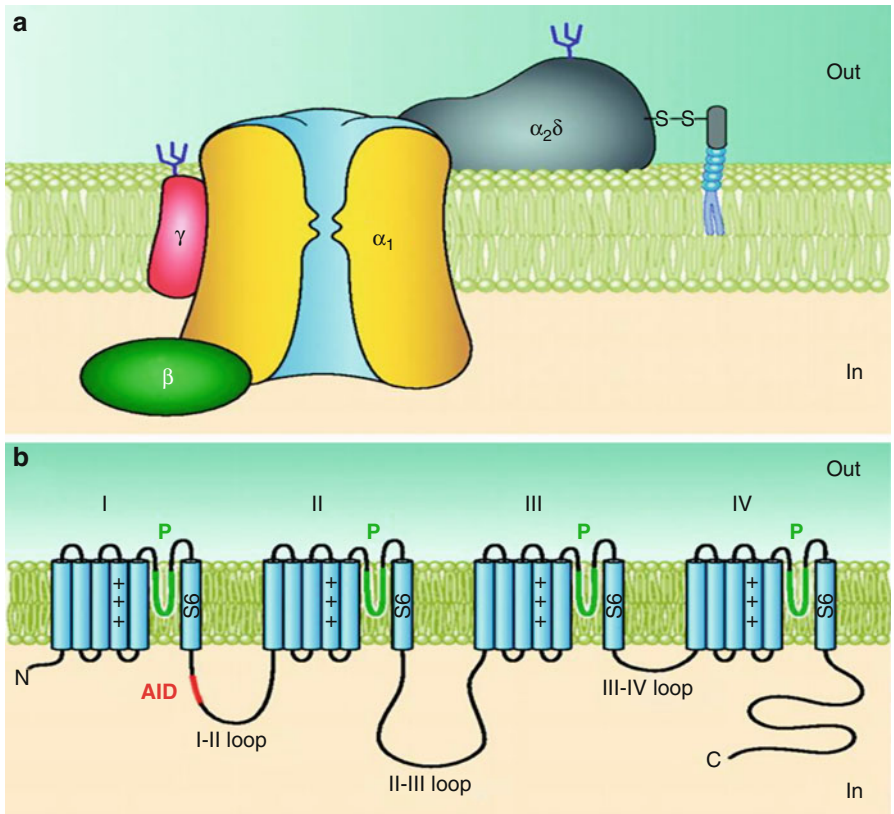
Voltage-gated calcium channels (CaV) are a family of complex proteins that conduct Ca^{2+} into the cell cytoplasm through a large pore-forming α_1 subunit of 190–250 kDa. They are divided into three major families CaV1.x, CaV2.x, and CaV3.x based on sequence homology of the α_1 subunit. In turn, each subfamily is comprised of four (CaV1.1–CaV1.4) or three (CaV2.1–2.3; CaV3.1–3.3) members derived from separate genes (Catterall et al. 2005). The α_1 subunits are variably associated with β, $\alpha_2\delta$, and γ accessory proteins that modulate expression, targeting, voltage dependence, and kinetic characteristics (Fig. 1a) (Catterall et al. 2005; Buraei and Yang 2010). Moreover, splice variants of the α_1 subunit can account for tissue-specific behavior. Alternate classification schemes exist. Table 1 shows the early nomenclature; L-, P/Q-, N-, R-, and T-type, and its relationship to more modern schemes based on sequence identification. The order of molecular identification of the α_1 subunit defines the skeletal muscle α_1 subunit as α_{1S}, and subsequent genes as α_{1A}–α_{1I} (i.e., a total of ten genes: α_{1S}, α_{1A}–α_{1I}). Amino acid homology can exceed ~80% within families but may be less than 30% between families (Fig. 2). In all cases, α_1 subunit structure includes four homologous domains (I–IV) each of which is comprised of six (S1–S6) membrane-spanning regions (Fig. 1b). Voltage sensing resides in the S4 regions of the domains, and the reentrant "P-loop" between S5 and S6 provides the selectivity filter. Other important regions include the inactivation gate and, in some channels, a site for calmodulin binding. The site of CaM binding to the IQ domain of the C-terminus has been proposed to confer local (near the channel pore) versus global (cytoplasmic) sensing of calcium (Liang et al. 2003). Both N-terminal and C-termini are intracellular. CaV bear structural resemblance to voltage-gated potassium and sodium channels, implying a common ancestry. Indeed, minor modifications within the pore-forming sequences of CaV can yield Na^+ selectivity and CaV commonly conduct Na^+ ions in Ca^{2+}-free extracellular solutions. These channels are of ancient origin; examples of each subfamily are found in *Caenorhabditis elegans*.

Voltage-Dependent Gating: Activation, Inactivation, Deactivation

As implied by the name, the open probability of CaV channels is regulated or "gated" by membrane potential. The electrical field within the plasma membrane is "sensed" by displacement of positively charged residues of the S4 transmembrane segment. The macroscopic behavior of CaV currents in whole cell voltage clamp experiments is complex. The CaV channels in a cell can be coordinately induced to conduct divalent ions by sequentially clamping the transmembrane potential to a low value (typically −90 to −110 mV) where "*inactivation*" is removed, making the channels available to open. From that available state, a rapid membrane depolarization to voltage that exceeds threshold level "*activates*" channel opening. Some CaV are "high voltage activated" (HVA) meaning that activation requires a large depolarization. In contrast, others are "low voltage activated" (LVA) such that only a small depolarization to more negative membrane potentials is sufficient. Invariably, whether HVA or LVA characterizes the subgroup, channel opening is followed by spontaneous closure to reenter the "*inactivated*" state. The rate at which channels inactivate is often characteristic of CaV families and subfamilies. Finally, activated channels can be "deactivated" (distinct from inactivation). Deactivation occurs when open channels are induced to close by repolarizing the membrane before the channels have entered the inactivated state. Following deactivation, in contrast to channels in the inactivated state, rapid sequential repolarization-depolarization can activate channel opening. In addition to voltage-dependent gating, CaV can be inhibited or activated by phosphorylation events involving protein kinase C (PKC), cyclic nucleotide–dependent protein kinases (e.g., cGMP-PKG, cAMP-PKA), ▶ MAP kinases, and calmodulin-dependent kinases Camk (Buraei and Yang 2010; Huc et al. 2009).

Channel Pharmacology

Prior to the elucidation of sequence information that led to CaVX.x and α_{1X} designations in Table 1, CaV classification was partially based on electrophysiological study of voltage dependence and kinetics. For example, the L-type channel is high voltage activated



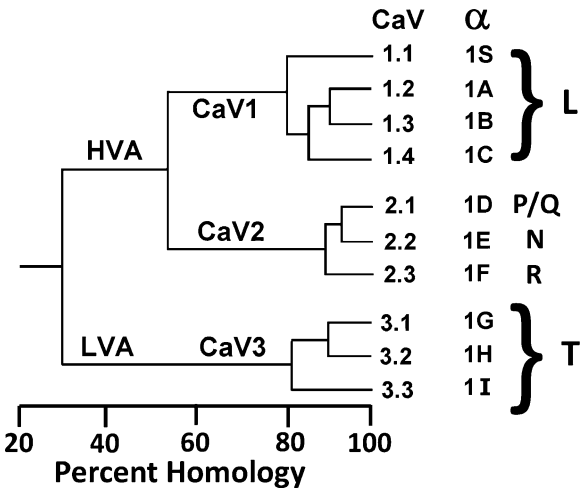
Voltage-Gated Calcium Channels: Structure and Function (CACNA), Fig. 1 Voltage-gated calcium channel (CaV) structure. (**a**) CaV are comprised of the α_1 pore-forming subunit and accessory $\alpha_2\delta$, β, and γ accessory proteins. (**b**) Topology of CaV α_1 subunit. The α_1 subunit is comprised of four domains (I–IV) each of which contains six membrane-spanning regions (S1–S6) and an intramembranous "P" loop that forms the selectivity filter. Voltage sensing is attributed to membrane-potential-dependent displacement of positively charged residues in the S4 region. The α interaction domain (AID, *red*) on intracellular loop I-II binds the β subunit (Adapted with permission from Zafir, B. and Yang, J., Phys Rev 90:1461, 2010)

Voltage-Gated Calcium Channels: Structure and Function (CACNA), Table 1 Voltage-gated Ca^{2+} channel (CaV) classification. Listing and comparison of voltage-gated calcium channel (CaV) classifications in common use

		CaV	α_1 subunit	Gene symbol	Human chromosome
HVA	L	1.1	1S	CACNA1S	1q32
	L	1.2	1C	CACNA1C	12p13.3
	L	1.3	1D	CACNA1D	3p14.3
	L	1.4	1F	CACNA1F	Xp11.23
HVA	P/Q	2.1	1A	CACNA1A	19p13
	N	2.2	1B	CACNA1B	9q34
	R	2.3	1E	CACNA1E	1q25-q31
LVA	T	3.1	1G	CACNA1G	17q22
	T	3.2	1H	CACNA1H	16p13.3
	T	3.3	1I	CACNA1I	22q13.1

HVA high voltage activated *LVA* low voltage activated

(i.e., requires a **L**arge depolarization) and has a slowly inactivating current, thus L- or "*L*ong-lasting" behavior. In contrast, low voltage activated T-type channels (i.e., LVA requiring a small depolarization) rapidly inactivate, thus T- or "*T*ransient" behavior. The "T" of T-type CaV can also be thought of as representing the *T*iny conductance of individual channels. Early separation of CaV classes depended on such kinetic information and the efficacy with which pharmaceuticals induce blockade. Probably the



Voltage-Gated Calcium Channels: Structure and Function (CACNA), Fig. 2 Evolutionary relationship between human voltage-gated calcium channels (CaV). Structural homologies are based on alignments of human channels. *HVA* high voltage activated, *LVA* low voltage activated

best-known and therapeutically exploited agents are the dihydropyridine, phenylalkylamine, and benzothiazepine compounds that provide relatively specific CaV1.x, L-type channel inhibition. Moreover, agonists such as BAYK8644 and FPL64176 serve as L-type channel openers. T-type, CaV3.x channels are relatively insensitive to those agents. Modifications of structure to generate compounds such as mibefradil, pimozide, and efonidipine have yielded alternate organic compounds that can block T-type channels although none of them are fully specific at high concentrations (Catterall et al. 2005; Kochegarov 2003; Striessnig and Koschak 2008). In many cases, where organic compound specificity is absent or lacking, peptide inhibitors isolated from venoms have filled roles in the study and identification of CaV subfamilies. A non-exhaustive summary of organic agents and venomous peptides that block CaV subclasses is in provided in Table 2.

Human Disease Associations and Effects of Gene Deletion

CaV subtypes are widely expressed in neural- and extraneural-excitable cells, including myocardium, skeletal muscle, and smooth muscle. In addition, they are variably expressed in a wide variety of other tissues where their functions are often poorly understood. Table 3 provides a summary of known human disease associations and phenotypic effects of gene deletion. These are summarized in following paragraphs.

CaV1.x, L-Type Channels: Expression and Disease Association

The CaV1.1 (α_{1S}) L-type voltage-gated calcium channel carries a slowly activating inward current and acts as the voltage sensor for calcium signaling via the type 1 ►ryanodine receptor (RyR1, ►RyR) in skeletal muscle. Association of CaV1.1 with RyR1 in the plasmalemmal junction facilitates rapid Ca^{2+} release from sarcoplasmic reticulum (SR) stores (Striessnig et al. 2010; Cannon 2010). Hypokalemic period paralysis type 1 (HPP1) is characterized by attacks of weakness or paralysis, often precipitated by stress, temperature change, exercise, or carbohydrate meals. HPP1 has been most frequently traced to neutralizing point mutations of arginine residues within the S4 voltage-sensing region of CaV1.1. It has been proposed that this modification gives rise to an abnormal "gating pore" current when the S4 region is realigned during depolarization. The role of hypokalemia may be related to its ability to reduce conductance of inward rectifier K^+ channels Kir (potassium inwardly rectifying channel) favoring a depolarizing shift in membrane. Malignant hyperthermia (MH) is a potentially lethal disorder in which affected individuals develop skeletal muscle contractures, ATP depletion, lactic acidosis, and fever upon exposure to anesthetics or muscle relaxants. The majority of mutations that induce MH are found in the RYR1 SR Ca^{2+} release channel, but others have been traced to CaV1.1. In the latter case, point mutations within the cytoplasmic region that links repeats III and IV or modifies the inner arginine of I-S4 are at fault. The III-IV linker region couples CaV1.1 to RYR1 and confers voltage sensing. Depolarization-induced conformational changes lead to rapid RYR1-mediated Ca^{2+} release. In mice, CaV1.1 deficiency is manifest as muscular dysgenesis, an autosomal recessive phenotype that leads to early death of pups by asphyxiation (Striessnig and Koschak 2008).

CaV1.2 (α_{1C}) is a widely expressed channel subject to extensive alternate splicing. It is present in brain, where it is the dominant L-type isoform; in heart,

Voltage-Gated Calcium Channels: Structure and Function (CACNA), Table 2 Voltage-gated calcium channel (CaV) pharmacology

		Pharmaceutical	Peptide blocker (source; species)	Agonist
CaV1.1-1.4	L-type (HVA)	Dihydroperidine Phenylakylamine Benzothiazepines	Calciseptine (black mamba)	BAYK8644 FPL64176
CaV2.1	P/Q-type (HVA)	Gabapentin Mibefradil	ω-agatoxin IVA (funnel web spider)	
CaV2.2	N-type (HVA)	Gabapentin	ω-conotoxins (cone snail) SNX325 (spider)	
CaV2.3	R-type (HVA)		SNX482 (spider)	
CaV3.1-3.3	T-type (LVA)[a]	Mibefradil Pimozide Efonidipine	Kurtoxin (scorpion)	

HVA high voltage activated, *LVA* low voltage activated
[a]Fully selective T-type channel blockers have not been identified

Voltage-Gated Calcium Channels: Structure and Function (CACNA), Table 3 Voltage-gated calcium channels (CaV), human disease, and murine deficiency. Insights into voltage-gated calcium channel (CaV) function are derived from known human syndromes attributable to mutations. Phenotypes of global knockout or conditional tissue-specific vascular smooth muscle (VSM) knockout mice are listed for comparison

CaV	Gene symbol	Human Association	Murine Deficiency
1.1	CACNA1S	HPP1, MH	Muscular dysgenesis
1.2	CACNA1C	Timothy syndrome Brugada syndrome	Embryonic lethality Conditional VSM: hypotension
1.3	CACNA1D	Deafness, bradycardia	Deafness, bradycardia
1.4	CACNA1F	CSNB2 CORDX3 Aland Island eye disease	Blindness
2.1	CACNA1A	FHM1, EA2, SA6	Dystonia, weakness ataxia Neurodegeneration
2.2	CACNA1B	NR	Decreased pain response Enhanced locomotion Enhanced aggression
2.3	CACNA1E	NR	Impaired spatial memory Glucose intolerance
3.1	CACNA1G	NR	Enhanced visceral pain Bradycardia
3.2	CACNA1H	Epilepsy, absence, generalized (association)	Enhanced peripheral pain Contracted coronary arteries Myocardial Fibrosis
3.3	CACNA1I	NR	NR

HPP1 hypokalemic periodic paralysis type 1, *MH* malignant hyperthermia, *VSM* vascular smooth muscle, *CSNB2* congenital stationary night blindness, *CORDX3* X-linked cone-rod dystrophy-3, *FHM1* familial hemiplegic migraine type 1, *EA2* episodic ataxia type 2, *SA6* spinocerebellar ataxia type 6, *NR* not reported

where it conducts Ca^{2+} during the plateau phase of the action potential; in smooth muscle, where it mediates the Ca^{2+} entry required for contraction; in neuroendocrine cells, where it is involved in insulin secretion; and in T-lymphocytes, where it affects immunity (Striessnig et al. 2010; Liao and Soong 2010). The relative predominance of separate exons in heart and smooth muscle accounts for variable ability of organic L-type channel blockers to modify contraction and affect heart rate. Gain-of-function and loss-of-function mutations in CaV1.2 underlie human Timothy and Brugada syndromes, respectively (Liao and Soong 2010).

Timothy syndrome, previously known as the long QT syndrome, is associated with syndactyly, neurological manifestations such as autism and depression, facial malformations, structural cardiac defects, early death, and predisposition to infection. The gain of function results in prolonged inward current due to slowing of inactivation and thus, enhanced Ca^{2+} entry through affected channels. It is inherited in an autosomal dominant pattern, as new mutations, or from an affected parent who is mosaic for the channel. Mutation in the mutually exclusive cardiac CaV1.2 exon yields the type 2 variant of Timothy syndrome (TS type 2, TS2) characterized by cardiac arrhythmias and absence of syndactyly. Brugada syndrome arises most often from mutations in the α_1 subunit of the NaV1.5 voltage-gated sodium channel NaV (sodium channel, voltage-gated, type V) but can also result from mutations in CaV1.2 α_1 or β_{2b} accessory subunits. Brugada patients are prone to sudden death from cardiac arrhythmias and exhibit short QT intervals and elevated ST segments on electrocardiogram. Extracardiac manifestations are generally mild or absent. Complete deletion of CaV1.2 yields embryonic lethality at day 12.5 when it is required to sustain myocardial contraction. As expected, conditional knockout of CaV1.2 from smooth muscle yields hypotension. Conditional knockout of the channel from cerebral cortex and hippocampus leads to loss of spatial memory (Striessnig and Koschak 2008).

Both voltage-dependent inhibition (VDI) and calcium-dependent inhibition (CDI) are characteristics of CaV1.2 behavior. The former is best studied as the spontaneous inactivation that occurs when Ba^{2+} (rather than Ca^{2+}, which affects CDI) is the current-carrying ion. VDI may depend upon interactions of the pore with the I-II intracellular loop. In calcium signaling, CDI depends upon interactions of Ca^{2+} ion with the calcium-sensing accessory subunit calmodulin (CaM) that binds to the IQ motif on the C-terminus of the channel (Minor and Findeisen 2010). Thus, CaM acts as an inhibitory sensor of Ca^{2+} that limits Ca^{2+} entry. Ba^{2+} cannot substitute for Ca^{2+} in CaM binding so that inactivation is prolonged when Ba^{2+} is charge carrier. Stated another way, with Ba^{2+}, CDI is lost and VDI can be studied in isolation.

CaV1.3 (α_{1D}) is expressed in brain, heart, and retina. The behavior of CaV1.3 is distinct from CaV1.2 in that the former activates more rapidly and at more negative potentials. Moreover, CaV1.3 lacks the calcium-dependent inactivation (CDI) exhibited by CaV1.2 and some other CaV channels. Only recently has a human channelopathy been traced to CaV1.3 manifest as deafness and bradycardia. That phenotype parallels deafness and sinoatrial node dysfunction of CaV1.3 knockout mice (Baig et al. 2011). Deletion of CaV1.3 in the heterozygous state may be without consequence. The homozygous knockout mouse, however, is deaf and bradycardic but viable and fertile (Striessnig and Koschak 2008). Deafness occurs because CaV1.3 is vital to proper function of inner ear hair cells of the cochlea. The associated bradycardia is benign and overcome during exercise (Striessnig and Koschak 2008; Striessnig et al. 2010).

CaV1.4 (α_{1F}) is found in retina and human deficiency leads to X-linked congenital stationary night blindness, type 2 (CSNB2) and its variants, X-linked cone-rod dystrophy-3 (CORDX3), and Aland Island eye disease or Forsius-Eriksson syndrome (Striessnig et al. 2010). The channel is characterized by rapid activation, occurring at relatively low membrane potentials below −40 mV, and slow inactivation which, like CaV1.3, is related to absence of CDI. CSNB2 patients exhibit variable manifestations of decreased visual acuity, particularly in dim light, myopia, and nystagmus. Associated mutations may yield loss of function, gain of function, or abnormalities in gating. Mutations in other proteins such as calcium-binding protein-4 can yield the CSNB2 phenotype. CaV1.4 is involved in control of neurotransmission in retinal photoreceptors (like CaV1.3 in cochlear hair cells). The relatively low voltage-dependent activation threshold of CaV1.3 and CaV1.4 may be vital to enhance their window current (Ca^{2+} influx related to the membrane potential range over which channels can activate and escape durable inactivation). The window current of CaV1.3 and CaV1.4 probably facilitates tonic neurotransmitter release in cochlear hair cells and retina, respectively. Structurally, absence of CDI results from the presence of an inhibitory domain near the calmodulin-binding site (IQ domain) on the channel C-terminus. Mutation or elimination of that motif can restore CaM-dependent CDI. Moreover, deletion of the inhibitory domain may occur as a normal splice variant in CaV1.3. An autoinhibitory domain of CaV1.2, generated by proteolytic cleavage of the C-terminus, may serve an analogous inhibitory function in cardiac myocytes (Hulme et al. 2006).

CaV2.x, P/Q-, N-, and R-Type Channels: Expression and Disease Association

CaV2.1 (α_{1A}) P/Q-type channels are predominantly expressed in the central nervous system where they are coupled to exocytosis at synapses. Human loss of function is associated with three autosomal dominant disorders: familial hemiplegic migrane type 1 (FHM1), episodic ataxia type 2 (EA2), and spinocerebellar ataxia type 6 (SA6) (Pietrobon 2010). FHM1 patients suffer from migraine headache with aura along with motor weakness or frank hemiplegia. Severely affected individuals may exhibit seizures, depression of consciousness, confusion, and prolonged weakness. Some FHM1 families exhibit cerebellar atrophy, progressive ataxia, cognitive impairment, and nystagmus. EA2 manifests in early life with symptoms such as episodic ataxia, loss of coordination, dysarthria, vertigo, and nausea. SA6 manifests later in life as progressive ataxia and cerebellar atrophy that associates with expansion of C-terminal polyglutamine repeats of splice variants. P/Q variants have been proposed to confer susceptibility to generalized seizures (Khosravani and Zamponi 2006) Homozygous CaV2.1 deficiency in mice leads to severe forms of neurological dysfunction involving dystonia, weakness, and ataxia (e.g., Tottering mice). The majority of pups do not survive to weaning and progressive decrease in cerebellar volume is associated with loss of Purkinje and granular cells. Heterozygous mice do not suffer similar neurodegeneration (Striessnig and Koschak 2008).

CaV2.2 (α_{1B}), N-type channels are predominantly expressed in the central nervous system and syndromes traced to their deficiency in humans have not been reported. Mice deficient in the α_{1B} subunit are viable and fertile. Reduced responses to some pain stimuli (Kim et al. 2001), enhanced aggression, and enhanced locomotor activity have been documented (Striessnig and Koschak 2008; Nakagawasai et al. 2010).

CaV2.3 (α_{1E}), R-type channels are widely expressed in neuronal tissue, and have also been found in retina, kidney, pancreatic islets, neuroendocrine cells of the GI tract, and spleen. CaV2.3 has several splice variants and is involved in diverse physiological functions, neurotransmitter release, neuronal plasticity, responses to fear and pain, insulin release, and reproduction. Human disorders traceable to α_{1B} mutations have not been reported; however, the channel is believed to be involved in predisposition to seizure and seizure propagation (Weiergraber et al. 2006). These and other CaV isoforms are targeted by many antiepileptic pharmaceuticals. Mice deficient in α_{1B} have been generated and studied extensively. They show altered responses to some forms of noxious stimuli, impaired spatial memory in maze testing, and disturbances in glucose tolerance and insulin release (Striessnig and Koschak 2008).

CaV3.x, T-Type Channels: Expression and Disease Association

CaV3.x, T-type channels are widely expressed in neural and extraneural tissues. Their behavior is generally distinguished from CaV1.x, L-type channels by their low activation thresholds and tendency toward rapid activation and inactivation (CaV3.3 is an arguable exception). They are also distinguished by their lower Ba^{2+} to Ca^{2+} conductance ratio, small single channel currents, and insensitivity to dihydropyridines (Huc et al. 2009; Perez-Reyes 2006; Zamponi et al. 2010). Their best-known roles involve pacemaker functions in heart and brain. They are known to be modulated by cyclic nucleotide–dependent protein kinases (PKA, PKC) (Huc et al. 2009).

CaV3.1 (α_{1G}), T-type channels are expressed predominantly in brain, and notably in thalamocortical relay neurons (TRN) where they participate in spike and wave discharges. CaV3.1 has also been reported in diverse tissues such as sinoatrial node, vascular smooth muscle, endothelia, myometrium, preadipocytes, outer hair cells, and testis. Human diseases associated with CaV3.1 have not been reported and murine deficiency yields a viable phenotype without gross abnormalities. CaV3.1 deficiency leads to inability to induce spike and wave discharges in TRN and, when superimposed by cross breeding, renders mouse models of absence seizures resistant to seizure events (Striessnig and Koschak 2008). Loss of CaV3.1 also leads to enhanced visceral pain, loss of T-type currents in sinoatrial node, and bradycardia in freely moving mice.

CaV3.2 (α_{1H}), T-type channels are expressed predominantly in brain, kidney, liver, and heart. In humans, CaV3.2 variants have been associated with susceptibility to absence epilepsy in Chinese Han children. Other human variants have been proposed to increase susceptibility to generalized seizures.

Murine homozygous deletion of CaV3.2 yields a viable and fertile phenotype that experiences delayed growth. Interestingly, CaV3.2 deficient mice exhibit constitutively contracted coronary arteries and myocardial fibrosis (Chen et al. 2003). A physical association between CaV3.2 and large conductance calcium-dependent potassium (BKCa) channels in coronary artery was found in wildtype mice but absent in CaV3.2 knockouts. Thus, it is possible that elimination of stimulation of BKCa might underlie enhanced coronary vasomotor tone in CaV3.2$^{-/-}$. In a manner analogous to increase in visceral pain induced by CaV3.1 deficiency, knockout of CaV3.2 leads to increase in peripheral pain from thermal or mechanical stimuli (Striessnig and Koschak 2008).

CaV3.3 (α_{1I}), T-type channels are expressed in brain and spinal cord with limited expression in adrenal and thyroid. When compared to either CaV3.1 or CaV3.2, CaV3.3 exhibits slower activation and inactivation rates (Huc et al. 2009; Perez-Reyes 2006). Information on its tissue-specific functions is sparse. Neither human disease association nor effects of murine gene deletion have been reported.

Summary

Voltage-gated calcium channels are large complex proteins evolved from ancient origins. Over the last 30 years, enormous progress has been made in their molecular identification (Table 1). Their predominant expression in excitable tissues such as the nervous system, skeletal muscle, and smooth muscle is consistent with regulation of those tissues through modulation of membrane potential and the important role of Ca^{2+} ions in intracellular calcium signaling. Much information has also been derived from associations between naturally occurring mutations and human disease. The associated gain-of-function and loss-of-function phenotypes that lead to alteration of neuromuscular functions are consistent with their expected roles. In many cases, the diseases are mimicked by effects of targeted murine gene deletions (Table 3). Pharmacological study remains challenging due to lack of selective organic compounds that are capable of blocking conduction of ions through the various subclasses (Table 2). Efforts to identify such compounds through high-throughput screening methods may bridge the gap and lead to new probes of physiological function as well as pharmaceutical antiarrhythmic, antiepileptic, and antihypertensive agents.

References

Baig SM, Koschak A, Lieb A, Gebhart M, Dafinger C, Nurnberg G, et al. Loss of Ca(v)1.3 (CACNA1D) function in a human channelopathy with bradycardia and congenital deafness. Nat Neurosci. 2011;14(1):77–84.

Buraei Z, Yang J. The ss subunit of voltage-gated Ca^{2+} channels. Physiol Rev. 2010;90(4):1461–506.

Cannon SC. Voltage-sensor mutations in channelopathies of skeletal muscle. J Physiol. 2010;588(Pt 11):1887–95.

Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J. International union of pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev. 2005;57(4):411–25.

Chen CC, Lamping KG, Nuno DW, Barresi R, Prouty SJ, Lavoie JL, et al. Abnormal coronary function in mice deficient in alpha1H T-type Ca^{2+} channels. Science. 2003;302(5649):1416–8.

Huc S, Monteil A, Bidaud I, Barbara G, Chemin J, Lory P. Regulation of T-type calcium channels: signalling pathways and functional implications. Biochim Biophys Acta. 2009;1793(6):947–52.

Hulme JT, Yarov-Yarovoy V, Lin TW, Scheuer T, Catterall WA. Autoinhibitory control of the CaV1.2 channel by its proteolytically processed distal C-terminal domain. J Physiol. 2006;576(Pt 1):87–102.

Khosravani H, Zamponi GW. Voltage-gated calcium channels and idiopathic generalized epilepsies. Physiol Rev. 2006;86(3):941–66.

Kim C, Jun K, Lee T, Kim SS, McEnery MW, Chin H, et al. Altered nociceptive response in mice deficient in the alpha (1B) subunit of the voltage-dependent calcium channel. Mol Cell Neurosci. 2001;18(2):235–45.

Kochegarov AA. Pharmacological modulators of voltage-gated calcium channels and their therapeutical application. Cell Calcium. 2003;33(3):145–62.

Liang H, DeMaria CD, Erickson MG, Mori MX, Alseikhan BA, Yue DT. Unified mechanisms of Ca^{2+} regulation across the Ca^{2+} channel family. Neuron. 2003;39(6):951–60.

Liao P, Soong TW. CaV1.2 channelopathies: from arrhythmias to autism, bipolar disorder, and immunodeficiency. Pflugers Arch. 2010;460(2):353–9.

Minor Jr DL, Findeisen F. Progress in the structural understanding of voltage-gated calcium channel (CaV) function and modulation. Channels (Austin). 2010;4(6):459–74.

Nakagawasai O, Onogi H, Mitazaki S, Sato A, Watanabe K, Saito H, et al. Behavioral and neurochemical characterization of mice deficient in the N-type Ca^{2+} channel alpha1B subunit. Behav Brain Res. 2010;208(1):224–30.

Perez-Reyes E. Molecular characterization of T-type calcium channels. Cell Calcium. 2006;40(2):89–96.

Pietrobon D. CaV2.1 channelopathies. Pflugers Arch. 2010;460(2):375–93.

Striessnig J, Koschak A. Exploring the function and pharmacotherapeutic potential of voltage-gated Ca^{2+}

channels with gene knockout models. Channels (Austin). 2008;2(4):233–51.
Striessnig J, Bolz HJ, Koschak A. Channelopathies in Cav1.1, Cav1.3, and Cav1.4 voltage-gated L-type Ca^{2+} channels. Pflugers Arch. 2010;460(2):361–74.
Weiergraber M, Kamp MA, Radhakrishnan K, Hescheler J, Schneider T. The Ca(v)2.3 voltage-gated calcium channel in epileptogenesis–shedding new light on an enigmatic channel. Neurosci Biobehav Rev. 2006;30(8):1122–44.
Zamponi GW, Lory P, Perez-Reyes E. Role of voltage-gated calcium channels in epilepsy. Pflugers Arch. 2010;460(2):395–403.

v-Raf

▶ RAF-1 (C-RAF)

v-Raf Murine Sarcoma 3611 Viral Oncogene Homolog

▶ A-RAF

v-Raf-1 Leukemia Viral Oncogene 1

▶ RAF-1 (C-RAF)

VRK1

Alberto Valbuena, Marta Sanz-García, Inmaculada López-Sánchez, Francisco M. Vega, Ana Sevilla and Pedro A. Lazo
Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Synonyms

Vaccinia-related kinase 1; Vaccinia virus B1R-related kinase 1; VRK-1

Historical Background

VRK proteins were identified as two human EST sequences whose translation products have homology with the kinase domain in vaccinia virus B1R kinase, from where its name is derived. During its evolution and adaptation to its host, the virus incorporated this cellular sequence required for viral DNA replication.

VRK1 Gene Structure and Expression

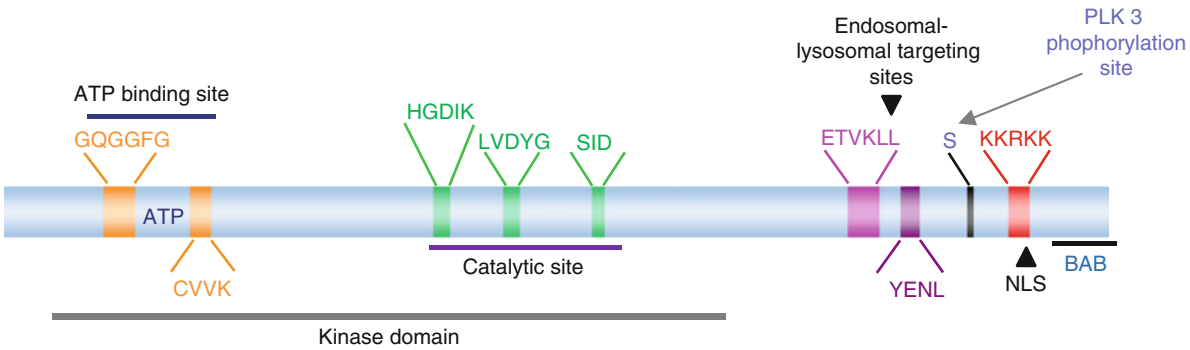
The human *VRK1* gene is located on the chromosomal region 14q32.2, containing 14 exons and coding for a protein with 396 amino acids. This gene has a polymorphism, marker rs722869, which is useful in the identification of population structure and genetic ancestry (Lao et al. 2006). Chromosomal translocations in the region do not affect VRK1. No mutation in this gene has been reported in human cancer.

VRK1 is expressed in all types of human and murine cells tested (Nichols and Traktman 2004), particularly at very high levels if they are proliferating (Nezu et al. 1997; Santos et al. 2006). In murine embryonic hematopoietic development, the highest level of expression is achieved between days E11.5 and E13.5, a time of massive hematopoietic-liver expansion (Vega et al. 2003).

Loss of VRK1 by siRNA results in a block in cell cycle progression, since VRK1 is an early response gen following stimulation by growth factors and is necessary for G0 exit (Valbuena et al. 2008b). VRK1 knockdown mice, which have a 15% residual level, are infertile because of loss of spermatogonia and have chromosomal meiotic aberrations (Wiebe et al. 2010).

VRK1 Protein Structure and Subcellular Localization

VRK (vaccinia-related kinases) are a new group of Ser-Thr kinases in the human kinome (Manning et al. 2002) and have homology to the catalytic domain of the B1R kinase of vaccinia virus that is required for viral DNA replication (Nichols and Traktman 2004). In yeast, there is no orthologous gene, and in invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans*, there is only one VRK protein (Klerkx et al. 2009). In mammals, there are three members, two of which



VRK1, Fig. 1 Human VRK1 protein structure. *BAB* basic-acid-basic motif, *NLS* Nuclear localization signal, *PLK3* polo-like kinase 3

(VRK1 and VRK2) are catalytically active (Lopez-Borges and Lazo 2000; Nichols and Traktman 2004), and a third one, VRK3, which is not active and might function as a scaffold protein (Kang and Kim 2006). The VRK catalytic domain has substitutions in several key residues making it an atypical kinase (Nichols and Traktman 2004). The relative conservation of the catalytic domain of VRK proteins indicates they can have an overlap of potential substrates in vitro, and the in vivo substrates are likely to be determined by subcellular localization.

Structurally, the three VRK proteins differ in their regulatory region located C-terminal on VRK1(3OP5) (Fig. 1) and VRK2, and N-terminal on VRK3. These regulatory regions are unrelated among them and have no homology to any known feature identified in other proteins. The length of the regulatory region is variable. It is approximately 100 amino acids in VRK1 and 200 amino acids in VRK2; thus, their subcellular localization and regulation is likely to be different among mammalian VRK proteins and those of lower eukaryotes.

VRK1 interacts by its C-terminal region with nuclear RAN, a small GTPase, which can regulate the kinase activity; RAN bound to GDP, but not to GTP, inhibits its kinase activity (Sanz-Garcia et al. 2008). This region also contains nuclear import and export localization signals, an endosomal-lysosomal targeting sequence, as well as a BAB (basic-acid-basic) motif of unknown function.

VRK1 is a protein mostly located within the nucleus, and it is outside the nucleolus (Lopez-Borges and Lazo 2000; Vega et al. 2004), a major fraction of which is interacting with chromatin (Kang et al. 2007). However, there is a minor cytoplasmic subpopulation located in the Golgi apparatus (Lopez-Sanchez et al. 2009). In some cell types, particularly in secretory epithelial cells, VRK1 is also present in the cytosol (Valbuena et al. 2007a), but the regulation of VRK1 subcellular localization is not known.

VRK1 Phosphorylation Targets and Role in Nuclear Dynamics

VRK1 regulates several transcription factors associated with cellular responses to stress and cell cycle progression. VRK1 phosphorylates the JUN oncogene in residues Ser63 and Ser73; these residues are the same targeted by JNK (Jun N-terminal kinase). This permits an additive activation of JUN-dependent transcription in situations of suboptimal activation of the JNK and VRK1 signaling pathways (Sevilla et al. 2004a).

The transcription factor ATF2 is phosphorylated in residues Ser62 and Thr73 by VRK1 (Sevilla et al. 2004b). These residues are in the same region, but are different from the Thr69 and Thr71 targeted by JNK. Thus they can have an additive effect even at maximum stimulation of VRK1 and JNK signaling pathways (Sevilla et al. 2004a). Furthermore, residues Ser62 and Thr72 are activated in response to cAMP signals mediated by PKA, which can cooperate with VRK1 in case of suboptimal stimulation. VRK1 phosphorylates CREB1 in Ser133 and is integrated in the transcriptional protein complex required for cyclin D1 (CCND1) gene expression (Kang et al. 2008), consistent with a VRK1 role in cell cycle entry and progression (Valbuena et al. 2008b).

VRK1 has been shown in several processes required for cell division. VRK1 phosphorylates histone H3 in Thr3 and Ser10 inducing chromatin condensation in mitosis (Kang et al. 2007; Sanz-Garcia et al. 2008). VRK1 protein level is higher in mitosis and is detected in chromatin, where it regulates the interaction of H3 with gamma heterochromatin protein 1 (HP1γ) (Kang et al. 2007).

VRK1 phosphorylates BAF, a protein regulating assembly and disassembly of the nuclear envelope (Nichols et al. 2006). BAF phosphorylation by VRK1 can inhibit the preintegration complex of retroviruses (Suzuki et al. 2010).

VRK1 interacts with RAN, the only nuclear small GTPase (Sanz-Garcia et al. 2008). RAN is implicated in nuclear transport and has an asymmetric distribution in the nucleus determined by its binding to either GDP or GTP. In mitosis, RAN-GTP is proximal to the spindle. RAN is an allosteric regulator of VRK1 activity. RAN-GDP inhibits VRK1 kinase activity on substrates such as p53 and histone H3, and VRK1 is active when it is bound to RAN-GTP (Sanz-Garcia et al. 2008). This effect indicates that VRK1 activity in the nucleus is likely to be asymmetric and dependent on its localization, despite an apparent homogeneous distribution of this protein. In some cells, VRK1 is detected in the nucleolus, but its significance is not known.

VRK1 Regulating p53 and Cell Cycle Processes

VRK1 phosphorylates p53 uniquely in Thr18 (Lopez-Borges and Lazo 2000). This phosphorylation prevents p53 interaction with HDM2 and favors its interaction with transcriptional cofactors. Thus VRK1 induces a stabilization and accumulation of p53 that exerts its known biological effects (Vega et al. 2004). A persistent accumulation of p53 is deleterious for the cell. Thus it is necessary to downregulate p53, but this requires also removing its VRK1 stabilizer. p53, with P300 as coactivator, activates transcription of DRAM (Valbuena et al. 2008a), a new gene that targets VRK1 for proteolytic degradation in the lysosome (Valbuena et al. 2006). Its mechanism involves part of the autophagic pathway implicated in removal of stable proteins, such as VRK1. In tumors with p53 mutations, this autoregulatory loop is not induced and there is an accumulation of VRK1 (Valbuena et al. 2007b). VRK1 appears to play a fundamental role allowing cells to respond to DNA damage and initiate protective responses (Vega et al. 2004).

VRK1 gene expression is regulated by entry in cell cycle after stimulation with serum and is expressed at the same time as MYC and FOS, two early genes and positively correlated with phospho-RB. Serum withdrawal results in loss of VRK1 gene expression (Valbuena et al. 2008b). VRK1 knockdown prevents cell division (Vega et al. 2004), causes loss of RB phosphorylation, and there is an accumulation of p27, suggesting a cell cycle blockade (Valbuena et al. 2008b). VRK1 gene expression precedes that of Cyclin D1 (CCND1gene) before the (restriction point in) G1 (Valbuena et al. 2008b), and VRK1 protein forms part of the transcriptional complex on the CCND1 gene promoter (Kang et al. 2008). In mitosis, there is a fragmentation of the Golgi apparatus required for its distribution into daughter cells; this process is induced by MEK1 and PLK3, and in which VRK1 is a downstream step. The VRK1 subpopulation located in Golgi apparatus is an essential component of the MEK1-Plk3 signaling pathway required for Golgi fragmentation in mitosis, in which Plk3 directly phosphorylates VRK1 in Ser342 (Lopez-Sanchez et al. 2009). Consistent with cell cycle defects is the phenotype of hypomorphic VRK1 knockdown mice (15% residual normal protein) of murine VRK1 that have a loss of spermatogonia and sterility (Wiebe et al. 2010).

VRK1 in Human Diseases

VRK1 is expressed at high level in most tumors, probably reflecting its association with proliferation, since it positively correlates with cell cycle markers cdk2, p63, and survivin, as shown in head and neck squamous cell carcinomas (Santos et al. 2006). In lung cancer with p53 mutations, there are also very high levels of VRK1, reflecting the disruption of its autoregulatory loop (Valbuena et al. 2007b). In breast cancer, VRK1 overexpression identifies a subgroup with poorer prognosis (Finetti et al. 2008).

The homozygous mutation R358Stop causes a syndrome characterized by pontocerebellar hypoplasia, spinal-muscular atrophy, mild mental retardation, and death in childhood (Renbaum et al. 2009). Heterozygous carriers have no pathology.

Summary

VRK1 is a Ser-Thr kinase functionally associated with entry in cell cycle, and is required for nuclear envelope dynamics, chromatin condensation, and Golgi fragmentation.

VRK1 is mostly located in the nucleus and in the Golgi apparatus. Among its substrates are several transcription factors: p53, c-Jun, ATF2, and CREB1. VRK1 probably acts cooperating with other signaling pathways that also phosphorylate these transcription factors. VRK1 stabilizes p53 by a specific phosphorylation in Thr18 and after induction of p53 responses, there is a VRK1 downregulation in the lysosome and in which the autophagic pathway participates in a p53-dependent manner. VRK1 contributes to chromatin condensation and nuclear envelop kinetics by phosphorylation of histone H3 and BAF. VRK1 is downstream of MEK1 and Plk3 in the pathway inducing Golgi fragmentation during mitosis. VRK1 is expressed in most tissues, is necessary in the early G1 phase for entry in cell cycle, and is associated with proliferation markers (Klerkx et al. 2009).

References

Finetti P, Cervera N, Charafe-Jauffret E, Chabannon C, Charpin C, Chaffanet M, Jacquemier J, Viens P, Birnbaum D, Bertucci F. Sixteen-kinase gene expression identifies luminal breast cancers with poor prognosis. Cancer Res. 2008;68:767–76.

Kang TH, Kim KT. Negative regulation of ERK activity by VRK3-mediated activation of VHR phosphatase. Nat Cell Biol. 2006;8:863–9.

Kang TH, Park DY, Choi YH, Kim KJ, Yoon HS, Kim KT. Mitotic histone H3 phosphorylation by vaccinia-related kinase 1 in mammalian cells. Mol Cell Biol. 2007;27:8533–46.

Kang TH, Park DY, Kim W, Kim KT. VRK1 phosphorylates CREB and mediates CCND1 expression. J Cell Sci. 2008;121:3035–41.

Klerkx EP, Lazo PA, Askjaer P. Emerging biological functions of the vaccinia-related kinase (VRK) family. Histol Histopathol. 2009;24:749–59.

Lao O, van Duijn K, Kersbergen P, de Knijff P, Kayser M. Proportioning whole-genome single-nucleotide-polymorphism diversity for the identification of geographic population structure and genetic ancestry. Am J Hum Genet. 2006;78:680–90.

Lopez-Borges S, Lazo PA. The human vaccinia-related kinase 1 (VRK1) phosphorylates threonine-18 within the mdm-2 binding site of the p53 tumour suppressor protein. Oncogene. 2000;19:3656–64.

Lopez-Sanchez I, Sanz-Garcia M, Lazo PA. Plk3 interacts with and specifically phosphorylates VRK1 in Ser342, a downstream target in a pathway that induces Golgi fragmentation. Mol Cell Biol. 2009;29:1189–201.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298:1912–34.

Nezu J, Oku A, Jones MH, Shimane M. Identification of two novel human putative serine/threonine kinases, VRK1 and VRK2, with structural similarity to vaccinia virus B1R kinase. Genomics. 1997;45:327–31.

Nichols RJ, Traktman P. Characterization of three paralogous members of the Mammalian vaccinia related kinase family. J Biol Chem. 2004;279:7934–46.

Nichols RJ, Wiebe MS, Traktman P. The vaccinia-related kinases phosphorylate the N′ terminus of BAF, regulating its interaction with DNA and its retention in the nucleus. Mol Biol Cell. 2006;17:2451–64.

Renbaum P, Kellerman E, Jaron R, Geiger D, Segel R, Lee M, King MC, Levy-Lahad E. Spinal muscular atrophy with pontocerebellar hypoplasia is caused by a mutation in the VRK1 gene. Am J Hum Genet. 2009;85:281–9.

Santos CR, Rodriguez-Pinilla M, Vega FM, Rodriguez-Peralto JL, Blanco S, Sevilla A, Valbuena A, Hernandez T, van Wijnen AJ, Li F, et al. VRK1 signaling pathway in the context of the proliferation phenotype in head and neck squamous cell carcinoma. Mol Cancer Res. 2006;4:177–85.

Sanz-Garcia M, Lopez-Sanchez I, Lazo PA. Proteomics identification of nuclear Ran GTPase as an inhibitor of human VRK1 and VRK2 (vaccinia-related kinase) activities. Mol Cell Proteomics. 2008;7:2199–214.

Sevilla A, Santos CR, Barcia R, Vega FM, Lazo PA. c-Jun phosphorylation by the human vaccinia-related kinase 1 (VRK1) and its cooperation with the N-terminal kinase of c-Jun (JNK). Oncogene. 2004a;23:8950–8.

Sevilla A, Santos CR, Vega FM, Lazo PA. Human vaccinia-related kinase 1 (VRK1) activates the ATF2 transcriptional activity by novel phosphorylation on Thr-73 and Ser-62 and cooperates with JNK. J Biol Chem. 2004b;279:27458–65.

Suzuki Y, Ogawa K, Koyanagi Y. Functional disruption of the moloney murine leukemia virus preintegration complex by vaccinia-related kinases. J Biol Chem. 2010;285:24032–43.

Valbuena A, Blanco S, Vega FM, Lazo PA. The C/H3 domain of p300 is required to protect VRK1 and VRK2 from their downregulation induced by p53. PLoS ONE. 2008a;3:e2649.

Valbuena A, Lopez-Sanchez I, Lazo PA. Human VRK1 is an early response gene and its loss causes a block in cell cycle progression. PLoS ONE. 2008b;3:e1642.

Valbuena A, Lopez-Sanchez I, Vega FM, Sevilla A, Sanz-Garcia M, Blanco S, Lazo PA. Identification of a dominant epitope in human vaccinia-related kinase 1 (VRK1) and detection of different intracellular subpopulations. Arch Biochem Biophys. 2007a;465:219–26.

Valbuena A, Suarez-Gauthier A, Lopez-Rios F, Lopez-Encuentra A, Blanco S, Fernandez PL, Sanchez-Cespedes M, Lazo PA. Alteration of the VRK1-p53 autoregulatory loop in human lung carcinomas. Lung Cancer. 2007b;58:303–9.

Valbuena A, Vega FM, Blanco S, Lazo PA. p53 downregulates its activating vaccinia-related kinase 1, forming a new autoregulatory loop. Mol Cell Biol. 2006;26:4782–93.

Vega FM, Gonzalo P, Gaspar ML, Lazo PA. Expression of the VRK (vaccinia-related kinase) gene family of p53 regulators in murine hematopoietic development. FEBS Lett. 2003; 544:176–80.
Vega FM, Sevilla A, Lazo PA. p53 Stabilization and accumulation induced by human vaccinia-related kinase 1. Mol Cell Biol. 2004;24:10366–80.
Wiebe MS, Nichols RJ, Molitor TP, Lindgren JK, Traktman P. Mice deficient in the serine/threonine protein kinase VRK1 are infertile due to a progressive loss of spermatogonia. Biol Reprod. 2010;82:182–93.

VRK-1

▶ VRK1

VRK2

Sandra Blanco, Isabel F. Fernández, Marta Vázquez-Cedeira, Diana M. Monsalve, Triana Merced and Pedro A. Lazo
Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Salamanca, Salamanca, Spain

Synonyms

Vaccinia-related kinase 2; Vaccinia virus B1R-related kinase 2; VRK-2

Historical Background

Mammalian VRK (vaccinia-related kinases) were originally identified as two human EST, ▶ VRK1 and VRK2, expressed in proliferating cell lines and regenerating liver. These EST have homology to the catalytic region of the B1R kinase of vaccinia virus (Nezu et al. 1997) that is expressed early in viral infection, and is required for viral DNA replication. These VRK proteins were classified as a new group of Ser-Thr kinases in the human kinome diverging very early from the same branch that led to casein kinase I (CK1) (Manning et al. 2002). This family has only one ortholog gene in invertebrates, such as *Caenorhabditis elegans* or *Drosophila melanogaster*, and is not present in yeast (Klerkx et al. 2009).

VRK2 Gene and Expression

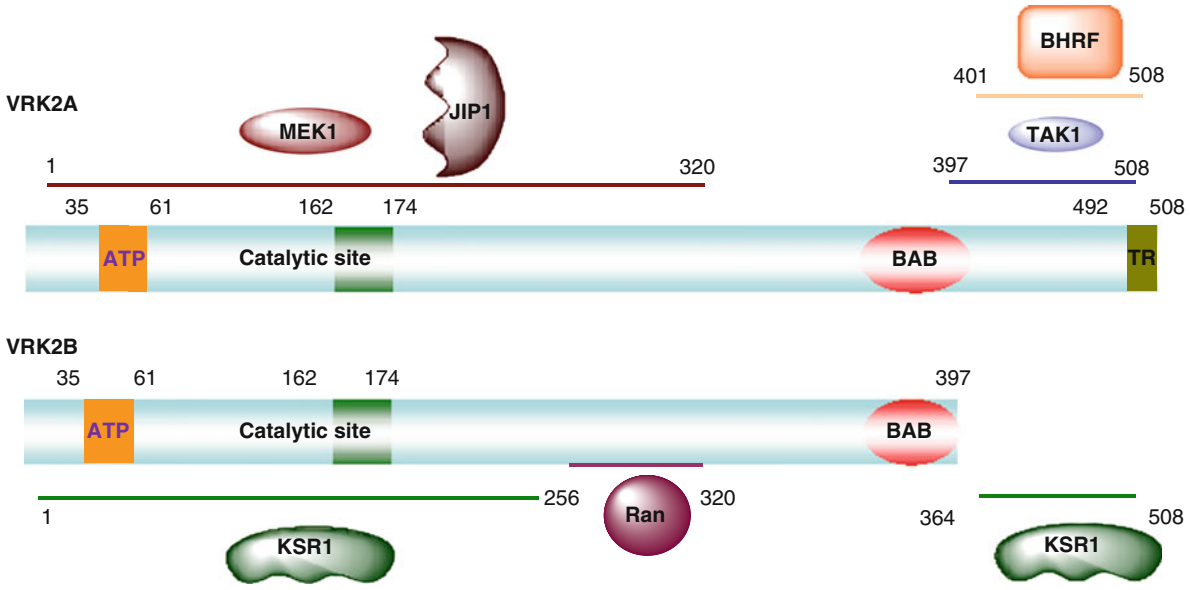
The human *VRK2* gene is located in chromosome 2p16.1 and generates two messages by alternative splicing. Their translation generates two protein isoforms, VRK2A (also known as VRK2) and VRK2B. The VRK2A corresponds to the full-length protein and is expressed from a message of 1,833 nucleotides coding for a protein of 508 amino acids. The alternatively spliced variant includes a novel exon and generates a message of 1,877 nucleotides. The additional exon has 44 nucleotides, and is the new exon 13. The message for isoform B has an inframe termination codon, generating a shorter protein of 397 amino acids that is truncated and lacks the C-terminal region that is present in isoform A (Blanco et al. 2006; Nichols and Traktman 2004).

Human VRK2 RNA is detected in many tissues, particularly those with high proliferation activity such as tumor cell lines. Fetal tissues express higher levels than adult organs (Nezu et al. 1997; Nichols and Traktman 2004).VRK2A has been detected in all cell types examined, whereas VRK2B has only been detected in cells in which VRK1 is cytoplasmic. Therefore, VRK2B might be functionally redundant with VRK1 in some roles not yet identified (Blanco et al. 2006).

During hematopoietic development in murine embryos, the highest level of VRK2 expression is achieved at days E11.5 to E13.5, in thymus, spleen, and liver, at a time of massive hematopoietic-liver expansion (Vega et al. 2003). Expression increases again at day 17.5, just before delivery. In adult mice high RNA levels are present in liver, kidney, and muscle (Vega et al. 2003).

VRK2 Protein Structure and Subcellular Localization

The amino acid sequence of human VRK2 is slightly different from other VRK kinases and contains amino acid substitutions in several key residues, but it is still



VRK2, Fig. 1 Structure of human VRK2A and VRK2B isoforms and their protein interactions. ATP (position of ATP binding site). Catalytic site is indicated. *BAB* basic-acid-basic region, *TM* transmembrane region, *VEA* three terminal amino acids in VRK2B isoform, which are different, from VRK2A. The interacting proteins and the location of the regions of interaction with other proteins are also indicated. Some of the interactions are common to both isoforms

catalytically active (Nichols and Traktman 2004; Blanco et al. 2006). The crystal structure of the catalytic domain of human VRK2 (PDB: 2V62) has been determined and it differs in the activation segment from the catalytically inactive VRK3 (Scheeff et al. 2009). VRK2A and VRK2B proteins have a strong autophosphorylation activity in multiple residues (Blanco et al. 2006; Nichols and Traktman 2004). However, both VRK2 proteins are also likely to be regulated and be the target of other kinases not yet identified. The C-terminal region is very different between VRK2A and VRK2B. VRK2A is the normal full-length protein and has 508 amino acids. VRK2A has a conserved BAB (basic-acid-basic motif) of unknown function in its C-terminal region, and the last amino acids are hydrophobic and form a typical transmembrane anchoring region. VRK2B is the result of alternative splicing in some cells and has 397 residues, of which the first 394 are identical to the corresponding region in VRK2A (Fig.1), and its last three residues are different (VEA), thus it lacks most of the C-terminal region present in VRK2A. Neither isoform has a nuclear localization signal. Several proteins have been identified interacting with different regions of VRK2A or VRK2B (Fig. 1).

The human VRK2A protein is located in the endoplasmic reticulum (ER), partly in the perinuclear area (Nichols and Traktman 2004). The VRK2A isoform has a hydrophobic membrane-anchoring domain that is not present in isoform VRK2B. VRK2A localizes to endoplasmic reticulum (ER) membranes and colocalizes with the ER markers calnexin and calreticulin, and is the isoform present in all cell types (Blanco et al. 2006; Nichols and Traktman 2004). VRK2A also colocalizes with mitochondria, as detected by its colocalization with the Mitotracker reagent (Blanco et al. 2006). VRK2B lacks the C-terminal region and its transmembrane hydrophobic tail, thus it is not bound to membranes. Thus, VRK2B isoform is detected as granular deposits in both cytoplasm and nucleus, and its expression is restricted to some cells by unknown reasons, but it is a regulated expression (Blanco et al. 2006).

VRK2 Phosphorylation Substrates and Protein Interactions

The two VRK2 isoforms, which differ in their subcellular location, seem to have similar substrate

specificity in vitro. Both VRK2A and VRK2B phosphorylate human ▶ TP53 at Thr 18 (equivalent to mouse Thr 21) (Blanco et al. 2006). It is not known if the mitochondrial VRK2A phosphorylates mitochondrial p53.

VRK2A, both murine and human, as well as VRK1 are able to phosphorylate the N-terminal region of ▶ BAF, a small protein that interacts with DNA, and is required for nuclear envelope assembly. Phosphorylation of BAF by VRK1 results in loss of binding to DNA and inhibits nuclear envelope assembly (Nichols et al. 2006). VRK2, like VRK1, is also able to phosphorylate the amino terminus of BAF (barrier to autointegration factor) protein, which is required for assembly of the nuclear envelope, and also causes loss of BAF binding to DNA (Nichols et al. 2006). The unique *C. elegans* orthologue for the VRK family, Vrk1-1, also phosphorylates BAF (Gorjanacz et al. 2008). This BAF phosphorylation impairs the formation of retroviral MoMLV (Moloney-murine leukemia virus) integration complexes (Suzuki et al. 2010).

VRK2A phosphorylates the JIP1 scaffold protein, but its significance is unknown since its effects on MAPK signaling are independent of phosphorylation and only require the protein interaction (Blanco et al. 2007, 2008).

The autophosphorylation activity of both VRK2 proteins can be inhibited in vitro by a stable interaction between the small nuclear GTPase ▶ Ran in a nucleotide-free or GDP-bound conformation. The GTP-bound form of Ran has no apparent effect on VRK2 kinase activity (Sanz-Garcia et al. 2008). VRK2A has a membrane-targeting domain that allows VRK2A to localize to intracellular membranes in the endoplasmic reticulum and mitochondria. VRK2B lacks this domain.

The large carboxy-terminal region of VRK2A can interact with other proteins, such as the JIP1 (Jun amino-terminal kinase (JNK)-interacting protein 1) (Blanco et al. 2007, 2008) and KSR1 scaffold proteins (Fernandez et al. 2010), and thus inhibits signal transduction, but they interact in a different manner. In these complexes, VRK2A also directly interacts with different mitogen-associated protein kinases, such as MEK1 (Fernandez et al. 2010) and TAK1 (Blanco et al. 2007, 2008).

VRK2A also directly interacts with the scaffold KSR1, implicated in signal transmission from EGF, ERBB2, KRAS, and BRAF signals that activate AP1-dependent transcription (Fernandez et al. 2010). In this complex, VRK2 also directly interacts with MEK1 (Fernandez et al. 2010).

The C-terminal region of VRK2A interacts with an Epstein-Barr Virus (EBV) protein, BHRR1, which is a homologue of BCL2 and enhances cell survival, but no interaction with human BCL2 has been detected (Li et al. 2006).

VRK2 Modulates MAPK Signaling

VRK2A isoform modulates signaling mediated by MAPK pathways. This action is mediated by a direct interaction between a scaffold protein and VRK2A, and does not depend on its kinase activity. Stress responses, such as hypoxia (Blanco et al. 2007), and response to inflammatory cytokines, such as interleukin-1β (IL-1β) (Blanco et al. 2008), mediate signals by activation of Jun-dependent transcription. These responses are inhibited by VRK2A which forms a macromolecular complex, signalosome, which is anchored to endoplasmic reticulum membranes. In this complex, VRK2A interacts with TAK1, the first MAP kinase (MAPKKK) and the JIP1 scaffold protein (Blanco et al. 2007, 2008). This complex prevents the incorporation of JNK (c-Jun N-terminal kinase), which cannot be activated by phosphorylation, inhibiting c-Jun dependent transcription. The amount of VRK2A in the cells seems to control the fraction of JIP1 free and bound, thus creating a compartmentalization with two complexes, one active without VRK2A, and another inactive with VRK2A.

Many proliferative responses mediated by oncogenes are transmitted by MAPK complexes resulting in the activation of RAF-MEK-ERK pathway, which is assembled on the KSR1 scaffold protein. Among the activated oncogenes that can be modulated by VRK2A are ERBB2, RAS, and RAF. VRK2A directly interacts with MEK1 and KSR1 preventing the activation of ERK1 and ▶ p90RSK (Fernandez et al. 2010). VRK2A does not affect signals that partly use this pathway, such as AKT. In this way, VRK2A inhibits at a downstream level the signal initiated in ERBB2,

which is amplified in many tumor types. Also reduced level of P-ERK was observed in the presence of VRK2, and VRK3, in the analysis of the EGFR response pathway (Komurov et al. 2010).

VRK2 in Human Pathology: Breast Cancer and Viral Infections

VRK2 levels in human breast cancer correlated positively with estrogen and progesterone receptors. VRK2 was inversely correlated with ErbB2, thus Erb2+ breast cancer have low levels of VRK2. VRK2 can block the signal mediated by MAPK in response to EGF or its receptors (ERBB family), as well as signals from mutated RAS or BRAF. By reducing VRK2 levels, inhibition of MAPK pathway transcriptional signaling is partly removed in these tumors (Fernandez et al. 2010). This downstream inhibitory effect of VRK2A on MAPK can also be occurring in other tumors with mutations along this signaling pathway, but it needs to be demonstrated.

VRK2 received its name from the homology of its catalytic domain and that of the unique vaccinia virus kinase B1, which is required for Vaccinia virus DNA replication early in infection. Despite their divergence, VRK2 and ▶ VRK1 are able to partially rescue replication of vaccinia virus strains with defective B1 kinase (Boyle and Traktman 2004),

Epstein-Barr Virus (EBV) expresses the BHRF1 protein that is a homologue of mammalian Bcl2, which has a protective role and favors cell survival. BHRF1 interacts with the C-terminal region of VRK2A, and if VRK2A levels are high cells are protected against apoptosis, and this protection depends on the interaction and not on the kinase activity of VRK2 (Li et al. 2006).

Summary

VRK2 is a Ser-Thr kinase and has two isoforms, cytosolic and nuclear. The full-length VRK2 cytosolic isoform (A) inhibits MAPK signaling by a direct interaction with the corresponding JIP1 or KSR1 scaffold proteins. As a consequence, high levels of VRK2A inhibit in a dose-dependent manner signals mediated by the TAK1-MKK7-JNK in response to hypoxia or interleukin, and the signal of the ERBB2-RAS-RAF-MEK-ERK pathway. Downregulation of VRK2A relieves this inhibition and allows signal transmission in response to stimuli initiated in receptor-tyrosine kinases (RTK), such as ERBB2. Thus, in breast cancer VRK2A and ERBB2 present an inverse correlation.

References

Blanco S, Klimcakova L, Vega FM, Lazo PA. The subcellular localization of vaccinia-related kinase-2 (VRK2) isoforms determines their different effect on p53 stability in tumour cell lines. FEBS J. 2006;273:2487–504.

Blanco S, Santos C, Lazo PA. Vaccinia-related kinase 2 modulates the stress response to hypoxia mediated by TAK1. Mol Cell Biol. 2007;27:7273–83.

Blanco S, Sanz-Garcia M, Santos CR, Lazo PA. Modulation of interleukin-1 transcriptional response by the interaction between VRK2 and the JIP1 scaffold protein. PLoS ONE. 2008;3:e1660.

Boyle KA, Traktman P. Members of a novel family of mammalian protein kinases complement the DNA-negative phenotype of a vaccinia virus ts mutant defective in the B1 kinase. J Virol. 2004;78:1992–2005.

Fernandez IF, Blanco S, Lozano J, Lazo PA. VRK2 Inhibits mitogen-activated protein kinase signaling and inversely correlates with ErbB2 in human breast cancer. Mol Cell Biol. 2010;30:4687–97.

Gorjanacz M, Klerkx EP, Galy V, Santarella R, Lopez-Iglesias C, Askjaer P, Mattaj IW. *Caenorhabditis elegans* BAF-1 and its kinase VRK-1 participate directly in post-mitotic nuclear envelope assembly. EMBO J. 2008;26:132–43.

Klerkx EP, Lazo PA, Askjaer P. Emerging biological functions of the vaccinia-related kinase (VRK) family. Histol Histopathol. 2009;24:749–59.

Komurov K, Padron D, Cheng T, Roth M, Rosenblatt KP, White MA. Comprehensive mapping of the human kinome to epidermal growth factor receptor signaling. J Biol Chem. 2010;285:21134–42.

Li LY, Liu MY, Shih HM, Tsai CH, Chen JY. Human cellular protein VRK2 interacts specifically with Epstein-Barr virus BHRF1, a homologue of Bcl-2, and enhances cell survival. J Gen Virol. 2006;87:2869–78.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298:1912–34.

Nezu J, Oku A, Jones MH, Shimane M. Identification of two novel human putative serine/threonine kinases, VRK1 and VRK2, with structural similarity to vaccinia virus B1R kinase. Genomics. 1997;45:327–31.

Nichols RJ, Traktman P. Characterization of three paralogous members of the mammalian vaccinia related kinase family. J Biol Chem. 2004;279:7934–46.

Nichols RJ, Wiebe MS, Traktman P. The vaccinia-related kinases phosphorylate the N' terminus of BAF, regulating its interaction with DNA and its retention in the nucleus. Mol Biol Cell. 2006;17:2451–64.
Sanz-Garcia M, Lopez-Sanchez I, Lazo PA. Proteomics identification of nuclear ran GTPase as an inhibitor of human VRK1 and VRK2 (vaccinia-related kinase) activities. Mol Cell Proteomics. 2008;7:2199–214.
Scheeff ED, Eswaran J, Bunkoczi G, Knapp S, Manning G. Structure of the pseudokinase VRK3 reveals a degraded catalytic site, a highly conserved kinase fold, and a putative regulatory binding site. Structure. 2009;17:128–38.
Suzuki Y, Ogawa K, Koyanagi Y. Functional disruption of the moloney murine leukemia virus preintegration complex by vaccinia-related kinases. J Biol Chem. 2010;285:24032–43.
Vega FM, Gonzalo P, Gaspar ML, Lazo PA. Expression of the VRK (vaccinia-related kinase) gene family of p53 regulators in murine hematopoietic development. FEBS Lett. 2003;544:176–80.

VRK-2

► VRK2

VRK-3

► Vaccinia-Related Kinase-3 (VRK3)

W

WASH

Joshua T. Piotrowski[1] and Daniel D. Billadeau[1,2]
[1]Department of Immunology, College of Medicine, Mayo Clinic, Rochester, MN, USA
[2]Division of Oncology Research, Schulze Center for Novel Therapeutics, College of Medicine, Mayo Clinic, Rochester, MN, USA

Synonyms

Wiskott-Aldrich Syndrome Protein and SCAR Homolog; WASP and SCAR Homolog

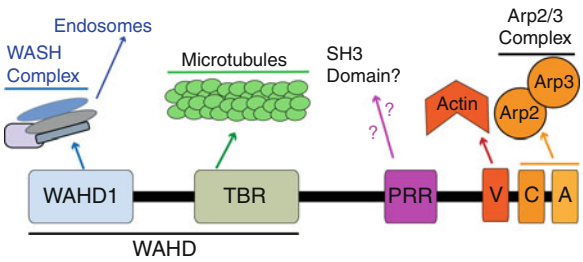
Historical Background

WASH (Wiskott-Aldrich Syndrome Protein and SCAR Homolog) was initially identified in 2007 as a novel member of the WASP (Wiskott-Aldrich Syndrome Protein) superfamily of nucleation-promoting factors (NPFs) (Linardopoulou et al. 2007). In addition to WASH, this family includes the founding member WASP as well as N-WASP (Neuronal Wiskott–Aldrich Syndrome Protein), three WAVE/SCAR (WASP family Verprolin-homologous protein/Suppressor of cAR) isoforms, WHAMM (WASP Homolog associated with Actin, Membranes, and Microtubules), and JMY (Junction Mediating and Regulatory Protein) (Campellone and Welch 2010). WASH orthologs are evolutionarily conserved from humans through *Entameba* and WASH mRNA (Messenger RNA) is ubiquitously expressed in human tissues (Linardopoulou et al. 2007). Further work comparing known genome sequences revealed that WASH is conserved down to the green algae *Ostreocuccus* and that divergent WASH homologs can even be found in members of the plant kingdom (Veltman and Insall 2010). The *wash* gene was found to be essential for *Drosophila melanogaster* viability and reduction of WASH levels in female *Drosophila melanogaster* rendered them sterile (Linardopoulou et al. 2007; Liu et al. 2009). While most species contain a single WASH ortholog, higher order primates and humans were found to posses multiple *wash* gene copies located in subtelomeric chromosomal regions (Linardopoulou et al. 2007). Significantly, WASH contains an evolutionarily conserved C-terminal VCA (verprolin homology, central hydrophobic, acidic region) domain, which is required for both profilin G-actin sequestration and Arp2/3 binding and activation leading to subsequent actin polymerization (Fig. 1) (Linardopoulou et al. 2007). A C-terminal VCA domain is a hallmark of the WASP family of NPFs (Campellone and Welch 2010). Similar to other NPFs of the WASP superfamily, WASH was found to induce Arp2/3-mediated actin polymerization and branched actin network formation in vitro (Linardopoulou et al. 2007; Liu et al. 2009).

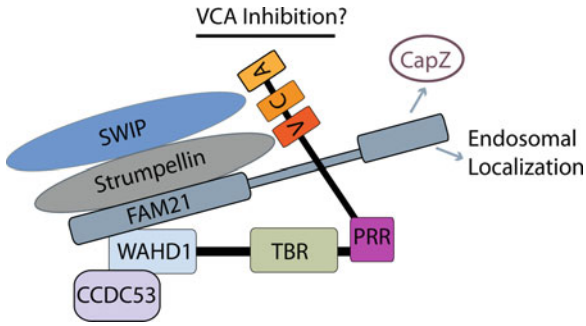
WASH Protein Structure

In addition to the conserved C-terminal VCA domain found in all WASP superfamily members, WASH also contains a proline-rich domain (PRD) located adjacent to the VCA, as well as a distinct N-terminal regulatory region (Linardopoulou et al. 2007; Rottner et al. 2010). As depicted in Fig. 1, this unique N-terminus of

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,



WASH, Fig. 1 *Functional Domains of WASH*. The N-terminal domain of WASH has been termed the WAHD (WASH Homology Domain) and is composed of the WAHD1 (WASH Homology Domain 1) and the TBR (Tubulin-Binding Region). The WAHD1 has been shown to be responsible for binding to the SHRC member FAM21 which is responsible for localizing WASH to endosomes. The TBR has been shown to bind to tubulin. The centrally located PRR (proline-Rich Region) currently has no identified binding partners. The V (verprolin homology) C (central hydrophobic) A (acidic region) at the C-terminus of WASH binds actin monomers and the Arp2/3 complex and induces branched actin network formation



WASH, Fig. 2 *Members and Function of the WASH Regulatory Complex (SHRC)*: The SHRC is composed of five members. WASH, FAM21, CCDC53, Strumpellin, and SWIP (Strumpellin- and WASH-Interacting Protein). It appears that the N-terminus of FAM21 is responsible for integration of WASH into the SHRC while a capping protein interaction motif in the C-terminus is responsible for binding to CapZ outside of the SHRC. It also currently appears that the C-terminal region of FAM21 is responsible for the endosomal localization of the SHRC. It is currently thought that the actin polymerization activity of the VCA domain of WASH is auto-inhibited by incorporation into the SHRC

WASH is comprised of the WASH homology domain 1 (WAHD1) followed by the tubulin-binding region (TBR), and these two domains are collectively referred to as the WASH homology domain (WAHD) due to conservation among WASH orthologs (Gomez and Billadeau 2009). The WAHD1 is critical for assembly into the WA*SH R*egulatory *C*omplex (SHRC) and will be discussed in detail below. WASH was found to co-immunoprecipitate with tubulin following T cell receptor ligation and the TBR was identified to directly interact with microtubule dimers in vitro (Gomez and Billadeau 2009). Significantly, *Drosophila* WASH has been shown to bundle microtubules and mammalian WASH has been reported to interact with g-tubulin (Liu et al. 2009; Monfregola et al. 2010). Unlike other NPF family members, WASH demonstrates endosomal localization in a variety of cell lines (Derivery et al. 2009; Duleh and Welch 2010; Gomez and Billadeau 2009). The N-terminal WAHD1 of WASH mediates its targeting to endosomes via interaction with the the SHRC member, FAM21 (Family number 21 (WASH Complex Subunit FAM21C)) (Fig. 1) (Gomez and Billadeau 2009). Currently, the PRD of WASH has not been linked to any specific functions, but the PRDs of other WASP superfamily members have been reported to be responsible for binding to a number of SH3 (SRC Homology 3 Domain)-containing proteins (Stradal et al. 2004). However, the proline-rich region of WASH does not harbor any canonical type I or type II SH3-binding motifs. Importantly, these domains appear to be conserved throughout most of the currently identified WASH homologs suggesting that all of these domains may play an evolutionarily important role in WASH function (Veltman and Insall 2010).

WASH Regulatory Complex

Two members of the WASP family, WASP and WAVE (WASP family Verprolin-homologous protein), exist in unique macromolecular complexes that are responsible for the stabilization and regulation of these NPFs as well as their subcellular localization (Campellone and Welch 2010). Similar to these WASP NPFs, WASH also exists in a large pentameric macromolecular complex (Fig. 2) (Derivery et al. 2009; Gomez and Billadeau 2009; Jia et al. 2010). Using bovine, murine, *Drosophila*, and human models, the components of the SHRC have been identified as: FAM21, Strumpellin, coil-coil-domain-containing protein 53 (CCDC53), and the previously undefined KIAA1033, now designated SWIP (*S*trumpellin- and *W*ASH-*I*nteracting *P*rotein) (Derivery et al. 2009; Jia et al. 2010). Detailed genetic analysis has found a significant degree of evolutionary co-conservation between WASH and members of the SHRC.

Specifically, of the 33 organisms genomes found to contain a WASH homolog, 90% or more also contained the other four SHRC components (Veltman and Insall 2010). Significantly, this suggests that the function of WASH is dependent upon the presence and proper assembly of the SHRC.

While the WASH complex also interacts with the actin capping protein CapZ, it currently appears that CapZ is a peripheral component of the complex, as the stability of CapZ is not subject to the expression of the core SHRC members. However, the core complex members do exhibit structural interdependency for their stability (Derivery et al. 2009; Gomez and Billadeau 2009; Jia et al. 2010). The C-terminus of the SHRC member FAM21 has been shown to bind to CapZ via a capping protein interaction motif, while FAM21 itself assembles directly into the SHRC via its N-terminus (Fig. 2) (Gomez and Billadeau 2009; Jia et al. 2010). Importantly, expression of a FAM21 mutant that could not interact with CapZ was still able to organize the SHRC and localize to endosomes (Gomez and Billadeau 2009; Jia et al. 2010). This suggests that CapZ is a peripherally associated member of the SHRC that might be critical for the activities of the SHRC at endosomal structures. While the function of each individual member of the SHRC is yet to be elucidated, it is important to note that suppression of individual complex members by RNA (Ribonucleic Acid) interference in HeLa cells results in downregulation of other complex components, with suppression of FAM21, Strumpellin, and SWIP affecting all complex components, while suppression of WASH or CCDC53 results in the downregulation of each other with only limited effect on the stability of the other members of the complex (Jia et al. 2010). However, it should be noted that mouse embryonic fibroblasts and T cells deficient in WASH show marked depletion of all complex members (J.T. Piotrowski, T.S. Gomez and D.D. Billadeau, unpublished observation).

Activation of Arp2/3 by WASP and WAVE is constitutively inhibited by either auto-inhibition (WASP) or by assembly of WAVE into the pentameric WRC (WAVE Regulatory Complex) (Ramesh and Geha 2009; Stradal and Scita 2006). As would be expected, recombinant full length WASH, which is highly unstable and aggregates in solution, or the C-terminal VCA domain was found to strongly induce Arp2/3-mediated actin polymerization (Derivery et al. 2009; Duleh and Welch 2010; Linardopoulou et al. 2007; Liu et al. 2009). However, it has been subsequently shown that assembly of WASH into the recombinant SHRC abrogates WASH-mediated Arp2/3 actin polymerization suggesting that like other WASP superfamily members, WASH is constitutively inhibited (Fig. 2) (Jia et al. 2010). While it is currently unclear how the actin polymerization activity of WASH is activated in vivo, it appears that the complex may exist in both active and inactive conformations as the denatured SHRC allowed WASH-mediated Arp2/3 actin polymerization to proceed in vitro (Jia et al. 2010).

Significantly, the SHRC is composed of proteins that have been subject to limited investigation. However, it has been recently demonstrated that the SHRC components contain significant structural homology to members of the WRC with similarity found between SHRC/WRC members: SWIP/SRA1 (Specifically Rac-associated protein 1), Strumpellin/NAP1, and helical regions within the N-termini of WASH and WAVE, as well as CCDC53 and HSPC300 (Heat-Shock Protein C300) (Jia et al. 2010). No significant structural conservation was found between the remaining SHRC member FAM21 and the WRC member Abi1/2 (Jia et al. 2010). However, it should be noted that both members contain highly helical N-terminal regions, which are necessary for complex assembly (Jia et al. 2010). Importantly, it was found that the conserved region in WASH was required for SHRC assembly and the structurally conserved region in CCDC53 was both necessary and sufficient for complex stability. In the same report, it was also found via electron microscopy that the WASH complex and the WAVE complex take on similar structures suggesting the possibility that they assemble in a similar fashion (Jia et al. 2010). Furthermore, the previous structural predictions of WRC assembly were recently confirmed by analysis of the actual crystal structure of the WRC, which demonstrated that the two large highly helical proteins, Sra1 and Nap1, interact and form an elongated pseudo-symmetric dimer forming a platform upon which a trimer containing WAVE1, Abi2, and HSPC300 assembles (Chen et al. 2010). Importantly, the two structurally conserved helical regions of WAVE1 and HSPC300, found in WASH and CCDC53 respectively, interact with two helices of Abi2, as well as making extensive interactions with Sra1. Mutation of key residues aimed at disrupting this four helical bundle

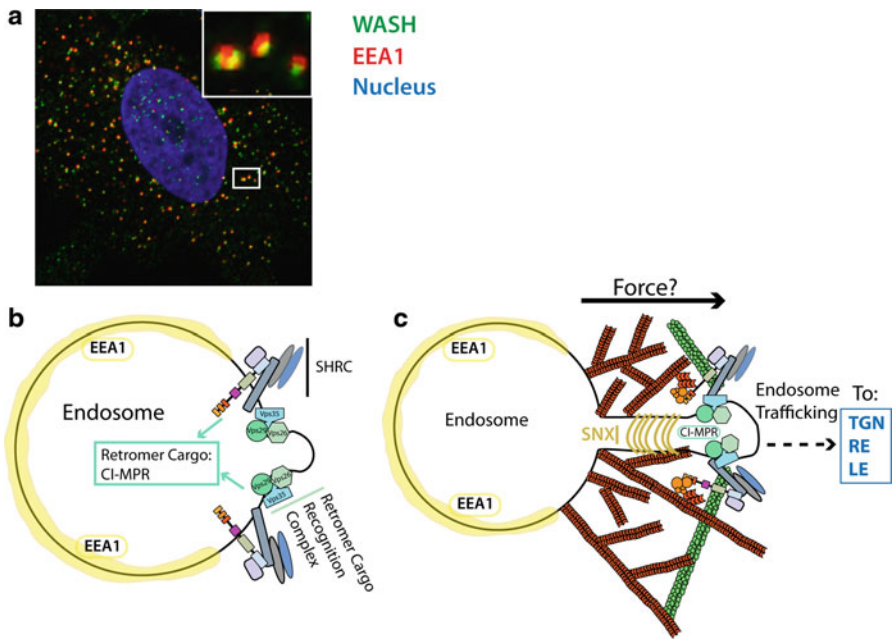
destabilizes the entire WRC (Chen et al. 2010). Lastly, the structure of the WRC indicates that the VCA of WAVE1 makes significant contacts with Sra1 as well as intramolecular associations resulting in inhibited activity toward Arp2/3 (Chen et al. 2010). Although a high-resolution structure of the SHRC remains to be made, these many structural similarities, coupled with the overall similar shape and size of the SHRC and the WRC, suggest that the SHRC is analogously organized as a large SWIP:Strumpellin platform upon which a helical bundle consisting of the N-termini of WASH:CCDC53:FAM21 assemble, with the WASH VCA sequestered by a similar mechanism (Fig. 2).

WASH Localization and Function

It was initially demonstrated that *Drosophila wash* was required for viability and that flies lacking *wash* were unable to reach the prepupal stage (Linardopoulou et al. 2007). *Drosophila* WASH has been further shown to bundle and crosslink both actin filaments and microtubules (MT) at the fly oocyte cortex as well as playing a critical role in maintaining the actin cytoskeleton in the nurse cells surrounding the oocyte (Liu et al. 2009). Work in this same model has reported that WASH, via the N-terminal WAHD1 domain, associates directly with the GTPase Rho1 and actin nucleation factor Spire while associating indirectly with the protein Cappuccino (Liu et al. 2009). While *Drosophila* WASH was reported to function as a Rho effector, mammalian WASH does not appear to function as a Rho effector (Jia et al. 2010; Liu et al. 2009). However, the GTPase Rac1 has been shown to have a weak interaction with the mammalian SHRC, yet it appears that Rac1 is unable to activate the SHRC (Jia et al. 2010). Thus it is currently unclear how the SHRC or WASH itself is activated in vivo. Future studies aimed at identifying the signaling pathways regulating WASH activity will greatly enhance our understanding of the function of WASH in cellular processes.

Three independent studies have confirmed that mammalian WASH possesses a punctate localization pattern in numerous mammalian cell lines and that WASH specifically colocalizes to early and recycling endosomes, which is consistent with the finding that both F-actin and Arp2/3 localize with early endosomes (Fig. 3a) (Derivery et al. 2009; Duleh and Welch 2010; Gomez and Billadeau 2009). It is possible that WASH regulates this specific localization of Arp2/3 as suppression of WASH results in reduced Arp2/3 levels at the early endosome (Derivery et al. 2009). Consistent with the role of WASH in recruiting Arp2/3 and accumulating F-actin at endosomal subdomains, MEFS (Mouse Embryonic Fibroblasts) genetically depleted of WASH lacks Arp2/3 and F-actin at endosomes (T.S. Gomez and D.D. Billadeau, unpublished observation). It currently appears that the C-terminal region of SHRC member, FAM21, is responsible for the localization of WASH to endosomes as expression of the N-terminus of FAM21 is capable of assembling the SHRC, but it no longer localizes to endosomal subdomains. Conversely, expression of the C-terminal region of FAM21 lacking the WASH-interacting region (amino acids 1–220) efficiently localizes to endosomes (Gomez and Billadeau 2009). Interestingly, it has been reported that FAM21 interacts with phospholipids via its C-terminus suggesting that WASH localization may be in part mediated by FAM21 binding to endosomal membranes or via other protein–protein interactions (Jia et al. 2010).

WASH may function in a variety of endosomal sorting and maturation pathways but there is much to clarify in the field. WASH has been reported to colocalize with epidermal growth factor (EGF) following internalization, and suppression of WASH may result in defective EGF transport to late endosomes, but there have been conflicting reports over whether WASH actually plays a role in the degradation of EGFR (Epidermal Growth Factor Receptor) (Duleh and Welch 2010; Gomez and Billadeau 2009). WASH has also been implicated in the regulation of transferrin recycling in mouse fibroblasts suppressed for WASH, but no such role was found using transformed human cells (Derivery et al. 2009; Duleh and Welch 2010). Additionally, WASH has been reported to regulate the trafficking of the cation-independent mannose-6-phosphate receptor (CI-MPR) (Gomez and Billadeau 2009). Under steady-state conditions, the CI-MPR is primarily localized to the trans-Golgi network (TGN) due to the action of the mammalian retromer complex, consisting of Vps35 (Vacuolar protein sorting-associated protein 35), Vps26 (Vacuolar protein sorting-associated protein 26), Vps29 (Vacuolar protein sorting-associated protein 29), and various sorting nexin (SNX) proteins, which is responsible for "retrograde"



WASH, Fig. 3 *Localization and Function of WASH*. (**a**) *WASH Localizes with Early Endosomes*. HeLa cells with the nucleus stained in blue are stained for the early endosome marker EEA1 (Early Endosome Antigen 1) in red and WASH in green. WASH localizes in a punctate pattern demonstrating endosomal localization. The white box in the upper right shows an enhanced view of WASH and EEA1 staining. (**b**) *Model of WASH Recruitment to Early Endosomes*. In a proposed model, WASH and the SHRC are localized to EEA1-coated endosomes by an interaction with the Retromer Cargo Recognition Complex of Vps26, 29, and 35. This Vps trimer is responsible for recruiting retromer cargo such as CI-MPR. It is proposed that this localization of the SHRC takes place at the site of endosomal membrane "budding." (**c**) *Model of WASH-Facilitated Endosomal Trafficking*. In this proposed model WASH is required to initiate Arp2/3-mediated actin polymerization at the site of endosomal sorting. The WASH-induced actin polymerization provides the physical force required to drive the scission of endosomal membranes that is required for trafficking of endosomal cargo. It is proposed that sorting nexins (SXN) may also play an important role by driving endosomal tubulation

endosome to Golgi transport (Anitei et al. 2010; Bonifacino and Hurley 2008). Importantly, the SHRC localizes with VPS35, SNX1, and SNX2 (Gomez and Billadeau 2009), and depletion of Vps35, which effectively removes Vps26 and Vps29 from endosomes, and results in SHRC dispersal (Harbour et al. 2010) (T.S. Gomez and D.D. Billadeau, unpublished observation). This suggests that retromer accumulation on endosomal subdomains leads to SHRC recruitment and function at these specific endosomal structures. Importantly, suppression of WASH results in CI-MPR dispersal from its normal localization near the TGN to endosomes (Gomez and Billadeau 2009). Consistent with missorting of CI-MPR by the retromer in WASH-depleted cells, the total levels of CI-MPR were also reduced indicating that CI-MPR was likely being shunted into the lysosomal pathway, a feature consistent with retromer deregulation (Gomez and Billadeau 2009). Thus it appears that WASH participates in retromer-mediated endosomal transport of cargo to the Golgi (Fig. 3b, c). It is important to note that so far WASH does not appear to regulate endocytosis, as it has been shown that WASH fails to localize with clathrin at the site of the plasma membrane or affect TCR (T cell Receptor) internalization rate (Gomez and Billadeau 2009). While there is no clear consensus on the exact function of WASH in each of these cellular trafficking pathways, the current reports are suggestive of WASH plays a key role in the regulation of actin assembly on early endosomes and the subsequent membrane remodeling that occurs during endosomal sorting and trafficking (Fig. 3b, c).

Interestingly, all three current reports on the function of mammalian WASH noted that upon suppression of WASH, the normally spherical early endosomes became enlarged and elongated resulting

in the endosomal network taking on a tubulation phenotype (Derivery et al. 2009; Duleh and Welch 2010; Gomez and Billadeau 2009). It was reported that depolymerization of microtubules in WASH-depleted cells prevented this endosomal tubulation from occurring (Derivery et al. 2009). Additionally, blocking the VCA domain of WASH in vivo induced endosome tubulation (Derivery et al. 2009). Coupled with the fact that WASH is known to interact with microtubules via its TBR and the C-terminal VCA domain has been shown to induce Arp2/3-mediated actin nucleation, it seems likely that the ability of WASH to interact with these two key cytoskeleton components is a critical part of its ability to influence endosomal membrane shape and dynamics (Derivery et al. 2009; Gomez and Billadeau 2009). Finally, it has been reported that the GTPase dynamin, which possesses a well-characterized role in membrane fission, immunoprecipitates with endogenous WASH, which suggests the possibility that dynamin may also play a role in WASH-mediated endosomal fission (Derivery et al. 2009). It has been widely proposed that WASH is required to mediate actin polymerization at the site of endosomal sorting and that this polymerization provides the physical force required to drive the constriction and eventual scission of endosomal membranes allowing for the proper cellular sorting and trafficking of endosomal cargo (Fig. 3b, c) (Derivery et al. 2009; Duleh and Welch 2010; Gomez and Billadeau 2009). This model, which requires further elucidation, if proved correct, could closely mimic the role of N-WASP-mediated Arp2/3 activity in driving membrane scission during endocytosis at the plasma membrane (Takenawa and Suetsugu 2007).

It should be briefly noted that WASH has been found to localize to the site of *Salmonella* cellular entry and that depletion of WASH in fibroblasts reduced the level of *Salmonella* invasion (Hänisch et al. 2010). Furthermore, the SHRC member, FAM21, has been reported to facilitate vaccina virus entry into HeLa cells via dynamin-mediated endocytosis and that knockdown of FAM21 resulted in reduced cellular entry of vaccina virus (Huang et al. 2008). Consistent with other reports of WASH localization, it appears that FAM21-mediated vaccina virus penetration occurs through clathrin-independent endocytosis (Gomez and Billadeau 2009; Huang et al. 2008). It is possible that actin polymerization mediated by the WASH complex may play an important role in pathogen-directed membrane remolding and subsequent intracellular invasion. It is unclear if this function of WASH is related to its regulation of endosomal sorting.

Summary

WASH is a recently identified member of the WASP superfamily of Arp2/3 NPFs. A highly evolutionarily conserved protein, the gene for WASH in humans and higher order primates has variable copy numbers with gene copies located within subtelomeric regions. The protein itself has been shown to posses several conversed domains: an N-terminal WAHD1, a C-terminal VCA, and a central PRD preceded by a TBR (Fig. 1). Like other WASP superfamily members, WASH is integrated into a multiprotein complex and the activity and stabilization of WASH is regulated and dependent upon the pentameric SHRC (Fig. 2). The SHRC has been found to contain a high degree of homology to the pentameric WRC, which regulates its subcellular localization and activity toward Arp2/3. Unlike the WRC however, it does not appear that the SHRC functions downstream of Rac1. While no activator of WASH has been established, the intrinsic activity of WASH toward Arp2/3 is inhibited upon assembly into the SHRC, suggesting that WASH activation is likely to be regulated in vivo by currently undefined molecular mechanisms. Unique among the WASP superfamily, WASH localizes to early endosomal subdomains enriched in retromer subunits and is responsible for endosomal localization and activity of Arp2/3 (Fig. 3a). The N-terminal region of the SHRC member FAM21 mediates this endosomal localization. Depletion of WASH has been shown to result in a tubulation phenotype in which endosomes lose their spherical shape and become enlarged and elongated. It appears that WASH plays a key role in the sorting and trafficking of early endosomes, and that as a result WASH may specifically help regulate EGFR and transferrin trafficking and degradation pathways. It is also clear that WASH is involved in the proper function of retromer-mediated transport from endosomes to the Golgi. As a recently discovered NPF, there is much to be learned in the future about the role of WASH in cellular signaling and function. As the

extent of endosomal-based signaling becomes better understood, the mechanisms by which WASH regulates cytoskeleton dynamics and endosomal trafficking will be even more important to understand.

References

Anitei M, Wassmer T, Stange C, Hoflack B. Bidirectional transport between the trans-Golgi network and the endosomal system. Mol Membr Biol. 2010;27:443–56.

Bonifacino JS, Hurley JH. Retromer. Curr Opin Cell Biol. 2008;20:427–36.

Campellone KG, Welch MD. A nucleator arms race: cellular control of actin assembly. Nat Rev Mol Cell Biol. 2010;11:237–51.

Chen Z, Borek D, Padrick SB, Gomez TS, Metlagel Z, Ismail AM, et al. Structure and control of the actin regulatory WAVE complex. Nature. 2010;468:533–8.

Derivery E, Sousa C, Gautier JJ, Lombard B, Loew D, Gautreau A. The Arp2/3 activator WASH controls the fission of endosomes through a large multiprotein complex. Dev Cell. 2009;17:712–23.

Duleh SN, Welch MD. WASH and the Arp2/3 complex regulate endosome shape and trafficking. Cytoskeleton. 2010;67:193–206.

Gomez TS, Billadeau DD. A FAM21-containing WASH complex regulates retromer-dependent sorting. Dev Cell. 2009;17:699–711.

Hänisch J, Ehinger J, Ladwein M, Rohde M, Derivery E, Bosse T, et al. Molecular dissection of Salmonella-induced membrane ruffling versus invasion. Cell Microbiol. 2010;12:84–98.

Harbour ME, Breusegem SYA, Antrobus R, Freeman C, Reid E, Seaman MNJ. The cargo-selective retromer complex is a recruiting hub for protein complexes that regulate endosomal tubule dynamics. J Cell Sci. 2010;123:3703–17.

Huang C-Y, Lu T-Y, Bair C-H, Chang Y-S, Jwo J-K, Chang W. A novel cellular protein, VPEF, facilitates vaccinia virus penetration into HeLa cells through fluid phase endocytosis. J Virol. 2008;82:7988–99.

Jia D, Gomez TS, Metlagel Z, Umetani J, Otwinowski Z, Rosen MK, et al. WASH and WAVE actin regulators of the Wiskott-Aldrich syndrome protein (WASP) family are controlled by analogous structurally related complexes. Proc Natl Acad Sci USA. 2010;107:10442–7.

Linardopoulou EV, Parghi SS, Friedman C, Osborn GE, Parkhurst SM, Trask BJ. Human subtelomeric WASH genes encode a new subclass of the WASP family. PLoS Genet. 2007;3:e237.

Liu R, Abreu-Blanco MT, Barry KC, Linardopoulou EV, Osborn GE, Parkhurst SM. Wash functions downstream of Rho and links linear and branched actin nucleation factors. Development. 2009;136:2849–60.

Monfregola J, Napolitano G, D'urso M, Lappalainen P, Ursini MV. Functional characterization of Wiskott-Aldrich Syndrome Protein and Scar Homolog (WASH), a bi-modular nucleation-promoting factor able to interact with biogenesis of lysosome-related organelle subunit 2 (BLOS2) and -Tubulin. J Biol Chem. 2010;285:16951–7.

Ramesh N, Geha R. Recent advances in the biology of WASP and WIP. Immunol Res. 2009;44:99–111.

Rottner K, Hänisch J, Campellone KG. WASH, WHAMM and JMY: regulation of Arp2/3 complex and beyond. Trends Cell Biol. 2010;20:650–61.

Stradal TEB, Scita G. Protein complexes regulating Arp2/3-mediated actin assembly. Curr Opin Cell Biol. 2006;18:4–10.

Stradal TEB, Rottner K, Disanza A, Confalonieri S, Innocenti M, Scita G. Regulation of actin dynamics by WASP and WAVE family proteins. Trends Cell Biol. 2004;14:303–11.

Takenawa T, Suetsugu S. The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat Rev Mol Cell Biol. 2007;8:37–48.

Veltman DM, Insall RH. WASP family proteins: their evolution and its physiological implications. Mol Biol Cell. 2010;21:2880–93.

WASP and SCAR Homolog

▶ WASH

Wiskott-Aldrich Syndrome Protein and SCAR Homolog

▶ WASH

Wiskott–Aldrich Syndrome-Like (WASL)

▶ N-WASP

Wyatt

▶ Toll-like Receptor Adaptor Protein Family Members

Y

Ypt7p (Yeast Ortholog)

► Rab7a in Endocytosis and Signaling

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

Z

ZAP70

▶ ZAP-70

ZAP-70

M. Ferro, N. Giommoni and C. T. Baldari
Department of Evolutionary Biology, Universty of Siena, Siena, Italy

Synonyms

FLJ17670; FLJ17679; SRK; STD; TZK; ZAP70

Historical Background

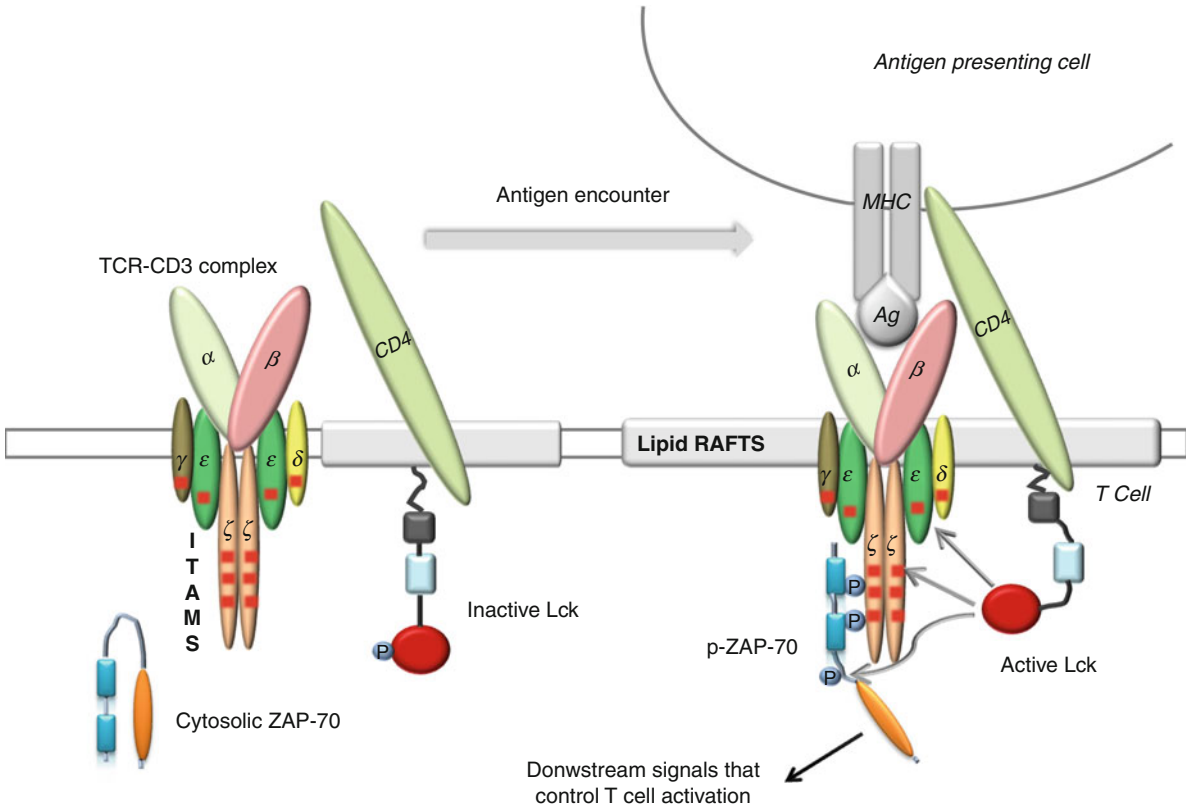
Since its initial discovery in 1992 by the Weiss group, ZAP-70 (ζ-associated protein of 70 kDa) has attracted significant attention as a central player in the early steps of T-cell antigen receptor (TCR) signaling during T-cell development and activation.

TCR engagement results in a dynamic phosphorylation cascade primarily regulated by two different families of *P*rotein *T*yrosine *K*inases (PTKs), the Src-(*SaRC*oma) and the Syk/ZAP-70 PTKs. When the TCR αβ heterodimer binds *p*eptide-MHC (pMHC), the non-covalently associated ▶ CD3ζζ homodimer is phosphorylated by the Src kinase Lck on two specific tyrosine residues located in the consensus sequence known as *I*mmunoreceptor *T*yrosine-based *A*ctivation *M*otif (ITAM). These motifs, present in the cytosolic tail of all invariant signaling subunits of the TCR complex (CD3γ, δ, ε and η/ζ chains), consist of the consensus amino acid sequence [(D/E)xxYxxI/Lx(6–8)YxxI/L]. ZAP-70 binds the doubly phosphorylated ITAMs through its tandem *S*rc *H*omology *2* (SH2) domains and is thereby recruited to the activated TCR complex near the plasma membrane. This allows its activation by relieving the autoinhibited conformation of the tyrosine kinase, which is further stabilized by Lck-mediated phosphorylation. Thus, the catalytic function of ZAP-70 is activated both through interaction with and phosphorylation by Lck and by autophosphorylation. Active ZAP-70 can then phosphorylate the downstream molecules in the TCR signaling pathway (Fig. 1).

Among the most important substrates of ZAP-70 are two adapter proteins, *S*H2-domain-containing *L*eukocyte *P*hosphoprotein of 76 kDa (▶ SLP-76) and *L*inker of *A*ctivated *T* cells (▶ LAT). When phosphorylated, these adapters mediate the recruitment of several signaling molecules that can lead to T-cell activation, proliferation, cytokine production, and differentiation.

The key role of ZAP-70 in T-cell development and activation, dramatically documented by a *S*evere *C*ombined *I*mmuno*D*eficiency Syndrome (SCID) in humans, has resulted in quantum advances in our understanding of its structure and regulation. This interest has been further kindled by the discovery of its implication in human diseases as diverse as a primary severe combined immunodeficiency disorder and B-cell *C*hronic *L*ymphocytic *L*eukemia (B-CLL) (Pezzicoli and Baldari 2005) (Au-Yeung et al. 2009) (Bene 2006) (Wang et al. 2010).

S. Choi (ed.), *Encyclopedia of Signalling Molecules*, DOI 10.1007/978-1-4419-0461-4,

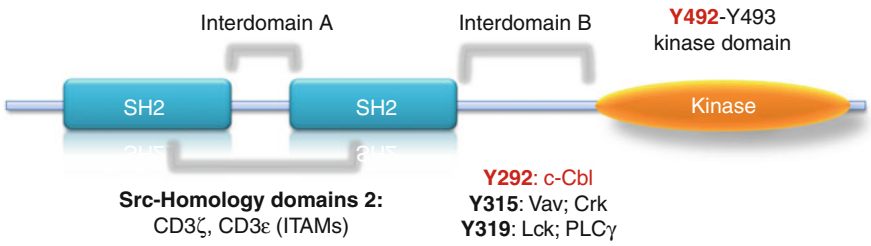


ZAP-70, Fig. 1 *ZAP-70-dependent TCR proximal signaling*. In quiescent conditions ZAP-70 is a cytosolic protein in an autoinhibited conformation. After TCR engagement by p-MHC ZAP-70 is recruited to phophorylated CD3ζ and is activated by the Src kinase Lck and autophosphorylation. This event is crucial for the downstream signaling events that lead to gene transcription and cellular responses

Structure and Regulation

ZAP-70 is closely related to *s*pleen t*y*rosine *k*inase (Syk) and together they constitute the Syk/ZAP-70 family of PTKs, sharing approximately 70% of amino acid sequence similarity. These proteins are characterized by the presence of two tandemly arranged SH2 domains at the N-terminus, spaced by an interdomain A and followed by an interdomain B and a C-terminal catalytic domain (Fig. 2).

ZAP-70 coordinates, together with the Src kinase Lck, the onset the TCR signaling cascade. At variance with Lck, which is provided of a lipid moiety constitutively targeting it to the plasma membrane, in quiescent T lymphocytes ZAP-70 is predominantly cytosolic. ZAP-70 activation and its juxtaposition to its protein substrates occur however in a membrane-proximal localization. This change in subcellular localization is mediated by its interaction with the doubly phosphorylated ITAMs on the ζ chains of the CD3 complex. The phosphorylation event within the cytosolic tails of the multiple ITAMs of the ζ chains is critical for ZAP-70 recruitment and activation and requires the binding of both SH2 domains. Binding studies have shown that the tandem SH2 domains of ZAP-70 have a high affinity for the doubly tyrosine-phosphorylated CD3ζ ITAMs, although other SH2-domain-containing molecules are able to transiently bind monophophorylated ITAMs at early steps (e.g., Grb2, ▶ phosphoinositide 3-kinase, p52Shc). The importance of the SH2 domains in the stable recruitment of ZAP-70 to the activated TCR and ZAP-70 activation is underlined by the decrease in total numbers of peripheral T lymphocytes and impaired in vivo peripheral T-cell responses in a transgenic mouse expressing a truncated dominant



ZAP-70, Fig. 2 *ZAP-70 structure*. Schematic representation of the domain organization of ZAP-70 and its tyrosine phosphorylation sites

negative form of ZAP-70 (ZAP-NC) consisting of only the tandem SH2 domains (Au-Yeung et al. 2009) (Humphrey et al. 2005).

Once recruited, ZAP-70 is activated by phosphorylation on a number of tyrosine residues. These events are important not only for its activation but also for recruitment of the other components of the proximal TCR signaling machinery. In this respect, interdomain B plays a critical role as it contains three tyrosine residues, Y292, Y315, and Y319, that have been identified as phosphorylation sites interacting with important downstream signaling molecules such as ▶ Vav, PLCγ, and CrkII.

Y292 is the best characterized negative regulatory site, initially identified as a site of interaction with the ubiquitin-ligase Cbl (*C*asitas *B*-lineage *l*ymphoma) in a screen of a degenerate phosphopeptide library using Cbl-PTB. Consistent with this finding, ZAP-70 failed to bind Cbl in a *knockin* mouse expressing a ZAP-70 mutant lacking Y292. T cells expressing the ZAP-70Y292F mutant display enhanced TCR signaling, resulting in increased interleukin-2 (IL-2) and interferon-γ (▶ IFN-γ) production. Moreover, ZAP-70Y292F expressing T cells have a slower rate of ligand-induced TCR downmodulation. This may be accounted for by the fact that, by interacting with ZAP-70, Cbl may promote degradation of the CD3ζ chains. The low molecular weight phosphotyrosine phosphatase (LMPTP) specifically dephosphorylates Y292 of ZAP-70, thereby preventing its inactivation.

Both Y315 and Y319 have a positive regulatory function in ZAP-70 activity and have been discovered as autophosphorylation sites. Y315 is a site of interaction for the proto-oncogene product, Vav. The point mutation Y315F not only abrogates Vav/ZAP-70 interaction but also prevents Vav phosphorylation and activation of the Ras-▶ MAP kinase pathway. More recently, ZAP-70 has been implicated in cell adhesion, as shown by the fact that ZAP-70, upon Vav phosphorylation, promotes the dissociation of Vav1 from the actin adapter, talin, which activates α4β1 integrin for T-cell adhesion. Furthermore, it has been shown that Y315 interacts with *C*T10 *r*egulator of *k*inase *II* (CrkII) and that this interaction is regulated by Lck, suggesting that this tyrosine residue may be phosphorylated by Lck as well as autophosphorylated. The CrkL-ZAP-70 complex is implicated in WASP recruitment to immunological synapse to initiate actin polymerization. A mutation in the CrkL SH3.1 domain results in the disruption of the complex and impairment of F-actin reorganization and IL-2 production.

On another hand, Y319 has been linked to *p*hospho-*l*ipase Cγ (PLCγ) and calcium signaling. Expression of a ZAP-70 mutant carrying a Y319F substitution results in a decrease in PLCγ phosphorylation that determines an alteration in TCR-induced Ca^{2+} mobilization. This mutant is also characterized by an impairment in the interaction with Lck due to the fact that Y319 mediates an SH2-dependent interaction between Lck and ZAP-70. Moreover, recent mutational and crystallographic studies have highlighted the possibility that both Y315 and Y319 may control the autoinhibited conformation of ZAP-70 that regulates its catalytic activity. In fact, mutation of these two tyrosine residues to phenylalanine stabilizes the autoinhibited structure of ZAP-70 and alanine substitution partially restores the phenotype. Interestingly, the crystal structure of recombinant autoinhibited ZAP-70 (YY315/319FF), solved at 2.6 Ångström, showed that the catalytic domain has a conformation similar to inactive Src kinases.

The kinase domain contains two important tyrosine residues, Y492 and Y493, that are *trans*-phosphorylated by Lck or autophosphoryled. Y493 was initially identified as a site of phosphorylation by Src PTKs. Mutational analysis has provided evidence for a crucial role for this residue in ZAP-70 activation and TCR-mediated induction of IL-2 expression in T lymphocytes. Conversely, the ZAP-70Y492F mutant displays an increased kinase activity which suggests

the possibility that Y492 may be a negative regulatory site. However, the fact that Y492 is phosphorylated under conditions in which ZAP-70 is fully activated may be compatible with the loss of the ability to bind another residue in the kinase domain, thereby preventing the formation of a structure less accessible to the substrate. Finally, a more recent crystallographic study showed that a truncated form of ZAP-70 (rhZAP-$70_{327–606}$) in complex with staurosporine can acquire an open active conformation even in the absence of phosphorylation of Y492 and Y493. Two other residues in the catalytic domain, Y593 or Y598, are important for the acquisition of the open conformation, as shown by the impairment in kinase activity in ZAP-70 mutants lacking either of these residues.

After receptor engagement, ZAP-70 also associates to the Shc adapter which couples the activated TCR to Ras. This association, which is mediated by the Shc PTB domain, contributes to TCR signaling. The putative Shc binding site on ZAP-70 is Y474 and a point mutation in this residue prevents ZAP-70 interaction with Shc and the subsequent binding of Shc to phospho-ζ, resulting in impaired TCR-dependent gene activation.

Phosphorylated ZAP-70 kinase can therefore interact with downstream molecules, participating in the assembly of a multimolecular cluster of proteins provided by phosphotyrosine binding domains that activates the downstream cascade of TCR signaling. In this cascade, two well-characterized substrates of ZAP-70 are LAT and SLP-76. These proteins are the two major adapters in TCR signaling which mediate the recruitment of crucial components such as *g*rowth factor *r*eceptor *b*ound protein *2* (Grb2), PLCγ, and Vav, which in turn mediate the activation of the Ras-MAPK pathway, the calcium signaling pathway, and the actin-remodeling network, respectively. These signaling modules are integrated to promote to T-cell activation.

Summarizing, following TCR stimulation activated Lck phosphorylates the ITAMs in the CD3 complex. Phosphorylation of the CD3ζ chains is the critical event for ZAP-70 recruitment and auto/*trans*phosphorylation. Activated ZAP-70 phosphorylates LAT and SLP-76 which recruit numerous other molecules involved in the regulation of downstream TCR signaling events that allow the T-cell response (Fischer et al. 2010) (Smith-Garvin et al. 2009).

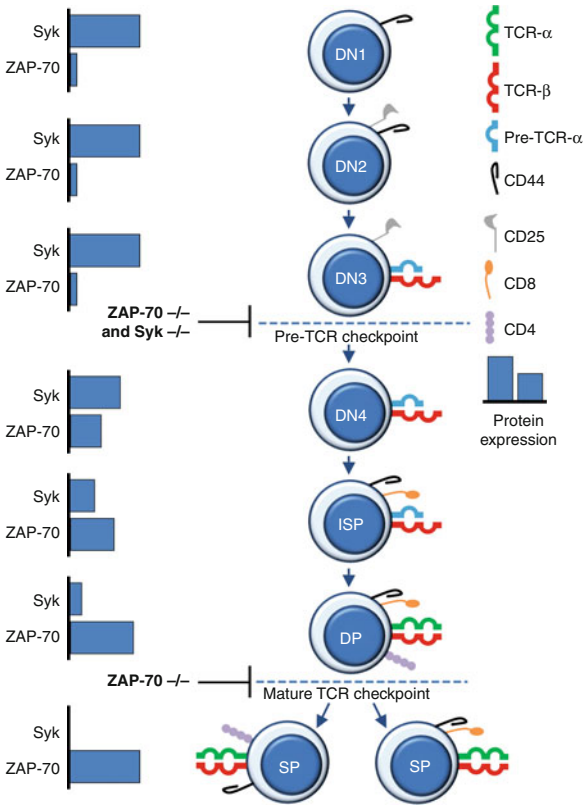
ZAP-70 in T-Cell Development

In the thymus, the developing thymocytes pass through a series of distinct phases characterized by changes in the status of protein expression, both in the cytoplasm and at the cell surface. These changes reflect the state of functional maturation of the cell, so that a particular combination of cell surface proteins distinguishes thymocytes at different stages of differentiation. In this developmental process two critical checkpoints, regulated by TCR signaling, allow the correct selection of thymocytes.

Syk and ZAP-70 have different expression patterns and roles in this process. ZAP-70 expression increases during thymocyte development, in particular after the DN4 ($CD4^-CD8^-$ Double Negative 4) stage, defined by the $CD25^-CD44^-$ phenotype. Conversely, Syk expression is very high during the early steps of thymocyte development (from DN1 to DN4), followed by a decrease in the protein levels beginning from pre-TCR selection until it becomes undetectable in mature T cell.

The first checkpoint is the pre-TCR β-chain selection at the DN3 ($CD4^-CD8^-$ Double Negative 3) stage, defined by the $CD25^+CD44^-$ phenotype. At this stage, ZAP-70 and Syk appear to have a redundant function, as neither ZAP-70 nor Syk knockout thymocytes have any evident defect. Nevertheless, mice knockout for both ZAP-70 and Syk display a total block of thymocyte maturation at the DN3 stage, indicating a fundamental function of these kinases in this process.

The second checkpoint is the positive/negative selection at the DP ($CD4^+CD8^+$ Double Positive) stage. To successfully pass through this checkpoint, the mature αβ-TCR needs to recognize with intermediate affinity a self-peptide presented by the MHC complex. The signal triggered by the engaged TCR must be indeed strong enough to induce positive selection of the thymocyte, but not so strong to induce thymocyte deletion by apoptosis (negative selection), which occurs when the TCR encounters a high-affinity (autoreactive) self-peptide. At this stage of thymocyte development, Syk expression is not required, as documented by the fact that Syk deficient mice show a normal progression in thymocyte maturation. In contrast, ZAP-70 deficient mice harbor a complete block of both positive and negative selection, and hence fail to progress beyond the DP stage, which results in the



ZAP-70, Fig. 3 *ZAP-70/Syk in T-cell development*. The expression profile of ZAP-70 and Syk throughout thymocyte development in schematized. Double knockout of ZAP-70 and Syk causes thymocyte arrest at the pre-TCR checkpoint (DN3), while ZAP-70 knockout causes their arrest at the mature TCR checkpoint (DP)

lack of both in mature single positive (SP) $CD4^+$ and $CD8^+$ thymocytes and peripheral T cells (Fig. 3) (Au-Yeung et al. 2009) (Wang et al. 2010).

Expression and Immunopathological Phenotype

ZAP-70 is mainly a T cell restricted gene and it is essentially expressed in thymocytes and peripheral T cells. The crucial importance of this gene is confirmed by the discovery, in 1994, of a form of severe combined immunodeficiency (SCID), which is due to the lack of functional ZAP-70, as well as by the study of *zap-70*-null allele mouse model.

In humans, this primary immunodeficiency is characterized by the selective absence of peripheral $CD8^+$ T cells and by a normal number of circulating $CD4^+$ T cells that are however non-functional, as they fail to respond to TCR-mediated stimuli in vitro. Conversely ZAP-70-deficient mice have neither $CD4^+$ nor $CD8^+$ single positive T cells in the periphery, because thymocyte development is blocked at the double positive (DP) stage. However, in humans the mechanism for a selective $CD8^+$ selection remains unclear and could depend on a stronger interaction between Lck and the coreceptor CD4 determining a sort of signaling compensation.

In Inbred Strange (ST) mice, a spontaneously arising point mutation has been identified within the highly conserved consensus DLAARN of the kinase domain of ZAP-70. Mice homozygous for this mutation are devoid of mature T cells because T-cell development is arrested at the DP stage, similar to the *ZAP-70* gene targeted mouse model. A human patient with SCID caused by a similar mutation has been subsequently reported. Similar to the ZAP-70 deficient SCID patients, this patient was characterized by a normal number of circulating $CD4^+$ T cells. The mechanism that causes the selective defect in $CD8^+$ cells in human SCID patients remains so far unclear (Fischer et al. 2010) (Au-Yeung et al. 2009).

A point mutation in the *ZAP-70* gene has been also associated to autoimmunity. In 2003, Sakaguchi and colleagues characterized a new mouse strain, named SKG, derived from BALB/c mice and naturally predisposed to develop chronic arthritis. These mice showed the typical features of rheumatoid arthritis (RA) with severe synovitis and hypergammaglobulinemia, the former due to a massive infiltration of leukocytes into the synovia. They also developed high titers of rheumatoid factor (RF), resembling RA in human patients. Moreover, adoptive transfer of spleen and lymph node $CD4^+$ T cells from arthritic SKG mice produced a similar arthritis in BALB/c nude mice, indicating that T cells are responsible for the priming of the disease. It must be highlighted that SKG mice develop spontaneous inflammatory arthritis when kept in a conventional animal facility where there is a certain level of pathogen exposure contrarily to what happens in SPF conditions, where the onset of arthritis in SKG mice requires fungal challenge. These SKG mice carry a missense mutation (W163C) within the C-terminal SH2 domain of ZAP-70 which causes an attenuation of TCR signaling. In particular, phosphorylation of ZAP-70, LAT, and PLCγ is reduced in SKG thymocytes and T cells following TCR

engagement, leading to an attenuation in downstream events, such as intracellular calcium influx and MAPK activation. The attenuation of TCR signaling compromises both positive and negative selection of SKG thymocytes, leading to a dangerous positive selection of autoreactive T cells.

Recently a hypomorphic ZAP-70 mutant has been reported. This mouse has a knockin alanine substitution of YY315/319 in interdomain B. YYAA mice have autoantibodies against rheumatoid factor however, at variance with SGK mice, they do not develop arthritis. The milder phenotype may be accounted for by differences in the T-cell repertoire, due to a less severe impairment in negative selection compared to SKG mice.

Last year, ZAP-70 has been proposed as a biomarker of B-cell activation in a scenario of autoimmunity. Ectopic expression of ZAP-70 is indeed increased in synovial fluid B cells from RA patients, and this correlates with improved B-cell survival in vitro, further strengthening the link between ZAP-70 and autoimmunity, and specifically RA (Thomas et al. 2008).

In addition to T-cell lineage, ZAP-70 has been identified in the sibling cell type Natural Killer (NK) cells. This cell type plays a crucial role in the antivirus and antitumor immune response and contributes to regulate helper T-cell differentiation through the production of IFNγ. NK cells express a multitude of germline-encoded receptors and ZAP-70/Syk are involved in positive signaling downstream of the activating receptors Fcγ receptor IIIA (CD16) and Fcγ receptor IIC (CD32). Following stimulation with specific antibodies, FcγIIIA or IIC become phosphorylated within their ITAMs, causing the recruitment and phosphorylation of ZAP-70/Syk. Interestingly, this also applies to the c3 isoform of FcγII, which lacks a canonical ITAM within its cytosolic tail. CD16 mediates another important aspect for NK-cell activation because it induces the ubiquitination of ZAP-70 and Syk, leading to their degradation via the proteasome, a mechanism probably involved in the fine-tuning of positive signals and in the eventual biological output.

NK cells also express another class of receptor, the KIRs (Killer cell Inhibitory Receptors), among which Ly49D and H have a non-inhibitory activity. In fact, they do not contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) but are able to associate with the coreceptor DAP12, involved in the positive regulation of NK cells. DAP12 recruits ZAP-70 and Syk via their SH2 domains, although the stimulation of Ly49D only induces the activation of Syk.

In the last few years, the interest of ZAP-70 has been predominantly focused on its involvement in B-cell chronic lymphocytic leukemia (B-CLL) as a prognostic marker. B-CLL is a B-cell malignancy characterized by the accumulation of monoclonal B cells with a morphology characteristic of small mature lymphocytes. These cells are $CD19^+$, $CD5^+$, and $CD23^+$ and they accumulate in blood, bone marrow, lymph nodes, and spleen, leading, in some cases, to a progressive enlargement of these lymphoid organs, anemia, and trombocytopenia. This type of leukemia occurs with a heterogeneous clinical course which is strictly related to some molecular and cellular markers. Among these, one of the most important prognostic markers is the mutational status of the immunoglobulin heavy chains (IgVH). A significant proportion of B-CLL patients have somatic hypermutations in the rearranged variable regions of the IgH genes, while the others have unmutated IgVH genes. The latter are characterized by a more rapid disease progression and shorter survival. A poorer prognosis is also frequently associated with surface expression of CD38. The discovery that ZAP-70 is ectopically expressed in the leukemic cells from CLL patients with poor prognosis, in frequent association with unmutated IgVH genes, has provided an important new prognostic marker for this disease. Very recently it has been reported that p66Shc, a pro-apoptotic adapter which interacts with ZAP-70 in TCR signaling, is downregulated in B-CLL patients, with the lowest expression levels associated to an unfavorable prognosis.

The finding that ZAP-70 is expressed in CLL B-cells has suggested the possibility that it might play a role in BCR signaling. ZAP-70 is in fact able to associate with the BCR complex and leads to a more sustained BCR signaling in CLL B-cells compared to normal B cells. However, this feature of CLL $ZAP\text{-}70^+$ B-cells is independent of ZAP-70 kinase activity and is related instead to its adapter function. ZAP-70 expression is however not limited to leukemic B cells. It has in fact been recently reported that ZAP-70 is also expressed in normal B cells and is implicated in B-cell development. This process occurs predominantly in the bone marrow where immature B cells cross different checkpoints which control the

rearrangement of immunoglobulin gene segments (VDJ). The transition from pro-B cells to pre-B cells requires positive signals that are critically dependent on the receptor-associated transmembrane molecule Igα and Igβ, which contain an ITAM. Similar to T cells, BCR stimulation induces ITAM phosphorylation and recruitment of Syk, leading to cell activation. Nevertheless, B-cell development is only partially blocked in the absence of Syk, which was taken as a proof that another kinase is able to propagate the signals independently of Syk. This has been identified as ZAP-70, and in fact the absence of both ZAP-70 and Syk results in a complete block in B-cell development at the pro-B cell stage. Consistent with this finding, ZAP-70 is expressed in all immature B cells. ZAP-70 expression has also been found in tonsillar and splenic B lymphocytes which show an activated phenotype. In this study, the authors demonstrate that ZAP-70 becomes phosphorylated in response to BCR engagement, further supporting a role for ZAP-70 in normal B cells.

The implication of ZAP-70 in the pathogenesis of diseases of abnormal T-cell activation, as well as its restricted tissue specificity, underscores it as an attractive drug target. Although at present there is no drug which can selectively inhibit ZAP-70, an inhibitor modeled to specifically target an analogue-sensitive ZAP-70AS was recently found to potently inhibit TCR signaling and T-cell activation, thereby providing the proof-of-concept that ZAP-70 manipulation may provide the basis for the treatment of autoimmune diseases and hematological malignancies characterized by alterations in ZAP-70 expression or activity (Montserrat 2006; Wiestner 2005; Wang et al. 2010).

Summary

The protein tyrosine kinase ZAP-70 is a crucial molecule for T-cell activation, where it orchestrates the early steps in antigen receptor signaling. This entry provides a brief overview of the structure and regulation of ZAP-70, as well as of its role in T-cell development and activation. The alterations in ZAP-70 expression and/or activity in a number of pathological settings, together with its restricted tissue specificity, identify this molecule as a potential valuable drug target.

References

Au-Yeung BB, Deindl S, Hsu LY, Palacios EH, Levin SE, Kuriyan J, et al. The structure, regulation, and function of ZAP-70. Immunol Rev. 2009;228(1):41–57.

Bene MC. What is ZAP-70? Cytom B Clin Cytom. 2006;70(4):204–8.

Fischer A, Picard C, Chemin K, Dogniaux S, le Deist F, Hivroz C. ZAP70: a master regulator of adaptive immunity. Semin Immunopathol. 2010;32(2):107–16.

Humphrey MB, Lanier LL, Nakamura MC. Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. Immunol Rev. 2005;208:50–65.

Montserrat E. New prognostic markers in CLL. Hematol Am Soc Hematol Educ Program. 2006;279–84.

Pezzicoli A, Baldari CT. ZAP-70. AfCS Nat Molecule Pages. 2005. doi:10.1038/mp.a002396.01.

Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol. 2009;27:591–619.

Thomas R, Turner M, Cope AP. High avidity autoreactive T cells with a low signalling capacity through the T-cell receptor: central to rheumatoid arthritis pathogenesis? Arthritis Res Ther. 2008;10(4):210.

Wang H, Kadlecek TA, Au-Yeung BB, Goodfellow HE, Hsu LY, Freedman TS, et al. ZAP-70: an essential kinase in T-cell signaling. Cold Spring Harb Perspect Biol. 2010;2(5):a002279.

Wiestner A. More ZAP for chronic lymphocytic leukemia (CLL). Blood. 2005;105(5):1839–40.

zDHHC Proteins

► DHHC Proteins

Zfp202

► ZNF202

ZFYVE9

► SARA

Zinc Finger Protein 202

► ZNF202

Zinc Transport in the Pancreatic β-Cell: Roles of ZnT (*SLC30A*) and ZiP (*SLC39A*) Family Members

Elisa A. Bellomo and Guy A. Rutter
Section of Cell Biology, Division of Diabetes, Endocrinology and Metabolism, Faculty of Medicine, Imperial College London, South Kensington, London, UK

Synonyms

SLC30A; SLC39A; ZiP; ZnT

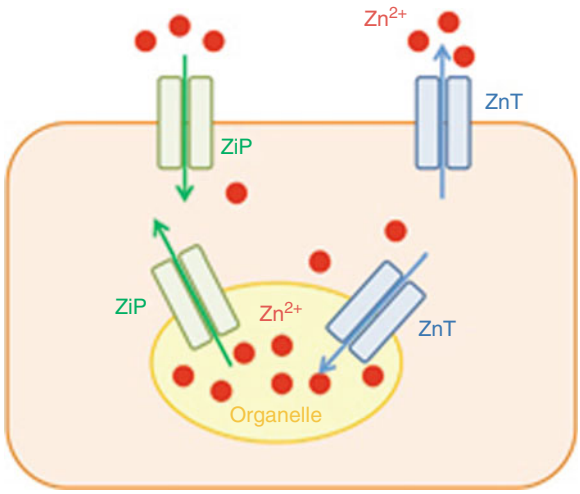
Historical Background

Zinc is an essential component of many proteins and is crucial for several cellular functions such as cell mitosis, gene expression, and modulation of cellular receptors (Lichten and Cousins 2009). Failure to regulate zinc homeostasis thus leads to the development of a variety of pathologies, and mutations in several zinc transporter genes are linked to diseases in man. Hypozincemia is a condition where insufficient zinc is available for metabolic needs. It is usually the result of inadequate dietary intake of zinc but can also be associated with malabsorption. Diarrhea, *acrodermatitis enteropathica*, chronic liver and renal disease, diabetes and other chronic illnesses are all associated with zinc deficiency.

Increasing evidence suggests that changes in the expression or activity of specific zinc transporters might play a role in regulating the general zinc homeostasis. In this entry, the function of zinc transporters and zinc-binding proteins will be reviewed, with particular attention to the role that these molecules may play in the endocrine pancreas and in the development of diabetes mellitus.

Zinc Transporters

Under normal physiological conditions, the intracellular free Zn^{2+} concentration ($[Zn^{2+}]_{cyt}$) is thought to be in the picomolar range (Vinkenborg et al. 2009), while the level in the plasma can reach micromolar level



Zinc Transport in the Pancreatic β-Cell: Roles of ZnT (*SLC30A*) and ZiP (*SLC39A*) Family Members, Fig. 1 Mechanisms involved in controlling Zn^{2+} homeostasis. Zn^{2+} homeostasis is tightly controlled in all cell types. The main proteins involved in maintaining low cytosolic Zn^{2+} levels include the Zn^{2+} transporters ZnT (which transport Zn^{2+} from the cytosol inside intracellular organelles on in the extracellular space) and ZiP (which concentrate Zn^{2+} in the cytosol moving the ion from intracellular compartments or from the extracellular space)

(Simons 1991) (though free plasma concentrations of Zn^{2+} are thought to be much lower, possibly in the nanomolar range). Therefore, the cellular Zn^{2+} content needs to be tightly regulated by both zinc transporters and zinc-binding proteins (Fig. 1).

There are two main classes of zinc transporters in mammalian cells: the ZnT family (coded by the *Slc30a* genes) is thought to move Zn^{2+} from the cytosol in the extracellular space or inside storage organelles, therefore decreasing the intracellular $[Zn^{2+}]$; the ZiP (Zrt-, Irt-like proteins) family (coded by the *Slc39a* genes), on the other hand, is believed to transport Zn^{2+} into the cytosoplasm, hence increasing the $[Zn^{2+}]_{cyt}$ (Lichten and Cousins 2009). Fifteen members of the ZiP family and ten members of the ZnT family have so far been identified. While ZiP importers possess eight transmembrane (TM) domains and extracytosolic N- and C- termini, ZnT transporters possess six TM domains with both the N- and C-termini situated in the cytosol (Lichten and Cousins 2009). For both families, the TM domains form the pore of the transporter and Zn^{2+} binds to the histidine situated between the domains (Lichten and Cousins 2009). Several ZnTs and ZiPs are ubiquitously

expressed while others display tissue specificity and are localized on specific intracellular membranes or on the plasma membrane. The physiological importance of these transporters is highlighted by the fact that some physiopathological conditions are the result of mutation in genes coding for Zn^{2+} transporters. For instance, mutations in the ZiP4 coding gene are linked to the autosomal recessive disorder *acrodermatitis enteropathica*, while alteration in ZnT2 gene expression have also been linked to Alzheimer's disease (Lichten and Cousins 2009). Finally, voltage-dependent Ca^{2+} channels (VDCCs), expressed in human tissues and involved in regulating insulin secretion, are also able to transport Zn^{2+} (Huang et al. 2000).

Zinc and Diabetes

The original observation that the total pancreatic Zn^{2+} concentration was reduced to about 50% in diabetic compared to nondiabetic cadavers (Scott and Fisher 1938) focused attention on the relationship between Zn^{2+}, the pancreas and diabetes and subsequent observations confirmed the link between Zn^{2+} and this disease (Williams et al. 1995). Several reports have shown that type 2 diabetic (T2D) patients display a marked decrease in total plasma Zn^{2+} compared to control patients (Williams et al. 1995), indicating that hyperglycemia may interfere with Zn^{2+} absorption in the kidney causing hyperzincuria. However, an important question is whether the hyperzincuria is a cause or an effect of T2D. There is evidence from clinical studies indicating that Zn^{2+} deficiency (due to dietary insufficiency) might predispose to diabetes mellitus and some of its complications (Kanoni et al. 2011). However, interpretation of these results is challenging, given technical difficulties in defining the onset of the disease, measurements of free versus bound Zn^{2+}, as well as factoring in variations resulting from different long-term dietary status and different treatment regimes.

Zinc Transporters in Pancreatic Islets

The pancreas contains one of the highest total Zn^{2+} concentrations of the body (0.14 mg of Zn^{2+} per g of pancreas) (Scott and Fisher 1938), consistent with the fact that these ions play an important role in insulin storage and secretion in pancreatic β-cells. In these cells, Zn^{2+} is complexed with the insulin hexamer in the dense core granules, where the concentration of the ion reaches very high levels (Hutton et al. 1990).

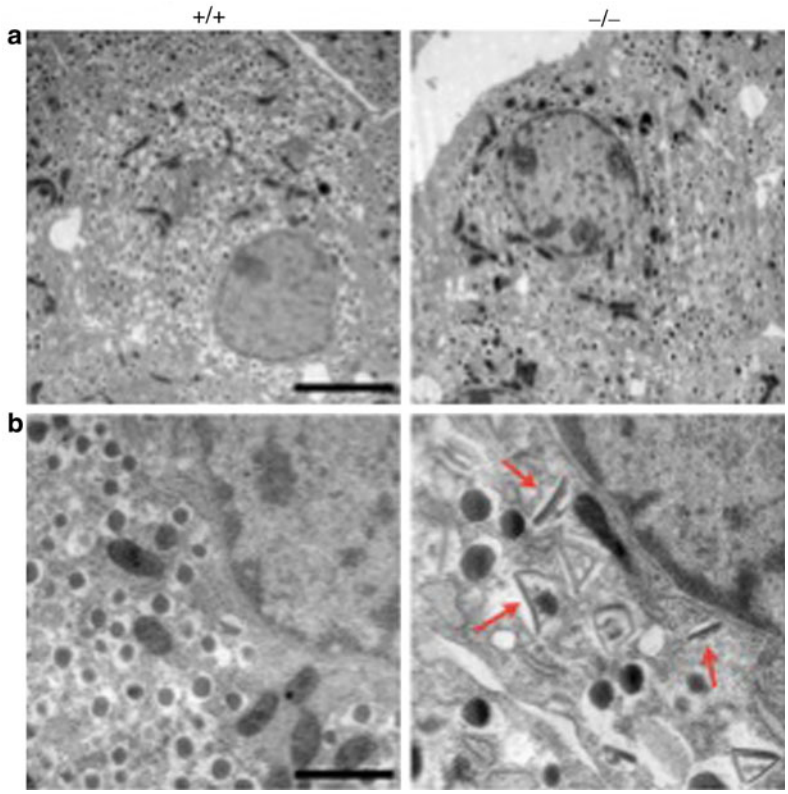
Evidence for ZiP and ZnT transporters' expression in the pancreas is quite limited and very controversial, although most studies agree on the fact that ZiP6 and ZiP7 coding genes are the most abundantly expressed in mouse and human islets as well as in the MIN6 mouse insulinoma β-cell line (Wijesekara et al. 2009). Similarly, ZnT1 and ZnT4-9 genes are also expressed in human and mouse islets and in MIN6 β-cells (Wijesekara et al. 2009); however, ZnT5 and ZnT8 appear to be the most abundant (Wijesekara et al. 2009).

ZnT3

Recently, ZnT3 has been shown to be expressed in the INS-1 β-cell line, but the cellular localization of this particular protein has not yet been established. In the hippocampus, however, ZnT3 has been found to localize to synaptic vesicles (Palmiter et al. 1996). *Smidt* and colleagues (2009) have demonstrated that ZnT3 may play an important role in the regulation of Zn^{2+} homeostasis with effect on insulin production and glucose metabolism. Indeed, silencing of this protein lowered insulin secretion in vitro and affected in vivo glucose metabolism after mice were rendered diabetic with streptozotocin treatment (Smidt et al. 2009). When ZnT3 was silenced in a rat β-cell line, an increased apoptosis was observed, while insulin secretion was reduced (Smidt et al. 2009). Despite these findings, others including ourselves (Nicolson et al. 2009) have found that ZnT3 expression in the endocrine pancreas is almost undetectable (Wijesekara et al. 2009). The reasons for these discrepancies are unclear.

ZnT5

Despite being expressed in many tissues, ZnT5 levels are more abundant in the endocrine pancreas, being, together with ZnT8, the most abundant zinc transporter within islets. In pancreatic β-cells, ZnT5 immunoreactivity is localized to both the Golgi apparatus and to insulin-containing vesicles (Kambe et al. 2002) where the transporter may serve to transport the ion inside the granule. ZnT5 expression is also present in pancreatic α-cells (Gyulkhandanyan et al. 2008), although its intracellular localization and role in the latter cell type, still remains to be elucidated.



Zinc Transport in the Pancreatic β-Cell: Roles of ZnT (*SLC30A*) and ZiP (*SLC39A*) Family Members, Fig. 2 Electron micrographs of $ZnT8^{+/+}$ and $ZnT8^{-/-}$β-cells. Transmission electron microscopy images of isolated islets from $ZnT8^{+/+}$ and $ZnT8^{-/-}$12-week-old male mice at different magnifications. Three islets each from three pairs of littermate mice were serially sectioned to a depth of 20 ± 1 μm. (**a**) Overview (scale bar 10 μm) of a β-cell from $ZnT8^{-/-}$ and control animals. (**b**) Higher magnification (scale bar 1 μm) reveals the appearance of rod-shaped core granules in $ZnT8^{-/-}$ cells, indicated by *red arrows* (see also (Nicolson et al. 2009))

ZnT8

ZnT8, which shares many structural properties with the other ZnTs, was recently cloned by *Chimienti* and colleagues (2006). *SLC30A8* expression is highly restricted to the pancreas, specifically to the islets, as demonstrated by oligonucleotide microarray analysis of mouse tissues which demonstrated that ZnT8 is essentially an endocrine pancreas-specific Zn^{2+} transporter (Seve et al. 2004). Expression of a ZnT8-EGFP chimera revealed close colocalization with insulin-containing granules in the rat insulinoma cell line INS-1 (832/13) (Chimienti et al. 2006), and argued that Zn^{2+} uptake by these organelles may be essential for insulin storage and processing. Correspondingly, overexpression of this transporter also led to increase in both total cellular Zn^{2+} concentration and glucose-stimulated insulin secretion (Chimienti et al. 2006).

Supporting a role for ZnT8 in the storage and/or secretion of insulin in man, a single nucleotide polymorphism (SNP) in the *SLC30A8* gene (rs13266634 C/T), coding for ZnT8, is associated with an elevated risk of T2D (Sladek et al. 2007). The risk variant incorporates an arginine residue at position 325 in the C-terminus of ZnT8 as opposed to a tryptophan, a change which lowers the activity of the transporter (Nicolson et al. 2009).

The above polymorphism is associated with metabolic phenotypes that differ depending on the population studied. In a German cohort of diabetic patients, the SNP has been associated with reduced insulin secretion stimulated intravenously and impaired proinsulin conversion, while insulin resistant appeared normal. Studies on French subjects showed that patients carrying the "at-risk" form of ZnT8 have increased fasting glycemia and decreased insulin secretion. Interestingly, no relationship between *SLC30A8* polymorphism and β-cell function was found in other studies (Rutter 2010).

ZnT8 is thus important as it provides Zn^{2+} for insulin maturation and storage in pancreatic β-cells (Hutton et al. 1990). Indeed, through the generation of mice bearing null alleles it has also been demonstrated that the absence of ZnT8 leads to the formation of amorphous β-cell granules (frequently lacking a dense core of crystalline insulin, Fig. 2) and mild glucose intolerance (Nicolson et al. 2009), data that are consistent with a role for abnormal β-cell Zn^{2+}

homeostasis in the pathogenesis of diabetes mellitus (Scott and Fisher 1938). Although there are apparent differences in the phenotype of the systemically null mice (see review by Rutter (2010)), it is noteworthy that β-cell-specific deletion leads to a striking metabolic phenotype and changes in the expression of β-cell restricted genes (Wijesekara et al. 2010).

The expression of the above zinc transporters varies considerably among species (mouse, rat, and human) and also between different strains of mice. It is therefore important to consider these differences while discussing and investigating human diseases such as diabetes. CD1 mouse islets, for instance, appear to contain higher level of ZnT4 and ZnT5 than human, while the relative abundance of ZnT8 is higher in human compared to mouse pancreas. This would in part explain why a single nucleotide polymorphism in the gene coding for ZnT8 in human increase the risk of developing type 2 diabetes, while the complete ablation in mouse only give such a mild phenotype.

ZiP Importers

The ZiP family of transporters can be subdivided into four subfamilies named subfamilies I and II, LIV-1 and gufA. The LIV-1 family, whose founding member is ZiP6 (coded by the *Slc39a6* gene), contains nine members in mice, of which only some have been characterized in details. There are not many information about ZiP proteins in pancreatic β-cells. This field is still in its primordial stage and almost certainly new and interesting result will appear soon. However, some preliminary evidence exists for the role of ZiP4 in pancreatic cancer and of ZiP6, 7, and 8 in the regulation of β-cell zinc homeostasis (Bellomo et al. 2011).

ZiP4

ZiP4 plays a role in Zn^{2+} homeostasis by sensing the dietary zinc levels. However, recent studies indicate that ZiP4 is overexpressed in human pancreatic cancer, and aberrant expression of ZiP4 contributes to pancreatic cancer growth (Li et al. 2007). Zinc availability is also important for tumor growth and progression as Zn^{2+} is a cofactor for many enzymes involved in processes such as hypoxia, angiogenesis, cell proliferation, and metastasis (Li et al. 2007). ZiP4, by regulating interleukin-6 (IL-6) and subsequently STAT3 and Cyclin D1, might regulate these events (Zhang et al. 2010). Indeed, several studies indicate that the silencing of ZiP4 decrease the metastatic ability of pancreatic cells, suggesting that this could be a new drug target for pancreatic cancer treatment (Zhang et al. 2010).

ZiP6

Functional analysis of cells overexpressing ZiP6 indicate that this protein localizes to the plasma membrane where it acts as a zinc importer (Lichten and Cousins 2009). Elevated expression of ZiP6 is observed in tissues sensitive to steroid hormones and the abundant expression of ZiP6 in HeLa cells and lung carcinoma cell lines, as well as in breast cancer with metastatic ability, indicate a role for this protein in breast cancer progression (Taylor and Nicholson 2003).

ZiP7

ZiP7 appears to be ubiquitously expressed (Lichten and Cousins 2009). Overexpression of ZiP7 in a breast cancer cell line causes an increase in intracellular Zn^{2+}, as expected for a Zn^{2+} importer. However, ZiP7, in contrast to ZiP6 and the majority of other ZiP proteins, localizes to the Golgi apparatus/endoplasmic reticulum, suggesting that a zinc-release mechanism can indeed take place in cells (Taylor et al. 2007).

ZiP8

First identified as a cadmium transporter, ZiP8 expression is found in lung, kidney, testis, liver, brain, and the small intestine. When overexpressed in cells, ZiP8 localization was found primarily on the plasma membrane. However, the localization of the protein appeared to change depending on the zinc concentration in the medium, consistent with an effect of Zn^{2+} to cause the transporter to internalize (Wang et al. 2007).

ZiP6, 7, and 8 have recently been suggested to play an important role in the regulation of Zn^{2+} metabolism in pancreatic β-cells (Bellomo et al. 2011). Indeed, when mouse islets were incubated with high (16.7 *vs* 3 mM) concentrations of glucose, the mRNA level of these three transporters increased. These changes were accompanied by an increased concentration of cytosolic Zn^{2+}, as measured using a highly selective recombinant probe for the cytosolic compartment ("eCALWY-4") (Vinkenborg et al. 2009). Further experiments demonstrated that the observed increase in cytosolic [Zn^{2+}] was likely to be a consequence of the increased expression of these transporters, notably

ZiP6, as an increased expression was observed not only at the mRNA but also at the protein level. Despite ZiP8 expression, in pancreatic islets, is low, it is important to note that under high stress, achieved by treatment with high glucose and the potassium channel opener diazoxide, its expression increased hugely. This was correlated with a much higher increase in cytosolic zinc than that observed after incubation in high glucose alone.

These data suggest an interesting hypothesis. First of all, upon stimulation with glucose, pancreatic β-cells not only promote insulin secretion but also insulin granule biogenesis. A newly formed granule not only needs a complete complement of intragranular proteins, but also requires high concentration of Zn^{2+} in order to properly store newly synthesized insulin. In this context, the fact that an increase in ZiPs together with cytosolic Zn^{2+} occurs after glucose stimulation can be considered a physiological adaptation. However, if the concentration of glucose remains elevated for longer period of time or if the stress level of pancreatic islets increase (events observed in the pathogenesis of type 2 diabetes), then the Zn^{2+} level could increase over a "safe" threshold leading, perhaps, to events such apoptosis to occur and therefore increasing the risk for diabetes.

Future Perspectives

While the role of ZnT8 in the mammalian β-cell is now difficult to refute, those of ZiP family members will require future work involving gene silencing ex vivo or knockout strategies in mice. An intriguing unanswered question is the extent to which these proteins influence not only insulin secretion, but also the storage and release of other islets hormones, including glucagon, given the reasonably abundant expression of ZnT8 in the α-cells (Nicolson et al. 2009). The extent to which the activity of these proteins is modified posttranslationally (by phosphorylation or other means) in either cell type is also completely unknown at present, and represents an exciting area for potential future exploration. The development of pharmacological agents able to modify the expression or activity of these proteins (and notably that of ZnT8, given its remarkably restricted expression to islet cells (Chimienti et al. 2006)) may also offer important new approaches toward controlling the release of endocrine pancreatic hormones and thus offer novel treatment for certain forms of diabetes.

Summary

Zinc plays a fundamental role in the biology of insulin secretion, acting in particular to stabilize the insulin crystal, but also potentially to regulate a variety of cellular processes ranging from transcription to ion channel activity. Here, we review the biology of the two key families of zinc transporters (ZnTs) and importers (ZiPs) with a particular focus on their role in the pancreatic β-cell. Recent genetic data in man, and the results of mouse knockout studies, have highlighted the importance of the secretory-granule-localized zinc transporter, ZnT8, encoded by *SLC30A8* in the pathology of both type 1 and type 2 diabetes. Likewise, cellular studies have revealed that the expression of several key ZiP family members and the free cytosolic concentration of zinc are dynamically regulated in response to the chief physiological stimulus for insulin secretion, glucose. Zinc transporters may thus represent potential interesting targets for pharmacological intervention in certain forms of diabetes mellitus, by controlling insulin and/or glucagon release.

References

Bellomo EA, Meur G et al (2011) Glucose regulates free cytosolic Zn2+ concentration, Slc39 (ZiP), and metallothionein gene expression in primary pancreatic islet {beta}-cells. J Biol Chem 286(29):25778–89

Chimienti F, Devergnas S et al (2006) In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. J Cell Sci 119(Pt 20):4199–206

Gyulkhandanyan AV, Lu H et al (2008) Investigation of transport mechanisms and regulation of intracellular Zn2+ in pancreatic alpha-cells. J Biol Chem 283(15):10184–97

Huang X, Cuajungco MP et al (2000) Alzheimer's disease, beta-amyloid protein and zinc. J Nutr 130(5S Suppl):1488S–92

Hutton JC, Bailyes EM et al (1990) Biosynthesis and storage of insulin. Biochem Soc Trans 18(1):122–4

Kambe T, Narita H et al (2002) Cloning and characterization of a novel mammalian zinc transporter, zinc transporter 5, abundantly expressed in pancreatic beta cells. J Biol Chem 277(21):19049–55

Kanoni S, Nettleton JA, et al (2011) Total Zinc Intake May Modify the Glucose-Raising Effect of a Zinc Transporter (SLC30A8) Variant: A 14-Cohort Meta-Analysis. Diabetes. 60(9):2407–16. Epub 2011 Aug 1

Li M, Zhang Y et al (2007) Aberrant expression of zinc transporter ZIP4 (SLC39A4) significantly contributes to human pancreatic cancer pathogenesis and progression. Proc Natl Acad Sci USA 104(47):18636–41

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr 29: 153–76
Nicolson TJ, Bellomo EA et al (2009) Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. Diabetes 58(9):2070–83
Palmiter RD, Cole TB et al (1996) ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci USA 93(25):14934–9
Rutter GA (2010) Think zinc: new roles for zinc in the control of insulin secretion. Islets 2(1):49–50
Scott DA, Fisher AM (1938) The insulin and the zinc content of normal and diabetic pancreas. J Clin Invest 17(6):725–8
Seve M, Chimienti F et al (2004) In silico identification and expression of SLC30 family genes: an expressed sequence tag data mining strategy for the characterization of zinc transporters' tissue expression. BMC Genomics 5(1):32
Simons TJ (1991) Calcium-dependent zinc efflux in human red blood cells. J Membr Biol 123(1):73–82
Sladek R, Rocheleau G et al (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 445(7130):881–5
Smidt K, Jessen N et al (2009) SLC30A3 responds to glucose- and zinc variations in beta-cells and is critical for insulin production and in vivo glucose-metabolism during beta-cell stress. PLoS One 4(5):e5684
Taylor KM, Nicholson RI (2003) The LZT proteins; the LIV-1 subfamily of zinc transporters. Biochim Biophys Acta 1611(1–2):16–30
Taylor KM, Morgan HE et al (2007) The emerging role of the LIV-1 subfamily of zinc transporters in breast cancer. Mol Med 13(7–8):396–406
Vinkenborg JL, Nicolson TJ et al (2009) Genetically encoded FRET sensors to monitor intracellular Zn2+ homeostasis. Nat Method 6(10):737–40
Wang B, Schneider SN et al (2007) Enhanced cadmium-induced testicular necrosis and renal proximal tubule damage caused by gene-dose increase in a Slc39a8-transgenic mouse line. Am J Physiol Cell Physiol 292(4):C1523–35
Wijesekara N, Chimienti F et al (2009) Zinc, a regulator of islet function and glucose homeostasis. Diabetes Obes Metab 11(Suppl 4):202–14
Wijesekara N, Dai FF et al (2010) Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion. Diabetologia 53(8):1656–68
Williams NR, Rajput-Williams J et al (1995) Plasma, granulocyte and mononuclear cell copper and zinc in patients with diabetes mellitus. Analyst 120(3):887–90
Zhang Y, Bharadwaj U et al (2010) ZIP4 regulates pancreatic cancer cell growth by activating IL-6/STAT3 pathway through zinc finger transcription factor CREB. Clin Cancer Res 16(5):1423–30

ZiP

▶ Zinc Transport in the Pancreatic β-Cell: Roles of ZnT (SLC30A) and ZiP (SLC39A) Family Members

Zipper Protein Kinase

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

ZNF202

Gerd Schmitz
Institute of Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany

Synonyms

Zfp202; Zinc finger protein 202

Historical Background

In 1998, ZNF202 was initially cloned and described as testis-specific zinc finger transcription factor (Monaco et al. 1998). Two years later, in 2000, a linkage analysis in Utah pedigrees suffering from hypoalphalipoproteinemia could identify a new low HDL locus on chromosome 11q23 distinct from the apolipoprotein AI/CIII/AIV/AV gene cluster (Kort et al. 2000). In addition to low HDL cholesterol, family members very frequently exhibited early onset of coronary heart disease (Kort et al. 2000). The newly identified locus contains the gene of the zinc finger transcription factor ZNF202 (Monaco et al. 1998; Wagner et al. 2000). A bioinformatics approach screening for ZNF202 transcription factor binding sites revealed that the ZNF202 gene product is a transcriptional repressor that binds to elements found predominantly in genes that either participate in lipid metabolism or being involved in processes related to energy metabolism and vascular disease (Wagner et al. 2000). Based on published linkage analysis and apparent transcriptional function of ZNF202, ZNF202 was proposed as a candidate susceptibility gene for human dyslipidemia and atherosclerosis for the first time.

Genomic Structure and Functional Domains

The ZNF202 gene consists of ten exons with a 4 kb mRNA. Two different ZNF202 isoforms m1 and m3

can arise by alternative splicing between exons 5 and 6 (Monaco et al. 1998;Wagner et al. 2000). The ZNF202 m1 form encodes a full-length 648 amino acid protein containing an N-terminal SCAN domain, a Krüppel-associated box (KRAB), and eight C_2–H_2-C-terminal zinc fingers. In contrast, the ZNF202m3 isoform encodes a 142 amino acid protein solely consisting of an isolated SCAN domain (Wagner et al. 2000). SCAN domains have been identified in various C_2–H_2-zinc finger transcription factors including ZNF191, ZNF202m1, SCAN domain protein 1 (SDP1), and NIRF where they are usually located in the N-terminal domain (Honer et al. 2001). Yeast-two hybrid studies revealed that SCAN motifs associate selectively with each other and that SCAN-mediated oligomerization may play a regulatory role in DNA binding activity as well as in transcriptional modulation (Schumacher et al. 2000).

The transcriptional repression capacity is mainly mediated by the KRAB domain which is present in one third of all zinc finger transcription factors. This domain consists of approximately 75 amino acid residues including two predicted amphiphatic α-helices (Margolin et al. 1994), which are highly conserved from yeast to man. For sufficient repression a 45 aa segment containing one of the two α-helices is necessary and sufficient. KRAB-mediated gene silencing requires the universal corepressor KRAB-associated protein-1 (KAP-1) which is able to bind the KRAB domain via its RING-B box-coiled coil (RBCC) domain. KAP-1 in turn recruits nonhistone chromosomal proteins of the heterochromatin protein-1 (HP-1) family (Lechner et al. 2000) and thereby prevents promoter activation.

The binding of ZNF202 binding to specific promoter sequences is mediated by its eight consecutive C_2-H_2 zinc finger motifs located in the C-terminal region. The consensus binding sequence for ZNF202 is a GnT (5′-GGGGT-3′) motif, which is found in a variety of promoters of genes associated with lipid metabolism (Wagner et al. 2000).

Regulation of ZNF202 Expression and Modulation of ZNF202 Function

Although initially identified as a testis-specific transcription factor (Monaco et al. 1998), ZNF202 mRNA is also expressed in numerous other tissues including heart, intestine, liver, kidney, and leukocytes (Porsch-Ozcurumez et al. 2001). In THP-1 cells as well as in native human macrophages, ZNF202 mRNA is inversely regulated with its target genes ABCA1 and ApoE during macrophage differentiation and foam cell formation. Upregulation of both genes was accompanied by a simultaneous downregulation of both ZNF202 mRNA isoforms (Langmann et al. 2003). Conversely, deloading of macrophage foam cells with HDL_3 caused upregulation of ZNF202 mRNA. Consistent with those mRNA expression data, reporter gene assays revealed a highly active ZNF202 core promoter region (247 bp upstream of the transcription initiation site) in THP-1 monocytes, which loses its activity upon macrophage differentiation. A GC-box and an Ets motif binding PU.1 has been identified (Langmann et al. 2003) as crucial elements for ZNF202 promoter activity.

Besides the control of ZNF202 gene expression, ZNF202 function may also be regulated via interactions with other SCAN domain proteins. In yeast two-hybrid studies, the zinc finger protein ZNF191 and SCAN domain protein 1 (SDP1) as well as the ZNF202 m3 isoform encoding an isolated SCAN domain were identified as ZNF202 interacting proteins (Schumacher et al. 2000). Data from other transcription factors like GAGA which self-oligomerizes via its BTB/POZ domain provide evidence that the oligomerization status of transcription factors modulates their DNA binding affinities (Espinas et al. 1999).

In addition to ZNF202 SCAN domain interactions, SDP-1 has been shown to prevent binding of the universal corepressor KAP-1 to ZNF202 thereby abolishing its repressional capacity (Porsch-Ozcurumez et al. 2001). Binding of SDP1 to ZNF202 was confirmed in another study demonstrating high amino acid identity of human SDP1 protein with the murine PPARγ2 coactivator PGC-2 (Babb and Bowen 2003). PGC-2 contains a partial SCAN domain and increases transcriptional activity of PPARγ and the estrogen receptor. Similar to PGC-2, SDP-1 was able to bind PPARγ2 through its SCAN domain and to enhance PPARγ2-induced gene transcription without altering the affinity of PPARγ2 for its ligands (Babb and Bowen 2003). The capability of SDP1 to interact with ZNF202 and PPARγ2 as two crucial transcription factors involved in lipid metabolism suggests that SCAN domain proteins such as SDP1 could function as important coregulators in lipid metabolism. Moreover, this interaction may define new regulatory

loops for target genes of both ZNF202 and PPARγ2 like ABCA1 playing a key role in reverse cholesterol transport. One the one hand ZNF202/SDP1 interactions prevent binding of KAP-1 resulting in a derepression of ABCA1. Otherwise, SDP1 coactivation of PPARγ2 may further induce ABCA1 gene transcription via activation of the liver X receptor α (LXR) promoter (Chinetti et al. 2001). These regulatory pathways may also involve other LXR target genes like SREBP1c, a master regulator of fatty acid and triglyceride biosynthesis.

The role of the SCAN encoding ZNF202 isoform m3 and ZNF191 remains still unclear. ZNF202m3 may impair repressional activity of the ZNF202 full-length form by modulating the formation of functional SCAN domain zinc finger transcription factors that is able to bind KAP-1. However, in contrast to the m1 form ZNF202m3 does not coactivate PPARγ (Babb and Bowen 2003).

The related factor ZNF191 contains a SCAN domain and a zinc functional zinc finger region (Honer et al. 2001). The physiological role of a potential interaction of ZNF202 with ZNF191 has not been elucidated so far.

ZNF202 Target Genes

The majority of ZNF202 target genes was identified by a whole genome bioinformatic survey of ZNF202 binding sites. Up to now, several of these targets have been confirmed by EMSA and functional promoter assays. The constantly growing list of ZNF202 regulated genes includes receptors, signaling molecules, transcription factors and enzymes and apolipoproteins involved in glucose and lipid metabolism. The ZNF202 target genes and related disease are listed in Table 1 and have been reviewed in Schmitz et al. (2004). The central ZNF202 target genes in lipid and glucose metabolism and in vascular growth are briefly emphasized below.

Apo AI/CIII/AIV Gene Cluster

ApoAIV together with apoAI and apoE represent the major apolipoprotein components of mature HDL-particles. The *apoAIV* gene is localized in a gene cluster with apoAI and apoCIII on chromosome 11q23-q24. ApoAIV is preferentially expressed in intestinal mucosa and brain and is suggested to play a role in intestinal lipid absorption. Additional functions of apoAIV include LCAT activation and promotion of cellular cholesterol efflux as well as modulation of LPL activation by apoCII. ApoCIII which is expressed in liver and to a minor amount in the intestine participates in the regulation of triglyceride-rich lipoproteins and is a major component of chylomicrons and VLDL. Similar to apoAIV, apoCIII is able to modulate LPL activity by a noncompetitive inhibition. GnT motifs acting as ZNF202 binding sites were identified within the apoAIV promoter region as well as within the apoCIII enhancer (Wagner et al. 2000), and in vitro binding to both promoter sites as well as transcriptional repression has been verified (Porsch-Ozcurumez et al. 2001; Wagner et al. 2000). Both, the apoAIV promoter and the apoCIII enhancer are induced by the ZNF202 target gene HNF4.

Apolipoprotein E

Together with PLTP, CETP, and apoCI, apoE facilitates cellular cholesterol efflux. In addition, it forms a major constituent of chylomicrons, VLDL, and their remnants, as well as certain subclasses of HDL and serve as ligands for receptor-mediated uptake by the liver and adipocytes. ApoE is expressed in liver and in a variety of peripheral tissues including monocytes/macrophages, astrocytes, microglia, and keratinocytes. ApoE is inversely regulated with its repressor ZNF202 during monocyte differentiation and foam cell formation (Langmann et al. 2003).

Phospholipid Transfer Protein

Two main functions contribute to the regulatory role of PLTP in HDL metabolism. First, PLTP is able to transfer phospholipids and triglycerides mainly from triglyceride-rich lipoproteins to nascent HDL particles upon lipoprotein lipase-mediated lipolysis. Second, PLTP activity results in the release of small lipid-poor pre-β-HDL particles, which are efficient acceptors of cholesterol from peripheral cells. Concomitantly, large α-HDL particles are produced. An increase in ApoE HDL and PLTP activity and a decrease in ApoAI/CETP containing HDL was found in patients with severe sepsis. These findings suggest that PLTP contributes to the delivery of phospholipids and energy substrates to tissues during inflammation.

The PLTP promoter contains a confirmed binding site for ZNF202 (Wagner et al. 2000) and members of the nuclear receptor family.

ZNF202, Table 1 ZNF202 target genes and related diseases

Gene		Chromosomal locus	OMIM	Human disease association
Lipid metabolism				
Apolipoprotein AI/CIII/AIV/AV gene cluster		11q23	*107680 *107720 *107690 *606368	ApoAI/CIII deficiency
Apolipoprotein E/CI/CIV/CII gene cluster		19q13.2	*107741 *107710 #207750	ApoE polymorphisms, atherosclerosis, Alzheimer's disease; Hyperlipoproteinemia type IB
Phospholipid transfer protein	PLTP	20q12-q13.1	*172425	
ATP binding cassette transporter A1	ABCA1	9q31.1	*600046	Genetic HDL deficiency syndrome (e.g., Tangier disease
ATP binding cassette transporter G1	ABCG1	21q22.3	*603076	
Hepatic triglyceride lipase	HTGL	15q21-q23	*151670	Hepatic lipase deficiency
Lipoprotein lipase	LPL	8p22	*238600	Hyperlipoproteinemia, type I
Lecithin cholesterol acyltransferase	LCAT	16q22.1	#245900 #136120	LCAT deficiency; fish eye disease
Phosphatidyl ethanolamine *N*-methyltransferase	PEMT	17p11.2	*602391	
Proteolipid protein	PLP	Xq22	#312080 #312920	Pelizaeus-Merzbacher disease; X-linked spastic paraplegia 2
Cellular retinoic acid binding protein 2	CRABP2	1q21.3	*180231	
Glucose metabolism				
Hepatic nuclear factor 4	HNF4	20q12-q13.1	#125850	Maturity onset diabetes of the young type I (MODY1)
B3 adrenergic receptor	b3AR	8p12-p11.3	*109691	NIDDM in Pima Indians, obesity
Insulinoma-associated protein	IA-1	20p11.2	*600010	
Growth, differentiation, and signaling				
Vascular endothelial growth factor	VEGF	6p12	*192240	
Calretinin	CALRT	16q22.2	*114051	
Lymphocyte-activation gene 3	LAG3	12p13.32	*153337	
PILOT/Egr-3 zinc finger transcription factor	EGR-3	8p23-p21	*602419	
GOS24		19q13.1	*190700	

ATP Binding Cassette Transporters A1 and G1

ABCA1 functions as a key regulator of cellular lipid efflux and of plasma HDL pool size, and mutations of ABCA1 have been causatively linked to familial HDL deficiency syndromes. Macrophage cholesterol and phospholipid efflux also involves the half-size transporter ABCG1. For both ABCA1 and ABCG1 promoters, specific binding of ZNF202 and a dose-dependent inhibition of promoter activity by ZNF202 was shown. Moreover, HDL and apoAI-mediated cholesterol and phospholipid efflux was significantly reduced in ZNF202 transfected RAW264.7 cells. This strong inhibition of cellular lipid efflux was not only observed at the basal level, but is also almost completely abolished by oxysterol dependent induction of ABCA1-mediated lipid efflux (Porsch-Ozcurumez et al. 2001). These data nicely demonstrate that the repressor capacity of ZNF202 is even able to abolish the LXR/RXR sensitivity of both promoters.

Hepatic Triglyceride Lipase

Similar to lipoprotein lipase (LPL), hepatic triglyceride lipase (HTGL) reveals not only enzymatic activity with both triglyceride lipase and phospholipase A1 activity but also is able to enhance LDL receptor-mediated uptake of remnant lipoproteins as an enzymatically inactive protein. HTGL binding is able to bind the LDL receptor and thereby enhances VLDL catabolism. Distinct HTGL promoter polymorphisms are associated with dyslipidemia and insulin resistance as well as response to lipid-lowering treatment.

Lipoprotein Lipase

Triglyceride-rich lipoproteins are major targets of LPL-mediated generation of fatty acids. In addition to the hydrolytic activity, LPL may promote particle binding to the GPI-anchored HDL-binding protein, the LDL-receptor, VLDL-receptor, and members of the LDL-related receptor family resulting in uptake of lipoprotein particles. Enzymatically inactive LPL is also able to mediate selective uptake of lipoprotein-associated cholesteryl esters.

Lecithin Cholesterol Acyltransferase

Lecithin cholesterol acyltransferase (LCAT) is a key enzyme in HDL metabolism converting ApoAI containing pre-β-HDL precursors to mature cholesteryl ester-rich αHDL catalyzing the transfer of linoleic acid from the sn-2 position of lecithin to free cholesterol, generating cholesteryl ester and lysolecithin. Mutations of LCAT in humans with either partial or complete absence of plasma LCAT activity lead to fish eye disease and familial LCAT deficiency, which are characterized by markedly reduced plasma HDL levels and corneal clouding. Thus, downregulation of LCAT gene expression by ZNF202 via the identified GnT motif (-1101/-1087) (Wagner et al. 2000) in the LCAT promoter may be of clinical importance.

Phosphatidyl Ethanolamine *N*-Methyltransferase

The phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes the conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) in three consecutive methylation reactions using *S*-adenosylmethionin as a substrate. PC is the primary phospholipid of all classes of lipoproteins in mammals and is required for secretion of lipoproteins and bile salt micelles, and is additionally involved in cellular signaling.

Proteolipid Protein

Myelin proteolipid protein (PLP) is an integral membrane protein and the most abundant protein of the central nerve system. The main source of PLP are oligodendrocytes, but small amounts are also synthesized by Schwann cells in the peripheral nervous system. PLP functions involve membrane adhesion and compaction of myelin, maturation of oligodendrocytes, and maintenance and survival of axons. Diseases resulting from PLP gene abnormalities include Pelizaeus-Merzbacher disease and spastic paraplegia type 2. Pelizaeus-Merzbacher disease is characterized by dysmyelination of the CNS and reduced number of mature oligodendrocytes leading to symptoms like nystagmus, psychomotor retardation, spasticity, and ataxia which develop shortly after birth. Spastic paraplegia type 2 shares the same clinical features, but has a later onset and milder phenotype. As ZNF202 is significantly expressed in the central nerve system, repression of ZNF202 might be of clinical relevance for these disorders. This is supported by recent GWA-studies where a ZNF202 SNP was associated with movement-related adverse antipsychotic effects {Aberg et al. 2010 241/id}.

Hepatic Nuclear Factor 4

In addition to genes involved in the metabolism of triglyceride-rich lipoproteins and HDL, ZNF202 target genes include several genes suggested to contribute broadly to glucose metabolism and diabetes. One of these genes is hepatic nuclear factor 4 (HNF4), a member of the family of nuclear hormone receptors binding fatty acids as endogenous ligands. HNF4 is involved in the regulation of a number of other identified ZNF202 targets. These include ZNF202-dependent suppression of the ApoAI/CIII/AIV/AV and the ApoE/CIV/CII gene cluster, and in addition, the downregulation of HNF4 as a well-known activator of these genes. Besides these ZNF202 targets HNF4 also activates sterol 27-hydroxylase which generates the endogenous LXR-ligand in cholesterol-loaded cells, amino acid and glucose metabolism, and other transcription factors like PPARα and HNF1α. Mutations in the human *HNF4α* gene cause maturity onset diabetes of the young (MODY) 1, a rare form of non-insulin-dependent diabetes mellitus and, in addition to HNF1α, HNF4α is able to directly activate the insulin promoter. In addition, HNF4α is one of the transcription factors required for full transcriptional

activation of the hepatic PEPCK gene, a rate-controlling enzyme of hepatic gluconeogenesis. Induction of PEPCK gene expression by HNF4 and GR is strongly coactivated by PGC-1, another key modulator of hepatic gluconeogenesis and central target of the insulin-cAMP axis in hepatocytes. In brown fat and skeletal muscle PGC-1 is dramatically elevated upon cold exposure and increases UCP-1 transcription via PPARγ and thyroid hormone receptor activation. Moreover, PGC-1α was shown to drive the formation of slow twitch muscle fibers.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is well-known to play a central role in angiogenesis during embryogenesis, wound healing, tumor growth, and chronic inflammatory diseases, e.g., rheumatoid arthritis. The biological activities of VEGF are mediated through two receptor protein tyrosine kinases, Flt-1 (VEGF-R1) and KDR/Flk-1 (VEGF-R2). mRNA levels of both receptors are reduced upon treatment with PPARγ ligands accompanied by an inhibition of VEGF-induced angiogenesis. *VEGF* gene expression is repressed by the oncogenes *p53* and *p73* in different tumors and together with repression by ZNF202 may influence tumor angiogenesis.

ZNF202 in Disease

First evidence for a role of ZNF202 in vivo was given by data from the Copenhagen City Heart Study demonstrating an association between a common variant in ZNF202, A154V, and increased risk for ischemic heart disease and myocardial infarction in women. However, the A154 genotype did not predict risk in men and findings for ZNF202 A154V were independent of further adjustment for lipid profile (Stene et al. 2006a), In addition, a homozygosity for a common promoter variant in the *ZNF202* (g.-660A>G) gene was demonstrated to be associated with decreased transcriptional activity in vitro predicting severe atherosclerosis and increased risk of ischemic heart disease (Stene et al. 2008). Comparable to the A154V genotype, ZNF202 g.-660A>G genotype was not associated with variation in plasma levels of total cholesterol, apoB and LDL-cholesterol, HDL-cholesterol, apoAI and triglycerides (Stene et al. 2008). Similarly, no major contribution of single nucleotide polymorphisms in the protein-coding region to serum HDL cholesterol levels in Danish adults could be detected (Stene et al. 2006b) suggesting that the observed effect on vascular disease may be due to other than lipid-related ZNF202 targets.

More recent studies identified ZNF202 as a candidate gene in other diseases independent of atherosclerosis. In a genome-wide association study searching for genetic susceptibility to extrapyramidal side effects as response to antipsychotic drugs, a SNP located in ZNF202 (rs2126709) reached genome-wide significance (Aberg et al. 2010). Since the major myelin protein, proteolipid protein (PLP), which is mutated in Pelizaeus-Merzbacher disease is a target of ZNF202 this could be a first feasible link between ZNF202 and disorders of the central nervous system.

Another work identified the alpha-methylacyl-coenzyme A racemase (AMACR) as a novel ZNF202 target gene. AMACR regulates peroxisomal β-oxidation of phytol-derived branched-chain fatty acids that may serve as energy source for malignancies and is established as a diagnostic marker in prostate cancer and also suspected to be involved in colon cancer. In colon adenocarcinoma an in vivo deletion hotspot of the ZNF202 binding site has been identified that is supposed to influence the grade of differentiation of tumor cells (Zhang et al. 2009).

By high-throughput SNP and expression analysis, ZNF202 was also identified as candidate gene for non-syndromic oral clefts (Park et al. 2006) suggesting a role in embryonic development.

Summary

In terms of the physiological importance of the in vitro identified ZNF202 target genes in the cellular transport of lipids and lipid metabolism in general ZNF202 was mainly investigated as master regulator of lipid metabolisms. The effects of ZNF202 as transcriptional repressor of lipid efflux by affecting ABCA1 and ABCG1 expression and the functional impact on apolipoprotein E, AIV and AV expression clearly substantiated these findings.

However, in vivo ZNF202 may have a much broader role that differs from the in vitro data, e.g., human angiogenesis or energy metabolism by controlling key genes of glucose and fatty acid metabolism. This is clearly supported by the so far published studies that could demonstrate a relationship between ZNF202 and vascular or neurodegenerative disease but not with lipid metabolism.

Studies working out the in vivo effect in particular in animal models of ZNF202 deletion/overexpression will be needed to provide further insight into the functions of this transcriptional repressor. If it is possible to identify a phenotype in such animals, we may get a better picture of the role of ZNF202. This could also be the basis for pharmacological studies for successful ZNF202 modulation.

Acknowledgments This work was supported by EU Framework 7 project "LipidomicNet".

References

Aberg K, Adkins DE, Bukszar J, Webb BT, Caroff SN, Miller DD, et al. Genomewide association study of movement-related adverse antipsychotic effects. Biol Psychiatry. 2010;67(3):279–82.

Babb R, Bowen BR. SDP1 is a peroxisome-proliferator-activated receptor gamma 2 co-activator that binds through its SCAN domain. Biochem J. 2003;370(Pt 2):719–27.

Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, et al. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nat Med. 2001;7(1):53–8.

Espinas ML, Jimenez-Garcia E, Vaquero A, Canudas S, Bernues J, Azorin F. The N-terminal POZ domain of GAGA mediates the formation of oligomers that bind DNA with high affinity and specificity. J Biol Chem. 1999;274(23):16461–9.

Honer C, Chen P, Toth MJ, Schumacher C. Identification of SCAN dimerization domains in four gene families. Biochim Biophys Acta. 2001;1517(3):441–8.

Kort EN, Ballinger DG, Ding W, Hunt SC, Bowen BR, Abkevich V, et al. Evidence of linkage of familial hypoalphalipoproteinemia to a novel locus on chromosome 11q23. Am J Hum Genet. 2000;66(6):1845–56.

Langmann T, Schumacher C, Morham SG, Honer C, Heimerl S, Moehle C, et al. ZNF202 is inversely regulated with its target genes ABCA1 and apoE during macrophage differentiation and foam cell formation. J Lipid Res. 2003;44(5):968–77.

Lechner MS, Begg GE, Speicher DW, Rauscher III FJ. Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. Mol Cell Biol. 2000;20(17):6449–65.

Margolin JF, Friedman JR, Meyer WK, Vissing H, Thiesen HJ, Rauscher III FJ. Kruppel-associated boxes are potent transcriptional repression domains. Proc Natl Acad Sci USA. 1994;91(10):4509–13.

Monaco C, Helmer CM, Caprini E, Vorechovsky I, Russo G, Croce CM, et al. Molecular cloning and characterization of ZNF202: a new gene at 11q23.3 encoding testis-specific zinc finger proteins. Genomics. 1998;52(3):358–62.

Park JW, Cai J, McIntosh I, Jabs EW, Fallin MD, Ingersoll R, et al. High throughput SNP and expression analyses of candidate genes for non-syndromic oral clefts. J Med Genet. 2006;43(7):598–608.

Porsch-Ozcurumez M, Langmann T, Heimerl S, Borsukova H, Kaminski WE, Drobnik W, et al. The zinc finger protein 202 (ZNF202) is a transcriptional repressor of ATP binding cassette transporter A1 (ABCA1) and ABCG1 gene expression and a modulator of cellular lipid efflux. J Biol Chem. 2001;276(15):12427–33.

Schmitz G, Heimerl S, Langmann T. Zinc finger protein ZNF202 structure and function in transcriptional control of HDL metabolism. Curr Opin Lipidol. 2004;15(2):199–208.

Schumacher C, Wang H, Honer C, Ding W, Koehn J, Lawrence Q, et al. The SCAN domain mediates selective oligomerization. J Biol Chem. 2000;275(22):17173–9.

Stene MC, Frikke-Schmidt R, Nordestgaard BG, Steffensen R, Schnohr P, Tybjaerg-Hansen A. Zinc finger protein 202: a new candidate gene for ischemic heart disease: the Copenhagen city heart study. Atherosclerosis. 2006a;188(1):43–50.

Stene MC, Frikke-Schmidt R, Nordestgaard BG, Tybjaerg-Hansen A. Zinc finger protein 202, genetic variation, and HDL cholesterol in the general population. J Lipid Res. 2006b;47(5):944–52.

Stene MC, Frikke-Schmidt R, Nordestgaard BG, Grande P, Schnohr P, Tybjaerg-Hansen A. Functional promoter variant in zinc finger protein 202 predicts severe atherosclerosis and ischemic heart disease. J Am Coll Cardiol. 2008;52(5):369–77.

Wagner S, Hess MA, Ormonde-Hanson P, Malandro J, Hu H, Chen M, et al. A broad role for the zinc finger protein ZNF202 in human lipid metabolism. J Biol Chem. 2000;275(21):15685–90.

Zhang X, Leav I, Revelo MP, Deka R, Medvedovic M, Jiang Z, et al. Deletion hotspots in AMACR promoter CpG island are *cis*-regulatory elements controlling the gene expression in the colon. PLoS Genet. 2009;5(1):e1000334.

ZnT

► Zinc Transport in the Pancreatic β-Cell: Roles of ZnT (SLC30A) and ZiP (SLC39A) Family Members

ZPK

► DLK (Dual Leucine Zipper-Bearing Kinase)